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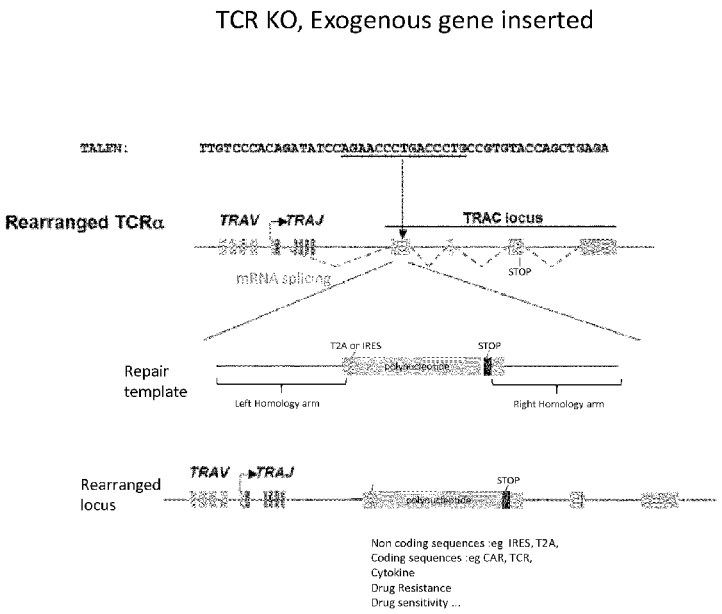

Figure 1

(57) Abstract: The invention relates to the fields of immunotherapy, molecular biology and recombinant nucleic acid technology. In particular, the invention relates to a TALEN-modified human primary cell comprising in its genome, a modified human T cell receptor alpha gene with an insertion comprising at least, from 5' to 3', a polynucleotide encoding a self-cleaving peptide, a chimeric antigen receptor, wherein the cell has undetectable cell-surface expression of the endogenous alpha beta T cell receptor as compared to a TCR positive control cell and expresses a receptor to target a pathological cell, use of said cell for treating a disease, including cancer. The invention further relates to methods for producing such a TALEN-modified cell, and to means for detecting such an engineered human primary cell or other genetically modified human primary cell obtained using alternative and/or additional rare cutting endonucleases.

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TAL- effector nuclease (TALEN) -MODIFIED ALLOGENIC CELLS SUITABLE FOR THERAPY

FIELD OF THE INVENTION

The invention relates to the fields of immunotherapy, molecular biology and recombinant nucleic acid technology. In particular, the invention relates to a TALEN-modified human primary cell comprising in its genome, a modified human T cell receptor alpha gene with an insertion comprising at least, from 5' to 3', a polynucleotide encoding a self-cleaving peptide, a chimeric antigen receptor, wherein the cell has undetectable cell-surface expression of the endogenous alpha beta T cell receptor as compared to a TCR positive control cell and expresses a receptor to target a pathological cell, use of said cell for treating a disease, including cancer. The invention further relates to methods for producing such a TALEN-modified cell, and to means for detecting such an engineered human primary cell or other genetically modified human primary cell obtained using alternative and/or additional rare cutting endonucleases.

BACKGROUND OF THE INVENTION

T cell adoptive immunotherapy is a promising approach for cancer treatment. This strategy utilizes isolated human T cells, usually obtained from patients themselves that have been genetically-modified to enhance their specificity for a specific tumor associated antigen. Genetic modification may involve the expression of a chimeric antigen receptor or of an exogenous T cell receptor, the inactivation of particular cell surface proteins, to prevent inhibition of the cytolytic activity by cancer cells, gene modification to make cells sensitive or resistant to a drug.

By contrast to exogenous T cell receptors, chimeric antigen receptors derive their specificity from the variable domains of a monoclonal antibody. Thus, T cells expressing chimeric antigen receptors (CAR T cells) induce tumor immunoreactivity in a major histocompatibility complex non-restricted manner. To date, T cell adoptive immunotherapy has been utilized as a clinical therapy for a number of cancers, including B cell malignancies (e.g., acute lymphoblastic leukemia (ALL), B cell non-

Hodgkin lymphoma (NHL), and chronic lymphocytic leukemia), multiple myeloma, neuroblastoma, glioblastoma, advanced gliomas, ovarian cancer, mesothelioma, melanoma, and pancreatic cancer.

Despite its potential usefulness as a cancer treatment, adoptive immunotherapy with CAR T cells has been limited, in part, by expression of the endogenous T cell receptor on the cell surface. CAR T cells expressing an endogenous T cell receptor may recognize major and minor histocompatibility antigens following administration to an allogeneic patient, which can lead to the development of graft-versus-host-disease (GVHD). As a result, clinical trials have largely focused on the use of autologous CAR T cells, wherein a patient's T cells are isolated, genetically-modified to incorporate a chimeric antigen receptor, and then re-infused into the same patient. An autologous approach provides immune tolerance to the administered CAR T cells; however, this approach is constrained by both the time and expense necessary to produce patient-specific CAR T cells after a patient's cancer has been diagnosed.

Thus, "off the shelf CAR T cells" or so called "allogenic CAR T cells" or universal CART (UCART), prepared using cells from a third-party donor, that have reduced or better, no expression of the endogenous T cell receptor and do not initiate GVHD upon administration have been prepared.

To reduce or eliminate cell surface expression of the TCR in primary T cells (isolated from healthy donors), various methods were used.

Genetic modification of genomic DNA can be performed using site-specific, rare-cutting endonucleases that are engineered to recognize DNA sequences in the locus of interest. Methods for producing engineered, site-specific endonucleases are known in the art. For example, zinc-finger nucleases (ZFNs) can be engineered to recognize and cut predetermined sites in a genome. ZFNs are chimeric proteins comprising a zinc finger DNA-binding domain fused to the nuclease domain of the FokI restriction enzyme. The zinc finger domain can be redesigned through rational or experimental means to produce a protein that binds to a pre-determined DNA sequence -18 basepairs in length. By fusing this engineered protein domain to the FokI nuclease, it is possible to target DNA breaks with

genome-level specificity. ZFNs have been used extensively to target gene addition, removal, and substitution in a wide range of eukaryotic organisms (reviewed in Durai et al. (2005), Nucleic Acids Res 33, 5978). Likewise, TAL-effector nucleases (TALENs) can be generated to cleave specific sites in genomic DNA. Like a ZFN, a TALEN comprises an engineered, site-specific DNA-binding domain fused to the FokI nuclease domain (reviewed in Mak et al. (2013), Curr Opin Struct Biol. 23:93-9). In this case, the DNA binding domain comprises a tandem array of TAL-effector domains, each of which specifically recognizes a single DNA basepair. Thus, TALENs are heterodimeric, so that the production of a single functional nuclease in a cell requires co-expression of two proteins, making it more reliable and specific for the locus targeted than other techniques (less off sites is measured). Compact TALENs have an alternative endonuclease architecture (Beurdeley et al. (2013), Nat Commun. 4: 1762). A Compact TALEN comprises an engineered, site-specific TAL-effector DNA-binding domain fused to the nuclease domain from the I-TevI homing endonuclease. Unlike FokI, I-TevI does not need to dimerize to produce a double-strand DNA break so a Compact TALEN is functional as a monomer.

Engineered endonucleases based on the CRISPR/Cas9 system are also known in the art (Ran et al. (2013), Nat Protoc. 8:2281-2308; Mali et al. (2013), Nat Methods 10:957-63). A CRISPR endonuclease comprises two components: (1)CRISPR-associated Protein9, typically microbial Cas9; and (2) a short "guide RNA" comprising a 20 nucleotides targeting sequence that directs the nuclease to a location of interest in the genome. By expressing multiple guide RNAs in the same cell, each having a different targeting sequence, it is possible to target DNA breaks simultaneously to multiple sites in the genome. The primary drawback of the CRISPR/Cas9 system is its reported high frequency of off-target DNA breaks, which could limit the utility of the system for treating human patients (Fu et al. (2013), Nat Biotechnol . 31 :822-6).

Homing endonucleases are a group of naturally-occurring nucleases that recognize 15-40 base-pair cleavage sites commonly found in the genomes of plants and fungi. They are frequently associated with parasitic DNA elements, such as group 1 self-splicing introns and inteins. They

naturally promote homologous recombination or gene insertion at specific locations in the host genome by producing a double -stranded break in the chromosome, which recruits the cellular DNA-repair machinery (Stoddard (2006), Q. Rev. Biophys. 38: 49-95). Homing endonucleases are commonly grouped into four families: the LAGLIDADG family, the GIY-YIG family, the His-Cys box family and the HNH family. These families are characterized by structural motifs, which affect catalytic activity and recognition sequence. For instance, members of the LAGLIDADG family are characterized by having either one or two copies of the conserved LAGLIDADG motif (see Chevalier et al. (2001), Nucleic Acids Res. 29(18): 3757-3774). The LAGLIDADG homing endonucleases with a single copy of the LAGLIDADG motif form homodimers, whereas members with two copies of the LAGLIDADG motif are found as monomers.

I-CreI is a member of the LAGLIDADG family of homing endonucleases that recognizes and cuts a 22 basepair recognition sequence in the chloroplast chromosome of the algae Chlamydomonas reinhardtii. Genetic selection techniques have been used to modify the wild-type I-CreI cleavage site preference (Sussman et al. (2004), J. Mol. Biol. 342: 31-41; Chames et al. (2005), Nucleic Acids Res. 33: el78; Seligman et al. (2002), Nucleic Acids Res. 30: 3870-9, Arnould et al. (2006), J. Mol. Biol. 355: 443-58). A method of rationally-designing mono-LAGLIDADG homing endonucleases was described that is capable of comprehensively redesigning I-CreI and other homing endonucleases to target widely-divergent DNA sites, including sites in mammalian, yeast, plant, bacterial, and viral genomes (WO 2007/047859).

As first described in WO 2009/059195, I-CreI and its engineered derivatives are normally dimeric but can be fused into a single polypeptide using a short peptide linker that joins the C-terminus of a first subunit to the N-terminus of a second subunit (Li et al. (2009), Nucleic Acids Res. 37: 1650-62; Grizot e/1 al. (2009), Nucleic Acids Res. 37:5405-19). Thus, a functional "single-chain" meganuclease can be expressed from a single transcript. The use of engineered meganucleases for cleaving DNA targets in the human T cell receptor alpha constant region was previously disclosed

(WO 2014/191527). WO 2014/191527 discloses variants of the I-Onul meganuclease that are engineered to target a recognition sequence within exon 1 of the TCR alpha constant region gene. Although the WO 2014/191527 publication discusses that a chimeric antigen receptor can be expressed in TCR knockout cells, the authors do not disclose the insertion of the chimeric antigen receptor coding sequence into the meganuclease cleavage site in the TCR alpha constant region gene.

The use of other nucleases and mechanisms for disrupting expression of the endogenous TCR has also been disclosed. For example, the use of zinc finger nucleases for disrupting TCR genes in human T cells was described by U.S. Patent No. 8,95,828 and by U.S. Patent Application Publication No. US2014/034902. U.S. Publication No.US2014/0301990 describes the use of zinc finger nucleases and transcription-activator like effector nucleases (TALENs), and of a CRISPR Cas system with an engineered single guide RNA for targeting TCR genes in an isolated T cell. U.S. Patent Application Publication No. US2012/0321667 discloses the use of small-hairpin RNAs that target nucleic acids encoding specific TCRs and/or CD3 chains in T cells.

Similarly, WO2017062451, WO2015057980, WO2017106528 or US7910332 B2 describes TCR negative T cells obtained using various tools of gene editing, in particular Crispr/cas 9 system, or a meganuclease targeting various sequences of the constant region of the T cell receptor gene (TRAC gene).

Each type of endonuclease, when used in optimized conditions, generates a double strand cut of the DNA and either a new DNA sequence at the site of insertion or a deletion is created. Further, the frequency of off target gene modifications is directly related to the endonuclease used and to the sequence of the TRAC gene bound and cut by the endonuclease used, making the final product more or less reliable as a medicament.

There is still a need for providing off the shelve products, in particular for immunotherapy, with more stable gene modification(s), less off site, and means of detecting engineered cells, measuring the quality of such products, identifying their integrity and stability.

The present inventors are the first to teach a TALEN-modified human primary cells with a TALEN –mediated specific insertion into the TCR-encoding gene allowing a CAR targeting CD123 or CD22 to be expressed at the cell surface and a TALEN-induced off site target below detection as measured by a guide seq technique. They are also the first to report means and method of detecting such genetically-modified human primary cells over cells engineered using other endonucleases designed to cleave the TCR, in particular on site and off site cleavages.

SUMMARY OF THE INVENTION

According to a first aspect, the present invention provides a method for producing an endonuclease-modified endogenous αβ-TCR negative human cell, said method comprising:

(a) introducing into a human cell:

(i) a first nucleic acid sequence encoding an engineered nuclease; or an engineered nuclease protein;

wherein said engineered nuclease produces a cleavage at a recognition sequence within said human TCR alpha constant region gene; said cleavage resulting in an inhibition of cell surface expression of the αβ-TCR to undetectable level;

(ii) a second nucleic acid sequence comprising an exogenous polynucleotide comprising a nucleic acid sequence encoding a self-cleaving peptide in frame with the genomic TRAC coding sequence and a nucleic acid sequence encoding a chimeric antigen receptor (CAR) or a recombinant TCR,

wherein the sequence of said exogenous polynucleotide is inserted into said genomic human TCR alpha constant region gene at said cleavage site; wherein said self-cleaving peptide is selected from a 2A peptide, a 2A like peptide, a P2A peptide, a E2A peptide and a F2A peptide, and further wherein said genetically-modified cell has reduced cell-surface expression of the endogenous TCR when compared to an unmodified control cell.

According to a second aspect, the present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell wherein the constant region of the genomic TCR gene (TRAC gene)

comprises a genetic modification generated by a TALEN and affecting cell surface expression of the endogenous αβ-TCR, said genomic TRAC gene comprising from 5' to 3':

(a) a 5' region of said human genomic TRAC gene,

(b) a first recognition domain for said TALEN,

(c) an insertion as compared to the wild-type TRAC gene affecting the cell surface expression of the extracellular domain or transmembrane domain of the αβ-TCR, said insertion comprising an exogenous polynucleotide comprising a sequence coding for a self-cleaving peptide in frame with the TRAC open reading frame, a sequence coding a chimeric antigen receptor or recombinant TCR, and a termination sequence,

(c') optionally a second TALEN recognition domain, and

(d) a 3' region of the genomic TRAC gene;

wherein said self-cleaving peptide is selected from a 2A peptide, a 2A like peptide, a P2A peptide, a E2A peptide and a F2A peptide.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell wherein the constant region of the genomic TCR gene (TRAC gene) comprises a genetic modification generated by a TALEN and affecting cell surface expression of the endogenous alpha beta TCR, said genomic TRAC gene comprising from 5' to 3':

(a) a 5' region of said human genomic TRAC gene upstream,

(b) a recognition domain for a TALEN,

(c) a gap or an insertion as compared to the wild type TRAC gene affecting the cell surface expression of the extracellular domain or transmembrane domain of the alpha beta TCR,

said insertion comprising an exogenous polynucleotide selected from a noncoding sequence such as, a stop codon, an IRES, a coding sequence such as a sequence coding for a self-cleaving peptide in frame with the TRAC open reading frame, a sequence coding a chimeric antigen receptor (CAR),

a sequence coding a TCR, a sequence coding a protein conferring sensitivity to a drug, a sequence coding a protein conferring resistance to a drug, a cytokine, a termination sequence, a combination thereof,

(c') optionally a second TALEN recognition domain,

(d) a 3' region of the genomic TRAC gene.

The present invention provides a TALEN-modified endogenous $\alpha\beta$-TCR negative human

primary cell wherein the constant region of the genomic TCR gene (TRAC gene) comprises a genetic modification generated by a TALEN and affecting cell surface expression of the endogenous alpha beta TCR, said genomic TRAC gene comprising from 5' to 3':

(a) a 5' region of said human genomic TRAC gene upstream,

(b) a recognition domain for a TALEN,

(c) a gap

(c') optionally a second TALEN recognition domain,

(d) a 3' region of the genomic TRAC gene.

The present invention provides a TALEN-modified endogenous αβ–TCR negative human primary cell wherein the constant region of the genomic TCR gene (TRAC gene) comprises a genetic modification generated by a TALEN and affecting cell surface expression of the endogenous alpha beta TCR, said genomic TRAC gene comprising from 5' to 3':

(a) a 5' region of said human genomic TRAC gene upstream,

(b) a recognition domain for a TALEN,

(c) an insertion as compared to the wild type TRAC gene affecting the cell surface expression of the extracellular domain or transmembrane domain of the alpha beta TCR,

said insertion comprising an exogenous polynucleotide selected from a noncoding sequence such as, a stop codon, an IRES, a coding sequence such as a sequence coding for a self-cleaving peptide in frame with the TRAC open reading frame, a sequence coding a chimeric antigen receptor (CAR), a sequence coding a TCR, a sequence coding a protein conferring sensitivity to a drug, a sequence coding a protein conferring resistance to a drug, a cytokine, a termination sequence, a combination thereof,

(c') optionally a second TALEN recognition domain,

(d) a 3' region of the genomic TRAC gene.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell wherein the constant region of the genomic TCR gene (TRAC gene) comprises a genetic modification generated by a TALEN and affecting cell surface expression of the endogenous alpha beta TCR, said genomic TRAC gene comprising from 5' to 3':

(a) a 5' region of said human genomic TRAC gene upstream,

(b) a recognition domain for a TALEN,

(c) an insertion as compared to the wild type TRAC gene affecting the cell surface expression of the extracellular domain or transmembrane domain of the alpha beta TCR,

said insertion comprising an exogenous polynucleotide comprising a sequence coding for a self-cleaving peptide in frame with the TRAC open reading frame, a sequence coding a chimeric antigen receptor (CAR), a termination sequence.

(c') optionally a second TALEN recognition domain,

(d) a 3' region of the genomic TRAC gene.

A CAR means chimeric antigen receptor and may be a recombinant TCR.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell wherein the constant region of the genomic TCR gene (TRAC gene) comprises a genetic modification generated by a TALEN and affecting cell surface expression of the endogenous alpha beta TCR, said genomic TRAC gene comprising from 5' to 3':

(a) a 5' region of said human genomic TRAC gene upstream,

(b) a recognition domain for a TALEN,

(c) an insertion as compared to the wild type TRAC gene affecting the cell surface expression of the extracellular domain or transmembrane domain of the alpha beta TCR,

said insertion comprising an IRES, a chimeric antigen receptor (CAR), a termination sequence, a combination thereof,

(c') optionally a second TALEN recognition domain,

(d) a 3' region of the genomic TRAC gene.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell wherein the constant region of the genomic TCR gene (TRAC gene) comprises a genetic modification generated by a TALEN and affecting cell surface expression of the endogenous alpha beta TCR, said genomic TRAC gene comprising from 5' to 3':

(a) a 5' region of said human genomic TRAC gene upstream,

(b) a recognition domain for a TALEN,

(c) an insertion as compared to the wild type TRAC gene affecting the cell surface expression of the extracellular domain or transmembrane domain of the alpha beta TCR,

said insertion comprising a self-cleaving peptide in frame with the TRAC open reading frame, a sequence coding a protein conferring sensitivity to a drug, a termination sequence,

(c') optionally a second TALEN recognition domain,

(d) a 3' region of the genomic TRAC gene.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell wherein the constant region of the genomic TCR gene (TRAC gene) comprises a genetic modification generated by a TALEN and affecting cell surface expression of the endogenous alpha beta TCR, said genomic TRAC gene comprising from 5' to 3':

(a) a 5' region of said human genomic TRAC gene upstream,

(b) a recognition domain for a TALEN,

(c) an insertion as compared to the wild type TRAC gene affecting the cell surface expression of the extracellular domain or transmembrane domain of the alpha beta TCR,

said insertion comprising a self-cleaving peptide in frame with the TRAC open reading frame, a sequence coding a protein conferring resistance to a drug, a termination sequence,

(c') optionally a second TALEN recognition domain,

(d) a 3' region of the genomic TRAC gene.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell wherein the constant region of the genomic TCR gene (TRAC gene) comprises a genetic modification generated by a TALEN and affecting cell surface expression of the endogenous alpha beta TCR, said genomic TRAC gene comprising from 5' to 3':

(a) a 5' region of said human genomic TRAC gene upstream,

(b) a recognition domain for a TALEN,

(c) an insertion as compared to the wild type TRAC gene affecting the cell surface expression of the extracellular domain or transmembrane domain of the alpha beta TCR,

said insertion comprising a self-cleaving peptide in frame with the TRAC open reading frame, a sequence coding a cytokine, a termination sequence,

(c') optionally a second TALEN recognition domain,

(d) a 3' region of the genomic TRAC gene.

Here; a "cytokine" encompasses any factor influencing the functioning (activity, capacity to migrate, adhere, resists to tumor environment) of immune cells.

In one aspect, the present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell according to the above embodiments, wherein said human primary cell is a human primary T cell or a human primary cell derived from a human primary T cell, or a human lymphoid primary cell, or a human primary stem cell, or a human primary progenitor cell.

In one aspect, the present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell according to any one of according to the above embodiments, wherein said human primary cell is a human primary T cell or a population of human primary T cells.

In one aspect, the present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell according to any one of according to the above embodiments, wherein said human T primary cell or population thereof comprises or consists in human primary CD8 T cell, human primary CD4 T cell, a combination thereof.

In still another aspect, the present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell according to any one of the above embodiments, wherein said recognition domain for a TALEN comprises the following sequence ttgtcccacagATATC, or ttgtcccacagATATCCAG, and optionally CCGTGTACCAGCTGAGA.

In one aspect, the present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell according to any one of claims 1 to 5 comprising the following sequence: AAGTAGCCCTGCATTTCAGGTTTCCTTGAGTGGCAGGCCAGGCCTGGCCGTGAACGTTCACTGAAATCATGGCCTCTTGGCCAAGATTGATAGCTTGTGCCTGTCCCTGAGTCCCAGTCCATCACGAGCAGCTGGTTTCTAAGATGCTATTTCCCGTATAAAGCATGAGACCGTGACTTGCCAGCCCCACAGAGCCCCGCCCTTGTCCATCACTGGCATCTGGACTCCAGCCTGGGTTGGGGCAAAGAGGGAAATGAGATCATGTCCTAACCCTGATCCTCTTGTCCCACAGATATCCAG***TCCGGTGAGGGCAGAGGAAGTCTTCTAACATGCGGTGACGTGGAGGAGAATCCGGGCCCC***GGATCC codingsequence TCTAGAGGGCCCGTTTAAACCCGCTGATCAGCCTCGA*CTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATG*ACTAGTGGCGAATTCCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTGCCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGACAAAACTGTGCTAGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCAACAAATCTGACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGACACCTTCTTCCCCAGCCCAGGTAAGGGCAGCTTTGGTGCCTTCGCAGGCTGTTTCCTTGCTTCAGGAA in the TRAC

gene, [wherein underlined sequences represent a homology arm,

italic bold sequences represent a self-cleaving peptide, preferably a 2A peptide,

italic sequences represent a termination sequence (BGH polyA),

The “codingsequence” represents a sequence coding a protein, such as a sequence coding for a self-cleaving peptide in frame with the TRAC open reading frame, a sequence coding a chimeric antigen receptor (CAR), a sequence coding a TCR, a sequence coding a protein conferring sensitivity to a drug, a sequence coding a protein conferring resistance to a drug, a cytokine, a combination thereof, preferably “codingsequence” represents a chimeric antigen receptor, even more preferably an anti-CD22 or anti-CD123 CAR, even more more preferably a sequence of SEQ ID N 9, 10, 11 or 12 (corresponding to an anti-CD22 CAR, an anti-CD22 CAR B-B7 QR3, an anti-CD22 CAR A-D4 QR3, anti-CD123 CAR, respectively).

The present invention contemplates any of these particular CARs comprising two epitopes recognized by the rituximab molecular antibody (R2) or 3 epitopes recognized by the rituximab and one recognized by QBEN (CD34), designed QR3 above.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell according to any one of, wherein said human T primary cell or population thereof comprising a sequence of SEQ ID N 9.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell according to any one of, wherein said human T primary cell or population thereof comprising a sequence of SEQ ID N 10.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell according to any one of, wherein said human T primary cell or population thereof comprising a sequence of SEQ ID N 11.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell

according to any one of, wherein said human T primary cell or population thereof comprising a sequence of SEQ ID N 12.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell according to any one of, wherein said human T primary cell or population thereof comprising a sequence having at least from 99 %, 98%, 97%, 96%, 95% 94% 93% 92% 91% 90% 89% 88% 87% 86% 85% 84% 83% 82% 81% to 80% identity with of SEQ ID N 9.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell according to any one of, wherein said human T primary cell or population thereof comprising a sequence having at least from 99 %, 98%, 97%, 96%,95% 94% 93% 92% 91% 90% 89% 88% 87% 86% 85% 84% 83% 82% 81% to 80% identity with of SEQ ID N 10.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell according to any one of, wherein said human T primary cell or population thereof comprising a sequence having at least from 99 %, 98%, 97%, 96%,95% 94% 93% 92% 91% 90% 89% 88% 87% 86% 85% 84% 83% 82% 81% to 80% identity with of SEQ ID N 11.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell according to any one of, wherein said human T primary cell or population thereof comprising a sequence having at least from 99 %, 98%, 97%, 96%,95% 94% 93% 92% 91% 90% 89% 88% 87% 86% 85% 84% 83% 82% 81% to 80% identity with of SEQ ID N 12.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human primary T cell according to any one of the above comprising a sequence of SEQ ID N° 9.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human primary T cell according to any one of the above comprising a sequence of SEQ ID N° 10.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human primary T cell according to any one of the above comprising a sequence of SEQ ID N° 11.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human primary T cell according to any one of the above comprising a sequence of SEQ ID N° 12.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human primary T cell according to any one of the above comprising a sequence of SEQ ID N° 9 and expressing undetectable level of MHC molecules.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human primary T cell according to any one of the above comprising a sequence of SEQ ID N° 10 and expressing undetectable level of MHC molecules.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human primary T cell according to any one of the above comprising a sequence of SEQ ID N° 11 and expressing undetectable level of MHC molecules.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human primary T cell according to any one of the above comprising a sequence of SEQ ID N° 12 and expressing undetectable level of MHC molecules.

In one aspect, the present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell according to any one of the above embodiments, wherein said CAR sequence comprises an anti-CD22 CAR sequence, anti-CD123 CAR sequence, anti-CD30 CAR sequence, anti-HSP-70 CAR sequence, anti-o-acetyl-GD2 CAR sequence, anti-CS-1 CAR sequence, anti-CLL-1 CAR sequence.

In one aspect, the present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell according to any one of the above embodiments, wherein said CAR sequence comprises an anti-CD22 CAR sequence, anti-CD123 CAR sequence, anti-CD30 CAR sequence, anti-HSP-70 CAR sequence, anti-o-acetyl-GD2 CAR sequence, anti-CS-1 CAR sequence, anti-CLL-1 CAR sequence.

In one aspect, the present invention provides a TALEN-modified endogenous ⍰⍰-TCR negative human primary cell according to any one of the above embodiments, wherein said CAR sequence comprises an Anti- BCMA CAR sequence, anti-CD33 CAR sequence, anti-EGFRVIII CAR sequence, anti

Flt3 CAR sequence, anti- WT1 CAR sequence, anti-CD70 CAR sequence, anti-MUC16 CAR sequence, anti-PRAME CAR sequence, anti-TSPAN10 CAR sequence, anti- CLAUDIN18.2 CAR sequence, anti-DLL3 CAR sequence, anti- LY6G6D CAR sequence. anti- Liv-1 CAR sequence, anti-CHRNA2 CAR sequence, anti-ADAM10 CAR sequence.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above embodiments, further comprising undetectable level of MHC molecules as compared to an unmodified (eg non-engineered) control cell and a deletion functionally affecting cell surface expression of a beta 2 microglobulin molecule or of a CIITA molecule.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above embodiments, wherein said insertion resulted in an inactivation of the gene coding the TCR alpha and an undetectable cell surface expression of endogenous αβ-TCR in at least 96% of the total cells, in at least 97% of the total cells, in at least 98% of the total cells, in at least 99% of the total cells, as compared to a positive control.

A positive control may be non-engineered mature T cells expressing a detectable level of alpha beta TCR at the surface that may be detected for example by flow cytometry using an antibody specific for the alpha TCR, beta TCR, alpha beta TCR.

The present invention provides the TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above embodiments, comprising a TALEN binding domain and or a sequence upstream a TALEN binding domain present in the wt TRAC gene.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above embodiments, wherein said TALEN comprises a first TALEN subunit:

MGDPKKKRKVIDIADLRTLGYSQQQQEKIKPKVRSTVAQHHEALVGHGFTHAHIVALSQHPAALGTVAVK
YQDMIAALPEATHEAIVGVGKQWSGARALEALLTVAGELRGPPLQLDTGQLLKIAKRGGVTAVEAVHAWRNALTG
APLNLTPQQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLTP
QQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIA

SHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQ
ALETVQALLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQALL
PVLCQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQALLPVLCQAH
GLTPQQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQALLPVLCQAHGLTPQQV
VAIASNGGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNG
GGRPALESIVAQLSRPDPALAALTNDHLVALACLGGRPALDAVKKGLGDPISRSQLVKSELEEKKSELRHKLKYVPHE
YIELIEIARNSTQDRILEMKVMEFFMKVYGYRGKHLGGSRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADE
MQRYVEENQTRNKHINPNEWWKVYPSSVTEFKFLFVSGHFKGNYKAQLTRLNHITNCNGAVLSVEELLIGGEMIKA
GTLTLEEVRRKFNNGEINFAAD, of SEQ ID N°3,

and a second TALEN subunit :

MGDPKKKRKVIDIADLRTLGYSQQQQEKIKPKVRSTVAQHHEALVGHGFTHAHIVALSQHPAALGTVAVKYQDMIA
ALPEATHEAIVGVGKQWSGARALEALLTVAGELRGPPLQLDTGQLLKIAKRGGVTAVEAVHAWRNALTGAPLNLTPE
QVVAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPEQVVAIASH
DGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQALLPVLCQAHGLTPQQVVAIASNNGGKQALE
TVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNGGGKQALETVQRLLPVL
CQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLT
PQQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQALLPVLCQAHGLTPEQVVAIAS
HDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQALLPVLCQAHGLTPEQVVAIASHDGGKQALE
TVQRLLPVLCQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNGGGRPALESIVAQLSRP
DPALAALTNDHLVALACLGGRPALDAVKKGLGDPISRSQLVKSELEEKKSELRHKLKYVPHEYIELIEIARNSTQDRILEM
KVMEFFMKVYGYRGKHLGGSRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMQRYVEENQTRNKHINP
NEWWKVYPSSVTEFKFLFVSGHFKGNYKAQLTRLNHITNCNGAVLSVEELLIGGEMIKAGTLTLEEVRRKFNNGEINF
AAD, or SEQ ID N°4,

In another embodiment, said

first TALEN subunit comprises a sequence having at least from 99 %, 98%, 97%, 96%,95% 94% 93% 92% 91% 90% 89% 88% 87% 86% 85% 84% 83% 82% 81% to 80% identity with SEQ ID N°3, provided

that said first TALEN subunit binds to said a TALEN recognition domain, preferably to ttgtcccacagATATC,

and a second TALEN subunit sequence having at least from 99 %, 98%, 97%, 96%,95% 94% 93% 92% 91% 90% 89% 88% 87% 86% 85% 84% 83% 82% 81% to 80% identity with SEQ ID N°4 provided that said second TALEN subunit binds to a second TALEN recognition domain, preferably to CCGTGTACCAGCTGAGA, and provided that the frequency of off target binding is below detection;

Off target may be measured by "guide-seq" analysis using for example an adapted version for TALEN guide seq. for TALEN engineered cells.

The present invention provides a TALEN-modified endogenous αβ–TCR negative human cell according to any one of the above embodiments, wherein said at least one insertion comprises an exogenous polynucleotide sequence located downstream a TALEN binding domain of sequence ttgtcccacagATATC, or ttgtcccacagATATCCAG, (present in the native TRAC).

In another embodiment, the present invention provides a TALEN-modified endogenous αβ–TCR negative human cell according to any one of the above embodiments, wherein said at least one insertion comprises an IRES, an exogenous polynucleotide sequence coding a chimeric antigen receptor, a terminator sequence of polyadenylation signal, optionally a TALEN binding domain.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above embodiments, wherein said at least one insertion comprises a sequence encoding a self-cleaving peptide in frame with the genomic TRAC coding sequence.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above embodiments, wherein said at least one insertion comprises a sequence encoding a self-cleaving peptide in frame with the genomic TRAC coding sequence, an exogenous

polynucleotide sequence coding a product conferring resistance to a drug, a terminator sequence of polyadenylation signal, optionally a TALEN binding domain.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above embodiments, wherein said at least one insertion comprises a sequence encoding a self-cleaving peptide in frame with the genomic TRAC coding sequence, an exogenous polynucleotide sequence coding a product conferring sensitivity to a drug, a terminator sequence of polyadenylation signal, optionally a TALEN binding domain.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above embodiments, wherein said at least one insertion comprises a sequence encoding a self-cleaving peptide in frame with the genomic TRAC coding sequence, an exogenous polynucleotide sequence coding a cytokine, a terminator sequence of polyadenylation signal, optionally a TALEN binding domain.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above embodiments, wherein said at least one insertion comprises a sequence encoding a self-cleaving peptide in frame with the genomic TRAC coding sequence, an exogenous polynucleotide sequence coding at least a chimeric antigen receptor, a terminator sequence of polyadenylation signal, optionally a TALEN binding domain.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above embodiments, wherein said at least one insertion comprises a sequence encoding a self-cleaving peptide in frame with the genomic TRAC coding sequence, said self-cleaving peptide is a self-cleaving peptide selected from a 2A peptide, a 2A like peptide, a P2A peptide, a E2A peptide, a F2A peptide, preferably a 2A peptide, more preferably a 2A peptide, of sequence GSGEGRGSLLTCGDVEENPGP, GSGATNFSLLKQAGDVEENPGP, GSGQCTNYALLKLAGDVESNPGP, GSGVKQTLNFDLLKLAGDVESNPGP, SGEGRGSLLTCGDVEENPGP, SGATNFSLLKQAGDVEENPGP, SGQCTNYALLKLAGDVESNPGP, SGVKQTLNFDLLKLAGDVESNPGP, even more preferably a 2A peptide of sequence SGEGRGSLLTCGDVEENPGP.

The present invention provides a TALEN-modified endogenous αβ–TCR negative human cell according to any one of the above embodiments, wherein said at least one insertion comprises a sequence coding a 2A peptide of sequence SGEGRGSLLTCGDVEENPGP encoded by a sequence in frame with the genomic TRAC coding sequence, an exogenous polynucleotide sequence coding a chimeric antigen receptor, a terminator sequence of polyadenylation signal, optionally a TALEN binding domain.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above embodiments, wherein said exogenous polynucleotide sequence comprises a chimeric antigen receptor (CAR) selected from a CAR specific for at least one of the following antigen The CAR molecule of the invention comprises an antigen binding domain wherein said antigen binding domain binds to the tumor antigen associated with a disease, and said tumor antigen is selected from a group consisting of: CD19 molecule (CD19); membrane spanning 4-domains A1 (MS4A1 also known as CD20); CD22 molecule (CD22); CD24 molecule (CD24); CD248 molecule (CD248); CD276 molecule (CD276 or B7H3); CD33 molecule (CD33); CD38 molecule (CD38); CD44v6; CD70 molecule (CD70); CD72; CD79a; CD79b; interleukin 3 receptor subunit alpha (IL3RA also known as CD123); TNF receptor superfamily member 8 (TNFRSF8 also known as CD30); KIT proto-oncogene receptor tyrosine kinase (CD117); V-set pre-B cell surrogate light chain 1 (VPREB1 or CD179a); adhesion G protein-coupled receptor E5 (ADGRE5 or CD97); TNF receptor superfamily member 17 (TNFRSF17 also known as BCMA); SLAM family member 7 (SLAMF7 also known as CS1); L1 cell adhesion molecule (L1CAM); C-type lectin domain family 12 member A (CLEC12A also known as CLL-1); tumor-specific variant of the epidermal growth factor receptor (EGFRvIII); thyroid stimulating hormone receptor (TSHR); Fms related tyrosine kinase 3 (FLT3); ganglioside GD3 (GD3); Tn antigen (Tn Ag); lymphocyte antigen 6 family member G6D (LY6G6D); Delta like canonical Notch ligand 3 (DLL3); Interleukin- 13 receptor subunit alpha-2 (IL-13RA2); Interleukin 11 receptor subunit alpha (IL11RA); mesothelin (MSLN); Receptor tyrosine kinase like orphan receptor 1 (ROR1); Prostate stem cell antigen (PSCA); erb-b2 receptor tyrosine kinase 2 (ERBB2 or Her2/neu); Protease Serine 21

(PRSS21); Kinase insert domain receptor (KDR also known as VEGFR2); Lewis y antigen (LewisY); Solute carrier family 39 member 6 (SLC39A6); Fibroblast activation protein alpha (FAP); Hsp70 family chaperone (HSP70); Platelet-derived growth factor receptor beta (PDGFR-beta); Cholinergic receptor nicotinic alpha 2 subunit (CHRNA2); Stage-Specific Embryonic Antigen-4 (SSEA-4); Mucin 1, cell surface associated (MUC1); mucin 16, cell surface associated (MUC16); claudin 18 (CLDN18); claudin 6 (CLDN6); Epidermal Growth Factor Receptor (EGFR); Preferentially expressed antigen in melanoma (PRAME); Neural Cell Adhesion Molecule (NCAM); ADAM metallopeptidase domain 10 (ADAM10); Folate receptor 1 (FOLR1); Folate receptor beta (FOLR2); Carbonic Anhydrase IX (CA9); Proteasome subunit beta 9 (PSMB9 or LMP2); Ephrin receptor A2 (EphA2); Tetraspanin 10 (TSPAN10); Fucosyl GM1 (Fuc-GM1); sialyl Lewis adhesion molecule (sLe); TGS5 ; high molecular weight- melanoma-associated antigen (HMWMAA); o-acetyl- GD2 ganglioside (OAcGD2); tumor endothelial marker 7-related (TEM7R); G protein-coupled receptor class C group 5, member D (GPRC5D); chromosome X open reading frame 61 (CXORF61); ALK receptor tyrosine kinase (ALK); Polysialic acid; Placenta-specific 1 (PLAC1); hexasaccharide portion of globoH glycoceramide (GloboH); NY-BR-1 antigen; uroplakin 2 (UPK2); Hepatitis A virus cellular receptor 1 (HAVCR1); adrenoceptor beta 3 (ADRB3); pannexin 3 (PANX3); G protein-coupled receptor 20 (GPR20); lymphocyte antigen 6 family member K (LY6K); olfactory receptor family 51 subfamily E member 2 (OR51E2); TCR Gamma Alternate Reading Frame Protein (TARP); Wilms tumor protein (WT1); ETV6-AML1 fusion protein due to 12;21 chromosomal translocation (ETV6-AML1); sperm autoantigenic protein 17 (SPA17); X Antigen Family, Member 1E (XAGE1E); TEK receptor tyrosine kinase (Tie2); melanoma cancer testis antigen- 1 (MAD-CT-1); melanoma cancer testis antigen-2 (MAD-CT-2); Fos-related antigen 1 ; p53 mutant; human Telomerase reverse transcriptase (hTERT); sarcoma translocation breakpoints; melanoma inhibitor of apoptosis (ML-IAP); ERG (transmembrane protease, serine 2 (TMPRSS2) ETS fusion gene); N- Acetyl glucosaminyl-transferase V (NA17); paired box protein Pax-3 (PAX3); Androgen receptor; Cyclin B 1 ; v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog (MYCN); Ras Homolog Family Member C (RhoC); Cytochrome P450 1B 1 (CYP1B 1); CCCTC-Binding Factor (Zinc Finger

Protein)-Like (BORIS); Squamous Cell Carcinoma Antigen Recognized By T Cells 3 (SART3); Paired box protein Pax-5 (PAX5); proacrosin binding protein sp32 (OY-TES 1); lymphocyte-specific protein tyrosine kinase (LCK); A kinase anchor protein 4 (AKAP-4); synovial sarcoma, X breakpoint 2 (SSX2); Leukocyte- associated immunoglobulin-like receptor 1 (LAIR1); Fc fragment of IgA receptor (FCAR); Leukocyte immunoglobulin-like receptor subfamily A member 2 (LILRA2); CD300 molecule-like family member f (CD300LF);; bone marrow stromal cell antigen 2 (BST2); EGF-like module-containing mucin-like hormone receptor- like 2 (EMR2); lymphocyte antigen 75 (LY75); Glypican-3 (GPC3); Fc receptor-like 5 (FCRL5); immunoglobulin lambda- like polypeptide 1 (IGLL1), and Heat shock protein 70 (HSP70).

Preferably said wherein said exogenous polynucleotide sequence comprises a CAR specific for CD22, CD123, CS-1, CLL-1, CD38, HSP70, MUC-1, CD30, o-acetyl-GD2 and a sequence SGEGRGSLLTCGDVEENPGP in frame with the genomic TRAC coding sequence, a terminator sequence of polyadenylation signal.

More Preferably said wherein said exogenous polynucleotide sequence comprises a CAR specific for CD123, and a sequence SGEGRGSLLTCGDVEENPGP in frame with the genomic TRAC coding sequence, a terminator sequence of polyadenylation signal.

More Preferably said wherein said exogenous polynucleotide sequence comprises a CAR specific for CD22, and a sequence SGEGRGSLLTCGDVEENPGP in frame with the genomic TRAC coding sequence, a terminator sequence of polyadenylation signal.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above embodiments wherein said (CAR) comprises an extracellular lig-and-binding domain comprising an epitope specific for a monoclonal antibody, a transmembrane domain and one or more intracellular signaling domains.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above embodiments, wherein said at least one insertion comprises an IRES, an exogenous polynucleotide sequence comprising a chimeric antigen receptor (CAR), a

terminator sequence of polyadenylation signal, optionally a TALEN binding domain.

The present invention provides the TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above embodiments, wherein said chimeric antigen receptor (CAR), comprise at least one antigen specific for a monoclonal antibody, preferably two antigens specific for a monoclonal antibody.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above embodiments wherein said exogenous polynucleotide comprises a transcription termination signal stopping the activity of RNA polymerase.

The present invention provides a TALEN-modified endogenous αβ–TCR negative human cell according to any one of the above embodiments comprising at least one additional disruption in an endogenous gene wherein said disrupted or inactivated endogenous gene is selected from the group consisting of an endogenous beta subunit gene of the TCR, an endogenous cytokine inducible SH2-containing (CISH) gene, an adenosine A2a receptor (ADORA) gene, a CD276 gene, a V-set domain containing T cell activation inhibitor 1 (VTCNl) gene, a B and T lymphocyte associated (BTLA) gene, a cytotoxic T-lymphocyte-associated protein 4 (CTLA4) gene, an indoleamine 2,3-dioxygenase 1 (IDO l) gene, a killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 1 (KIR3DL1) gene, a lymphocyte -activation gene 3 (LAG3) gene, a programmed cell death 1 (PD-1) gene, an hepatitis A virus cellular receptor 2 (HAVCR2) gene, a V-domain immunoglobulin suppressor of T-cell activation (VISTA) gene, a natural killer cell receptor 2B4 (CD244) gene , a hypoxanthine phosphoribosyltransferase 1 (HPRT) gene, an adeno-associated virus integration site (AAVS l), and chemokine (C-C motif) receptor 5 (gene/pseudogene) (CCR5) gene, a combination thereof.

The present invention provides a TALEN-modified endogenous αβ–TCR negative human cell according to any one of the above embodiments, for use to alter the survival of pathological cells responsible for a pathological condition, said pathological condition may be a cancer, a viral infection in any individual regardless of his MHC or TCR molecules.

The present invention provides a population of human cells comprising a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above embodiments.

The present invention provides a pharmaceutical composition comprising a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above embodiments or a population of human cells according to the above embodiments and a pharmaceutically acceptable excipient.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above embodiments or the population of human cells according to the above embodiments or the pharmaceutical composition according to the above embodiments for use as a medicament.

The present invention provides a TALEN-modified endogenous αβ–TCR negative human cell according to any one of the above embodiments or the population of human cells according to the above embodiments the above or the pharmaceutical composition according to the above, for use in the treatment of cancer.

The present invention provides the TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above embodiments or the population of human cells according to the above or the pharmaceutical composition according to the above for use in the treatment of a cancer selected from the group consisting of carcinoma, lymphoma, sarcoma, blastomas, and leukemia.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above embodiments or the population of human cells according to the above or the pharmaceutical composition according to the above for use in the treatment of cancer wherein the cancer is selected from the group consisting of a cancer of B-cell origin, a cancer of T cell origin, breast cancer, gastric cancer, neuroblastoma, osteosarcoma, lung cancer, melanoma, prostate cancer, colon cancer, renal cell carcinoma, ovarian cancer, rhabdomyo sarcoma, leukemia, and Hodgkin's lymphoma.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above or the population of human cells according to the above embodiments or the pharmaceutical composition according to the above embodiments for use in the treatment of cancer wherein the cancer of B-cell origin is selected from the group consisting of B-lineage acute lymphoblastic leukemia, B-cell chronic lymphocytic leukemia, and B-cell non-Hodgkin's lymphoma.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above embodiments or a population of human cells according to the above embodiments or a pharmaceutical composition according to the above embodiments for use in the treatment of a cancer wherein the cancer is AML, ALL, a T cell lymphoma, CLL.

The present invention provides a means for detecting an endonuclease-modified endogenous TCR negative human cell.

More particularly, the present invention provides a means for detecting an endonuclease-modified endogenous αβ–TCR negative human cell, wherein said endonuclease-modified endogenous αβ-TCR negative human cell comprises an endonuclease modified genomic TRAC gene as compared to the wild type TRAC gene.

In advantageous embodiments, a means according to the invention allows detecting a modified human genomic TRAC gene comprising from 5' to 3': a gap or an insertion as compared to the wild type TRAC gene, said insertion comprising an exogenous polynucleotide selected from a noncoding sequence, such as a stop codon, a termination sequence, an IRES, a sequence coding for a protein such as a self-cleaving peptide in frame with the TRAC open reading frame, a sequence coding a chimeric antigen receptor (CAR), a sequence coding a TCR, a sequence coding a protein conferring sensitivity to a drug, a sequence coding a protein conferring resistance to a drug, a combination thereof.

In one embodiment said gap or insertion is affecting the expression of the extracellular domain of the alpha beta TCR, or affecting the transmembrane domain of the alpha beta TCR, and ultimately affecting the cell surface expression of the alpha beta TCR.

The present invention provides a means for detecting an endonuclease-modified endogenous αβ–TCR negative human cell, said endonuclease-modified endogenous αβ-TCR negative human cell comprising an endonuclease modified genomic TRAC gene as compared to the wild type TRAC gene, wherein said modified human genomic TRAC gene comprises from 5' to 3':

(a) a 5' region of said human genomic TRAC gene upstream a recognition domain for a rare cutting endonuclease present in the wild type TRAC gene,

(b) a gap or an insertion as compared to the wild type TRAC gene affecting the cell surface expression of the extracellular domain or transmembrane domain of the alpha beta TCR,

said insertion comprising an exogenous polynucleotide selected from a noncoding sequence, a stop codon, a sequence coding for a self-cleaving peptide in frame with the TRAC open reading frame, an IRES, a sequence coding a chimeric antigen receptor (CAR), a sequence coding a TCR, a sequence coding a protein conferring sensitivity to a drug, a sequence coding a protein conferring resistance to a drug, a termination sequence, a combination thereof,

(c') optionally a second rare cutting endonuclease recognition domain,

(d) a 3' region of the genomic TRAC gene.

The present invention provides a means according to the above wherein said endonuclease is selected from the group consisting of: Crispr/Cas 9, CpfI, TALEN, transposase, ZEN, Zinc finger endonuclease, meganuclease, MegaTAL, a combination thereof, and said means binds to a sequence of the endonuclease-modified TRAC gene specific for said endonuclease and/or upstream a sequence specific for said endonuclease.

The present invention provides a means according to the above wherein said endonuclease is selected from the group consisting of: Crispr/Cas 9, TALEN, Zinc finger endonuclease, meganuclease,

MegaTAL, a combination thereof, and said means binds to a sequence of the endonuclease modified TRAC, which is specific for said nuclease or located upstream said sequence specific for said endonuclease.

In advantageous embodiments, the TALEN-modified endogenous αβ–TCR negative human cell according to the above comprises an inactivated genomic TCRA gene wherein an exogenous sequence coding a CAR was integrated into the genomic TCRA gene using a vector, such as for example a lentiviral vector or a AAV vector, and said genomic disruption(s) performed using an endonuclease selected from a CRISPR/CAS9, meganuclease, MegaTAL, Zn Finger, TALEN, combination thereof.

The present invention provides a means according to the above embodiments wherein said endonuclease is a TALEN.

The present invention provides a means according to the above embodiments for detecting a TALEN modified endogenous αβ-TCR negative human cell comprising a probe wherein said probe binds to a sequence in the modified genomic TRAC gene, preferably to a sequence in the modified genomic TRAC gene upstream the endonuclease binding domain, or to a sequence in the modified genomic TRAC gene at the endonuclease recognition domain, and/or to a sequence encoding a tag.

The present invention provides a means according to the above embodiments wherein said TALEN modified genomic TRAC gene comprises,

(a) a 5' region of said human genomic TRAC gene;

(b) a recognition domain for a TALEN, preferably a recognition domain for a TALEN comprising the following sequence ttgtcccacagATATC, or ttgtcccacagATATCCAG, or

(c) a gap or an insertion as compared to the wild type TRAC gene affecting the cell surface expression of the extracellular domain or transmembrane domain of the alpha beta TCR,

said insertion comprising an exogenous polynucleotide selected from a stop codon, a sequence coding for a self-cleaving peptide in frame with the TRAC open reading frame, an IRES, a sequence cod-

ing a chimeric antigen receptor (CAR), a sequence coding a TCR, a sequence coding a protein conferring sensitivity to a drug, a sequence coding a protein conferring resistance to a drug, a termination sequence, a combination thereof,

(c') optionally a second TALEN recognition domain.

(d) a 3' region of the genomic TRAC gene;

and said means binds specifically to said TALEN-modified genomic TRAC gene.

The present invention provides a means according to any one of the above embodiments for detecting a TALEN modified endogenous αβ-TCR negative human cell comprising a probe wherein said probe binds to at least 10 bases of the sequence ttgtcccacagATATC, or ttgtcccacagATATCCAG, in the modified genomic TRAC gene.

The present invention provides a means according to any one of the above embodiments for detecting an endonuclease-modified endogenous αβ-TCR negative human cell by polymerase chain reaction (pcr) or off sites modifications, preferably by guide sequence analysis.

The present invention provides a means according to any one of the above embodiments for detecting a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above embodiments.

The present invention provides a TALEN-modified endogenous αβ–TCR negative human cell according to any one of the above embodiments comprising an inactivated genomic TCRA gene wherein an exogenous coding sequence was integrated into the genomic TCRA gene using one or more endonucleases and/or a viral vector.

The present invention provides a TALEN-modified endogenous αβ–TCR negative human cell according to any one of the above embodiments comprising an inactivated genomic TCRA gene wherein an exogenous sequence coding a CAR was integrated into the genomic TCRA gene using a lentiviral vector or a AAV vector and said genomic disruptions are performed using a CRISPR/CAS9, meganuclease, MEGATAL or TALEN endonuclease system.

Under particular aspects, the present invention provides a method for treating a patient in need thereof, the method comprising administering a cell according to any one of the preceding embodiments.

Under particular aspects, the present invention provides a kit comprising at least one TALEN that binds to the genomic TRAC gene, and a TALEN that binds a gene coding one of the products selected from an endogenous cytokine inducible SH2 -containing (CISH), adenosine A2a receptor (ADORA), CD276, V-set domain containing T cell activation inhibitor 1 (VTCN1), B and T lymphocyte associated (BTLA), cytotoxic T-lymphocyte -associated protein 4 (CTLA4), indoleamine 2,3-dioxygenase 1 (IDOl), killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 1 (KIR3DL1), lymphocyte -activation gene 3 (LAG3), programmed cell death 1 (PD-1), hepatitis A virus cellular receptor 2 (HAVCR2), V-domain immunoglobulin suppressor of T-cell activation (VISTA), natural killer cell receptor 2B4 (CD244), hypoxanthine phosphoribosyltransferase 1 (HPRT), adeno-associated virus integration site (AAVS SITE (E.G. AAVS 1, AAVS2, ETC.)), or chemokine (C-C motif) receptor 5 (gene/pseudogene) (CCR5).

The present invention provides a method of producing an endonuclease-modified endogenous αβ-TCR negative human cell said method comprising:

(a) introducing into a human cell:

(i) a first nucleic acid sequence encoding an engineered nuclease; or an engineered nuclease protein; optionally, a nucleic acid guiding said endonuclease,

wherein said engineered nuclease produces a cleavage at a recognition sequence within said human TCR alpha constant region gene; said cleavage resulting in an inhibition of cell surface expression of the αβ-TCR to undetectable level as compared to adequate control (eg non engineered immune T cell).

(ii) a second nucleic acid sequence comprising an exogenous polynucleotide encoding a CAR,

(iii) optionally a probe,

wherein the sequence of said exogenous polynucleotide is inserted into said human TCR alpha constant region gene at said cleavage site; by homologous recombination (HR) and further wherein said genetically-modified cell has reduced cell-surface expression of the endogenous TCR when compared to an unmodified control cell.

In particular embodiments, the present invention provides a method of producing an endonuclease-modified endogenous αβ-TCR negative human cell wherein a probe is introduced into the human cell at the same time as the endonuclease so that said probe may be inserted into any dsDNA cutting sites, unless a sequence is inserted by HR. This is performed (in parallel to or during the manufacturing of T cells as an internal control for identifying any off site cutting of the endonuclease.

Above a threshold of integration of said probe into the genome, the endonuclease-modified endogenous αβ-TCR negative human cell may not be used for immunotherapy.

The present invention provides a method for producing an endonuclease-modified endogenous αβ-TCR negative human cell according to any one of the above embodiments said method comprises a step of detecting endonuclease-induced on site and off site(s), preferably by pcr and/or by guide sequence.

The present invention provides a method of any one of the above embodiments, wherein said engineered nuclease is a meganuclease, a zinc-finger nuclease (ZFN), a transcription activator-like effector nuclease (TALEN), a CRISPR/Cas nuclease, or a megaTAL nuclease.

The present invention provides a method for producing an endonuclease-modified endogenous αβ-TCR negative human cell according to any one of the above embodiments said method comprising the following steps:

Providing a cell according to the invention and a control cell(s)

Removing DNA and RNA using a mixture of nucleases,

Extracting Genomic DNA, (gDNA),

Incubating gDNA with a forward primer, a reverse primer and a probe.

Performing a PCR with an annealing/extension time of more than 45 seconds, preferably of more than 60 seconds, more preferably of more than 90 seconds.

A control cell(s) may be a non-engineered T cell.

The step of providing a cell according to the invention comprises a step of :

Providing a primary cell,

Introducing a mRNA encoding said endonuclease and /or a guide conferring specificity to a cutting site in the genomic DNA (that may be an endonuclease as such and/or a polynucleotide), said cutting site resulting in a KO of the TRAC gene and undetectable level of cell surface expression of alpha beta TCR,

Introducing a polynucleotide comprising at least a self cleaving peptide or an IRES, a gene encoding a CAR, sequence homology with the gene to be KO, resulting in a KO by KI of the TRAC gene and the production of a TCR negative CAR positive cells

Purifying said resulting TCR negative CAR positive cells,

Providing said resulting TCR negative CAR positive cells according to the invention and a control cell(s)

Removing DNA and RNA using a mixture of nucleases,

Extracting Genomic DNA, (gDNA),

Incubating gDNA with a forward primer, a reverse primer and a probe.

Performing a PCR with an annealing/extension time of more than 45 seconds, preferably of more than 60 seconds, more preferably of more than 90 seconds.

detecting endonuclease- specific on and off site cutting.

In a preferred embodiment,

introducing a mRNA encoding said endonuclease conferring specificity to a cutting site in the genomic DNA (that may be an endonuclease as such and/or a polynucleotide), said cutting site resulting in a KO of the TRAC gene and undetectable level of cell surface expression of alpha beta TCR, is introducing a mRNA encoding a TALEN conferring specificity to a cutting site in the genomic DNA (that may be an endonuclease as such and/or a polynucleotide), said cutting site resulting in a KO of the TRAC gene and undetectable level of cell surface expression of alpha beta TCR.

The same method applies to any genes that is intended to be KO and in which an exogenous coding sequence is introduced. In that embodiment, the step of

introducing a mRNA encoding said endonuclease and /or a guide conferring specificity to a cutting site in the genomic DNA (that may be an endonuclease as such and/or a polynucleotide), said cutting site resulting in a KO of the TRAC gene and undetectable level of cell surface expression of alpha beta TCR, is generalized to introducing a mRNA encoding said endonuclease and /or a guide conferring specificity to a cutting site in the genomic DNA (that may be an endonuclease as such and/or a polynucleotide), said cutting site resulting in a KO of the said gene and undetectable level expression of the product encoded by said gene.

The present invention also provides a method for detecting an endonuclease-modified endogenous αβ-TCR negative human cell according to any one of the above embodiments said method comprises:

-Providing a cell according to the invention, a control cell(s)

-Removing DNA and RNA using benzonase,

-Extracting Genomic DNA, (gDNA),

-Incubating gDNA with a forward primer, a reverse primer and a probe.

-Performing a PCR with an annealing/extension time increased by 2.

The present invention provides a method for detecting an endonuclease-modified endogenous αβ-TCR negative human cell according to any one of the above embodiments said method comprising:

-Providing a cell according to the invention, a control cell(s)

-Removing DNA and RNA using a mixture of nucleases, such as benzonase,

-Extracting Genomic DNA, (gDNA),

-Incubating from 20 to 30 ng of gDNA, with 300 nM of forward primer, 900 nM of reverse primer and 220 nM of probe.

-Performing a PCR with an annealing/extension time increased by 2, preferably of more than 45 sec.

The present invention provides a method for detecting an endonuclease-modified endogenous αβ-TCR negative human cell according to any one of the above embodiments said method comprises

-Providing a cell according to the invention, a control cell(s)

-Removing DNA and RNA using benzonase,

-Extracting Genomic DNA, (gDNA),

-Incubating gDNA with a forward primer of SEQ ID N° 5, a reverse primer of SEQ ID N°6 and a probe of SEQ ID N°7.

-Performing a PCR with an annealing/extension time increased by 2.

The present invention provides a method as disclosed in the above embodiments, wherein said endonuclease is a TALEN.

The present invention provides a method as disclosed in the above embodiments, wherein said exogenous polynucleotide comprises a nucleic acid sequence encoding a self-cleaving peptide and a chimeric antigen receptor.

The present invention provides a method as disclosed in the above embodiments wherein said chimeric antigen receptor comprises an extracellular ligand-binding domain and one or more intracellular signaling domains.

The present invention provides a method as disclosed in the above embodiments wherein said chimeric antigen receptor comprises an extracellular ligand-binding domain and one or more intracellular signaling domains and a costimulatory domain.

The present invention provides a method as disclosed in the above embodiments, wherein said exogenous polynucleotide comprises a first promoter sequence that drives expression of said exogenous polynucleotide.

The present invention provides the method as disclosed in the above embodiments, wherein at least said second nucleic acid sequence is introduced into said cell by contacting said cell with a recombinant adeno-associated virus (AAV) vector comprising said second nucleic acid sequence.

The present invention provides the method of any one of the above embodiments, wherein said recombinant AAV vector is a self-complementary AAV vector.

A method of any one of the above is provided, wherein said recombinant AAV vector derived at least in part from an AAV6.

The method of any one of the above, wherein said recombinant AAV vector derived from an AAV6/AAV2 particles.

The method of any one of the above, wherein said recombinant AAV vector comprises AAV6 particles and a DNA sequence between inverted terminal repeats (ITRs) from AAV2.

The method of any one of the above, wherein said recombinant TALEN comprises a first subunit and a second subunit, wherein said first subunit binds to a first recognition half-site, and wherein said second subunit binds to a second recognition half-site.

The method as any one of the above is provided, wherein said recombinant TALEN recognizes a sequence ttgtcccacagATATCCAG in the wild- type human TCR alpha constant region.

The present invention further provides a method of as the general method above, wherein said meganuclease recognizes and cleaves a recognition sequence within residues 93-208 of the wild-type human TCR alpha constant region, wherein said recombinant meganuclease comprises a first subunit and a second subunit, wherein said first subunit binds to a first recognition half-site of said recognition sequence and comprises a first hypervariable (HVR1) region, and wherein said second subunit binds to a second recognition half-site of said recognition sequence and comprises a second hypervariable (HVR2) region.

The present invention provides a method as any one of the above, wherein said meganuclease is a single-chain meganuclease comprising a linker, wherein said linker covalently joins said first subunit and said second subunit.

In general, the present invention provides a means of detection of cells obtained according to any of one the methods above.

Others embodiments

The present invention is useful to improve the therapeutic outcome of CAR T-cell (TCRneg, CAR+) therapies by integrating exogenous genetic attributes/circuits under the control of endogenous T-cell promoters influenced by tumor microenvironment (TME). TME features, including as non-limiting examples, arginine, cysteine, tryptophan and oxygen deprivation as well as extracellular acidosis (lactate build up), are known to upregulate specific endogenous genes. Pursuant to the invention, upregulation of endogenous genes can be “hijacked” to re-express relevant exogenous coding sequences to improve the antitumor activity of CAR T-cells in certain tumor microenvironment.

In preferred embodiments, the method of the invention comprises the step of generating a double-strand break at a locus highly transcribed under tumor microenvironment, by expressing sequence-specific nuclease reagents, such as TALEN, ZFN or RNA-guided endonucleases as non-limiting

examples, in the presence of a DNA repair matrix preferably set into an AAV6 based vector. This DNA donor template generally includes two homology arms embedding unique or multiple Open Reading Frames and regulatory genetic elements (stop codon and polyA sequences) referred to herein as exogenous coding sequences.

In another aspect, said exogenous sequence is introduced into the genome by deleting or modifying the endogenous coding sequence(s) present at said locus (knock-out by knock-in), so that a gene inactivation is combined with transgenesis.

Depending on the locus targeted and its involvement in immune cells activity, the targeted endogenous gene may be inactivated or maintained in its original function. Should the targeted gene be essential for immune cells activity, this insertion procedure can generate a single knock-in (KI) without gene inactivation. In the opposite, if the targeted gene is deemed involved in immune cells inhibition/exhaustion, the insertion procedure is designed to prevent expression of the endogenous gene, preferably by knocking-out the endogenous sequence, while enabling expression of the introduced exogenous coding sequence(s).

In more specific aspects, the invention relies on up-regulating, with various kinetics, the target gene expression upon activation of the CAR signaling pathway by targeted integration (with or without the native gene disruption) at the specific loci such as, as non-limiting example, PD1, PDL1, CTLA-4, TIM3, LAG3, TNFalpha or IFNgamma.

In an even more specific aspect, it is herein described engineered immune cells, and preferably primary immune cells for infusion into patients, comprising exogenous sequences encoding IL-15 or IL-12 polypeptide(s), which are integrated at the PD1, CD25 or CD69 endogenous locus for their expression under the control of the endogenous promoters present at these loci.

In an even more specific aspect, it is herein described engineered immune cells, and preferably primary immune cells for infusion into patients, comprising exogenous sequences encoding

IL2
IL12
IL15
IL15_IL15R
Tbet
CTLA4 AB soluble
PD1 AB soluble
CD40L (CD154)
NGR-TNF

IL-7, an antibody, preferably a neutralizing antibody.

The immune cells according to the present invention can be [CAR]positive,[CAR]negative,[TCR]positive,or [TCR]negative, depending on the therapeutic indications and recipient patients. In one preferred aspect, the immune cells are further made [TCR]negative for allogeneic transplantation. This can be achieved especially by genetic disruption of at least one endogenous sequence encoding at least one component of TCR, such as TRAC (locus encoding TCRalpha), preferably by integration of an exogenous sequence encoding a chimeric antigen receptor (CAR) or a recombinant TCR, or component(s) thereof.

According to a further aspect of the invention, the immune cells are transfected with an exogenous sequence coding for a polypeptide which can associate and preferably interfere with a cytokine receptor of the IL-6 receptor family, such as a mutated GP130, In particular, the invention provides immune cells, preferably T-cells, which secrete soluble mutated GP130, aiming at reducing

cytokine release syndrome (CRS) by interfering, and ideally block, interleukine-6 (IL-6) signal transduction. CRS is a well-known complication of cell immunotherapy leading to auto immunity that appears when the transduced immune cells start to be active in-vivo. Following binding of IL-6 to its receptor IL-6R, the complex associate with the GP130 subunit, initiating signal transduction and a cascade of inflammatory responses. According to a particular aspect, a dimeric protein comprising the extracellular domain of GP130 fused to the Fc portion of a IgG1 antibody (sgp130Fc) is expressed in the engineered immune cells to bind specifically soluble IL-R/IL-6 complex to achieve partial or complete blockade of IL-6 trans signaling. The present invention thus refers to a method for limiting CRS in immunotherapy, wherein immune cells are genetically modified to express a soluble polypeptide which can associate and preferably interfere with a cytokine receptor of the IL-6 receptor family, such as sgp130Fc. According to a preferred aspect, this sequence encoding said soluble polypeptide which can associate and preferably interfere with a cytokine receptor of the IL-6 receptor family, is integrated under control of an endogenous promoter, preferably at one locus responsive to T-cells activation, such as one selected from Tables below, more especially PD1, CD25 or CD69. Polynucleotide sequences of the vectors, donor templates comprising the exogenous coding sequences and/or sequences homologous to the endogenous loci, the sequences pertaining to the resulting engineered cells, as well as those permitting the detection of said engineered cells are all part of the present disclosure.

The present invention provides an endonuclease-modified endogenous αβ-TCR negative human cell wherein the constant region of the genomic TCR gene (TRAC gene) comprises a genetic modification generated by a rare a cutting endonuclease and affecting cell surface expression of the alpha beta TCR, said genomic TRAC gene further comprising from 5' to 3':

(a) a 5' region of said human genomic TRAC gene upstream

(b) a recognition domain for a rare cutting endonuclease,

(c) a gap or an insertion as compared to the wild type TRAC gene affecting the cell surface expression of the extracellular domain or transmembrane domain of the alpha beta TCR,

said insertion comprising an exogenous polynucleotide selected from a noncoding sequence such as, a stop codon, an IRES, a sequence coding such as a sequence coding for a self-cleaving peptide in frame with the TRAC open reading frame, a sequence coding a chimeric antigen receptor (CAR), a sequence coding a TCR, a sequence coding a protein conferring sensitivity to a drug, a sequence coding a protein conferring resistance to a drug, a cytokine, a termination sequence, a combination thereof,

(c') optionally a second rare cutting endonuclease recognition domain,

(d) a 3' region of the genomic TRAC gene.

TCR means T cell receptor. TRAC means T cell receptor alpha constant region.

According to the present invention, the endonuclease-modified endogenous αβ-TCR negative human cell (hereafter "a cell" or "a human cell") may be "a population of human cells", preferably, a primary cell or a population of human primary cells, or more preferably a primary endonuclease-modified endogenous αβ-TCR negative human cell or a population of primary endonuclease-modified endogenous αβ-TCR negative human cells.

In particular embodiments, a cell of the invention is a primary, endonuclease-modified, endogenous αβ-TCR negative, human T cell or a population of primary endonuclease-modified endogenous αβ-TCR negative human T cells.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell wherein the constant region of the genomic TCR gene (TRAC gene) comprises a genetic modification generated by a TALEN and affecting cell surface expression of the alpha beta TCR, said genomic TRAC gene comprising from 5' to 3':

(a) a 5' region of said human genomic TRAC gene upstream

(b) a recognition domain for a TALEN,

(c) a gap or an insertion as compared to the wild type TRAC gene affecting the cell surface expression of the extracellular domain or transmembrane domain of the alpha beta TCR,

said insertion comprising an exogenous polynucleotide selected from a noncoding sequence such as, a stop codon, an IRES, a sequence coding such as a sequence coding for a self-cleaving peptide in frame with the TRAC open reading frame, a sequence coding a chimeric antigen receptor (CAR), a sequence coding a TCR, a sequence coding a protein conferring sensitivity to a drug, a sequence coding a protein conferring resistance to a drug, a cytokine, a termination sequence, a combination thereof,

(c') optionally a second TALEN recognition domain,

(d) a 3' region of the genomic TRAC gene.

In particular embodiments, the TALEN-modified endogenous αβ–TCR negative primary human cell obtained comprises undetectable level of off-site cut as determined using an adapted guide seq technique and a TALEN binding to the following sequence: TTGTCCCACAGA-TATCCagaaccctgaccctgCCGTGTACCAGCTGAGAGA.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell wherein the constant region of the genomic TCR gene (TRAC gene) comprises a genetic modification generated by a TALEN and affecting cell surface expression of the alpha beta TCR, said genomic TRAC gene comprising from 5' to 3':

(a) a 5' region of said human genomic TRAC gene upstream

(b) a recognition domain for a TALEN comprising the following sequence ttgtcccacagATATC, or ttgtcccacagATATCCAG

(c) a gap or an insertion as compared to the wild type TRAC gene affecting the cell surface expression of the extracellular domain or transmembrane domain of the alpha beta TCR,

said insertion comprising an exogenous polynucleotide selected from a noncoding sequence such as, a stop codon, an IRES, a sequence coding such as a sequence coding for a self-cleaving peptide in frame with the TRAC open reading frame, a sequence coding a chimeric antigen receptor (CAR), a sequence coding a TCR, a sequence coding a protein conferring sensitivity to a drug, a sequence coding a protein conferring resistance to a drug, a cytokine, a termination sequence, a combination thereof,

(c') optionally a second TALEN recognition domain, comprising the following sequence CCGTGTACCAGCTGAGA

(d) a 3' region of the genomic TRAC gene.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell wherein the constant region of the genomic TCR gene (TRAC gene) comprises an insertion generated by a TALEN and affecting cell surface expression of the alpha beta TCR, said genomic TRAC gene comprising from 5' to 3':

(a) a 5' region of said human genomic TRAC gene upstream

(b) a recognition domain for a TALEN comprising the following sequence ttgtcccacagATATC, or ttgtcccacagATATCCAG

(c) an insertion as compared to the wild type TRAC gene affecting the cell surface expression of the extracellular domain or transmembrane domain of the alpha beta TCR,

said insertion comprising an exogenous polynucleotide selected from a noncoding sequence such as, a stop codon, an IRES, a sequence coding such as a sequence coding for a self-cleaving peptide in frame with the TRAC open reading frame, a sequence coding a chimeric antigen receptor (CAR), a sequence coding a TCR, a sequence coding a protein conferring sensitivity to a drug, a sequence coding a protein conferring resistance to a drug, a cytokine, a termination sequence, a combination thereof,

(c') optionally a second TALEN recognition domain, comprising the following sequence CCGTGTAC-CAGCTGAGA

(d) a 3' region of the genomic TRAC gene.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell wherein the constant region of the genomic TCR gene (TRAC gene) comprises an insertion generated by a TALEN and affecting cell surface expression of the alpha beta TCR, said genomic TRAC gene comprising from 5' to 3':

(a) a 5' region of said human genomic TRAC gene upstream

(b) a recognition domain for a TALEN comprising the following sequence ttgtcccacagATATC, or ttgtcccacagATATCCAG

(c) an insertion as compared to the wild type TRAC gene affecting the cell surface expression of the extracellular domain or transmembrane domain of the alpha beta TCR,

said insertion comprising an exogenous polynucleotide comprising an IRES, a coding sequence coding a chimeric antigen receptor (CAR), a termination sequence,

(c') optionally a second TALEN recognition domain, comprising the following sequence CCGTGTAC-CAGCTGAGA

(d) a 3' region of the genomic TRAC gene.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell wherein the constant region of the genomic TCR gene (TRAC gene) comprises an insertion generated by a TALEN and affecting cell surface expression of the alpha beta TCR, said genomic TRAC gene comprising from 5' to 3':

(a) a 5' region of said human genomic TRAC gene upstream

(b) a recognition domain for a TALEN comprising the following sequence ttgtcccacagATATC, or ttgtcccacagATATCCAG

(c) an insertion as compared to the wild type TRAC gene affecting the cell surface expression of the extracellular domain or transmembrane domain of the alpha beta TCR,

said insertion comprising a self-cleaving peptide in frame with the TRAC open reading frame, a sequence coding a chimeric antigen receptor (CAR), a termination sequence,

(c') optionally a second TALEN recognition domain, comprising the following sequence CCGTGTACCAGCTGAGA

(d) a 3' region of the genomic TRAC gene.

More particularly, the present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell comprising in the genomic TRAC gene one of the following sequences:

AAGTAGCCCTGCATTTCAGGTTTCCTTGAGTGGCAGGCCAGGCCTGGCCGTGAACGTTCACTGAAATCATGGCC
TCTTGGCCAAGATTGATAGCTTGTGCCTGTCCCTGAGTCCCAGTCCATCACGAGCAGCTGGTTTCTAAGATGCTAT
TTCCCGTATAAAGCATGAGACCGTGACTTGCCAGCCCCACAGAGCCCCGCCCTTGTCCATCACTGGCATCTGGAC
TCCAGCCTGGGTTGGGGCAAAGAGGGAAATGAGATCATGTCCTAACCCTGATCCTCTTGTCCCACAGATATCCAG
TCCGGTGAGGGCAGAGGAAGTCTTCTAACATGCGGTGACGTGGAGGAGAATCCGGGCCCCGGATCCcodingse
quenceTCTAGAGGGCCCGTTTAAACCCGCTGATCAGCCTCGA*CTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTT*
GCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCA
TCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGG
*AAGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATG*ACTAGTGGCGAATTCCCGTGTACCAGCTGAGAG
ACTCTAAATCCAGTGACAAGTCTGTCTGCCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGA
TTCTGATGTGTATATCACAGACAAAACTGTGCTAGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGC
CTGGAGCAACAAATCTGACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGACACCTTCTTCCCC
AGCCCAGGTAAGGGCAGCTTTGGTGCCTTCGCAGGCTGTTTCCTTGCTTCAGGAA,

wherein underlined sequences are sequences of endogenous genomic TRAC gene that may be used for homologous recombination, bold italic sequence corresponds to a sequence encoding a self-cleaving peptide, Italic sequence corresponds to termination signal such as poly A sequences, cod-

ingsequence corresponds to at least one open reading frame encoding a protein, said protein may be for example a chimeric antigen receptor (CAR);

AAGTAGCCCTGCATTTCAGGTTTCCTTGAGTGGCAGGCCAGGCCTGGCCGTGAACGTTCACTGAAATCATGGCC
TCTTGGCCAAGATTGATAGCTTGTGCCTGTCCCTGAGTCCCAGTCCATCACGAGCAGCTGGTTTCTAAGATGCTAT
TTCCCGTATAAAGCATGAGACCGTGACTTGCCAGCCCCACAGAGCCCCGCCCTTGTCCATCACTGGCATCTGGAC
TCCAGCCTGGGTTGGGGCAAAGAGGGAAATGAGATCATGTCCTAACCCTGATCCTCTTGTCCCACAGATATCCAG
TCCGGTGAGGGCAGAGGAAGTCTTCTAACATGCGGTGACGTGGAGGAGAATCCGGGCCCCGGATCCGCTCTG
CCCGTCACCGCTCTGCTGCTGCCACTGGCCCTGCTGCTGCACGCAGCAAGACCAGGAGGGGGAGGCAGCTGCC
CCTACAGCAACCCCAGCCTGTGCAGCGGAGGCGGCGGCAGCGGCGGAGGGGGTAGCCAGGTGCAGCTGCAG
CAGAGCGGCCCTGGCCTGGTGAAGCCAAGCCAGACACTGTCCCTGACCTGCGCCATCAGCGGCGATTCCGTGA
GCTCCAACTCCGCCGCCTGGAATTGGATCAGGCAGTCCCCTTCTCGGGGCCTGGAGTGGCTGGGAAGGACATAC
TATCGGTCTAAGTGGTACAACGATTATGCCGTGTCTGTGAAGAGCAGAATCACAATCAACCCTGACACCTCCAAG
AATCAGTTCTCTCTGCAGCTGAATAGCGTGACACCAGAGGACACCGCCGTGTACTATTGCGCCAGGGAGGTGAC
CGGCGACCTGGAGGATGCCTTTGACATCTGGGGCCAGGGCACAATGGTGACCGTGTCTAGCGGAGGAGGAGG
ATCCGGAGGAGGAGGATCTGGCGGCGGCGGCAGCGATATCCAGATGACACAGTCCCCATCCTCTCTGAGCGCCT
CCGTGGGCGACAGAGTGACAATCACCTGTAGGGCCTCCCAGACCATCTGGTCTTACCTGAACTGGTATCAGCAG
AGGCCCGGCAAGGCCCCTAATCTGCTGATCTACGCAGCAAGCTCCCTGCAGAGCGGAGTGCCATCCAGATTCTCT
GGCAGGGGCTCCGGCACAGACTTCACCCTGACCATCTCTAGCCTGCAGGCCGAGGACTTCGCCACCTACTATTGC
CAGCAGTCTTATAGCATCCCCCAGACATTTGGCCAGGGCACCAAGCTGGAGATCAAGGGAAGCGGAGGGGGAG
GCAGCTGCCCCTACAGCAACCCCAGCCTGTGCAGCGGAGGCGGCGGCAGCGAGCTGCCCACCCAGGGCACCTT
CTCCAACGTGTCCACCAACGTGAGCCCAGCCAAGCCCACCACCACCGCCTGTCCTTATTCCAATCCTTCCCTGTGT
GCTCCCACCACAACCCCAGCACCAAGGCCACCTACACCTGCACCAACCATCGCCTCTCAGCCCCTGAGCCTGAGA
CCTGAGGCATGTAGGCCAGCAGCAGGAGGAGCAGTCCATACAAGGGGTCTGGATTTTGCATGCGACATCTACAT
CTGGGCACCTCTGGCAGGAACATGTGGCGTGCTCCTGCTCAGCCTGGTCATCACCCTGTACTGCAAGAGAGGCA
GGAAGAAGCTGCTGTATATCTTCAAGCAGCCCTTCATGCGCCCCGTGCAGACAACCCAGGAGGAGGATGGCTGC
TCCTGTAGGTTCCCAGAAGAGGAGGAGGGAGGATGTGAGCTGCGCGTGAAGTTTTCCCGGTCTGCCGACGCAC

CTGCATACCAGCAGGGCCAGAACCAGCTGTATAACGAGCTGAATCTGGGCCGGAGAGAGGAGTACGATGTGCT
GGACAAGAGGCGCGGCAGAGATCCAGAGATGGGCGGCAAGCCCCGGAGAAAGAACCCTCAGGAGGGCCTGT
ACAATGAGCTGCAGAAGGATAAGATGGCCGAGGCCTATTCTGAGATCGGCATGAAGGGAGAGAGGCGCCGGG
GCAAGGGACACGACGGACTGTACCAGGGACTGAGCACAGCCACCAAGGATACCTATGACGCCCTGCATATGCAG
GCACTGCCTCCAAGGTGATCTAGAGGGCCCGTTTAAACCCGCTGATCAGCCTCGA*CTGTGCCTTCTAGTTGCCAG*
CCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAAT
GAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGG
*GGGAGGATTGGGAAGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATG*ACTAGTGGCGAATTCCCGTGT
ACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTGCCTATTCACCGATTTTGATTCTCAAACAAATGTGTC
ACAAAGTAAGGATTCTGATGTGTATATCACAGACAAAACTGTGCTAGACATGAGGTCTATGGACTTCAAGAGCAA
CAGTGCTGTGGCCTGGAGCAACAAATCTGACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGA
CACCTTCTTCCCCAGCCCAGGTAAGGGCAGCTTTGGTGCCTTCGCAGGCTGTTTCCTTGCTTCAGGAA, (or SEQ
ID N° 9),

(CD22 B-B7 QR3)

AAGTAGCCCTGCATTTCAGGTTTCCTTGAGTGGCAGGCCAGGCCTGGCCGTGAACGTTCACTGAAATCATGGCC
TCTTGGCCAAGATTGATAGCTTGTGCCTGTCCCTGAGTCCCAGTCCATCACGAGCAGCTGGTTTCTAAGATGCTAT
TTCCCGTATAAAGCATGAGACCGTGACTTGCCAGCCCCACAGAGCCCCGCCCTTGTCCATCACTGGCATCTGGAC
TCCAGCCTGGGTTGGGGCAAAGAGGGAAATGAGATCATGTCCTAACCCTGATCCTCTTGTCCCACAGATATCCAG
TCCGGTGAGGGCAGAGGAAGTCTTCTAACATGCGGTGACGTGGAGGAGAATCCGGGCCCCGGATCCGCTCTG
CCCGTCACCGCTCTGCTGCTGCCACTGGCCCTGCTGCTGCACGCCGCCAGACCCGGCGGAGGAGGCTCTTGCCC
CTACAGCAACCCCAGCCTGTGCTCTGGCGGCGGCGGCAGCGGAGGCGGCGGCTCCCAGGTGCAGCTGCAGCA
GAGCGGCCCTGGCCTGGTGGAGCCAAGCCAGACACTGTCCCTGACCTGCGCCATCTCTGGCGACAGCGTGAGC
TCCAACAGCGCCGCATGGAATTGGATCAGGCAGTCCCCATCTCGGGGCCTGGAGTGGCTGGGCAGAACATACTA
TAGGTCCACCTGGTACAACGACTATGCCGGCTCCGTGAAGTCTCGCATCACAATCAACCCCGATACCAGCAAGAA
TCAGTTCTCCCTGCAGCTGACATCTGTGACCCCTGAGGACACAGCCGTGTACTATTGCACCAGAAGCAGGCACAA
TACATTTCGGGGAATGGACGTGTGGGGACAGGGCACACTGGTGACCGTGAGCGGAGGAGGAGGATCCGGCGG
AGGAGGCTCTGGCGGCGGCGGCAGCGACATCCAGCTGACCCAGTCCCCTTCTAGCCTGAGCGCCTCCGTGGGC
GATAGAGTGACAATCACCTGTAGGGCCTCTCAGAGCATCTCCTCTTACCTGAACTGGTATCAGCAGAAGCCCGGC
AAGGCCCCTAAGCTGCTGATCTACGCAGCAAGCTCCCTGCAGTCTGGAGTGCCAAGCAGATTCTCCGGCTCTGG
CAGCGGCACCGACTTTACACTGACCATCTCTAGCCTGCAGCCTGAGGATTTCGCCACATACTATTGCCAGCAGTCC
TATTCTACACCACTGACCTTTGGCGGCGGCACCAAGGTGGAGATCAAGGGAAGCGGCGGCGGCGGAAGTTGTC
CATATTCAAACCCAAGTCTGTGCAGCGGCGGAGGAGGAAGCGAACTGCCTACTCAGGGAACCTTCAGCAACGT
GTCCACCAATGTGAGCCCAGCAAAGCCTACCACAACCGCATGCCCATACTCTAACCCCAGCCTGTGCACAACCAC
ACCAGCACCCAGGCCCCCTACCCCTGCACCAACAATCGCCTCCCAGCCTCTGTCTCTGCGGCCAGAGGCCTGCAG
ACCCGCCGCCGGCGGAGCAGTGCACACACGGGGCCTGGACTTTGCCTGTGATATCTATATCTGGGCACCACTGG
CCGGAACATGTGGCGTGCTGCTGCTGTCACTGGTCATTACACTGTACTGTAAGCGAGGCCGGAAGAAACTGCTGT
ATATTTTCAAACAGCCCTTTATGAGACCTGTGCAGACTACCCAGGAGGAAGACGGCTGCAGCTGTAGGTTCCCCG
AGGAAGAGGAAGGCGGGTGTGAGCTGAGGGTCAAGTTTAGCCGCTCCGCAGATGCCCCTGCTTACCAGCAGG
GGCAGAATCAGCTGTATAACGAGCTGAATCTGGGACGGAGAGAGGAATACGACGTGCTGGATAAAAGGCGCGG

GAGAGACCCCGAAATGGGAGGCAAGCCACGACGGAAAAACCCCCAGGAGGGCCTGTACAATGAACTGCAGAA
GGACAAAATGGCAGAGGCCTATAGTGAAATCGGGATGAAGGGAGAGAGAAGGCGCGGCAAAGGGCACGATG
GCCTGTACCAGGGGCTGTCTACTGCCACCAAGGACACCTATGATGCTCTGCATATGCAGGCACTGCCTCCAAGGT
GATCTAGAGGGCCCGTTTAAACCCGCTGATCAGCCTCGA*CTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCC*
CTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGC
ATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGA
*CAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATG*ACTAGTGGCGAATTCCCGTGTACCAGCTGAGAGACTCT
AAATCCAGTGACAAGTCTGTCTGCCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTG
ATGTGTATATCACAGACAAAACTGTGCTAGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGA
GCAACAAATCTGACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGACACCTTCTTCCCCAGCCC
AGGTAAGGGCAGCTTTGGTGCCTTCGCAGGCTGTTTCCTTGCTTCAGGAA, (SEQ ID N° 10),

(CD22 A-D4 QR3)

AAGTAGCCCTGCATTTCAGGTTTCCTTGAGTGGCAGGCCAGGCCTGGCCGTGAACGTTCACTGAAATCATGGCC
TCTTGGCCAAGATTGATAGCTTGTGCCTGTCCCTGAGTCCCAGTCCATCACGAGCAGCTGGTTTCTAAGATGCTAT
TTCCCGTATAAAGCATGAGACCGTGACTTGCCAGCCCCACAGAGCCCCGCCCTTGTCCATCACTGGCATCTGGAC
TCCAGCCTGGGTTGGGGCAAAGAGGGAAATGAGATCATGTCCTAACCCTGATCCTCTTGTCCCACAGATATCCAG
TCCGGTGAGGGCAGAGGAAGTCTTCTAACATGCGGTGACGTGGAGGAGAATCCGGGCCCCGGATCCGCTCTG
CCCGTCACCGCTCTGCTGCTGCCACTGGCCCTGCTGCTGCACGCCGCCAGACCCGGCGGAGGAGGCTCTTGCCC
CTACAGCAACCCCAGCCTGTGCTCTGGCGGCGGCGGCAGCGGAGGCGGCGGCTCCCAGGTGCAGCTGCAGCA
GAGCGGCCCCGGCCTGGTGAAGCCTAGCCAGACACTGTCCCTGACCTGCGCAATCTCCGGCGACAGCGTGTCCG
GAAACAGGGCCACATGGAATTGGATCAGACAGTCTCCAAGCAGGGGCCTGGAGTGGCTGGGAAGGACCTACTA
TCGGTCCGCCTGGTACAACGACTATGCCGTGTCTGTGAAGGGCCGCATCACATTCAACCCAGATACCAGCAAGAA
TCAGTTTTCCCTGCAGCTGAATTCTGTGACACCCGAGGATACCGCCGTGTACTATTGCGCCAGAGGCGAGAGCG
GAGCAGCAGCAGACGCCTTCGATATCTGGGGCCAGGGCACCACAGTGACAGTGAGCGGAGGAGGAGGATCCG
GCGGAGGAGGCTCTGGCGGCGGCGGCAGCGACATCCAGCTGACCCAGAGCCCACCTTCCCTGTCTGCCAGCGT

GGGCGATCGCGTGACAATCACCTGTCGGGCCTCCCAGTCTATCAGCTCCTACCTGAACTGGTATCAGCAGAAGCC
AGGCAAGGCCCCCAAGCTGCTGATCTACGCAGCATCTAGCCTGCAGTCTGGAGTGCCAAGCAGATTCAGCGGAT
CCGGATTCGGCACAGACTTTACACTGACCATCTCCTCTCTGCAGCCCGAGGATTTCGCCACCTACTATTGCCAGCA
GTCTTATAGCACACCTCAGACCTTTGGCCAGGGCACCAAGGTGGACATCAAGGGAAGTGGAGGAGGAGGAAG
TTGTCCCTACTCAAACCCATCTCTGTGCTCAGGAGGAGGAGGAAGTGAACTGCCTACTCAGGGAACATTCAGCA
ACGTGTCCACCAATGTGAGCCCAGCAAAGCCTACCACAACCGCATGCCCATACTCTAACCCCAGCCTGTGCACAA
CCACACCAGCACCCAGGCCCCCTACCCCTGCACCAACAATCGCCTCCCAGCCTCTGTCTCTGCGGCCAGAGGCCT
GCAGACCCGCCGCCGGCGGAGCAGTGCACACACGGGGCCTGGACTTTGCCTGTGATATCTATATCTGGGCACCA
CTGGCCGGAACATGTGGCGTGCTGCTGCTGTCACTGGTCATTACACTGTACTGTAAGCGAGGCCGGAAGAAACT
GCTGTATATTTTCAAACAGCCCTTTATGAGACCTGTGCAGACTACCCAGGAGGAAGACGGCTGCAGCTGTAGGTT
CCCCGAGGAAGAGGAAGGCGGGTGTGAGCTGAGGGTCAAGTTTAGCCGCTCCGCAGATGCCCCTGCTTACCAG
CAGGGGCAGAATCAGCTGTATAACGAGCTGAATCTGGGACGGAGAGAGGAATACGACGTGCTGGATAAAAGGC
GCGGGAGAGACCCCGAAATGGGAGGCAAGCCACGACGGAAAAACCCCCAGGAGGGCCTGTACAATGAACTGC
AGAAGGACAAAATGGCAGAGGCCTATAGTGAAATCGGGATGAAGGGAGAGAGAAGGCGCGGCAAAGGGCAC
GATGGCCTGTACCAGGGGCTGTCTACTGCCACCAAGGACACCTATGATGCTCTGCATATGCAGGCACTGCCTCCA
AGGTGATCTAGAGGGCCCGTTTAAACCCGCTGATCAGCCTCGA*CTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTT*
GCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCA
TCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGG
*AAGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATG*ACTAGTGGCGAATTCCCGTGTACCAGCTGAGAG
ACTCTAAATCCAGTGACAAGTCTGTCTGCCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGA
TTCTGATGTGTATATCACAGACAAAACTGTGCTAGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGC
CTGGAGCAACAAATCTGACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGACACCTTCTTCCCC
AGCCCAGGTAAGGGCAGCTTTGGTGCCTTCGCAGGCTGTTTCCTTGCTTCAGGAA, or (SEQ ID N° 11);

(CD123 K43 QR3)

AAGTAGCCCTGCATTTCAGGTTTCCTTGAGTGGCAGGCCAGGCCTGGCCGTGAACGTTCACTGAAATCATGGCC
TCTTGGCCAAGATTGATAGCTTGTGCCTGTCCCTGAGTCCCAGTCCATCACGAGCAGCTGGTTTCTAAGATGCTAT

TTCCCGTATAAAGCATGAGACCGTGACTTGCCAGCCCCACAGAGCCCCGCCCTTGTCCATCACTGGCATCTGGAC

TCCAGCCTGGGTTGGGGCAAAGAGGGAAATGAGATCATGTCCTAACCCTGATCCTCTTGTCCCACAGATATCCAG

TCCGGTGAGGGCAGAGGAAGTCTTCTAACATGCGGTGACGTGGAGGAGAATCCGGGCCCCGGATCCGCTCTG

CCCGTCACCGCTCTGCTGCTGCCTCTGGCCCTGCTGCTGCACGCAGCCAGACCAGGCGGAGGAGGCTCCTGCCC

TTACTCTAACCCAAGCCTGTGCTCCGGAGGAGGAGGATCCGGCGGAGGAGGCTCTGAGGTGAAGCTGGTGGA

GAGCGGAGGAGGCCTGGTGCAGCCTGGCGGCTCCCTGTCTCTGAGCTGCGCAGCATCCGGCTTCACCTTTACAG

ACTACTATATGTCTTGGGTGAGACAGCCCCCTGGCAAGGCCCTGGAGTGGCTGGCCCTGATCAGGTCCAAGGCC

GATGGCTACACCACAGAGTATTCCGCCTCTGTGAAGGGCAGATTCACCCTGTCTAGGGACGATAGCCAGTCCATC

CTGTACCTGCAGATGAATGCACTGCGCCCCGAGGACAGCGCCACATACTATTGTGCCAGAGACGCCGCCTACTAT

TCTTACTATAGCCCTGAGGGCGCTATGGACTACTGGGGCCAGGGCACCTCCGTGACAGTGAGCTCCGGAGGAGG

AGGAAGCGGAGGAGGAGGCTCCGGCGGCGGCGGCTCTATGGCCGACTATAAGGATATCGTGATGACCCAGAGC

CACAAGTTTATGTCTACAAGCGTGGGCGACCGCGTGAACATCACCTGCAAGGCCAGCCAGAATGTGGATTCCGC

CGTGGCCTGGTACCAGCAGAAGCCTGGCCAGAGCCCTAAGGCCCTGATCTATTCCGCCTCTTACCGGTATAGCGG

AGTGCCTGACCGCTTCACCGGAAGGGGATCCGGAACAGACTTCACCCTGACAATCTCTAGCGTGCAGGCCGAG

GATCTGGCCGTGTACTATTGTCAGCAGTACTATAGCACCCCCTGGACCTTCGGCGGAGGAACCAAGCTGGAGATC

AAGAGAGGATCTGGAGGAGGAGGAAGCTGCCCATACTCCAACCCCTCTCTGTGCAGCGGAGGAGGAGGATCTG

AGCTGCCAACCCAGGGCACATTTTCCAACGTGTCTACAAATGTGAGCCCAGCAAAGCCAACCACAACCGCATGC

CCTTATAGCAATCCATCCCTGTGCACAACCACACCTGCACCAAGACCACCAACCCCAGCACCTACAATCGCCTCTC

AGCCACTGAGCCTGCGCCCCGAGGCATGCCGGCCTGCAGCAGGCGGCGCCGTGCACACCAGGGGCCTGGACTT

CGCCTGCGATATCTACATCTGGGCACCTCTGGCAGGAACCTGTGGCGTGCTGCTGCTGAGCCTGGTCATCACCCT

GTACTGCAAGAGAGGCAGGAAGAAGCTGCTGTATATCTTCAAGCAGCCCTTTATGCGCCCTGTGCAGACCACAC

AGGAGGAGGACGGCTGCAGCTGTCGGTTCCCAGAAGAGGAGGAGGGCGGCTGTGAGCTGAGAGTGAAGTTT

AGCAGGTCCGCCGATGCACCAGCATACCAGCAGGGACAGAACCAGCTGTATAACGAGCTGAATCTGGGCCGGA

GAGAGGAGTACGACGTGCTGGATAAGAGGAGGGGAAGGGACCCCGAGATGGGAGGCAAGCCACGGAGAAA

GAACCCCCAGGAGGGCCTGTACAATGAGCTGCAGAAGGACAAGATGGCCGAGGCCTATTCCGAGATCGGCATG

AAGGGAGAGAGGCGCCGGGGCAAGGGACACGATGGCCTGTACCAGGGCCTGTCTACCGCCACAAAGGACACC

TATGATGCCCTGCATATGCAGGCACTGCCTCCAAGGTGATCTAGAGGGCCCGTTTAAACCCGCTGATCAGCCTCG

ACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCC

CACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGG

GTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATG

ACTAGTGGCGAATTCCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTGCCTATTCACCGATTT

TGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGACAAAACTGTGCTAGACATGAGG

TCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCAACAAATCTGACTTTGCATGTGCAAACGCCTTCAAC

AACAGCATTATTCCAGAAGACACCTTCTTCCCCAGCCCAGGTAAGGGCAGCTTTGGTGCCTTCGCAGGCTGTTTC

CTTGCTTCAGGAA, SEQ ID N° 12.

The insertion may comprise a sequence having at least from 99 %, 98%, 97%, 96%,95% 94% 93% 92% 91% 90% 89% 88% 87% 86% 85% 84% 83% 82% 81% to 80% identity with SEQ ID N° 9

The insertion may comprise a sequence having at least from 99 %, 98%, 97%, 96%,95% 94% 93% 92% 91% 90% 89% 88% 87% 86% 85% 84% 83% 82% 81% to 80% identity with SEQ ID N°10

The insertion may comprise a sequence having at least from 99 %, 98%, 97%, 96%,95% 94% 93% 92% 91% 90% 89% 88% 87% 86% 85% 84% 83% 82% 81% to 80% identity with SEQ ID N°11

The insertion may comprise a sequence having at least from 99 %, 98%, 97%, 96%,95% 94% 93% 92% 91% 90% 89% 88% 87% 86% 85% 84% 83% 82% 81% to 80% identity with SEQ ID N°12.

The insertion may comprise a sequence having at least 80% identity with SEQ ID N° 9

The insertion may comprise a sequence having at least 80% identity with SEQ ID N° 10

The insertion may comprise a sequence having at least 80% identity with SEQ ID N° 11

The insertion may comprise a sequence having at least 80% identity with SEQ ID N° 12

The insertion may comprise a sequence having at least 90% identity with SEQ ID N° 9

The insertion may comprise a sequence having at least 90% identity with SEQ ID N° 10

The insertion may comprise a sequence having at least 90% identity with SEQ ID N° 11

The insertion may comprise a sequence having at least 90% identity with SEQ ID N° 12

The insertion may comprise a sequence having at least 95% identity with SEQ ID N° 9

The insertion may comprise a sequence having at least 95% identity with SEQ ID N° 10

The insertion may comprise a sequence having at least 95% identity with SEQ ID N° 11

The insertion may comprise a sequence having at least 95% identity with SEQ ID N° 12.

In particular embodiments, the insertion may comprise a sequence encoding a CAR having the desired function of said CAR that is to say binding to any one of the target listed herein, preferably an antigen expressed at the surface of pathological cells directly or indirectly responsible for a disease.

CELL

By cell or cells is intended any eukaryotic living cell, any primary cell or cell line.

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

The cell of the invention (ie TALEN-modified endogenous αβ-TCR negative human primary cell) according to any one of the above embodiments may be a human T-cell, a human cell derived from a human T cell, a human lymphoid cell, a human stem cell, a human progenitor cell, a human induced pluripotent stem cell (iPSC), a human embryonic stem cell, a human mesenchymal stem cell (MSC), a human hematopoietic stem cell (HSC).

Preferably, the cell (TALEN-modified endogenous αβ-TCR negative human primary cell) according to any of the embodiments of the present invention, may be (ultimately) a human T-cell, more preferably a human lymphoid T cell, a human progenitor T cell, a human CD4+ T cell, a human CD8+ T cell, a human regulatory T cell, a human NKT cell, a human naïve T cell, a human memory T cell, a human TIL, a combination thereof.

In preferred embodiments, the cell or human cell of the invention is a TALEN-modified endogenous αβ-TCR negative human primary cell.

In lore preferred embodiments, the cell or human cell of the invention is a TALEN-modified endogenous αβ-TCR negative human primary T cell.

The human cell of the present invention may be even more preferably a primary human T cell isolated from the blood or from a tissue or a population of primary human T cell isolated from the blood or from a tissue and cultured in vitro for few passages before and/or after being engineered.

In particular embodiments, the human T cell of the present invention may be a human T cell line, a primary human T cell line, a primary human T cell line means derived from one human primary cell or a homogenous population of human primary cells.

In particular embodiments, the human cell of the invention is part of a population of human cells comprising said human cell.

In particular embodiments, the human cell of the invention is a population of human cells, preferably a homogenous population of human immune cells, more preferably a homogenous population of human T cells, even more preferably a homogenous population of human T cells with cytolytic activity.

In particular embodiments, the human cell of the invention is a population of human primary cells, preferably a homogenous population of human immune primary cells, more preferably a homogenous population of human primary T cells, even more preferably a homogenous population of human primary T cells with cytolytic activity.

The human cell of the present invention is engineered, meaning that said human cell comprises a genetic modification, in particular an insertion of an exogenous polynucleotide, into the genomic DNA, induced by an endonuclease, preferably a rare cutting endonuclease, more preferably a rare cutting endonuclease selected from TALEN, CRISPR CAS9, Meganuclease, MegaTAL, Zn Finger, or a combination thereof, even more preferably at least by a TALEN.

In preferred embodiments, the human cell of the present invention is engineered, meaning that said human cell comprises at least one genetic modification, preferably an insertion affecting the TRAC gene, said insertion comprising an exogenous polynucleotide encoding a CAR, said cell express a CAR at the cell surface, and undetectable level of alpha beta TCR at the cell surface.

In preferred embodiments, the human cell of the present invention is engineered, meaning that said human cell comprises at least one genetic modification, preferably an insertion affecting the TRAC gene, said insertion comprising an exogenous polynucleotide encoding a TCR (here an exogenous TCR), said cell express said exogenous TCR at the cell surface, and undetectable level of alpha beta TCR at the cell surface.

In particular embodiments, the human cell of the present invention is engineered, meaning that said human cell comprises at least one genetic modification, preferably an insertion within the TRAC gene, said insertion comprising an exogenous polynucleotide encoding a tag or a protein, said insertion does not prevent the endogenous TCR to be translated and transduced and expressed at the cell surface, said cell express said endogenous TCR at the cell surface.

1. A TALEN-modified endogenous αβ-TCR negative human primary cell wherein the constant region of the genomic TCR gene (TRAC gene) comprises a genetic modification generated by a TALEN and affecting cell surface expression of the endogenous alpha beta TCR, said genomic TRAC gene comprising from 5' to 3':

(a) a 5' region of said human genomic TRAC gene upstream,

(b) a recognition domain for a half TALEN,

(c) a gap or an insertion as compared to the wild type TRAC gene affecting the cell surface expression of the extracellular domain or transmembrane domain of the alpha beta TCR,

said insertion comprising an exogenous polynucleotide selected from a noncoding sequence such as, a stop codon, an IRES, a coding sequence such as a sequence coding for a self-cleaving peptide in

frame with the TRAC open reading frame, a sequence coding a chimeric antigen receptor (CAR), a sequence coding a TCR, a sequence coding a protein conferring sensitivity to a drug, a sequence coding a protein conferring resistance to a drug, a cytokine, a termination sequence, a combination thereof,

(c') optionally a recognition domain for another half TALEN,

(d) a 3' region of the genomic TRAC gene.

The TALEN-modified endogenous αβ-TCR negative human primary cell of claim 1 comprising a level of off target cutting below detection by a guide seq analysis is provided.

2. The TALEN-modified endogenous αβ-TCR negative human primary cell according to claim 1 wherein said human primary cell is a human primary T cell, or a human lymphoid primary cell, or a human primary stem cell, or a human primary progenitor cell.

3. The TALEN-modified endogenous αβ-TCR negative human primary cell according to any one of claims 1 to 2 wherein said human primary cell is a human primary T cell or a population of human primary T cells.

4. The TALEN-modified endogenous αβ-TCR negative human primary cell according to any one of claims 1 to 3 wherein said human T primary cell or population thereof comprises or consists in human primary CD8 T cell, human primary CD4 T cell, a combination thereof.

5. The TALEN-modified endogenous αβ-TCR negative human primary cell according to any one of claim 1 to 4 wherein said recognition domain for a TALEN comprises the following sequence ttgtcccacagATATC, or ttgtcccacagATATCCAG, and optionally CCGTGTACCAGCTGAGA.

6. The TALEN-modified endogenous αβ-TCR negative human primary cell according to any one of claims 1 to 5 comprising the following sequence:

AAGTAGCCCTGCATTTCAGGTTTCCTTGAGTGGCAGGCCAGGCCTGGCCGTGAACGTTCACTGAAATCATGGCC
TCTTGGCCAAGATTGATAGCTTGTGCCTGTCCCTGAGTCCCAGTCCATCACGAGCAGCTGGTTTCTAAGATGCTAT
TTCCCGTATAAAGCATGAGACCGTGACTTGCCAGCCCCACAGAGCCCCGCCCTTGTCCATCACTGGCATCTGGAC
TCCAGCCTGGGTTGGGGCAAAGAGGGAAATGAGATCATGTCCTAACCCTGATCCTCTTGTCCCACAGATATCCAG
TCCGGTGAGGGCAGAGGAAGTCTTCTAACATGCGGTGACGTGGAGGAGAATCCGGGCCCCGGATCCcodingse
quenceTCTAGAGGGCCCGTTTAAACCCGCTGATCAGCCTCGA*CTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTT*
GCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCA
TCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGG
*AAGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATG*ACTAGTGGCGAATTCCCGTGTACCAGCTGAGAG
ACTCTAAATCCAGTGACAAGTCTGTCTGCCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGA
TTCTGATGTGTATATCACAGACAAAACTGTGCTAGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGC
CTGGAGCAACAAATCTGACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGACACCTTCTTCCCC
AGCCCAGGTAAGGGCAGCTTTGGTGCCTTCGCAGGCTGTTTCCTTGCTTCAGGAA in the TRAC gene,

The TALEN-modified endogenous αβ-TCR negative human primary cell according to any one of claim 1 to 5 wherein said recognition domain for a TALEN comprises a homology arm, a sequence coding a protein, such as a sequence coding for a self-cleaving peptide in frame with the TRAC open reading frame, preferably a 2A peptide; a sequence coding a chimeric antigen receptor (CAR), preferably a CAR, more preferably an anti-CD123 CAR or an anti-CD22 CAR; a sequence coding a TCR, a sequence coding a protein conferring sensitivity to a drug, a sequence coding a protein conferring resistance to a drug, a cytokine, a termination sequence.

In a preferred embodiment said coding sequence is followed by a termination sequence such as a BGH poly A.

7. The TALEN-modified endogenous αβ-TCR negative human primary cell according to any one of claims 1 to 6 wherein said CAR sequence comprises an anti-CD22 CAR sequence, anti-CD123 CAR sequence, anti-CD30 CAR sequence, anti-HSP-70 CAR sequence, anti-o-acetyl-GD2 CAR sequence, anti-CS-1 CAR sequence, anti-CLL-1 CAR sequence, an anti-CD38 CAR sequence, anti-5T4 CAR sequence, anti-MUC1 CAR sequence, anti-FAP CAR sequence, anti-HER2 CAR sequence, anti- CD79a CAR sequence, anti-CD79b CAR.

The TALEN-modified endogenous αβ-TCR negative human primary cell according to any one of claims 1 to 7, wherein said polynucleotide sequence comprises one of the following sequences: SEQ ID N°9, SEQ ID N°10, SEQ ID N°11, SEQ ID N°12.

The TALEN-modified endogenous αβ-TCR negative human primary cell according to any one of claims 1 to 7, wherein said polynucleotide sequence comprises one of the following sequences: SEQ ID N°9, SEQ ID N°10, SEQ ID N°11, SEQ ID N°12 which are respectively:

AAGTAGCCCTGCATTTCAGGTTTCCTTGAGTGGCAGGCCAGGCCTGGCCGTGAACGTTCACTGAAATCATGGCC
TCTTGGCCAAGATTGATAGCTTGTGCCTGTCCCTGAGTCCCAGTCCATCACGAGCAGCTGGTTTCTAAGATGCTAT
TTCCCGTATAAAGCATGAGACCGTGACTTGCCAGCCCCACAGAGCCCCGCCCTTGTCCATCACTGGCATCTGGAC
TCCAGCCTGGGTTGGGGCAAAGAGGGAAATGAGATCATGTCCTAACCCTGATCCTCTTGTCCCACAGATATCCAG
TCCGGTGAGGGCAGAGGAAGTCTTCTAACATGCGGTGACGTGGAGGAGAATCCGGGCCCCGGATCCGCTCTG
CCCGTCACCGCTCTGCTGCTGCCACTGGCCCTGCTGCTGCACGCAGCAAGACCAGGAGGGGGAGGCAGCTGCC
CCTACAGCAACCCCAGCCTGTGCAGCGGAGGCGGCGGCAGCGGCGGAGGGGGTAGCCAGGTGCAGCTGCAG
CAGAGCGGCCCTGGCCTGGTGAAGCCAAGCCAGACACTGTCCCTGACCTGCGCCATCAGCGGCGATTCCGTGA
GCTCCAACTCCGCCGCCTGGAATTGGATCAGGCAGTCCCCTTCTCGGGGCCTGGAGTGGCTGGGAAGGACATAC
TATCGGTCTAAGTGGTACAACGATTATGCCGTGTCTGTGAAGAGCAGAATCACAATCAACCCTGACACCTCCAAG
AATCAGTTCTCTCTGCAGCTGAATAGCGTGACACCAGAGGACACCGCCGTGTACTATTGCGCCAGGGAGGTGAC
CGGCGACCTGGAGGATGCCTTTGACATCTGGGGCCAGGGCACAATGGTGACCGTGTCTAGCGGAGGAGGAGG

ATCCGGAGGAGGAGGATCTGGCGGCGGCGGCAGCGATATCCAGATGACACAGTCCCCATCCTCTCTGAGCGCCT
CCGTGGGCGACAGAGTGACAATCACCTGTAGGGCCTCCCAGACCATCTGGTCTTACCTGAACTGGTATCAGCAG
AGGCCCGGCAAGGCCCCTAATCTGCTGATCTACGCAGCAAGCTCCCTGCAGAGCGGAGTGCCATCCAGATTCTCT
GGCAGGGGCTCCGGCACAGACTTCACCCTGACCATCTCTAGCCTGCAGGCCGAGGACTTCGCCACCTACTATTGC
CAGCAGTCTTATAGCATCCCCCAGACATTTGGCCAGGGCACCAAGCTGGAGATCAAGGGAAGCGGAGGGGGAG
GCAGCTGCCCCTACAGCAACCCCAGCCTGTGCAGCGGAGGCGGCGGCAGCGAGCTGCCCACCCAGGGCACCTT
CTCCAACGTGTCCACCAACGTGAGCCCAGCCAAGCCCACCACCACCGCCTGTCCTTATTCCAATCCTTCCCTGTGT
GCTCCCACCACAACCCCAGCACCAAGGCCACCTACACCTGCACCAACCATCGCCTCTCAGCCCCTGAGCCTGAGA
CCTGAGGCATGTAGGCCAGCAGCAGGAGGAGCAGTCCATACAAGGGGTCTGGATTTTGCATGCGACATCTACAT
CTGGGCACCTCTGGCAGGAACATGTGGCGTGCTCCTGCTCAGCCTGGTCATCACCCTGTACTGCAAGAGAGGCA
GGAAGAAGCTGCTGTATATCTTCAAGCAGCCCTTCATGCGCCCCGTGCAGACAACCCAGGAGGAGGATGGCTGC
TCCTGTAGGTTCCCAGAAGAGGAGGAGGGAGGATGTGAGCTGCGCGTGAAGTTTTCCCGGTCTGCCGACGCAC
CTGCATACCAGCAGGGCCAGAACCAGCTGTATAACGAGCTGAATCTGGGCCGGAGAGAGGAGTACGATGTGCT
GGACAAGAGGCGCGGCAGAGATCCAGAGATGGGCGGCAAGCCCCGGAGAAAGAACCCTCAGGAGGGCCTGT
ACAATGAGCTGCAGAAGGATAAGATGGCCGAGGCCTATTCTGAGATCGGCATGAAGGGAGAGAGGCGCCGGG
GCAAGGGACACGACGGACTGTACCAGGGACTGAGCACAGCCACCAAGGATACCTATGACGCCCTGCATATGCAG
GCACTGCCTCCAAGGTGATCTAGAGGGCCCGTTTAAACCCGCTGATCAGCCTCGA*CTGTGCCTTCTAGTTGCCAG*
CCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAAT
GAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGG
*GGGAGGATTGGGAAGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATG*ACTAGTGGCGAATTCCCGTGT
ACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTGCCTATTCACCGATTTTGATTCTCAAACAAATGTGTC
ACAAAGTAAGGATTCTGATGTGTATATCACAGACAAAACTGTGCTAGACATGAGGTCTATGGACTTCAAGAGCAA
CAGTGCTGTGGCCTGGAGCAACAAATCTGACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGA
CACCTTCTTCCCCAGCCCAGGTAAGGGCAGCTTTGGTGCCTTCGCAGGCTGTTTCCTTGCTTCAGGAA,
AAGTAGCCCTGCATTTCAGGTTTCCTTGAGTGGCAGGCCAGGCCTGGCCGTGAACGTTCACTGAAATCATGGCC
TCTTGGCCAAGATTGATAGCTTGTGCCTGTCCCTGAGTCCCAGTCCATCACGAGCAGCTGGTTTCTAAGATGCTAT

TTCCCGTATAAAGCATGAGACCGTGACTTGCCAGCCCCACAGAGCCCCGCCCTTGTCCATCACTGGCATCTGGAC
TCCAGCCTGGGTTGGGGCAAAGAGGGAAATGAGATCATGTCCTAACCCTGATCCTCTTGTCCCACAGATATCCAG
TCCGGTGAGGGCAGAGGAAGTCTTCTAACATGCGGTGACGTGGAGGAGAATCCGGGCCCCGGATCCGCTCTGC
CCGTCACCGCTCTGCTGCTGCCACTGGCCCTGCTGCTGCACGCCGCCAGACCCGGCGGAGGAGGCTCTTGCCCC
TACAGCAACCCCAGCCTGTGCTCTGGCGGCGGCGGCAGCGGAGGCGGCGGCTCCCAGGTGCAGCTGCAGCAG
AGCGGCCCTGGCCTGGTGGAGCCAAGCCAGACACTGTCCCTGACCTGCGCCATCTCTGGCGACAGCGTGAGCTC
CAACAGCGCCGCATGGAATTGGATCAGGCAGTCCCCATCTCGGGGCCTGGAGTGGCTGGGCAGAACATACTATA
GGTCCACCTGGTACAACGACTATGCCGGCTCCGTGAAGTCTCGCATCACAATCAACCCCGATACCAGCAAGAATC
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TCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCAACAAATCTGACTTTGCATGTGCAAACGCCTTCAAC AACAGCATTATTCCAGAAGACACCTTCTTCCCCAGCCCAGGTAAGGGCAGCTTTGGTGCCTTCGCAGGCTGTTTC CTTGCTTCAGGAA.

8. The TALEN-modified endogenous αβ-TCR negative human cell according to any one of claims 1 to 7, further comprising undetectable level of MHC molecules as compared to an unmodified control cell and a deletion functionally affecting cell surface expression of a beta 2 microglobulin molecule or of a CIITA molecule.

9. The TALEN-modified endogenous αβ-TCR negative human cell according to any one of claims 1 to 8, wherein said insertion resulted in an inactivation of the gene coding the TCR alpha and an undetectable cell surface expression of endogenous αβ-TCR in at least 96% of the total cells, in at least 97% of the total cells, in at least 98% of the total cells, in at least 99% of the total cells.

10. The TALEN-modified endogenous αβ-TCR negative human cell according to any one of claim 1 to 9, comprising :

a first half TALEN having a sequence having at least from 100%, 99 %, 98%, 97%, 96%,95% 94% 93% 92% 91% 90% 89% 88% 87% 86% 85% 84% 83% 82% 81% to 80% identity with SEQ ID N°3, provided that said first TALEN binding domain binds to ttgtcccacagATATC, and a second half TALEN having a sequence having at least from 100%, 99 %, 98%, 97%, 96%,95% 94% 93% 92% 91% 90% 89% 88% 87% 86% 85% 84% 83% 82% 81% to 80% identity with SEQ ID N°4, provided that said second half TALEN binds to CCGTGTACCAGCTGAGA and the frequency of off target cleavage of said TALEN is below detection.

11. The TALEN-modified endogenous αβ-TCR negative human cell according to any one of claim 1 to

10, wherein said at least one insertion comprises an exogenous polynucleotide sequence located downstream a TALEN binding domain of sequence ttgtcccacagATATC, or ttgtcccacagATATCCAG, present in the wt TRAC.

12. The TALEN-modified endogenous αβ-TCR negative human cell according to any one of claim 1 to 11, wherein said at least one insertion comprises a sequence encoding a self-cleaving peptide in frame with the genomic TRAC coding sequence selected from a 2A peptide, a 2A like peptide, a P2A peptide, a E2A peptide, a F2A peptide, preferably a 2A peptide, more preferably a 2A peptide, of sequence GSGEGRGSLLTCGDVEENPGP, GSGATNFSLLKQAGDVEENPGP, GSGQCTNYALLKLAGDVESNPGP, GSGVKQTLNFDLLKLAGDVESNPGP, SGEGRGSLLTCGDVEENPGP, SGATNFSLLKQAGDVEENPGP, SGQCTNYALLKLAGDVESNPGP, SGVKQTLNFDLLKLAGDVESNPGP, even more preferably a 2A peptide of sequence SGEGRGSLLTCGDVEENPGP.

13. The TALEN-modified endogenous αβ–TCR negative human cell according to any one of claim 1 to 12, wherein said at least one insertion comprises a sequence coding a 2A peptide, of sequence SGEGRGSLLTCGDVEENPGP in frame with the genomic TRAC coding sequence, an exogenous polynucleotide sequence coding a chimeric antigen receptor, a terminator sequence of polyadenylation signal, optionally a TALEN binding domain.

14. The TALEN-modified endogenous αβ-TCR negative human cell according to any one of claim 1 to 13, wherein said exogenous polynucleotide sequence comprises a chimeric antigen receptor (CAR) selected from a CAR specific for at least one of the following antigen : Preferably CD22, CD123, CS-1, CLL-1, CD38, HSP70, MUC-1, CD30 , o-acetyl-GD2nd a sequence SGEGRGSLLTCGDVEENPGP in frame with the genomic TRAC coding sequence, a terminator sequence of polyadenylation signal.

15. The TALEN-modified endogenous αβ-TCR negative human cell according to any one of claim 1 to 14 wherein said (CAR) comprises an extracellular ligand-binding domain, an epitope specific for a monoclonal antibody, a transmembrane domain and one or more intracellular signaling domains.

16. The TALEN-modified endogenous αβ-TCR negative human cell according to any one of claim 1 to 10, wherein said at least one insertion comprises an IRES, an exogenous polynucleotide sequence comprising a chimeric antigen receptor (CAR), a terminator sequence of polyadenylation signal, optionally a TALEN binding domain.

17. The TALEN-modified endogenous αβ-TCR negative human cell according to any one of claim 1 to 15, wherein said chimeric antigen receptor (CAR), comprise at least one antigen specific for a monoclonal antibody, preferably two antigens specific for a monoclonal antibody.

18. The TALEN-modified endogenous αβ-TCR negative human cell according to any one of claim 1 to 12 wherein said exogenous polynucleotide comprises a transcription termination signal stopping the activity of RNA polymerase.

19. The TALEN-modified endogenous αβ–TCR negative human cell according to any one of claim 1 to 18 comprising at least one additional disruption in an endogenous gene wherein said endogenous gene is selected from the group consisting CD52, dCK GR, beta subunit gene of the TCR (TCRB1 or TCRB2), a cytokine inducible SH2- containing (CISH) gene, an adenosine A2a receptor (ADORA) gene, a CD276 gene, a V-set domain containing T cell activation inhibitor 1 (VTCNl) gene, a B and T lymphocyte associated (BTLA) gene, a cytotoxic T-lymphocyte-associated protein 4 (CTLA4) gene, an indoleamine 2,3-dioxygenase 1 (IDO l) gene, a killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 1 (KIR3DL1) gene, a lymphocyte -activation gene 3 (LAG3) gene, a programmed cell death 1 (PD-1) gene, an hepatitis A virus cellular receptor 2 (HAVCR2) gene, a V-domain

immunoglobulin suppressor of T-cell activation (VISTA) gene, a natural killer cell receptor 2B4 (CD244) gene , a hypoxanthine phosphoribosyltransferase 1 (HPRT) gene, and chemokine (C-C motif) receptor 5 (gene/pseudogene) (CCR5) gene, CXCR4, a combination thereof.

20. A population of human cells comprising a TALEN-modified endogenous αβ-TCR negative human cell according to any one of claim 1 to 19.

21. A pharmaceutical composition comprising a TALEN-modified endogenous αβ-TCR negative human cell according to any one of claim 1 to 19 or a population of human cells according to claim 20 and a pharmaceutically acceptable excipient.

22. The TALEN-modified endogenous αβ-TCR negative human cell according to any one of claim 1 to 19 or the population of human cells according to claim 20 or the pharmaceutical composition according to claim 21 for use as a medicament.

23. The TALEN-modified endogenous αβ–TCR negative human cell according to any one of claim 1 to 19 or the population of human cells according to claim 19 or the pharmaceutical composition according to claim 21 for use in the treatment of cancer.

24. The TALEN-modified endogenous αβ-TCR negative human cell according to any one of claim 1 to 19 or the population of human cells according to claim 20 or the pharmaceutical composition according to claim 21 for use in the treatment of a cancer selected from the group consisting of carcinoma, lymphoma, sarcoma, blastomas, and leukemia.

25. The TALEN-modified endogenous αβ-TCR negative human cell according to any one of claim 1 to 19 or the population of human cells according to claim 20 or the pharmaceutical composition accord-

ing to claim 21 for use in the treatment of cancer wherein the cancer is selected from the group consisting of a cancer of B-cell origin, a cancer of T cell origin, breast cancer, gastric cancer, neuroblastoma, osteosarcoma, lung cancer, melanoma, prostate cancer, colon cancer, renal cell carcinoma, ovarian cancer, rhabdomyo sarcoma, leukemia, and Hodgkin's lymphoma.

26. The TALEN-modified endogenous αβ-TCR negative human cell according to any one of claim 1 to 19 or the population of human cells according to claim 20 or the pharmaceutical composition according to claim 21 for use in the treatment of cancer wherein the cancer of B-cell origin is selected from the group consisting of B-lineage acute lymphoblastic leukemia, B-cell chronic lymphocytic leukemia, and B-cell non-Hodgkin's lymphoma.

27. The TALEN-modified endogenous αβ-TCR negative human cell according to any one of claim 1 to 19 or the population of human cells according to claim 20 or the pharmaceutical composition according to claim 21 for use in the treatment of a cancer wherein the cancer is AML, ALL, a T cell lymphoma, CLL.

28. A means for detecting an endonuclease-modified endogenous αβ–TCR negative human cell,

said endonuclease-modified endogenous αβ-TCR negative human cell comprising an endonuclease modified genomic TRAC gene as compared to the wild type TRAC gene,

wherein said modified human genomic TRAC gene comprises from 5' to 3':

(a) a 5' region of said human genomic TRAC gene upstream a recognition domain for a rare cutting endonuclease present in the wild type TRAC gene,

(b) a gap or an insertion as compared to the wild type TRAC gene affecting the cell surface expression of the extracellular domain or transmembrane domain of the alpha beta TCR,

said insertion comprising an exogenous polynucleotide selected from a noncoding sequence, a stop codon, a sequence coding for a self-cleaving peptide in frame with the TRAC open reading frame, an IRES, a sequence coding a chimeric antigen receptor (CAR), a sequence coding a TCR, a sequence coding a protein conferring sensitivity to a drug, a sequence coding a protein conferring resistance to a drug, a sequence coding for a cytokine, a termination sequence, a combination thereof,

(c') optionally a second rare cutting endonuclease recognition domain,

(d) a 3' region of the genomic TRAC gene.

29. The means according to claim 28 wherein said endonuclease is selected from the group consisting of: CRISPR/Cas 9, CRISPR/Cpfl, TALEN, transposase, ZEN, Zinc finger endonuclease, meganuclease, or MegaTAL and said means binds to a sequence of the endonuclease modified TRAC, specific for said nuclease and/or upstream said sequence specific for said nuclease.

30. The means according to claim 28 or 29 wherein said endonuclease is a TALEN.

31. The means according to claim 30 for detecting a TALEN modified endogenous αβ-TCR negative human cell comprising a probe wherein said probe binds to a sequence in the modified genomic TRAC gene, upstream the endonuclease binding domain or endonuclease recognition domain, and/or to a sequence encoding a tag.

32. The MEANS according to claim 30 or 31 wherein said TALEN modified genomic TRAC gene comprises,

(a) a 5' region of said human genomic TRAC gene;

(b) a recognition domain for a (half?) TALEN, preferably a recognition domain for a (half?) TALEN comprising the following sequence ttgtcccacagATATC, or ttgtcccacagATATCCAG, or

(c) a gap or an insertion as compared to the wild type TRAC gene affecting the cell surface expression of the extracellular domain or transmembrane domain of the alpha beta TCR,

said insertion comprising an exogenous polynucleotide selected from a noncoding sequence, s stop codon, a sequence coding for a self-cleaving peptide in frame with the TRAC open reading frame, an IRES, a sequence coding a chimeric antigen receptor (CAR), a sequence coding a TCR, a sequence coding a protein conferring sensitivity to a drug, a sequence coding a protein conferring resistance to a drug, a termination sequence, a combination thereof,

(c') optionally a second (half?) TALEN recognition domain.

(d) a 3' region of the genomic TRAC gene;

and said means binds specifically to said TALEN-modified genomic TRAC gene.

33.The means according to any one of claims 30 to 32 for detecting a TALEN modified endogenous αβ-TCR negative human cell comprising a probe wherein said probe binds to at least 10 bases of the sequence ttgtcccacagATATC, or ttgtcccacagATATCCAG, in the modified genomic TRAC gene.

34. The means according to any one of the claims to 30 to 33 for detecting an endonuclease-modified endogenous αβ–TCR negative human cell by polymerase chain reaction (pcr) or potential off sites modifications, preferably by guide sequence.

35. The means according to any one of the claims to 30 to 34 for detecting a TALEN-modified endogenous αβ–TCR negative human cell according to any one of the claims 1 to 19.

36. The TALEN-modified endogenous αβ–TCR negative human cell according to any one of claim 1 to 15 comprising an inactivated genomic TCRA gene wherein an exogenous sequence coding a CAR was integrated into the genomic TCRA gene using a lentiviral vector or a AAV vector and said genomic disruptions were performed using an endonuclease selected from a CRISPR/CAS9, CRISPR/Cpf1 meganuclease, MegaTAL, ZFN, TALEN, combination thereof.

37. A method for producing an endonuclease-modified endogenous αβ-TCR negative human cell said method comprising:

(a) introducing into a human cell:

(i) a first nucleic acid sequence encoding an engineered nuclease; or an engineered nuclease protein;

wherein said engineered nuclease produces a cleavage at a recognition sequence within said human TCR alpha constant region gene; said cleavage resulting in an inhibition of cell surface expression of the αβ-TCR to undetectable level.

(ii) a second nucleic acid sequence comprising an exogenous polynucleotide encoding a CAR,

(iii) optionally a probe,

wherein the sequence of said exogenous polynucleotide is inserted into said genomic human TCR alpha constant region gene at said cleavage site; and further wherein said genetically-modified cell has reduced cell-surface expression of the endogenous TCR when compared to an unmodified control cell.

38. A method for producing an endonuclease-modified endogenous αβ-TCR negative human cell according to claim 37 said method comprising:

detecting endonuclease(s)-induced on site and off site, preferably by pcr and/or by guide sequence.

39. The method of any one of claims 37 to 38, wherein said engineered nuclease is a meganuclease, a zinc-finger nuclease (ZFN), a transcription activator-like effector nuclease (TALEN), a CRISPR/Cas nuclease, or a megaTAL nuclease.

40. The method of claim 37 to 39, wherein said engineered nuclease is a TALEN.

41. The method of claim 37 to 40, wherein said exogenous polynucleotide comprises a nucleic acid sequence encoding a self-cleaving peptide and a chimeric antigen receptor.

42. The method of any one of claim 37 to 41 wherein said chimeric antigen receptor comprises an extracellular ligand-binding domain and one or more intracellular signaling domains.

43. The method of any one of claims 37 to 42, wherein said exogenous polynucleotide comprises a promoter sequence that drives expression of said exogenous polynucleotide.

44. The method of any one of claims 37 to 43, wherein at least said second nucleic acid sequence is introduced into said cell by contacting said cell with a recombinant adeno-associated virus (AAV6) vector comprising said second nucleic acid sequence.

45. The method of any one of claims 39 to 44, wherein said recombinant AAV vector is a self-complementary AAV vector.

46. The method of any one of claims 39 to 45, wherein said recombinant AAV vector derived at least in part from an AAV6.

47. The method of claim 39 to 46, wherein said recombinant TALEN comprises a first subunit and a second subunit, wherein said first subunit binds to a first recognition half-site, and wherein said second subunit binds to a second recognition half-site.

48. The method of claim 39 to 47, wherein said recombinant TALEN recognizes a sequence ttgtcccacagATATC in the wild- type human TCR alpha constant region.

49. The method of claim 37-39, wherein said endonuclease is a Zing finger that recognizes from 5' to 3' TGCTGTGGCCTGGAGCAAC and GACTTTGCATGTGCA and cleaves within AAATCT , a Crispr /Cas 9 that recognizes a complementary sequence to any one of the following sequences : AGAG-TCTCTCAGCTGGTACA, GCACCAAAGCTGCCCTTACC, AAGTTCCTGTGATGTCAAGC , TTCGGAACCCAATCACTGAC, GAT-TAAACCCGGCCACTTTC, CGTCATGAGCAGATTAAACC, CTCAAGGTTCAGATCAGAAG, TAGGCAGACAGACTTGTCAC, AACAAATGTGTCACAAAGTA, CACCAAAGCTGCCCTTACCT, CTGACAGGTTTT-

GAAAGTTT, TTCAAAACCTGTCAGTGATT, CCGAATCCTCCTCCTGAAAG, CCACTTTCAGGAGGAGGATT, TAAACCCGGCCACTTTCAGG, TCTCAAACAAATGTGTCACAAAGTA, CTTACAATCTTGCAGATCTGGAATG, TTAATCTGCTCATGACGCTG, GGAGAAGAGGGGCAATGCAG, TCTTCTCCCTCTCCAAACAG, AGCAGCTTTCACCTCCTTGG, GTAGCAGCTTTCACCTCCTT, AGTTGGTGGCATTGCCGGGG, TCTGTGATATACACATCAGAATC, TCTGTGATATACACATCAGAATCC, GAGTCTCTCAGCTGGTACACGGC, GAGTCTCTCAGCTGGTACACGGCA, ATTCTCAAACAAATGTGTCACAA, ATTCTCAAACAAATGTGTCACAAA, GTCTGTGATATACACATCAGAAT, GTCTGTGATATACACATCAGAATC, GAGAATCAAAATCGGTGAATAGG, TGTGCTAGACATGAGGTCTATGG , TCAGGGTTCTGGATATCTGTGGG, GTCAGGGTTCTGGATATCTGTGG, AAAGTCAGATTTGTTGCTCCAGG, AACAAATGTGTCACAAAGTAAGG, TGGATTTAGAGTCTCTCAGCTGG, TAGGCAGACAGACTTGTCACTGG, AGCTGGTACACGGCAGGGTCAGG, GCTGGTACACGGCAGGGTCAGGG, TCTCTCAGCTGGTACACGGCAGG, AGAGTCTCTCAGCTGGTACACGG, CTCTCAGCTGGTACACGGCAGGG, ACAAAACTGTGCTAGACATGAGG, ATTTGTTTGAGAATCAAAATCGG, TGGAATAATGCTGTTGTTGAAGG, AGAGCAACAGTGCTGTGGCCTGG, CTTCTTCCCCAGCCCAGGTAAGG, ACACGGCAGGGTCAGGGTTCTGG, CTTCAAGAGCAACAGTGCTGTGG, CTGGGGAAGAAGGTGTCTTCTGG, TTCTTCCCCAGCCCAGGTAAGGG, CTTACCTGGGCTGGGGAAGAAGG, GACACCTTCTTCCCCAGCCCAGG, TTCAAAACCTGTCAGTGATTGGG, CGTCATGAGCAGATTAAACCCGG, TTCGGAACCCAATCACTGACAGG, TAAACCCGGCCACTTTCAGGAGG, TTTCAAAACCTGTCAGTGATTGG, GATTAAACCCGGCCACTTTCAGG, CTCGACCAGCTTGACATCACAGG, AAGTTCCTGTGATGTCAAGCTGG

,ATCCTCCTCCTGAAAGTGGCCGG, TGCTCATGACGCTGCGGCTGTGG, CATCACAGGAACTTTCTAAAAGG, GTCGAGAAAAGCTTTGAAACAGG, CCACTTTCAGGAGGAGGATTCGG, CTGACAGGTTTTGAAAGTTTAGG, AGCTTTGAAACAGGTAAGACAGG, CTGTGGTCCAGCTGAGGTGAGGG, CTGCGGCTGTGGTCCAGCTGAGG, TGTGGTCCAGCTGAGGTGAGGGG, TCCTCCTCCTGAAAGTGGCCGGG, TTAATCTGCTCATGACGCTGCGG, ACCCGGCCACTTTCAGGAGGAGG, GCTGTGGTCCAGCTGAGGTGAGG, CCGAATCCTCCTCCTGAAAGTGG.

a MegaTAL, a meganuclease that recognizes and cleaves a recognition sequence within residues 93-208 of the wild- type human TCR alpha constant region, wherein said recombinant meganuclease comprises a first subunit and a second subunit, wherein said first subunit binds to a first recognition half-site of said recognition sequence and comprises a first hypervariable (HVR1) region, and wherein said second subunit binds to a second recognition half-site of said recognition sequence and comprises a second hypervariable (HVR2) region.

50. The method of any one of claims 49, wherein said meganuclease is a single-chain meganuclease comprising a linker, wherein said linker covalently joins said first subunit and said second subunit.

51. A means for detecting cells obtained according to any of one the method of claims 37-50.

52. The means of claims 51 selected from a sequence of SEQ ID N° 5, SEQ ID N° 6, SEQ ID N° 7, a combination thereof or any degenerated SEQ ID N° 5, SEQ ID N° 6, SEQ ID N° 7, a combination thereof.

53. The means of claims 51 for detecting on site and/or off site cleavage by endonuclease.

54. The means of claims 53 for detecting on site and/or off site cleavage by endonuclease having any one of SEQ ID N° 13 to 22, a combination thereof.

The means of claims 53 for detecting on site and/or off site cleavage by a TALEN comprising a sequence selected from any one GAGAATCAAAATCGGTGAATAGG, TTCAAAACCTGTCAGTGATTGGG,

TGTGCTAGACATGAGGTCTATGG, CGTCATGAGCAGATTAAACCCGG, TCAGGGTTCTGGATATCTGTGGG, GTCAGGGTTCTGGATATCTGTGG, TTCGGAACCCAATCACTGACAGG, TAAACCCGGCCACTTTCAGGAGG, AAAGTCAGATTTGTTGCTCCAGG, AACAAATGTGTCACAAAGTAAGG, TGGATTTAGAGTCTCTCAGCTGG, TAGGCAGACAGACTTGTCACTGG, AGCTGGTACACGGCAGGGTCAGG, GCTGGTACACGGCAGGGTCAGGG, TCTCTCAGCTGGTACACGGCAGG, TTTCAAAACCTGTCAGTGATTGG, GATTAAACCCGGCCACTTTCAGG, CTCGACCAGCTTGACATCACAGG, AGAGTCTCTCAGCTGGTACACGG, CTCTCAGCTGGTACACGGCAGGG, AAGTTCCTGTGATGTCAAGCTGG, ATCCTCCTCCTGAAAGTGGCCGG, TGCTCATGACGCTGCGGCTGTGG, ACAAAACTGTGCTAGACATGAGG, ATTTGTTTGAGAATCAAAATCGG, CATCACAGGAACTTTCTAAAAGG,GTCGAGAAAAGCTTTGAAACAGG, CCACTTTCAGGAGGAGGATTCGG, CTGACAGGTTTTGAAAGTTTAGG, AGCTTTGAAACAGGTAAGACAGG, TGGAATAATGCTGTTGTTGAAGG, AGAGCAACAGTGCTGTGGCCTGG, CTGTGGTCCAGCTGAGGTGAGGG, CTGCGGCTGTGGTCCAGCTGAGG, TGTGGTCCAGCTGAGGTGAGGGG, CTTCTTCCCCAGCCCAGGTAAGG, ACACGGCAGGGTCAGGGTTCTGG, CTTCAAGAGCAACAGTGCTGTGG, CTGGGGAAGAAGGTGTCTTCTGG, TCCTCCTCCTGAAAGTGGCCGGG, TTAATCTGCTCATGACGCTGCGG, ACCCGGCCACTTTCAGGAGGAGG, TTCTTCCCCAGCCCAGGTAAGGG, CTTACCTGGGCTGGGGAAGAAGG, GACACCTTCTTCCCCAGCCCAGG, GCTGTGGTCCAGCTGAGGTGAGG, CCGAATCCTCCTCCTGAAAGTGG, a complementary sequence thereof; said mean may be associated for example for a mean binding to the CAR sequence of peptide 2A sequence, and allowing a PCR according to the present invention.

55. A means comprising a sequence comprising at least one of the following sequences : AGAGTCTCTCAGCTGGTACA, GCACCAAAGCTGCCCTTACC, AAGTTCCTGTGATGTCAAGC , TTCGGAACCCAATCACTGAC, GATTAAACCCGGCCACTTTC, CGTCATGAGCAGATTAAACC, CTCAAGGTTCAGATCAGAAG, TAGGCAGACAGACTTGTCAC, AACAAATGTGTCACAAAGTA, CACCAAAGCTGCCCTTACCT, CTGACAGGTTTTGAAAGTTT, TTCAAAACCTGTCAGTGATT, CCGAATCCTCCTCCTGAAAG, CCACTTTCAGGAGGAGGATT, TAAACCCGGCCACTTTCAGG,

TCTCAAACAAATGTGTCACAAAGTA, CTTACAATCTTGCAGATCTGGAATG, TTAATCTGCTCATGACGCTG, GGAGAAGAGGGGCAATGCAG, TCTTCTCCCTCTCCAAACAG, AGCAGCTTTCACCTCCTTGG, GTAGCAGCTTTCACCTCCTT, AGTTGGTGGCATTGCCGGGG, TCTGTGATATACACATCAGAATC, TCTGTGATATACACATCAGAATCC, GAGTCTCTCAGCTGGTACACGGC, GAGTCTCTCAGCTGGTACACGGCA, ATTCTCAAACAAATGTGTCACAA, ATTCTCAAACAAATGTGTCACAAA, GTCTGTGATATACACATCAGAAT, GTCTGTGATATACACATCAGAATC, GAGAATCAAAATCGGTGAATAGG, TGTGCTAGACATGAGGTCTATGG , TCAGGGTTCTGGATATCTGTGGG, GTCAGGGTTCTGGATATCTGTGG, AAAGTCAGATTTGTTGCTCCAGG, AACAAATGTGTCACAAAGTAAGG, TGGATTTAGAGTCTCTCAGCTGG, TAGGCAGACAGACTTGTCACTGG, AGCTGGTACACGGCAGGGTCAGG, GCTGGTACACGGCAGGGTCAGGG, TCTCTCAGCTGGTACACGGCAGG, AGAGTCTCTCAGCTGGTACACGG, CTCTCAGCTGGTACACGGCAGGG, ACAAAACTGTGCTAGACATGAGG, ATTTGTTTGAGAATCAAAATCGG, TGGAATAATGCTGTTGTTGAAGG, AGAGCAACAGTGCTGTGGCCTGG, CTTCTTCCCCAGCCCAGGTAAGG, ACACGGCAGGGTCAGGGTTCTGG, CTTCAAGAGCAACAGTGCTGTGG, CTGGGGAAGAAGGTGTCTTCTGG, TTCTTCCCCAGCCCAGGTAAGGG, CTTACCTGGGCTGGGGAAGAAGG, GACACCTTCTTCCCCAGCCCAGG, TTCAAAACCTGTCAGTGATTGGG, CGTCATGAGCAGATTAAACCCGG, TTCGGAACCCAATCACTGACAGG, TAAACCCGGCCACTTTCAGGAGG, TTTCAAAACCTGTCAGTGATTGG, GATTAAACCCGGCCACTTTCAGG, CTCGACCAGCTTGACATCACAGG, AAGTTCCTGTGATGTCAAGCTGG ,ATCCTCCTCCTGAAAGTGGCCGG, TGCTCATGACGCTGCGGCTGTGG, CATCACAGGAACTTTCTAAAAGG, GTCGAGAAAAGCTTTGAAACAGG, CCAC-

TTTCAGGAGGAGGATTCGG, CTGACAGGTTTTGAAAGTTTAGG, AGCTTTGAAACAGGTAAGACAGG, CTGTGGTCCAGCTGAGGTGAGGG, CTGCGGCTGTGGTCCAGCTGAGG, TGTGGTCCAGCTGAGGTGAGGGG, TCCTCCTCCTGAAAGTGGCCGGG, TTAATCTGCTCATGACGCTGCGG, ACCCGGCCACTTTCAGGAGGAGG, GCTGTGGTCCAGCTGAGGTGAGG, CCGAATCCTCCTCCTGAAAGTGG and complementary sequence of any one thereof.

56. The present invention also provides a Method for preparing engineered primary immune cells for cell immunotherapy, said method comprising:
    - providing a population of primary CAR positive (CAR+) TCR negative (TCR-) immune cells as above (comprising within the endogenous TCR alpha gene an insertion encoding any one of the CAR described herein),
    - introducing into said CAR+TCR- primary immune cells:

    At least one nucleic acid comprising an exogenous nucleotide or polynucleotide sequence to be integrated at an endogenous locus to encode at least one molecule improving the therapeutic potential of said immune cells population;

    At least one sequence-specific reagent that specifically targets said selected endogenous locus,

    wherein said exogenous nucleotide or polynucleotide sequence is inserted by targeted gene integration into said endogenous locus, so that said exogenous nucleotide or polynucleotide sequence forms an exogenous coding sequence under transcriptional control of an endogenous promoter present at said locus.

57. In the Method according to item 1, said sequence specific reagent is a nuclease, preferably an endonuclease selected from the group consisting of: Crispr/Cas 9, TALEN, Zinc finger endonuclease, meganuclease, MegaTAL, a combination thereof.

58. Accordingly, the Method according to item 1 or 2, wherein said targeted gene integration is operated by homologous recombination or NHEJ into said CAR+TCR- primary immune cells.

59. The present invention provides a Method according to any one of items 1 to 3, wherein said endogenous promoter is selected to be active during CAR+TCR- primary immune cells activation.

60. The present invention provides a Method according to any one of items 1 to 4, wherein said molecule encoded by said exogenous coding sequence is a RNA transcript, such as a RNAi, or a polypeptide, such as a functional protein.

61. The present invention provides a Method according to any one of items 1 to 5, wherein said molecule improving the therapeutic potential activity or said population of CAR+TCR- primary immune cells, confers resistance of the CAR+TCR- immune cells to a drug, increases persistence of the CAR+TCR- immune cells (in-vivo or in-vitro) or its safety.

62. The present invention provides a Method according to item 6, wherein said molecule enhancing the persistence of the CAR+TCR- primary immune cells is selected from a cytokine receptor, a protein conferring resistance to a drug or a secreted antibody directed against inhibitory peptides or proteins.

63. The present invention provides a Method according to any one of item1 to 6, wherein said exogenous coding sequence encodes an IL-2, IL-12 or IL-15 receptor.

64. The present invention provides a Method according to any one of items 1 to 6, wherein said exogenous coding sequence conferring drug resistance encodes dihydrofolate reductase (DHFR), inosine monophosphate dehydrogenase 2 (IMPDH2), calcineurin or methylguanine transferase (MGMT), mTORmut and Lckmut.

65. The present invention provides a Method according to any one of items 1 to 6, wherein said exogenous sequence encodes a chemokine or a cytokine, such as IL-2, IL-12 and IL-15.

66. The present invention provides a Method according any one of items 1 to 6, wherein said exogenous sequence enhancing the therapeutic activity encodes an inhibitor of FOXP3.

67. The present invention provides a Method according to any one of items 1 to 6, wherein said exogenous sequence enhancing the therapeutic activity of the CAR+TCR- primary T-cell encodes a secreted inhibitor of Tumor Associated Macrophages (TAM), such as a CCR2/CCL2 neutralization agent.

68. The present invention provides a Method according to any one of items 1 to 6, wherein said exogenous sequence enhancing the safety of the CAR+TCR- primary immune cell encodes a component of a chimeric antigen receptor (CAR).

69. The present invention provides a Method according to item13, wherein said CAR is an inhibitory CAR that contributes to an improved specificity of the CAR+TCR- immune cell against a given cell type.

70. The present invention provides a Method according to any one of items 1 to 6, wherein said exogenous sequence enhancing the safety of the CAR+TCR- primary immune cell encodes a factor that has the capability to kill the cell, directly, in combination with, or by activating other compound(s).

71. The present invention provides a Method according to any one of items 1 to 6, wherein said exogenous sequence enhancing the safety of the CAR+TCR- primary immune cell encodes a component of an apoptosis CAR.

72. The present invention provides a Method according to item16, wherein said apoptosis CAR comprises FasL (CD95).

73. The present invention provides a Method according to any one of items 1 to 6, wherein said exogenous sequence enhancing the safety of the CAR+TCR- primary immune cell encodes cytochrome(s) P450, CYP2D6-1, CYP2D6-2, CYP2C9, CYP3A4, CYP2C19 or CYP1A2, conferring hypersensitivity of said immune cells to a drug, such as cyclophosphamide and/or isophosphamide.

The present invention provides a Method according to any one of items 1 to 6, wherein said exogenous sequence enhancing the safety of the CAR+TCR- primary immune cell encodes cy-

tochrome(s) P450, CYP2D6-1, CYP2D6-2, CYP2C9, CYP3A4, CYP2C19 or CYP1A2, conferring hypersensitivity of said immune cells to a drug, such as cyclophosphamide and/or isophosphamide combined to the drug to which said cell is hypersensitive.

74. The present invention provides a Method according to any one of claims 1 to 18, wherein said gene is under transcriptional control of an endogenous promoter that is constantly active during CAR+TCR- immune cell activation.

75. The present invention provides a Method according to item 19, wherein said gene is selected from CD3G, Rn28s1, Rn18s, Rn7sk, Actg1, β2m, Rpl18a, Pabpc1, Gapdh, Rpl17, Rpl19, Rplp0, Cfl1 and Pfn1.

76. The present invention provides a Method according to item 19, wherein the transcriptional activity of said endogenous promoter is stable and independent from immune cell activation.

77. The present invention provides a Method according to item 21, wherein said gene under control of said endogenous promoter stable and independent from CAR+TCR- immune cell activation is CD3.

78. The present invention provides a Method according to item 22, wherein said sequence introduced into said gene encodes a TCR binding domain, optionally in fusion with a polypeptide CD3, CD28 or 4-1BB.

79. The present invention provides a Method according to item21, wherein said coding sequence introduced into said gene under control of said endogenous promoter with an activity that is stable and independent from CAR+TCR- immune cell activation, encodes a cytokine, a chemokine receptor, a molecule conferring resistance to a product, a co-stimulation ligand, such as 4-1BRL and OX40L, or a secreted antibody.

80. The present invention provides a Method according to any one of items 1 to 18, wherein the transcriptional activity of said endogenous promoter is dependent from immune cell activation.

81. The present invention provides a Method according to item 25, wherein said transcriptional activity of said endogenous promoter is transient upon CAR+TCR- immune cell activation.

82. The present invention provides a Method according to item 25, wherein said transcriptional activity of said endogenous promoter is up-regulated.

83. The present invention provides a Method according to item27, wherein said transcriptional activity is strongly up-regulated.

84. The present invention provides a Method according to item28, wherein said exogenous sequence introduced into said gene whose transcriptional activity is up regulated more particularly encodes cytokine(s), immunogenic peptide(s) or a secreted antibody, such as an anti-IDO1, anti-IL10, anti-PD1, anti-PDL1, anti-IL6 or anti-PGE2 antibody.

85. The present invention provides a Method according to item27, wherein said transcriptional activity is weakly up-regulated.

86. The present invention provides a Method according to item30, wherein said sequence introduced into said gene whose transcriptional activity is transient up regulated more particularly encodes a constituent of an inhibitory CAR or an apoptotic CAR, to improve the specificity of the safety of said immune cells.

87. The present invention provides a Method according to item26, wherein said promoter is up-regulated over less than 12 hours upon immune cell activation.

Up regulated means that the gene expression is increased by at least a factor 2 as compared to a non activated T cellls

88. The present invention provides a Method according to item 32, wherein said gene is selected from *Spata6, Itga6, Rcbtb2, Cd1d1, St8sia4, Itgae* and *Fam214a*.

89. The present invention provides a Method according to item 26, wherein said promoter is up-regulated over less than 24 hours upon immune cell activation.

90. The present invention provides a Method according to item 34, wherein said gene is selected from *IL3, IL2, Ccl4, IL21, Gp49a, Nr4a3, Lilrb4, Cd200, Cdkn1a, Gzmc, Nr4a2, Cish, Ccr8, Lad1* and *Crabp2*.

91. The present invention provides a Method according to item26, wherein said gene is up-regulated over more than 24 hours upon CAR+TCR- immune cell activation.

92. he present invention provides a Method according to item36, wherein said gene is selected from *Gzmb, Tbx21, Plek, Chek1, Slamf7, Zbtb32, Tigit, Lag3, Gzma, Wee1, IL12rb2, Eea1* and *Dtl*.

93. The present invention provides a Method according to any one of items 1 to 37, wherein a modified TCR is being independently expressed in the transfected immune cells.

94. The present invention provides a Method according to item 42, wherein said CAR is directed against a CD22 antigen.

95. The present invention provides a Method according to item 38 or item39, wherein said endogenous promoter activity is dependent on said CAR expressed into the transfected immune cells.

96. The present invention provides a Method according to any one of items 1 to 40, wherein said specific endonuclease reagent is selected from a RNA or DNA-guided endonuclease, such as Cas9 or Cpf1, a RNA or DNA guide, a TAL-endonuclease, a zing finger nuclease, a homing endonuclease or any combination thereof.

97. The present invention provides a Method according to any one of items 1 to 41, wherein said specific endonuclease reagent is introduced by electroporation as a polypeptide or under a mRNA, which is translated into the cell.

98. The present invention provides a Method according to item 42, wherein said exogenous nucleic acid comprising said coding sequence is included in a DNA vector.

99. The present invention provides a Method according to item 43, wherein said DNA vector is a viral vector such as an AAV vector.

100. The present invention provides a Method according to item 43, wherein the nucleic acid encoding said sequence-specific endonuclease reagent and said exogenous nucleic acid are both included into said DNA vector.

101. The present invention provides a Method according to any one of items 1 to 45, wherein the gene sequence that is introduced into the CAR+TCR- immune cell is preceded or followed by a sequence encoding a 2A peptide to enable the transcription of said gene sequence along with at least one part of the endogenous gene.

102. The present invention provides a Method according to any one of items 1 to 46, wherein the gene sequence is introduced with the effect of inactivating the expression of at least one endogenous genomic sequence initially present in said gene.

103. The present invention provides a Method according to any one of items 1 to 47, wherein said endogenous promoter does not control the transcriptional activity of a TCR gene.

104. The present invention provides a Method according to item 47, wherein said endogenous genomic sequence that is being inactivated encodes suppressive cytokines, kinases or their receptors thereof, such as TGFb, TGFbR, IL-10, IL-10R, GCN2 or PRDM1.

105. The present invention provides a Method according to item 49, wherein said endogenous genomic sequence that is being inactivated encodes a protein acting as an immune checkpoint, such as PD1, PDL1, CTLA4, TIM3 or LAG3.

106. The present invention provides a Method according to item 47, wherein said endogenous genomic sequence that is being inactivated expresses an enzyme that activates a prodrug, such as DCK, HPRT or GGH.

The present invention provides a Method according to item 47, wherein said endogenous genomic sequence that is being inactivated is CD25, CD95 or PD1.

107. The present invention provides a Method according to item 47, wherein said endogenous genomic sequence that is being inactivated expresses a receptor to immune depletion treatments, such a Glucocorticoid receptors and CD52.

108. The present invention provides a Method according to item 47, wherein said endogenous genomic sequence that is being inactivated expresses a surface antigen which has an affinity with a CAR expressed by said CAR+TCR- immune cell or another CAR+TCR- immune cell from said population of immune cells.

109. The present invention provides a Method according to any one of items 1 to 53, wherein said CAR+TCR- immune cell is a hematopoietic stem cell HSC.

110. The present invention provides a Method according to any one of items 1 to 53, wherein said immune cell is a CAR+TCR- T-cell or CAR+TCR- NKT cell.

111. The present invention provides an engineered primary immune cell obtainable by the method of any of items 1 to 55.

112. The present invention provides an engineered primary immune CAR+TCR- cell, which comprises an exogenous coding sequence under transcriptional control of an endogenous gene promoter.

113. The present invention provides an engineered primary immune CAR+TCR- cell according to item59 or 60, wherein said endogenous gene promoter is active during the activation of said immune cell.

114. The present invention provides An engineered primary immune CAR+TCR- cell according to any one items 59 to 61, wherein said endogenous gene promoter is responsive to the activation of said immune cell, preferably up-regulated.

115. The present invention provides an engineered primary immune CAR+TCR- cell according to any one items 59 to 62, wherein said endogenous gene is selected among those listed herein below.

116. The present invention provides an engineered primary immune cell according to any one items 59 to 63, wherein said primary immune cell is a T-cell or a NKT-cell.

117. The present invention provides an engineered primary immune CAR+TCR- cell according to any one items 59 to 64, wherein said primary cell is endowed with a second chimeric antigen receptor

118. The present invention provides an engineered primary immune CAR+TCR- cell according to item 65, wherein said transcriptional control of said endogenous gene is responsive to the signal activation of said chimeric antigen receptor (CAR).

119. The present invention provides a therapeutically effective population of primary immune cells, comprising at least 30 %, preferably 50 %, more preferably 80 % of cells according to any one of items 59 to 66.

120. The present invention provides a population of primary immune cells according to item64, wherein at least 30 %, preferably 50 %, more preferably 80 % of cells originate from a donor, preferably one single donor.

121. The present invention provides a population of primary immune CAR+TCR- cells according to item64, wherein more than 50% of said immune cells are TCR negative CAR positive T-cells.

122. The present invention provides a population of primary immune CAR+TCR- cells according to any one of items 64 to 66, wherein more than 50% of said immune cells are CAR positive and TCR negative cells and comprise another inactivated gene comprising a coding sequence, preferably a coding sequence coding for IL-7, IL-12, IL-15, IL-21 or il-IL-27.

123. The present invention provides a pharmaceutical composition comprising an engineered primary immune CAR+TCR- cell or immune CAR+TCR- cell population according to any one of the above items 56 to 67.

124. The present invention provides a method for treating a patient in need thereof, wherein said method comprises:

preparing a population of TCR-CAR- engineered primary immune CAR+TCR- cells according to any one of items 56 to 67;

optionally, purifying or sorting said engineered primary immune CAR+TCR- cells;

activating said population of engineered primary immune CAR+TCR- cells upon or after infusion of said cells into said patient.

125. The present invention provides a method according to item 69, wherein said patient is treated for cancer.

126. The present invention provides a method according to item 69, wherein said patient is treated for an infection.

In particular embodiments, said engineered primary human cells of the invention temporarily expressed a TALEN specific for at least one genomic sequence that introduced a genetic modification into the TRAC gene and/or into another genomic sequence. Preferably human cells temporarily expressed a TALEN specific for the TRAC gene and/or an endonuclease such as a TALEN, a CRISPR/CAS9, meganuclease, Zn Finger nuclease, specific for in another genomic sequence. More preferably, human cells temporarily expressed a TALEN specific for the sequence TTGTCCCACAGATATCCagaaccctgaccctgCCGTGTACCAGCTGAGAGA of the TRAC gene and/or another TALEN specific for another genomic sequence selected from a genomic sequence coding a T cell antigen such as CD38, CS-1, CLL-1, CD70, a genomic sequence coding a molecule (or immune checkpoint) selected from CTLA4, PPP2CA, PPP2CB, PTPN6, PTPN22, PDCD1, LAG 3, HAVCR2, BTLA, CD160, TIGIT, CD96, CRTAM, LAIR1, SIGLEC7, SIGLEC9, CD244, TNFRSF10B, TNFRSF10A, CASP8,

CASP10, CASP3, CASP6, CASP7, FADD, FAS, TGFBRII, TGFBRI, SMAD2, SMAD3, SMAD4, SMAD10, SKI, SKIL, TGIF1, IL10RA, IL10RB, HMOX2, IL6R, IL6ST, CSK, PAG1, SIT1, FOXP3, PRDM1, BATF, GUCY1A2, GUCY1A3, GUCY1B2, GUCY1B3, SERCA3, IL27RA, STAT1, STAT3, ILT2 ILT4, SEMA7A, SHARPIN, STAT1, PEA15, RICTOR, JAK2, AURKA, DNMT3, miRNA31, MT1A, MT2A, PTGER2, a gene generating a miR101, mir26A, mir21.

PD1, PDL1, CTLA-4, TIM3, LAG3, TNFα or IFNγ may be preferred gene(s) engineered into the cells of the present invention.

Immune checkpoints are molecules that either turn up a signal (co-stimulatory molecules) or turn down a signal, said signal is involved in the functioning of immune cells, in particular the functioning of immune cells against cancer cells or against the connective tissue in solid cancer.

In particular embodiments, the human cell of the present invention may be a human cell line, a primary human cell line, a primary human cell line means derived from one human primary cell or from a homogenous population of human primary cells.

In general, the human cell of the present invention or the population of human cells of the present invention for used in immunotherapy comprises no or undetectable off target cleavage(s) in the genome.

In particular embodiments, off sites may be determined using an adapted GUIDE-Seq analysis for each of the TALEN moiety and/or for TALEN used to engineered human cells.

In particular embodiments, off sites may be determined using an adapted GUIDE-Seq analysis for each of the TALEN moiety and/or for TALEN used to engineered human cells and compared to off targets induced by another endonuclease.

In particular embodiments, off sites may be determined using an adapted GUIDE-Seq analysis for meganuclease(s) used to engineered human cells.

In particular embodiments, off sites may be determined using an adapted GUIDE-Seq analysis for megaTAL used to engineered human cells.

In particular embodiments, off sites may be determined using an adapted GUIDE-Seq analysis for Zn Finger(s) used to engineered human cells.

In particular embodiments, off sites may be determined using an adapted GUIDE-Seq analysis for Crispr/cas 9 used to engineered human cells.

Off target sites determination may be as determined for example using guide-seq analysis as described and adapted to TALEN (as in https://www.cellectis.com/uploads/files/ASGCT_2017.pdf).

Guide – sequence (or guide-seq) analysis was first described in GUIDE-sequences enables genome-wide profiling of off-target cleavage by CRISPR-Cas nucleases.

Tsai SQ, Zheng Z, Nguyen NT, Liebers M, Topkar VV, Thapar V, Wyvekens N, Khayter C, Iafrate AJ, Le LP, Aryee MJ, Joung JK. Nat Biotechnol. 2015 Feb;33(2):187-197. doi: 10.1038/nbt.3117. Epub 2014 Dec 16. and used as described in https://www.cellectis.com/uploads/files/ASGCT_2017.pdf adapted version to TALEN).

A GUIDE-Seq read count or GUIDE-Seq score for a given site by a given endonuclease, represents a quantitative measurement of the cleavage efficiency of that sequence by an RNA or protein guided nuclease. Preferably the RNA or protein guided nuclease of the invention affecting the TRAC gene have a score near zero (below detection threshold or undetectable).

The invention further provides a human cell which is an endonuclease-modified endogenous αβ-TCR negative human cell, preferably a TALEN- modified endogenous αβ-TCR negative human cell as in any of the preceding embodiments wherein said endonuclease-modified endogenous αβ-TCR negative human cell, or TALEN modified endogenous αβ–TCR negative human cell can be expanded at least over 10x in a period of 12 days.

The invention further provides a human cell which is an endonuclease-modified endogenous

αβ-TCR negative human cell, preferably a TALEN- modified endogenous αβ-TCR negative human cell as in any of the preceding embodiments wherein said endonuclease-modified endogenous αβ-TCR negative human cell, or TALEN modified endogenous αβ-TCR negative human cell can be expanded without clonal expansion.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to the above further comprising undetectable level of MHC molecules as compared to an unmodified (non engineered) control cell and a deletion functionally affecting cell surface expression of a beta 2 microglobulin molecule.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to the above further comprising undetectable level of MHC molecules as compared to an unmodified (non engineered) control cell and a genomic deletion, a mutation or an insertion affecting the expression of a CIITA molecule, preferably a CIITA molecule of Gene ID: 4261.

The present invention provides the TALEN-modified endogenous αβ-TCR negative human cell according to the above wherein said insertion resulted in an inactivation of the gene coding the TCR alpha and an undetectable cell surface expression of endogenous αβ-TCR in at least 96 % of the cells, in at least 97% of the cells, in at least 98% of the cells, or in at least 99% of the cells.

Inactivation of the TRAC gene means that cells with an inactivated TRAC gene have undetectable level of cell surface TCR alpha, preferably due to a disruption of the TRAC gene, more preferably a disruption of the sequence coding for an extracellular domain and/or transmembrane domain of the TRC alpha subunit of the alpha beta TCR, and therefore no extracellular alpha beta TCR can be addressed to the membrane, resulting in an undetectable level of cell surface TCR alpha beta as compared to a positive control.

In one embodiment, cell surface expression may be measured by flow cytometry using a monoclonal anti human alpha beta TCR, a control isotype antibody.

In addition, human cells (engineered human cells) of the invention express a detectable level

of the product encoded by the gene(s) inserted into the TRAC, (unless the expression is a conditional expression controlled by a promotor, itself activated by a protein or a drug), for example of a CAR or a TCR.

The present invention provides the TALEN-modified endogenous αβ-TCR negative human cell according to the above wherein said insertion resulted in an inactivation of the gene coding the TCR alpha and an undetectable cell surface expression of endogenous αβ-TCR in at least 96 % of the cells, in at least 97% of the cells, in at least 98% of the cells, in at least 99% of the cells as determined by flow cytometry 4 days after introduction of said TALEN cutting said TRAC into said human cells.

In particular embodiments, a TALEN-modified endogenous αβ-TCR negative human cell according to the above comprises a TALEN binding domain and/or a sequence upstream a TALEN binding domain present in the wild type (wt) TRAC gene, preferably said TALEN binding domain in the wt TRAC gene has the following sequence :TTGTCCCACAGATATCCagaaccctgaccctgCCGTGTACCAGCTGAGAGA.

In particular embodiments, the engineered TRAC gene may contain any one of the following of sequence TTGTCCCACAGATATCC,

TTGTCCCACAGATATCCa

TTGTCCCACAGATATCCag

TTGTCCCACAGATATCCaga

TTGTCCCACAGATATCCagaa

TTGTCCCACAGATATCCagaac

TTGTCCCACAGATATCCagaacc

TTGTCCCACAGATATCCagaaccc

TTGTCCCACAGATATCCagaaccct

TTGTCCCACAGATATCCagaaccctg

TTGTCCCACAGATATCCagaaccctga

TTGTCCCACAGATATCCagaaccctgac

TTGTCCCACAGATATCCagaaccctgacc

TTGTCCCACAGATATCCagaaccctgaccc

TTGTCCCACAGATATCCagaaccctgaccct

TTGTCCCACAGATATCCagaaccctgaccctg,

The present invention encompasses a TALEN-modified endogenous αβ-TCR negative human cell obtained using any rare cutting endonuclease binding to ttgtcccacagATATC, or to ttgtcccacagATATCCAG, preferably a TALEN as described in WO2014184741.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell according to any one of the above, obtained using a TALEN binding to ttgtcccacagATATC, or to ttgtcccacagATATCCAG or to a sequence having at least from 93,75 %, 87.5%, 81.25%, 75%, 68.75% 62.5% 56.25% 5% 43.75% 37.5% 31.25% 25% 18.75% 12.5% 6.25% identity with ttgtcccacagATATC, or with ttgtcccacagATATCCAG, provided that said TALEN cleaves the TRAC gene inducing an inactivation of the TCR alpha gene, undetectable cell surface expression of the TCR alpha beta , and off target sites for said TALEN below detection using a guide-seq method).

The present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell according to any one of the above, wherein said at least one insertion comprises an exogenous polynucleotide sequence located downstream

any one of the following of sequence TTGTCCCACAGATATCC,

TTGTCCCACAGATATCCa

TTGTCCCACAGATATCCag

TTGTCCCACAGATATCCaga

TTGTCCCACAGATATCCagaa

TTGTCCCACAGATATCCagaac

TTGTCCCACAGATATCCagaacc

TTGTCCCACAGATATCCagaaccc

TTGTCCCACAGATATCCagaaccct

TTGTCCCACAGATATCCagaaccctg

TTGTCCCACAGATATCCagaaccctga

TTGTCCCACAGATATCCagaaccctgac

TTGTCCCACAGATATCCagaaccctgacc

TTGTCCCACAGATATCCagaaccctgaccc

TTGTCCCACAGATATCCagaaccctgaccct

TTGTCCCACAGATATCCagaaccctgaccctg,

The present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell according to any one of the above, wherein said at least one insertion comprises an exogenous polynucleotide sequence located downstream any one of the following of sequence

TTGTCCCACAGATATCC,

TTGTCCCACAGATATCCa

TTGTCCCACAGATATCCag

TTGTCCCACAGATATCCaga

TTGTCCCACAGATATCCagaa

TTGTCCCACAGATATCCagaac

TTGTCCCACAGATATCCagaacc

TTGTCCCACAGATATCCagaaccc

TTGTCCCACAGATATCCagaaccct

TTGTCCCACAGATATCCagaaccctg

TTGTCCCACAGATATCCagaaccctga

TTGTCCCACAGATATCCagaaccctgac

TTGTCCCACAGATATCCagaaccctgacc

TTGTCCCACAGATATCCagaaccctgaccc

TTGTCCCACAGATATCCagaaccctgaccct, and

TTGTCCCACAGATATCCagaaccctgaccctg,

and upstream any one of the following sequence CCGTGTACCAGCTGAGAGA,

gCCGTGTACCAGCTGAGAGA,

tgCCGTGTACCAGCTGAGAGA,

ctgCCGTGTACCAGCTGAGAGA,

cctgCCGTGTACCAGCTGAGAGA,

ccctgCCGTGTACCAGCTGAGAGA,

accctgCCGTGTACCAGCTGAGAGA,

gaccctgCCGTGTACCAGCTGAGAGA,

tgaccctgCCGTGTACCAGCTGAGAGA,

ctgaccctgCCGTGTACCAGCTGAGAGA,

cctgaccctgCCGTGTACCAGCTGAGAGA,

ccctgaccctgCCGTGTACCAGCTGAGAGA,

accctgaccctgCCGTGTACCAGCTGAGAGA,

aaccctgaccctgCCGTGTACCAGCTGAGAGA,

gaaccctgaccctgCCGTGTACCAGCTGAGAGA, and

agaaccctgaccctgCCGTGTACCAGCTGAGAGA.

A means according to the present invention allows the detection of any one the sequences below.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell according to any one of the above wherein said at least one insertion comprises a sequence encoding a self-cleaving peptide in frame with the genomic TRAC coding sequence selected from a 2A peptide, a 2A like peptide, a P2A peptide, a E2A peptide, a F2A peptide, preferably a 2A peptide, more preferably a 2A peptide of sequence GSGEGRGSLLTCGDVEENPGP,

GSGATNFSLLKQAGDVEENPGP,

GSGQCTNYALLKLAGDVESNPGP,

GSGVKQTLNFDLLKLAGDVESNPGP,

SGEGRGSLLTCGDVEENPGP,

SGATNFSLLKQAGDVEENPGP,

SGQCTNYALLKLAGDVESNPGP,

SGVKQTLNFDLLKLAGDVESNPGP, even more preferably a 2A peptide of sequence SGEGRGSLLTCGDVEENPGP.

Any polynucleotide sequence encoding a 2A peptide, preferably 2A peptide of sequence SGEGRGSLLTCGDVEENPGP may be inserted into the genomic TRAC gene of a cell according to the present invention.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell according to any one of the above, wherein said at least one insertion comprises a sequence coding a 2A peptide, of sequence SGEGRGSLLTCGDVEENPGP in frame with the genomic TRAC coding sequence, an exogenous polynucleotide sequence coding a chimeric antigen receptor, a terminator sequence (a terminator sequence of polyadenylation signal), optionally a TALEN binding domain.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human primary cell according to any one of the above, wherein said exogenous polynucleotide sequence comprises a chimeric antigen receptor (CAR) ,specific for at least one of the following antigen : CD19, CD123, CD20, CD22, CD38, CD30, CS-1, CLL-1, HSP70, BCMA, VEGF, DR4, GD2, the cancer testis (CT) antigens, MUC1, GD2, o acetyl GD2, HM1.24 (CD317), CYP1B1, SP17, PRAME, Wilms' Tumour 1 (WT1), heat shock protein gp96, thyroid stimulating hormone receptor (TSHR); CD171 ; CS- 1 (CD2 subset 1 , CRACC, SLAMF7, CD319, and 19A24); C-type lectin-like molecule- 1 (CLL- 1); ganglioside GD3 (aNeu5Ac(2- 8)aNeu5Ac(2-3)bDGalp(] -4)bDGlcp(l-l)Cer); Tn antigen (Tn Ag); Fms-Like Tyrosine Kinase 3 (FLT3); CD38; CD44v6; B7H3 (CD276); KIT (CD117); Interleukin- 13 receptor subunit alpha-2 (IL- 13Ra2); Interleukin 11 receptor alpha (IL- l lRa); prostate stem cell antigen (PSCA); Protease Serine 21 (PRSS21); vascular endothelial growth factor receptor 2 (VEGFR2); Lewis(Y) antigen; CD24; Platelet-derived growth factor receptor beta (PDGFR-beta); stage-specific embryonic antigen-4 (SSEA-

4); Mucin 1, cell surface associated (MUC1); epidermal growth factor receptor (EGFR); neural cell adhesion molecule (NCAM); carbonic anhydrase IX (CAIX); Proteasome (Prosome, Macropain) Subunit, Beta Type, 9 (LMP2); ephrin type-A receptor 2 (EphA2); Fucosyl GM1 ; sialyl Lewis adhesion molecule (sLe); ganglioside GM3 (aNeu5Ac(2-3)bDGalp(l -4)bDGlcp(l - l)Cer; TGS5 ; high molecular weight- melanoma-associated antigen (HMWMAA); o-acetyl- GD2 ganglioside (OAcGD2); Folate receptor beta; tumor endothelial marker 1 (TEM1/CD248); tumor endothelial marker 7-related (TEM7R); claudin 6 (CLDN6); G protein-coupled receptor class C group 5, member D (GPRC5D); chromosome X open reading frame 61 (CXORF61); CD97; CD179a; anaplastic lymphoma kinase (ALK); Polysialic acid; placenta-specific 1 (PLAC1); hexasaccharide portion of globoH glycoceramide (GloboH); mammary gland differentiation antigen (NY-BR- 1); uroplakin 2 (UPK2); Hepatitis A virus cellular receptor 1 (HAVCR1); adrenoceptor beta 3 (ADRB3); pannexin 3 (PANX3); G protein-coupled receptor 20 (GPR20); lymphocyte antigen 6 complex, locus K 9 (LY6K); Olfactory receptor 51E2 (OR51E2); TCR Gamma Alternate Reading Frame Protein (TARP); Wilms tumor protein (WT1); ETS translocation-variant gene 6, located on chromosome 12p (ETV6-AML); sperm protein 17 (SPA17); X Antigen Family, Member 1A (XAGE1); angiopoietin-binding cell surface receptor 2 (Tie 2); melanoma cancer testis antigen- 1 (MAD-CT-1); melanoma cancer testis antigen-2 (MAD- CT-2); Fos-related antigen 1 ; p53 mutant; human Telomerase reverse transcriptase (hTERT); sarcoma translocation breakpoints; melanoma inhibitor of apoptosis (ML-IAP); ERG (transmembrane protease, serine 2 (TMPRSS2) ETS fusion gene); N- Acetyl glucosaminyl-transferase V (NA17); paired box protein Pax-3 (PAX3); Androgen receptor; Cyclin B 1 ; v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog (MYCN); Ras Homolog Family Member C (RhoC); Cytochrome P450 1B 1 (CYP1B 1); CCCTC-Binding Factor (Zinc Finger Protein)-Like (BORIS); Squamous Cell Carcinoma Antigen Recognized By T Cells 3 (SART3); Paired box protein Pax-5 (PAX5); proacrosin binding protein sp32 (OY-TES 1); lymphocyte-specific protein tyrosine kinase (LCK); A kinase anchor protein 4 (AKAP-4); synovial sarcoma, X breakpoint 2 (SSX2); CD79a; CD79b; CD72; Leukocyte- associated immunoglobulin-like receptor 1 (LAIR1); Fc fragment of IgA receptor (FCAR); Leukocyte

immunoglobulin-like receptor subfamily A member 2 (LILRA2); CD300 molecule-like family member f (CD300LF); C-type lectin domain family 12 member A (CLEC12A); bone marrow stromal cell antigen 2 (BST2); EGF-like module-containing mucin-like hormone receptor- like 2 (EMR2); lymphocyte antigen 75 (LY75); Glypican-3 (GPC3); Fc receptor-like 5 (FCRL5); and immunoglobulin lambda- like polypeptide 1 (IGLL1) and a combination thereof.

The CAR may preferably be a CAR selected from the list consisting of a CAR specific for CD19, CD123, CD20, CD22, CD38, CD30, CS-1, CLL-1, HSP70, BCMA, VEGF, DR4, GD2, O-acethyl GD2, the cancer testis (CT) antigens, MUC1, MUC16, HM1.24 (CD317), CYP1B1, SP17, PRAME, Wilms' tumour 1 (WT1), heat shock protein gp96, claudine18.2, CEA, FAP - HER2 - CD79a, CD79b and a combination thereof.

The CAR may be more preferably a CAR selected from the list consisting of a CAR specific for CD123, CD22, CD38, CD30, CS-1, CLL-1, HSP70, BCMA, GD2, O-acethyl GD2, MUC1, FAP, HER2, CD79a, CD79b and a combination thereof.

In a preferred embodiment, the CAR is directed against one of the following target antigen mesothelin FRα, L1-CAM, CAIX, GD2, O-acethyl GD2, FAP, Lewis Y, EGFRvIII, HER2, CD20, PSMA, kLC, CD30, CEA, FAP - HER2 - CD79a, CD79b.

In another preferred embodiment, the CAR is directed against one of the following target: α-folate receptor (FRα); L1-cell adhesion molecule (L1-CAM); carboxy-anhydrase-IX (CAIX,); Fibroblast activation protein (FAP), human epidermal growth factor receptor 2 (HER2); carcinoembryonic antigen (CEA); Prostate Specific Membrane Antigen (PSMA); CD79a, CD79b, CD20 or CD268, C type lectin domain family 14 member A; also EGFR5 (CLEC14a), Epithelial cell adhesion molecule (EPCAM), Liv-1, or Zinc transporter LIV-1 (SLC39A6), Cholinergic Receptor Nicotinic Alpha 2 Subunit (CHRNA2), A Disintegrin and metalloproteinase domain-containing protein 10, (ADAM10) or CDw156 or CD156cADAM10, Delta -like 3 (DLL3), C type lectin domain family 14 member A; also EGFR5, (CLEC14a).

CLEC14a is a 51 kDa (predicted) member of the C type lectin domain family of proteins. It is a type transmembrane protein, expressed in brain. Mature human CLEC14A is 469 amino acids in length.

In a more preferred embodiment, the CAR is directed against one of the following target antigen CD123, CD22, CD30, CLL-1, CS-1, O-Acetyl GD2, FAP, HER2, CD79a, CD79b.

On a more more preferred embodiment the CAR is directed against one of the following target antigen CD123, CD22, CD30, CLL-1, CS-1, O-Acetyl GD2, FAP, HER2, CD79a, CD79b expressed (or over expressed on tumor cells. This is achieved by using specific scfv domains, in the present invention.

The CAR molecule of the invention comprises an antigen binding domain, preferably a scfv, a transmembrane domain, and an intracellular domain comprising a costimulatory domain and/or a primary signaling domain.

The CAR molecule of the invention comprises an antigen binding domain wherein said antigen binding domain binds to the tumor antigen associated with a disease, and said tumor antigen is selected from a group consisting of: CD19 molecule (CD19); membrane spanning 4-domains A1 (MS4A1 also known as CD20); CD22 molecule (CD22); CD24 molecule (CD24); CD248 molecule (CD248); CD276 molecule (CD276 or B7H3); CD33 molecule (CD33); CD38 molecule (CD38); CD44v6; CD70 molecule (CD70); CD72; CD79a; CD79b; interleukin 3 receptor subunit alpha (IL3RA also known as CD123); TNF receptor superfamily member 8 (TNFRSF8 also known as CD30); KIT proto-oncogene receptor tyrosine kinase (CD117); V-set pre-B cell surrogate light chain 1 (VPREB1 or CD179a); adhesion G protein-coupled receptor E5 (ADGRE5 or CD97); TNF receptor superfamily member 17 (TNFRSF17 also known as BCMA); SLAM family member 7 (SLAMF7 also known as CS1); L1 cell adhesion molecule (L1CAM); C-type lectin domain family 12 member A (CLEC12A also known as CLL-1); tumor-specific variant of the epidermal growth factor receptor (EGFRvIII); thyroid stimulating hormone receptor (TSHR); Fms related tyrosine kinase 3 (FLT3); ganglioside GD3 (GD3); Tn antigen (Tn Ag); lymphocyte antigen 6 family member G6D (LY6G6D); Delta like canonical Notch ligand 3 (DLL3);

Interleukin- 13 receptor subunit alpha-2 (IL-13RA2); Interleukin 11 receptor subunit alpha (IL11RA); mesothelin (MSLN); Receptor tyrosine kinase like orphan receptor 1 (ROR1); Prostate stem cell antigen (PSCA); erb-b2 receptor tyrosine kinase 2 (ERBB2 or Her2/neu); Protease Serine 21 (PRSS21); Kinase insert domain receptor (KDR also known as VEGFR2); Lewis y antigen (LewisY); Solute carrier family 39 member 6 (SLC39A6); Fibroblast activation protein alpha (FAP); Hsp70 family chaperone (HSP70); Platelet-derived growth factor receptor beta (PDGFR-beta); Cholinergic receptor nicotinic alpha 2 subunit (CHRNA2); Stage-Specific Embryonic Antigen-4 (SSEA-4); Mucin 1, cell surface associated (MUC1); mucin 16, cell surface associated (MUC16); claudin 18 (CLDN18); claudin 6 (CLDN6); Epidermal Growth Factor Receptor (EGFR); Preferentially expressed antigen in melanoma (PRAME); Neural Cell Adhesion Molecule (NCAM); ADAM metallopeptidase domain 10 (ADAM10); Folate receptor 1 (FOLR1); Folate receptor beta (FOLR2); Carbonic Anhydrase IX (CA9); Proteasome subunit beta 9 (PSMB9 or LMP2); Ephrin receptor A2 (EphA2); Tetraspanin 10 (TSPAN10); Fucosyl GM1 (Fuc-GM1); sialyl Lewis adhesion molecule (sLe); TGS5 ; high molecular weight- melanoma-associated antigen (HMWMAA); o-acetyl- GD2 ganglioside (OAcGD2); tumor endothelial marker 7-related (TEM7R); G protein-coupled receptor class C group 5, member D (GPRC5D); chromosome X open reading frame 61 (CXORF61); ALK receptor tyrosine kinase (ALK); Polysialic acid; Placenta-specific 1 (PLAC1); hexasaccharide portion of globoH glycoceramide (GloboH); NY-BR-1 antigen; uroplakin 2 (UPK2); Hepatitis A virus cellular receptor 1 (HAVCR1); adrenoceptor beta 3 (ADRB3); pannexin 3 (PANX3); G protein-coupled receptor 20 (GPR20); lymphocyte antigen 6 family member K (LY6K); olfactory receptor family 51 subfamily E member 2 (OR51E2); TCR Gamma Alternate Reading Frame Protein (TARP); Wilms tumor protein (WT1); ETV6-AML1 fusion protein due to 12;21 chromosomal translocation (ETV6-AML1); sperm autoantigenic protein 17 (SPA17); X Antigen Family, Member 1E (XAGE1E); TEK receptor tyrosine kinase (Tie2); melanoma cancer testis antigen- 1 (MAD-CT-1); melanoma cancer testis antigen-2 (MAD-CT-2); Fos-related antigen 1 ; p53 mutant; human Telomerase reverse transcriptase (hTERT); sarcoma translocation breakpoints; melanoma inhibitor of apoptosis (ML-IAP); ERG (transmembrane protease, serine 2 (TMPRSS2) ETS fusion gene); N- Acetyl

glucosaminyl-transferase V (NA17); paired box protein Pax-3 (PAX3); Androgen receptor; Cyclin B 1 ; v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog (MYCN); Ras Homolog Family Member C (RhoC); Cytochrome P450 1B 1 (CYP1B 1); CCCTC-Binding Factor (Zinc Finger Protein)-Like (BORIS); Squamous Cell Carcinoma Antigen Recognized By T Cells 3 (SART3); Paired box protein Pax-5 (PAX5); proacrosin binding protein sp32 (OY-TES 1); lymphocyte-specific protein tyrosine kinase (LCK); A kinase anchor protein 4 (AKAP-4); synovial sarcoma, X breakpoint 2 (SSX2); Leukocyte- associated immunoglobulin-like receptor 1 (LAIR1); Fc fragment of IgA receptor (FCAR); Leukocyte immunoglobulin-like receptor subfamily A member 2 (LILRA2); CD300 molecule-like family member f (CD300LF);; bone marrow stromal cell antigen 2 (BST2); EGF-like module-containing mucin-like hormone receptor- like 2 (EMR2); lymphocyte antigen 75 (LY75); Glypican-3 (GPC3); Fc receptor-like 5 (FCRL5); immunoglobulin lambda- like polypeptide 1 (IGLL1), and Heat shock protein 70 (HSP70).

In another aspect, the CAR molecule of the invention comprises an antigen binding domain wherein said antigen binding domain binds to an antigen associated with a disease, and said tumor antigen is selected from a group consisting of: CD19 molecule (CD19); membrane spanning 4-domains A1 (MS4A1 also known as CD20); CD22 molecule (CD22); CD24 molecule (CD24); CD248 molecule (CD248); CD276 molecule (CD276 or B7H3); CD33 molecule (CD33); CD38 molecule (CD38); CD44v6; CD70 molecule (CD70); CD72; CD79a; CD79b; interleukin 3 receptor subunit alpha (IL3RA also known as CD123); TNF receptor superfamily member 8 (TNFRSF8 also known as CD30); KIT proto-oncogene receptor tyrosine kinase (CD117); V-set pre-B cell surrogate light chain 1 (VPREB1 or CD179a); adhesion G protein-coupled receptor E5 (ADGRE5 or CD97); TNF receptor superfamily member 17 (TNFRSF17 also known as BCMA); SLAM family member 7 (SLAMF7 also known as CS1); L1 cell adhesion molecule (L1CAM); C-type lectin domain family 12 member A (CLEC12A also known as CLL-1); tumor-specific variant of the epidermal growth factor receptor (EGFRvIII); thyroid stimulating hormone receptor (TSHR); Fms related tyrosine kinase 3 (FLT3); ganglioside GD3 (GD3); Tn antigen (Tn Ag); lymphocyte antigen 6 family member G6D (LY6G6D); Delta like canonical Notch

ligand 3 (DLL3); Interleukin- 13 receptor subunit alpha-2 (IL-13RA2); Interleukin 11 receptor subunit alpha (IL11RA); mesothelin (MSLN); Receptor tyrosine kinase like orphan receptor 1 (ROR1); Prostate stem cell antigen (PSCA); erb-b2 receptor tyrosine kinase 2 (ERBB2 or Her2/neu); Protease Serine 21 (PRSS21); Kinase insert domain receptor (KDR also known as VEGFR2); Lewis y antigen (LewisY); Solute carrier family 39 member 6 (SLC39A6); Fibroblast activation protein alpha (FAP); Hsp70 family chaperone (HSP70); Platelet-derived growth factor receptor beta (PDGFR-beta); Cholinergic receptor nicotinic alpha 2 subunit (CHRNA2); Stage-Specific Embryonic Antigen-4 (SSEA-4); Mucin 1, cell surface associated (MUC1); mucin 16, cell surface associated (MUC16); claudin 18 (CLDN18); claudin 6 (CLDN6); Epidermal Growth Factor Receptor (EGFR); Preferentially expressed antigen in melanoma (PRAME); Neural Cell Adhesion Molecule (NCAM); ADAM metallopeptidase domain 10 (ADAM10); Folate receptor 1 (FOLR1); Folate receptor beta (FOLR2); Carbonic Anhydrase IX (CA9); Proteasome subunit beta 9 (PSMB9 or LMP2); Ephrin receptor A2 (EphA2); Tetraspanin 10 (TSPAN10); Fucosyl GM1 (Fuc-GM1); sialyl Lewis adhesion molecule (sLe); TGS5 ; high molecular weight- melanoma-associated antigen (HMWMAA); o-acetyl- GD2 ganglioside (OAcGD2); tumor endothelial marker 7-related (TEM7R); G protein-coupled receptor class C group 5, member D (GPRC5D); chromosome X open reading frame 61 (CXORF61); ALK receptor tyrosine kinase (ALK); Polysialic acid; Placenta-specific 1 (PLAC1); hexasaccharide portion of globoH glycoceramide (GloboH); NY-BR-1 antigen; uroplakin 2 (UPK2); Hepatitis A virus cellular receptor 1 (HAVCR1); adrenoceptor beta 3 (ADRB3); pannexin 3 (PANX3); G protein-coupled receptor 20 (GPR20); lymphocyte antigen 6 family member K (LY6K); olfactory receptor family 51 subfamily E member 2 (OR51E2); TCR Gamma Alternate Reading Frame Protein (TARP); Wilms tumor protein (WT1); ETV6-AML1 fusion protein due to 12;21 chromosomal translocation (ETV6-AML1); sperm autoantigenic protein 17 (SPA17); X Antigen Family, Member 1E (XAGE1E); TEK receptor tyrosine kinase (Tie2); melanoma cancer testis antigen- 1 (MAD-CT-1); melanoma cancer testis antigen-2 (MAD-CT-2); Fos-related antigen 1 ; p53 mutant; human Telomerase reverse transcriptase (hTERT); sarcoma translocation breakpoints; melanoma inhibitor of apoptosis (ML-IAP); ERG (transmembrane protease, serine 2 (TMPRSS2) ETS fusion gene); N- Acetyl

glucosaminyl-transferase V (NA17); paired box protein Pax-3 (PAX3); Androgen receptor; Cyclin B 1 ; v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog (MYCN); Ras Homolog Family Member C (RhoC); Cytochrome P450 1B 1 (CYP1B 1); CCCTC-Binding Factor (Zinc Finger Protein)-Like (BORIS); Squamous Cell Carcinoma Antigen Recognized By T Cells 3 (SART3); Paired box protein Pax-5 (PAX5); proacrosin binding protein sp32 (OY-TES 1); lymphocyte-specific protein tyrosine kinase (LCK); A kinase anchor protein 4 (AKAP-4); synovial sarcoma, X breakpoint 2 (SSX2); Leukocyte- associated immunoglobulin-like receptor 1 (LAIR1); Fc fragment of IgA receptor (FCAR); Leukocyte immunoglobulin-like receptor subfamily A member 2 (LILRA2); CD300 molecule-like family member f (CD300LF);; bone marrow stromal cell antigen 2 (BST2); EGF-like module-containing mucin-like hormone receptor- like 2 (EMR2); lymphocyte antigen 75 (LY75); Glypican-3 (GPC3); Fc receptor-like 5 (FCRL5); immunoglobulin lambda- like polypeptide 1 (IGLL1), and Heat shock protein 70 (HSP70).

Under particular aspects, the cells of the invention are used for the treatment of a disease wherein said antigen against which the CAR is directed to is expressed or over expressed by pathological cells or tissue responsible for the disease.

The TCR molecule of the present invention comprises an antigen binding domain, wherein said antigen binding domain binds to a tumor antigen associated with a disease, and said tumor antigen is selected from a group consisting of PCTA-l/Galectin 8, CD171, TAG72, CEA, EPCAM, PSCA, PRSS21, PDGFR-beta, Prostase, PAP, ELF2M, Ephrin B2, IGF-I receptor, CAIX, ,gp100, bcr-abl, tyrosinase, GM3, NY-ESO-1, LAGE-la, MAGE-A1, legumain, HPV E6,E7, MAGE Al, prostein, survivin and telomerase, PCTA-l/Galectin 8, MelanA/MARTl, Ras mutant, TRP-2, RAGE-1, RU1, RU2, and intestinal carboxyl esterase.

Preferably the CAR of the invention comprises an antigen binding domain, that binds to a tumor antigen associated with a disease, and said tumor antigen is selected from a group consisting of CD22, CD123, CS-1, CLL-1, CD38, HSP70, MUC-1, CD30, FAP, HER2, CD79a and CD79b.

In preferred embodiments the CAR of the invention comprises an antigen binding domain, that binds to a tumor antigen associated with a disease, and said tumor antigen is selected from a group consisting of BCMA, CD33, EGFRVIII, Flt3, WT1, CD70, MUC16, PRAME, TSPAN10 CLAUDIN18.2, DLL3, LY6G6D, Liv-1, CHRNA2, ADAM10.

Preferably the CAR expressed at the cell surface of the cell of the invention comprises an antigen binding domain that binds to CD22.

Preferably the CAR expressed at the cell surface of the cell of the invention comprises an antigen binding domain that binds to CD123.

Preferably the CAR expressed at the cell surface of the cell of the invention comprises an antigen binding domain, that binds to CD30.

Preferably the CAR expressed at the cell surface of the cell of the invention comprises an antigen binding domain that binds to CD38.

Preferably the CAR expressed at the cell surface of the cell of the invention comprises an antigen binding domain that binds to CS-1.

Preferably the CAR expressed at the cell surface of the cell of the invention comprises an antigen binding domain that binds to CLL-1.

Preferably the CAR expressed at the cell surface of the cell of the invention comprises an antigen binding domain that binds to O-acethyl GD2.

Preferably the CAR expressed at the cell surface of the cell of the invention comprises an antigen binding domain that binds to FAP

Preferably the CAR expressed at the cell surface of the cell of the invention comprises an antigen binding domain that binds to HER2

Preferably the CAR expressed at the cell surface of the cell of the invention comprises an antigen binding domain that binds to CD79a

Preferably the CAR expressed at the cell surface of the cell of the invention comprises an antigen binding domain that binds to CD79b.

Preferably the CAR expressed at the cell surface of the cell of the invention comprises an antigen binding domain that binds to BCMA,

Preferably the CAR expressed at the cell surface of the cell of the invention comprises an antigen binding domain that binds to CD33,

Preferably the CAR expressed at the cell surface of the cell of the invention comprises an antigen binding domain that binds to EGFRVIII,

Preferably the CAR expressed at the cell surface of the cell of the invention comprises an antigen binding domain that binds to Flt3,

Preferably the CAR expressed at the cell surface of the cell of the invention comprises an antigen binding domain that binds to WT1,

Preferably the CAR expressed at the cell surface of the cell of the invention comprises an antigen binding domain that binds to CD70,

Preferably the CAR expressed at the cell surface of the cell of the invention comprises an antigen binding domain that binds to MUC16,

Preferably the CAR expressed at the cell surface of the cell of the invention comprises an antigen binding domain that binds to PRAME,

Preferably the CAR expressed at the cell surface of the cell of the invention comprises an antigen binding domain that binds to TSPAN10,

Preferably the CAR expressed at the cell surface of the cell of the invention comprises an antigen binding domain that binds to CLAUDIN18.2,

Preferably the CAR expressed at the cell surface of the cell of the invention comprises an antigen binding domain that binds to DLL3,

Preferably the CAR expressed at the cell surface of the cell of the invention comprises an antigen binding domain that binds to LY6G6D,

Preferably the CAR expressed at the cell surface of the cell of the invention comprises an antigen binding domain that binds to Liv-1,

Preferably the CAR expressed at the cell surface of the cell of the invention comprises an antigen binding domain that binds to CHRNA2,

Preferably the CAR expressed at the cell surface of the cell of the invention comprises an antigen binding domain that binds to ADAM10.

The invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of above embodiments, wherein said CAR comprises an extracellular ligand-binding domain, at least one epitope specific for a monoclonal antibody, a transmembrane domain and one or more intracellular signaling domains.

The invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of above embodiments, wherein said CAR comprises an extracellular ligand-binding domain, at least one epitope specific for a monoclonal antibody, a transmembrane domain from CD8 alpha, and one or more intracellular signaling domains from CD3 zeta and costimulatory domain from 4-1BB (human CD137).

The invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of above embodiments, wherein said CAR comprises an extracellular ligand-binding domain, a hinge from CD8alpha, at least one epitope specific for a monoclonal antibody, a transmembrane domain from CD8 alpha, and one or more intracellular signaling domains from CD3 zeta and costimulatory domain from 4-1BB.

The invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of above embodiments, wherein said CAR comprises an extracellular ligand-binding domain, a hinge from IgG1, at least one epitope specific for a monoclonal antibody, a transmembrane

domain from CD8 alpha, and one or more intracellular signaling domains from CD3 zeta and costimulatory domain from 4-1BB.

The invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of above embodiments, wherein said CAR comprises an extracellular ligand-binding domain, no hinge, a hinge from CD8alpha, a hinge from IgG1, a hinge from FcγRIIIα, at least one epitope specific for a monoclonal antibody, a transmembrane domain from CD8 alpha, and one or more intracellular signaling domains from CD3 zeta and costimulatory domain from 4-1BB.

The invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of above embodiments wherein said anti-CD123 CAR comprises an extracellular ligand-binding domain, at least one epitope specific for a monoclonal antibody, a transmembrane domain and one or more intracellular signaling domains.

The invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of above embodiments wherein said anti-CD22 CAR comprises an extracellular ligand-binding domain, at least one epitope specific for a monoclonal antibody, a transmembrane domain and one or more intracellular signaling domains.

The invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of above embodiments wherein said anti- CD30 CAR comprises an extracellular ligand-binding domain, at least one epitope specific for a monoclonal antibody, a transmembrane domain and one or more intracellular signaling domains.

The invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of above embodiments wherein said anti- CLL-1 CAR comprises an extracellular ligand-binding domain, at least one epitope specific for a monoclonal antibody, a transmembrane domain and one or more intracellular signaling domains.

The invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of above embodiments wherein said anti- CS-1 CAR comprises an extracellular ligand-

binding domain, at least one epitope specific for a monoclonal antibody, a transmembrane domain and one or more intracellular signaling domains.

In particular embodiments, the invention provides a TALEN-modified endogenous αβ-TCR negative human cell wherein said at least one insertion comprises a first a TALEN binding domain, an IRES, an exogenous polynucleotide sequence comprising a chimeric antigen receptor (CAR), a terminator sequence of polyadenylation signal, optionally a TALEN binding domain.

In particular embodiments, the invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to the above, wherein said chimeric antigen receptor (CAR), comprises at least one antigen specific for a monoclonal antibody, preferably two antigens specific for a monoclonal antibody.

In particular embodiments, the invention provides a TALEN-modified endogenous αβ-TCR negative human cell as above wherein said exogenous polynucleotide comprises a terminator sequence or a signal terminating the transcription by stopping the activity of RNA polymerase.

TERMINATOR SEQUENCE

The TALEN-modified endogenous αβ-TCR negative human cell according to the invention comprises an insertion into the TRAC gene comprising a termination or terminator sequence, preferably the TALEN-modified endogenous αβ-TCR negative human cell invention may comprise, from 5' to 3,' an insertion comprising an open reading frame encoding a protein, preferably a CAR and a termination sequence.

Thus, a termination or terminator sequence may be inserted into the TRAC locus allowing a CAR to be expressed and preventing cell surface expression of the endogenous alpha beta TCR.

In general, a terminator is a sequence-based element defining the end of a transcriptional unit (such as a gene) and initiating the process of releasing the newly synthesized RNA from the transcription machinery. Terminators are found downstream of a gene to be transcribed, and typically occur directly after any 3' regulatory elements, such as the polyadenylation or poly(A) signal.

Polyadenylation is the post-transcriptional additional of multiple adenine (A) nucleotides to the tail of a messenger RNA transcript. The purpose and mechanism of polyadenylation vary among cell types, but polyadenylation generally serves to promote transcript longevity in eukaryotes.

Accordingly, terminators regulate RNA processing and contribute to variability in RNA half-life, and ultimately gene-expression.

The following terminators available in eukaryotes at http://parts.igem.org/Terminators/Catalog#Eukaryotic_terminators is part of the present invention.

The terminators of the present invention include a terminator selected from SV40, hGH, BGH, and rbGlob terminators.

The terminators of the present invention include a terminator selected from SV40, hGH, BGH, and rbGlob terminators and comprise the sequence motif AAUAAA which promotes both polyadenylation and termination. Advantageously, the SV40 late polyA and rbGlob polyA are preferred when termination of transcription is due to the presence of additional helper sequences.

In a preferred embodiment terminator sequence of the present invention may be a terminator sequence selected from SV40 polyA, BGH polyA, hGH polyA, rbGlob polyA. In a more preferred embodiment, a terminator sequence of the present invention comprises a BGH polyA and includes the consensus sequence AATAAA, in an even more preferred embodiment,

A terminator sequence according to the present invention comprises a BGH polyA of sequence:

CTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCC

CACTGTCCTTTCCT**AATAAA**ATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGG

GTGGGGCAGGACAGCAAGGGGGAGGATTGGGAA**C**ACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATG

,

CTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTG-

GAAGGTGCCACTCCCACTGTCCTTTCCT**AATAAA**ATGAGGAAATTGCATCGCATTGTCTGAG-

TAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAA**G**A-

CAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATGG, or

CCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGC

CACTCCCACTGTCCTTTCCT**AATAAA**ATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGG

GGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAA**G**ACAATAGCAGGCATGCTGGGGATGCGGTGG

GCTCTATGGC***TTCTGAGGCGGAAAGAACCAGCTGGGGCTCTAGGGGGTATCCCC.*** The even more more preferred terminator sequence according to the present invention comprises the following sequence:

CTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCC

CACTGTCCTTTCCT**AATAAA**ATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGG

GTGGGGCAGGACAGCAAGGGGGAGGATTGGGAA**C**ACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTAT

IMMUNE CHECK POINT FACTORS

In particular embodiments, the invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above wherein at least one additional endogenous genomic gene is endonuclease-inactivated, preferably TALEN-inactivated said endogenous genomic gene is selected from the group consisting of an endogenous genomic beta subunit gene of the TCR, an endogenous genomic cytokine inducible SH2- containing (CISH) gene, an adenosine A2a receptor (ADORA) genomic gene, an endogenous genomic CD276 gene, an endogenous genomic V-set domain containing T cell activation inhibitor 1 (VTCNl) gene, an endogenous genomic B and T lymphocyte associated (BTLA) gene, an endogenous genomic cytotoxic T-lymphocyte-associated protein 4 (CTLA4) gene, an endogenous genomic indoleamine 2,3-dioxygenase 1 (IDO l) gene, an endogenous genomic killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 1 (KIR3DL1) gene, an endogenous genomic lymphocyte -activation gene 3 (LAG3) gene, an endogenous genomic

programmed cell death 1 (PD-1) gene, an endogenous genomic hepatitis A virus cellular receptor 2 (HAVCR2) gene, an endogenous genomic V-domain immunoglobulin suppressor of T-cell activation (VISTA) gene, an endogenous genomic natural killer cell receptor 2B4 (CD244) gene , an endogenous genomic hypoxanthine phosphoribosyltransferase 1 (HPRT) gene, an endogenous genomic adeno-associated virus integration site (AAVS I), and an endogenous genomic chemokine (C-C motif) receptor 5 (gene/pseudogene) (CCR5) gene, a combination thereof.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell expressing a CAR or a TCR, in particular any of the CAR described above, wherein one gene selected from the genes CTLA4, PPP2CA, PPP2CB, PTPN6, PTPN22, PDCD1, LAG 3, HAVCR2, BTLA, CD160, TIGIT, CD96, CRTAM, LAIR1, SIGLEC7, SIGLEC9, CD244, TNFRSF10B, TNFRSF10A, CASP8, CASP10, CASP3, CASP6, CASP7, FADD, FAS, TGFBRII, TGFBRI, SMAD2, SMAD3, SMAD4, SMAD10, SKI, SKIL, TGIF1, IL10RA, IL10RB, HMOX2, IL6R, IL6ST, CSK, PAG1, SIT1, FOXP3, PRDM1, BATF, GUCY1A2, GUCY1A3, GUCY1B2, GUCY1B3, is inactivated as in WO2014/184741 incorporated herein by reference.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above, expressing a CAR or a TCR, in particular any of the CAR described above, and wherein the level of expression of at least one additional factor selected from:

a) a factor which expression is involved in the reduction of glycolysis and/or calcium signaling, such as SERCA3, miR101 and mir26A to increase glycolysis, BCAT to mobilize glycolytic reserves; and/or

b) a factor which expression up regulate(s) immune checkpoint proteins (e.g.TIM3, CEACAM, LAG3, TIGIT), such as IL27RA , STAT1, STAT3; and/or

c) a factor which expression mediates interaction with HLA-G, such as ILT2 or ILT4; and/or

d) a factor which expression is involved into the down regulation of T-cell proliferation such as SEMA7A, SHARPIN to reduce Treg proliferation, STAT1 to lower apoptosis, PEA15 to increase IL-2 secretion and RICTOR to favor CD8 memory differentiation; and/or

e) polynucleotide sequence(s), which expression is(are) involved into the down regulation of T-cell activation, such as mir21; and/or

f) polynucleotide sequence(s), which expression is(are) involved in signaling pathways responding to cytokines, such as JAK2 and AURKA; and/or

g) polynucleotide sequence(s), which expression is(are) involved in T-cell exhaustion, such as DNMT3, miRNA31, MT1A, MT2A, PTGER2.

In particular embodiments, the invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above wherein said cell has been genetically modified, as described in PA2017 70603 which is incorporated herein by reference in its entirety.

In particular embodiments, the invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above wherein said cell has been genetically modified to reduce or inactivate the expression of SERCA3.

In particular embodiments, the invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above wherein said cell has been genetically modified to reduce or inactivate the expression of miR101.

In particular embodiments, the invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above wherein said cell has been genetically modified to reduce or inactivate the expression of mir26A to increase glycolysis.

In particular embodiments, the invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above wherein said cell has been genetically modified to reduce or inactivate the expression of BCAT to mobilize glycolytic reserves.

In particular embodiments, the invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above wherein said cell has been genetically modified to reduce or inactivate the expression of IL27RA.

In particular embodiments, the invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above wherein said cell has been genetically modified to reduce or inactivate the expression of STAT1.

In particular embodiments, the invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above wherein said cell has been genetically modified to reduce or inactivate the expression of STAT3.

In particular embodiments, the invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above wherein said cell has been genetically modified to reduce or inactivate the expression of ILT2.

In particular embodiments, the invention provides a TALEN-modified endogenous αβ–TCR negative human cell according to any one of the above wherein said cell has been genetically modified to reduce or inactivate the expression of ILT4.

In particular embodiments, the invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above wherein said cell has been genetically modified to reduce or inactivate the expression of SEMA7.

In particular embodiments, the invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above wherein said cell has been genetically modified to reduce or inactivate the expression of SHARPIN.

In particular embodiments, the invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above wherein said cell has been genetically modified to reduce or inactivate the expression of STAT1.

In particular embodiments, the invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above wherein said cell has been genetically modified to reduce or inactivate the expression PEA15.

In particular embodiments, the invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above wherein said cell has been genetically modified to reduce or inactivate the expression of RICTOR.

In particular embodiments, the invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above wherein said cell has been genetically modified to reduce or inactivate the expression of mir21

In particular embodiments, the invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above wherein said cell has been genetically modified to reduce or inactivate the expression of JAK2.

In particular embodiments, the invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above wherein said cell has been genetically modified to reduce or inactivate the expression of AURKA.

In particular embodiments, the invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above wherein said cell has been genetically modified to reduce or inactivate the expression of DNMT3.

In particular embodiments, the invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above wherein said cell has been genetically modified to reduce or inactivate the expression of miRNA31.

In particular embodiments, the invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above wherein said cell has been genetically modified to reduce or inactivate the expression of MT1A.

In particular embodiments, the invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above wherein said cell has been genetically modified to reduce or inactivate the expression of MT2A.

In particular embodiments, the invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above wherein said cell has been genetically modified to reduce or inactivate the expression of PTGER2.

In particular embodiments, the invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above wherein at least one additional endogenous genomic gene is overexpressed as in PCT/EP2017/058923 which is incorporated herein by reference in its entirety.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell as above for use to alter the survival of pathological cells responsible for a pathological condition, said pathological condition may be for example a cancer, a viral infection, in an individual regardless of his MHC or TCR molecules, preferably said TALEN-modified endogenous αβ-TCR negative human cell induces no GVHD, more preferably said TALEN-modified endogenous αβ-TCR negative human cell induces no GVHD and no HVGD .

The present invention provides a TALEN-modified endogenous αβ–TCR negative human cell as above for use as a medicament that induces no GVHD.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell as above for use as a medicament that induces no grade 1 GVHD.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell as above for use as a medicament that induces no grade 2 GVHD.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell as above for use as a medicament that induces no grade 3 GVHD.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell as above for use as a medicament that induces no grade 4 GVHD.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell as

above for use as a medicament that induces no acute GVHD.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell as above for use as a medicament that induces no chronical GVHD.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell as above for use as a medicament that induces neither acute nor chronical GVHD.

The present invention provides a population of human cells comprising a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above embodiments.

The present invention provides a pharmaceutical composition comprising a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above or a population of human cells as above and a pharmaceutically acceptable excipient.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to the above or a population of human cells as above or the pharmaceutical composition as above, for use as a medicament.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above or the population of human cells as above or the pharmaceutical composition according to the above for use in the treatment of cancer, in particular pediatric cancer.

The object of the present invention encompasses immunotherapy against a solid cancer; cells of the invention may be engineered to be resistant to hypoxia and/or for expressing a CAR under hypoxia as disclosed in WO2015092024 incorporated herein by reference.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above or the population of human cells as above or the pharmaceutical composition according to the above for use in the treatment of cancer. wherein said cancer is selected from the group consisting of a cancer of carcinoma, lymphoma, sarcoma, blastomas, and leukemia.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above or the population of human cells as above or the pharmaceutical composition according to the above for use in the treatment of a hematologic cancer, acute leukemia, B-cell acute lymphoid leukemia (BALL), T-cell acute lymphoid leukemia (TALL), small lymphocytic leukemia (SLL), acute lymphoid leukemia (ALL); chronic leukemia, chronic myelogenous leukemia (CML), chronic lymphocytic leukemia (CLL), non-Hodgkin lymphoma, or myeloma; or wherein the disease is a CD19-negative cancer, e.g., a CD19-negative relapsed cancer.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above or the population of human cells as above or the pharmaceutical composition according to the above for use in the treatment of cancer wherein the cancer is selected from the group consisting of a cancer of B-cell origin, breast cancer, gastric cancer, neuroblastoma, osteosarcoma, lung cancer, melanoma, prostate cancer, colon cancer, renal cell carcinoma, ovarian cancer, rhabdomyo sarcoma, leukemia, and Hodgkin's lymphoma.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above or the population of human cells as above or the pharmaceutical composition according to the above for use in the treatment of cancer wherein the cancer of B-cell origin is selected from the group consisting of B cell lymphoma, B-lineage acute lymphoblastic leukemia, B-cell chronic lymphocytic leukemia, and B-cell non-Hodgkin's lymphoma.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above or the population of human cells as above or the pharmaceutical composition according to the above for use in the treatment of cancer wherein the cancer is AML, ALL, a T cell lymphoma.

MEANS for detecting an endonuclease modified primary cell

The present invention provides a means or a degenerated means for detecting an endonuclease-modified endogenous αβ-TCR negative primary human cell wherein said endonuclease is selected from a TALEN, a Crispr/cas 9, meganuclease, TAL -nuclease, Zinc finger nuclease.

In a Polymerase Chain Reaction (PCR), a primer is used to bind to the DNA to start the replication. Usually, the objective is to amplify a specific piece of DNA, so primers are designed to only bind around that sequence of DNA. In contrast, in degenerate oligonucleotide primed PCR (DOP-PCR), one uses primers that can potentially bind to many sequences. This is accomplished by two ways: degenerate base pairing and/or using a low annealing temperature in the first two or so cycles. Degenerate base pairing is also accomplished by using bases like deoxyinosine that can bind to any other base pair. The low initial annealing temperatures stabilize the binding of this primer to the DNA, despite the fact that with only a 6bp match, the bond between the primer and the DNA is weak as compared to normal adequate condition with non degenerated primers

The degenerated primer generally has 6 regular base pairs on the 3' end, a bunch of degenerate nucleotides, and 6 regular base pairs again on the 5' end. The specificity of the probe may be given by the 6 bps on the 3' end. Since it is much easier to match 6 bps than 16 or more, as in a regular PCR primer, this degenerate primer amplifies many more sequences in the genome than regular PCR primers do.

The present invention provides a means for detecting an endonuclease-modified endogenous αβ-TCR negative primary human cell wherein the constant region of the genomic TCR gene (TRAC gene) comprises a genetic modification generated by a rare a cutting endonuclease and affecting cell surface expression of the alpha beta TCR, said genomic TRAC gene further comprising from 5' to 3':

(a) a 5' region of said human genomic TRAC gene upstream

(b) a recognition domain for a rare cutting endonuclease,

(c) a gap or an insertion as compared to the wild type TRAC gene affecting the cell surface expression of the extracellular domain or transmembrane domain of the alpha beta TCR,

said insertion comprising an exogenous polynucleotide selected from a noncoding sequence such as, a stop codon, an IRES, a sequence coding such as a sequence coding for a self-cleaving peptide in frame with the TRAC open reading frame, a sequence coding a chimeric antigen receptor (CAR), a sequence coding a TCR, a sequence coding a protein conferring sensitivity to a drug, a sequence coding a protein conferring resistance to a drug, a cytokine, a termination sequence, a combination thereof,

(c') optionally a second rare cutting endonuclease recognition domain,

(d) a 3' region of the genomic TRAC gene.

The present invention provides a means according to the above for detecting potential off site and on site endonuclease-induced events (double strand DNA cut).

The present invention provides a means according to the above wherein said endonuclease is selected from the group consisting of: Crispr/Cas 9, CpfI, TALEN, transposase, ZEN, Zinc finger endonuclease, meganuclease, or MegaTAL.

The present invention provides a means according to the above wherein said endonuclease is selected from the group consisting of: Crispr/Cas 9, TALEN, transposase, Zinc finger endonuclease, meganuclease, MegaTAL, a combination thereof.

The present invention provides a means for identifying a TALEN-modified endogenous αβ-TCR negative human primary cell wherein the constant region of the genomic TCR gene (TRAC gene)

comprises a genetic modification generated by a TALEN and affecting cell surface expression of the alpha beta TCR, said genomic TRAC gene comprising from 5' to 3':

(a) a 5' region of said human genomic TRAC gene upstream

(b) a recognition domain for a TALEN, preferably TTGTCCCACAGATATCC,

(c) a gap or an insertion as compared to the wild type TRAC gene affecting the cell surface expression of the extracellular domain or transmembrane domain of the alpha beta TCR,

said insertion comprising an exogenous polynucleotide selected from a noncoding sequence such as, a stop codon, an IRES, a sequence coding such as a sequence coding for a self-cleaving peptide in frame with the TRAC open reading frame, a sequence coding a chimeric antigen receptor (CAR), a sequence coding a TCR, a sequence coding a protein conferring sensitivity to a drug, a sequence coding a protein conferring resistance to a drug, a cytokine, a termination sequence, a combination thereof,

(c') optionally a second TALEN recognition domain,

(d) a 3' region of the genomic TRAC gene.

The present invention provides means according to the above wherein at least one means binds to a sequence inserted into the endonuclease modified TRAC gene and/or to the sequence upstream an endonuclease binding sequence.

The present invention provides a means according to the above for detecting off site and on site endonuclease-induced events in engineered human primary cells using at least one probe.

In the present invention, a probe may be designed based on known bona fide on-sites identified using a given endonuclease, preferably a TALEN, more preferably a TALEN binding to TTGTCCCACAGATATCCagaaccctgaccctgCCGTGTACCAGCTGAGAGA.

Under this preferred aspect, a first half TALEN moiety binds to TTGTCCCACAGATATCC, the second binds to CCGTGTACCAGCTGAGAGA and the dsDNA cut occurs within : agaaccctgaccctg.

Any of the known methods allowing detecting and identifying off sites sequences may be used, preferably the adapted guide seq technique of the present invention and method of detection of an insertion of the present invention.

In particular embodiments, the present invention provides a means according to the above for detecting potential off site and on site endonuclease-induced events in engineered human primary cells using at least one probe binding to a sequence inserted into off sequence(s) during the process of engineering human primary cells.

Detection of said such off-site insertion would ultimately allow to put aside and discard engineered human primary cells with off sites as off site events are not desired in endonuclease-engineered human primary cells used as a medicament for immunotherapy in human.

Alternatively, off site events in an endonuclease-engineered human primary cell may be analyzed using lists of bona fide off-site sequences in the human genome identified using any one of the known method for measuring endonuclease-induced off site events in human primary cells.

The present invention provides a means according to the above for detecting potential off site and on site endonuclease-induced events in human primary cells using at least one probe binding to a sequence corresponding to an endonuclease binding domain or a probe binding to a sequence upstream an endonuclease binding domain.

The complementary sequences may be used.

The present invention provides a means according to the above for detecting potential off site and on site endonuclease-induced events in human primary cells using at least one probe binding to a sequence corresponding to a defined bona fide off site sequence.

Preferably, the present invention provides a means according to the above for detecting off site and on site TRAC gene-specific endonuclease-induced events in human primary cells.

Preferably, the present invention provides a means according to the above for detecting human cells comprising on site TRAC-specific TALEN-induced events in human primary cells.

Preferably, the present invention provides a means according to the above for detecting on site TRAC-specific TALEN-induced events in human primary cells said probe binds to TTGTCCCACAGATATC and/or to CCGTGTACCAGCTGAGA.

Preferably, the present invention provides a means according to the above for detecting on site TRAC-specific TALEN-induced events in human primary cells said probe binds to TTGTCCCACAGATATC or GGCACATGGTCGACTCT, preferably to TTGTCCCACAGATATC, and to AACAGGGTGTCTATAG.

The present invention takes advantage of lists of bona fide off-site sequences identified in prior art in the human genome for an endonuclease(s) Crispr, TALEN, meganucleases, Zn Finger nuclease targeting the TRAC gene.

The initial method is those disclosed in Hendel et al., 2015. Trends Biotechnol. Feb; 33(2): 132–140; Pattanayak V. 2014. Methods Enzymol. 546: 47–78, preferably a guide seq method adapted to TALEN.

The present invention provides a means for identifying an endonuclease (preferably a TALEN-modified endogenous αβ-TCR negative human primary cell wherein at least one or at least two different endonucleases were used either together or successively as in PCT/EP2016/066355 and targeting the TRAC gene and at least one another gene selected from those listed in PA2016 70840 which is herein incorporated by reference.

The present invention provides a means for identifying an endonuclease modified αβ-TCR negative human primary cell, preferably a TALEN modified endogenous αβ-TCR negative human primary cell wherein at least one or at least two different endonucleases were used either together or successively as in PCT/EP2016/066355 and targeting the TRAC gene and at least one another gene

selected from CD38, CD70, dCK, CD52, beta 2 microglobulin, CIITA, TRBC, PD1, CTLA4, PPP2CA, PPP2CB, PTPN6, PTPN22, PDCD1, LAG 3, HAVCR2, BTLA, CD160, TIGIT, CD96, CRTAM, LAIR1, SIGLEC7, SIGLEC9, CD244, TNFRSF10B, TNFRSF10A, CASP8, CASP10, CASP3, CASP6, CASP7, FADD, FAS, TGFBRII, TGFBRI, SMAD2, SMAD3, SMAD4, SMAD10, SKI, SKIL, TGIF1, IL10RA, IL10RB, HMOX2, IL6R, IL6ST, CSK, PAG1, SIT1, FOXP3, PRDM1, BATF, GUCY1A2, GUCY1A3, GUCY1B2, GUCY1B3, SERCA3, BCAT, IL27RA, STAT1, STAT3, ILT2, ILT4, SEMA7, SHARPIN, STAT1, PEA15,RICTOR, JAK2, AURKA, DNMT3,MT1A,MT2A,PTGER2, gene generating miR101, gene generating mir26A, gene generating mir21, or gene generating miRNA31.

The present invention provides a means for identifying a TALEN-modified endogenous αβ-TCR negative human primary cell wherein at least one or at least two different endonucleases were used either together or successively as in PCT/EP2016/066355 and targeting the TRAC gene and at least one another gene selected from CD38, CD70, dCK, CD52, beta 2 microglobulin, CIITA, TRAC, TRBC, PD1, CTLA4, PPP2CA, PPP2CB, PTPN6, PTPN22, PDCD1, LAG 3, HAVCR2, BTLA, CD160, TIGIT, CD96, CRTAM, LAIR1, SIGLEC7, SIGLEC9, CD244, TNFRSF10B, TNFRSF10A, CASP8, CASP10, CASP3, CASP6, CASP7, FADD, FAS, TGFBRII, TGFBRI, SMAD2, SMAD3, SMAD4, SMAD10, SKI, SKIL, TGIF1, IL10RA, IL10RB, HMOX2, IL6R, IL6ST, CSK, PAG1, SIT1, FOXP3, PRDM1, BATF, GUCY1A2, GUCY1A3, GUCY1B2, GUCY1B3, SERCA3, BCAT, IL27RA, STAT1, STAT3, ILT2, ILT4, SEMA7, SHARPIN, STAT1, PEA15,RICTOR, JAK2, AURKA, DNMT3,MT1A,MT2A,PTGER2, gene generating miR101, gene generating mir26A, gene generating mir21, or gene generating miRNA31.

The present invention provides a means for identifying a TALEN-modified endogenous αβ-TCR negative human primary cell wherein at least one or at least two different TALEN were used targeting the TRAC gene and at least one another gene selected from: dCK, CD52, beta 2 microglobulin, CIITA, TRBC, PD1, CTLA4, PPP2CA, PPP2CB, PTPN6, PTPN22, PDCD1, LAG 3, HAVCR2, BTLA, CD160, TIGIT, CD96, CRTAM, LAIR1, SIGLEC7, SIGLEC9, CD244, TNFRSF10B, TNFRSF10A, CASP8, CASP10, CASP3, CASP6, CASP7, FADD, FAS, TGFBRII, TGFBRI, SMAD2, SMAD3, SMAD4, SMAD10, SKI, SKIL, TGIF1, IL10RA, IL10RB, HMOX2, IL6R, IL6ST, CSK, PAG1, SIT1, FOXP3, PRDM1, BATF, GUCY1A2, GUCY1A3,

GUCY1B2, GUCY1B3, SERCA3, BCAT, IL27RA, STAT1, STAT3, ILT2, ILT4, SEMA7, SHARPIN, STAT1, PEA15,RICTOR, JAK2, AURKA, DNMT3,MT1A,MT2A,PTGER2, gene generating miR101, gene generating mir26A, gene generating mir21, or gene generating miRNA31.

The present invention provides a means for identifying a TALEN-modified endogenous αβ-TCR negative human primary cell wherein at least one or at least two different TALEN were used targeting the TRAC gene and at least one another gene selected from: CD38, CD70, CS1, PD1 (Uniprot Q15116), CTLA4 (Uniprot P16410), PPP2CA (Uniprot P67775), PPP2CB (Uniprot P62714), PTPN6 (Uniprot P29350), PTPN22 (Uniprot Q9Y2R2), LAG3 (Uniprot P18627), HAVCR2 (Uniprot Q8TDQ0), BTLA (Uniprot Q7Z6A9), CD160 (Uniprot O95971), TIGIT (Uniprot Q495A1), CD96 (Uniprot P40200), CRTAM (Uniprot O95727), LAIR1 (Uniprot Q6GTX8), SIGLEC7 (Uniprot Q9Y286), SIGLEC9 (Uniprot Q9Y336), CD244 (Uniprot Q9BZW8), TNFRSF10B (Uniprot O14763), TNFRSF10A (Uniprot O00220), CASP8 (Uniprot Q14790), CASP10 (Uniprot Q92851), CASP3 (Uniprot P42574), CASP6 (Uniprot P55212), CASP7 (Uniprot P55210), FADD (Uniprot Q13158), FAS (Uniprot P25445), TGFBRII (Uniprot P37173), TGFRBRI (Uniprot Q15582), SMAD2 (Uniprot Q15796), SMAD3 (Uniprot P84022), SMAD4 (Uniprot Q13485), SMAD10 (Uniprot B7ZSB5), SKI (Uniprot P12755), SKIL (Uniprot P12757), TGIF1 (Uniprot Q15583), IL10RA (Uniprot Q13651), IL10RB (Uniprot Q08334), HMOX2 (Uniprot P30519), IL6R (Uniprot P08887), IL6ST (Uniprot P40189), EIF2AK4 (Uniprot Q9P2K8), CSK (Uniprot P41240), PAG1 (Uniprot Q9NWQ8), SIT1 (Uniprot Q9Y3P8), FOXP3 (Uniprot Q9BZS1), PRDM1 (Uniprot Q60636), BATF (Uniprot Q16520), GUCY1A2 (Uniprot P33402), GUCY1A3 (Uniprot Q02108), GUCY1B2 (Uniprot Q8BXH3) and GUCY1B3 (Uniprot Q02153) and for those disclosed in PA201770603 that is to say, human SERCA3, human BCAT, human IL27RA, human STAT1, human STAT3, human ILT2, human ILT4, human SEMA7, human SHARPIN, human STAT1, human PEA15, human RICTOR, human JAK2, human AURKA, human DNMT3, human MT1A, human MT2A, human PTGER2, the human gene generating miR101, mir26A, mir21, or miRNA31 is part of the present invention.

Means for identifying the following TALEN-engineered genes: CD38, CD70, CS1, CTLA4, PPP2CA, PPP2CB, PTPN6, PTPN22, PDCD1, LAG 3, HAVCR2, BTLA, CD160, TIGIT, CD96, CRTAM, LAIR1, SIGLEC7, SIGLEC9, CD244, TNFRSF10B, TNFRSF10A, CASP8, CASP10, CASP3, CASP6, CASP7, FADD, FAS, TGFBRII, TGFBRI, SMAD2, SMAD3, SMAD4, SMAD10, SKI, SKIL, TGIF1, IL10RA, IL10RB, HMOX2, IL6R, IL6ST, CSK, PAG1, SIT1, FOXP3, PRDM1, BATF, GUCY1A2, GUCY1A3, GUCY1B2, GUCY1B3, SERCA3, BCAT, IL27RA, STAT1, STAT3, ILT2, ILT4, SEMA7, SHARPIN, STAT1, PEA15,RICTOR, JAK2, AURKA, DNMT3,MT1A,MT2A,PTGER2, gene generating miR101, mir26A, mir21, or miRNA31, TRAC were generated.

Particularly, meganuclease-induced bona fide off-site sequences in primary cells were identified, as well as CRISPR-induced bona fide off-site sequences were identified, using said endonucleases targeting one of the following human genes or a combination of TRAC and one of the following human genes : TRBC, CD40L, adenosine A2a receptor (A2aR); B7-related protein 1 (B7RP1); B and T lymphocyte attenuator (BTLA); galectin 9 (GAL9), herpesvirus entry mediator (HVEM); inducible T cell co-stimulator (ICOS); interleukin-12, -IL-27 (IL-12, IL27); killer cell immunoglobulin-like receptor (KIR); lymphocyte activation gene 3 (LAG3); programmed cell death protein 1 (PD1); PD1 ligand (PDL); transforming growth factor-β (TGFβ); T cell membrane protein 3 (TIM3), CD38, CD70, CS1, CTLA4, PPP2CA, PPP2CB, PTPN6, PTPN22, PDCD1, LAG 3, HAVCR2, BTLA, CD160, TIGIT, CD96, CRTAM, LAIR1, SIGLEC7, SIGLEC9, CD244, TNFRSF10B, TNFRSF10A, CASP8, CASP10, CASP3, CASP6, CASP7, FADD, FAS, TGFBRII, TGFBRI, SMAD2, SMAD3, SMAD4, SMAD10, SKI, SKIL, TGIF1, IL10RA, IL10RB, HMOX2, IL6R, IL6ST, CSK, PAG1, SIT1, FOXP3, PRDM1, BATF, GUCY1A2, GUCY1A3, GUCY1B2, GUCY1B3, SERCA3, BCAT, IL27RA, STAT1, STAT3, ILT2, ILT4, SEMA7, SHARPIN, STAT1, PEA15, RICTOR, JAK2, AURKA, DNMT3,MT1A,MT2A,PTGER2, gene generating miR101, mir26A, mir21, or miRNA31.

The present invention provides Means or Probe(s) for detecting off sites and/or on sites generated during cell engineering using an endonuclease, preferably an endonuclease selected from a TALEN, a

Meganuclease, a Crispr /Cas 9, a Zinc Finger endonuclease, more preferably a TALEN and even more preferably a TALEN binding to the TRAC gene, preferably to a sequence in the exon 1 of the TRAC gene.

The present invention provides Means or Probe(s) for detecting off sites and/or on sites generated during cell engineering using a TALEN binding to the following sequence ttgtcccacagATATC.

The present invention provides Means or Probe(s) for detecting off sites and/or on sites generated during cell engineering using an endonuclease selected from a TALEN, a meganuclease, a Crispr /Cas 9, a Zinc Finger endonuclease, a megaTAL more preferably a TALEN and even more preferably a TAL-ENthat bind to a sequence located in any one of the genes identified in 62/410,187:

symbol	description
Il21	interleukin 21
Il3	interleukin 3
Ccl4	isopentenyl-diphosphate delta isomerase 2
Il21	granzyme C
Gp49a	chemokine (C-C motif) receptor 8
Cxcl10	interleukin 2
Nr4a3	interleukin 1 receptor, type I
Lilrb4	tumor necrosis factor (ligand) superfamily, member 4
Cd200	neuronal calcium sensor 1
Cdkn1a	CDK5 and Abl enzyme substrate 1
Gzmc	transmembrane and tetratricopeptide repeat containing 2
Nr4a2	LON peptidase N-terminal domain and ring

	finger 1
Cish	glycoprotein 49 A
Nr4a1	polo-like kinase 2
Tnf	lipase, endothelial
Ccr8	cyclin-dependent kinase inhibitor 1A (P21)
Lad1	grainyhead-like 1 (Drosophila)
Slamf1	cellular retinoic acid binding protein II
Crabp2	adenylate kinase 4
Furin	microtubule-associated protein 1B
Gadd45g	acyl-CoA synthetase long-chain family member 6
Bcl2l1	zinc finger E-box binding homeobox 2
Ncs1	CD200 antigen
Ciart	carboxypeptidase D
Ahr	thioredoxin reductase 3
Spry1	myosin IE
Tnfsf4	RNA binding protein with multiple splicing 2
Myo10	mitogen-activated protein kinase kinase 3, opposite strand
Dusp5	PERP, TP53 apoptosis effector
Myc	myosin X
Psrc1	immediate early response 3
St6galnac4	folliculin interacting protein 2
Nfkbid	leukocyte immunoglobulin-like receptor, subfamily B, member 4

Bst2	circadian associated repressor of transcription
Txnrd3	RAR-related orphan receptor gamma
Plk2	proline/serine-rich coiled-coil 1
Gfi1	cysteine rich protein 2
Pim1	cAMP responsive element modulator
Pvt1	chemokine (C-C motif) ligand 4
Nfkbib	nuclear receptor subfamily 4, group A, member 2
Gnl2	transglutaminase 2, C polypeptide
Cd69	synapse defective 1, Rho GTPase, homolog 2 (C, elegans)
Dgat2	sprouty homolog 1 (Drosophila)
Atf3	activating transcription factor 3
Tnfrsf21	pogo transposable element with KRAB domain
Lonrf1	tumor necrosis factor receptor superfamily, member 21
Cables1	cytokine inducible SH2-containing protein
Cpd	lymphotoxin A
Qtrtd1	FBJ osteosarcoma oncogene
Polr3d	signaling lymphocytic activation molecule family member 1
Kcnq5	syndecan 3
Fos	mitochondrial ribosomal protein L47

Slc19a2	ladinin
Hif1a	E2F transcription factor 5
Il15ra	ISG15 ubiquitin-like modifier
Nfkb1	aryl-hydrocarbon receptor
Phlda3	diacylglycerol O-acyltransferase 2
Mtrr	FBJ osteosarcoma oncogene B
Pogk	pleckstrin homology-like domain, family A, member 3
Map2k3os	potassium voltage-gated channel, subfamily Q, member 5
Egr2	tumor necrosis factor receptor superfamily, member 10b
Isg15	Mir17 host gene 1 (non-protein coding)
Perp	glucose-fructose oxidoreductase domain containing 1
Ipo4	plexin A1
Mphosph10	heat shock factor 2
Plk3	carbohydrate sulfotransferase 11
Ifitm3	growth arrest and DNA-damage-inducible 45 gamma
Polr1b	solute carrier family 5 (sodium-dependent vitamin transporter), member 6
Usp18	interferon induced transmembrane protein 3
Top1mt	DENN/MADD domain containing 5A
Dkc1	plasminogen activator, urokinase receptor

Polr1c	solute carrier family 19 (thiamine transporter), member 2
Cdk6	ubiquitin domain containing 2
Ier3	nuclear receptor subfamily 4, group A, member 3
Lta	zinc finger protein 52
Ptprs	SH3 domain containing ring finger 1
Fnip2	dihydrouridine synthase 2
Asna1	cyclin-dependent kinase 5, regulatory subunit 1 (p35)
Mybbp1a	processing of precursor 7, ribonuclease P family, (S, cerevisiae)
Il1r1	growth factor independent 1
Dennd5a	interleukin 15 receptor, alpha chain
E2f5	BCL2-like 1
Rcl1	protein tyrosine phosphatase, receptor type, S
Fosl2	plasmacytoma variant translocation 1
Atad3a	fos-like antigen 2
Bax	BCL2-associated X protein
Phf6	solute carrier family 4, sodium bicarbonate cotransporter, member 7
Zfp52	tumor necrosis factor receptor superfamily, member 4
Crtam	chemokine (C-X-C motif) ligand 10

Nop14	polo-like kinase 3
Rel	CD3E antigen, epsilon polypeptide associated protein
Gramd1b	tumor necrosis factor (ligand) superfamily, member 11
Ifi27l2a	polymerase (RNA) III (DNA directed) polypeptide D
Tnfrsf10b	early growth response 2
Rpl7l1	DnaJ (Hsp40) homolog, subfamily C, member 2
Eif1a	DNA topoisomerase 1, mitochondrial
Nfkb2	tripartite motif-containing 30D
Heatr1	DnaJ (Hsp40) homolog, subfamily C, member 21
Utp20	SAM domain, SH3 domain and nuclear localization signals, 1
Chst11	solute carrier family 5 (inositol transporters), member 3
Ddx21	mitochondrial ribosomal protein L15
Hsf2	dual specificity phosphatase 5
Bccip	apoptosis enhancing nuclease
Tagap	ets variant 6
Sdc3	DIM1 dimethyladenosine transferase 1-like (S, cerevisiae)
Sytl3	2'-5' oligoadenylate synthetase-like 1

Gtpbp4	UTP18, small subunit (SSU) processome component, homolog (yeast)
Crip2	BRCA2 and CDKN1A interacting protein
Sh3rf1	synaptotagmin-like 3
Nsfl1c	5-methyltetrahydrofolate-homocysteine methyltransferase reductase
Gtf2f1	URB2 ribosome biogenesis 2 homolog (S, cerevisiae)
Slc4a7	ubiquitin-conjugating enzyme E2C binding protein
Etv6	lysine (K)-specific demethylase 2B
Trim30d	queuine tRNA-ribosyltransferase domain containing 1
Ddx27	ubiquitin specific peptidase 31
Pwp2	eukaryotic translation initiation factor 2-alpha kinase 2
Chchd2	ATPase family, AAA domain containing 3A
Myo1e	adhesion molecule, interacts with CXADR antigen 1
Eif5b	SUMO/sentrin specific peptidase 3
Stat5a	ESF1, nucleolar pre-rRNA processing protein, homolog (S, cerevisiae)
Cops6	deoxynucleotidyltransferase, terminal, interacting protein 2
D19Bwg1357e	TGFB-induced factor homeobox 1

Aatf	eukaryotic translation initiation factor 1A
Aen	interferon-stimulated protein
Amica1	pleiomorphic adenoma gene-like 2
Wdr43	PWP2 periodic tryptophan protein homolog (yeast)
Cct4	furin (paired basic amino acid cleaving enzyme)
Nifk	tumor necrosis factor
Tgm2	apoptosis antagonizing transcription factor
Ero1l	interferon, alpha-inducible protein 27 like 2A
Gfod1	ST6 (alpha-N-acetyl-neuraminyl-2,3-beta-galactosyl-1,3)-N-acetylgalactosaminide alpha-2,6-sialyltransferase 4
Ak4	methyltransferase like 1
Sdad1	notchless homolog 1 (Drosophila)
Dimt1	mitochondrial ribosomal protein L3
Esf1	UBX domain protein 2A
Cd3eap	guanine nucleotide binding protein-like 2 (nucleolar)
Samsn1	programmed cell death 11
Tnfrsf4	cyclin-dependent kinase 8
Mettl1	eukaryotic translation initiation factor 5B
Cd274	RNA terminal phosphate cyclase-like 1
Ubtd2	NSFL1 (p97) cofactor (p47)
Icos	nuclear factor of kappa light polypeptide

	gene enhancer in B cells inhibitor, delta
Kdm2b	M-phase phosphoprotein 10 (U3 small nucleolar ribonucleoprotein)
Larp4	GRAM domain containing 1B
Eif3d	ERO1-like (S, cerevisiae)
Tnfaip3	nuclear receptor subfamily 4, group A, member 1
Map1b	surfeit gene 2
Cdv3	N(alpha)-acetyltransferase 25, NatB auxiliary subunit
Plac8	yrdC domain containing (E,coli)
Mrpl3	La ribonucleoprotein domain family, member 4
Surf2	SDA1 domain containing 1
Ubxn2a	importin 4
Utp18	inducible T cell co-stimulator
Isg20	solute carrier family 7 (cationic amino acid transporter, y+ system), member 1
Dnajc2	arsA arsenite transporter, ATP-binding, homolog 1 (bacterial)
Jak2	polymerase (RNA) I polypeptide C
Slc7a1	spermatogenesis associated 5
Syde2	ubiquitin specific peptidase 18
Slc5a6	placenta-specific 8
Dnttip2	general transcription factor IIF, polypeptide 1

Idi2	nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, beta
Dus2	PHD finger protein 6
Pitrm1	RRN3 RNA polymerase I transcription factor homolog (yeast)
Plxna1	cytotoxic and regulatory T cell molecule
Cdk5r1	COP9 (constitutive photomorphogenic) homolog, subunit 6 (Arabidopsis thaliana)
Ube2cbp	asparagine-linked glycosylation 3 (alpha-1,3-mannosyltransferase)
Tnfsf11	tryptophanyl-tRNA synthetase
Pop7	hypoxia up-regulated 1
Psme3	family with sequence similarity 60, member A
Mir17hg	bone marrow stromal cell antigen 2
Tsr1	nuclear factor of kappa light polypeptide gene enhancer in B cells 2, p49/p100
Rbpms2	UTP20, small subunit (SSU) processome component, homolog (yeast)
Mrpl47	CD274 antigen
Rab8b	proviral integration site 1
Plagl2	signal transducer and activator of transcription 5A
Grhl1	CD69 antigen
Zeb2	pitrilysin metallepetidase 1
sept-02	cyclin-dependent kinase 6

Slc5a3	DEAD (Asp-Glu-Ala-Asp) box polypeptide 27
Naa25	polymerase (RNA) I polypeptide B
Plaur	tumor necrosis factor, alpha-induced protein 3
Metap1	nodal modulator 1
Alg3	NOP14 nucleolar protein
Mrpl15	ribosomal protein L7-like 1
Oasl1	methionyl aminopeptidase 1
Rorc	hypoxia inducible factor 1, alpha subunit
Nomo1	Janus kinase 2
Tgif1	nuclear factor of kappa light polypeptide gene enhancer in B cells 1, p105
Lipg	reticuloendotheliosis oncogene
Rrn3	septin 2
Dnajc21	nucleolar protein interacting with the FHA domain of MKI67
Yrdc	elongation factor Tu GTP binding domain containing 2
Acsl6	myelocytomatosis oncogene
Spata5	dyskeratosis congenita 1, dyskerin
Urb2	carnitine deficiency-associated gene expressed in ventricle 3
Nle1	GTP binding protein 4
Wars	HEAT repeat containing 1
Crem	proteaseome (prosome, macropain) activator

	subunit 3 (PA28 gamma, Ki)
Larp1	La ribonucleoprotein domain family, member 1
Eif2ak2	DNA segment, Chr 19, Brigham & Women's Genetics 1357 expressed
Hyou1	eukaryotic translation initiation factor 3, subunit D
Senp3	TSR1 20S rRNA accumulation
Tmtc2	MYB binding protein (P160) 1a
Fosb	T cell activation Rho GTPase activating protein
Pdcd11	RAB8B, member RAS oncogene family
Usp31	DEAD (Asp-Glu-Ala-Asp) box polypeptide 21
Cdk8	chaperonin containing Tcp1, subunit 4 (delta)
Eftud2	coiled-coil-helix-coiled-coil-helix domain containing 2
Fam60a	WD repeat domain 43

Selection of preferred endogenous genes that are constantly active during immune cell activation (dependent or independent from T-cell activation).

Symbol	Gene description
CD3G	CD3 gamma
Rn28s1	28S ribosomal RNA
Rn18s	18S ribosomal RNA
Rn7sk	RNA, 7SK, nuclear
Actg1	actin, gamma, cytoplasmic 1
B2m	beta-2 microglobulin
Rpl18a	ribosomal protein L18A
Pabpc1	poly(A) binding protein, cytoplasmic 1
Gapdh	glyceraldehyde-3-phosphate dehydrogenase
Rpl19	ribosomal protein L19
Rpl17	ribosomal protein L17
Rplp0	ribosomal protein, large, P0
Cfl1	cofilin 1, non-muscle
Pfn1	profilin 1

Selection of genes that are transiently upregulated upon T-cell activation.

Symbol	Gene description
Il3	interleukin 3
Il2	interleukin 2
Ccl4	chemokine (C-C motif) ligand 4
Il21	interleukin 21
Gp49a	glycoprotein 49 A
Nr4a3	nuclear receptor subfamily 4, group A, member 3
Lilrb4	leukocyte immunoglobulin-like receptor, subfamily B, member 4
Cd200	CD200 antigen
Cdkn1a	cyclin-dependent kinase inhibitor 1A (P21)
Gzmc	granzyme C
Nr4a2	nuclear receptor subfamily 4, group A, member 2
Cish	cytokine inducible SH2-containing protein
Ccr8	chemokine (C-C motif) receptor 8
Lad1	ladinin
Crabp2	cellular retinoic acid binding protein II

Selection of genes that are upregulated

over more than 24 hours upon T-cell activation.

Symbol	Description
Gzmb	granzyme B
Tbx21	T-box 21
Pdcd1	programmed cell death 1
Plek	pleckstrin
Chek1	checkpoint kinase 1
Slamf7	SLAM family member 7
Zbtb32	zinc finger and BTB domain containing 32
Tigit	T cell immunoreceptor with Ig and ITIM domains
Lag3	lymphocyte-activation gene 3
Gzma	granzyme A
Wee1	WEE 1 homolog 1 (S. pombe)
Il12rb2	interleukin 12 receptor, beta 2
Ccr5	chemokine (C-C motif) receptor 5
Eea1	early endosome antigen 1
Dtl	denticleless homolog (Drosophila)

Selection of genes that are down-regulated upon immune cell activation.

Symbol	Gene description
Spata6	spermatogenesis associated 6
Itga6	integrin alpha 6
Rcbtb2	regulator of chromosome condensation (RCC1) and BTB (POZ) domain containing protein 2
Cd1d1	CD1d1 antigen
St8sia4	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 4
Itgae	integrin alpha E, epithelial-associated
Fam214a	family with sequence similarity 214, member A
Slc6a19	solute carrier family 6 (neurotransmitter transporter), member 19
Cd55	CD55 antigen
Xkrx	X Kell blood group precursor related X linked
Mturn	maturin, neural progenitor differentiation regulator homolog (Xenopus)
H2-Ob	histocompatibility 2, O region beta locus
Cnr2	cannabinoid receptor 2 (macrophage)
Itgae	integrin alpha E, epithelial-associated
Raver2	ribonucleoprotein, PTB-binding 2
Zbtb20	zinc finger and BTB domain containing 20
Arrb1	arrestin, beta 1
Abca1	ATP-binding cassette, sub-family A (ABC1), member 1

Tet1	tet methylcytosine dioxygenase 1
Slc16a5	solute carrier family 16 (monocarboxylic acid transporters), member 5
Trav14-1	T cell receptor alpha variable 14-1
Ampd3	adenosine monophosphate deaminase 3

Symbol	Gene description
Zfp640	zinc finger protein 640
LOC100038422	uncharacterized LOC100038422
Zfp600	zinc finger protein 600
Serpinb3a	serine (or cysteine) peptidase inhibitor, clade B (ovalbumin), member 3A
Tas2r106	taste receptor, type 2, member 106
Magea3	melanoma antigen, family A, 3
Omt2a	oocyte maturation, alpha
Cpxcr1	CPX chromosome region, candidate 1
Hsf3	heat shock transcription factor 3
Pbsn	Probasin
Sbp	spermine binding protein
Wfdc6b	WAP four-disulfide core domain 6B
Meiob	meiosis specific with OB domains
Dnm3os	dynamin 3, opposite strand
Skint11	selection and upkeep of intraepithelial T cells 11

Selection of human genes that are silent upon T-cell activation

(safe harbor gene targeted integration loci).

In particular, the present invention provides Means or Probe(s) for detecting off sites and/or on sites generated during cell engineering using a TALEN binding to the TCR alpha, the TCR beta, more preferably to the TCR alpha and to the following sequence ttgtcccacagATATC, even more preferably said means or probe comprise a sequence complementary to a sequence comprising ttgtcccacagATATC

and a sequence complementary to a sequence in the TRAC gene after the initiation codon and downstream the sequence CCGTGTACCAGCTGAGA.

In the present invention off sites cut using a TALEN binding to the following sequence ttgtcccacagATATC, said means or probe comprising a sequence complementary to a sequence comprising ttgtcccacagATATC and/or a sequence complementary to a sequence in the TRAC gene downstream the sequence CCGTGTACCAGCTGAGA, are below the level of detection using a guide seq analysis as adapted for TALEN.

In particular embodiments, the present invention provides means for detecting off site and on site of endonuclease-induced events wherein detecting off sites and on sites is performed by a guide-seq adapted technique implemented during the process of making said endogenous αβ-TCR negative human T cell comprising an endonuclease modified genomic TRAC gene.

In particular embodiments, the present invention provides a means according to any one of the above wherein said TALEN modified genomic TRAC gene comprises:

(a) a 5' region of said human genomic TRAC gene;

(b) a recognition domain for a TALEN, preferably a recognition domain for a TALEN comprising the following sequence ttgtcccacagATATC, or ttgtcccacagATATCCAG, or

(c) a gap or an insertion as compared to the wild type TRAC gene affecting the cell surface expression of the extracellular domain or transmembrane domain of the alpha beta TCR,

said insertion comprising an exogenous polynucleotide selected from a noncoding sequence, a stop codon, a sequence coding for a self-cleaving peptide in frame with the TRAC open reading frame, an IRES, a sequence coding a chimeric antigen receptor (CAR), a sequence coding a TCR, a sequence coding a protein conferring sensitivity to a drug, a sequence coding a protein conferring resistance to a drug, a termination sequence, a combination thereof,

(c') optionally a second TALEN recognition domain,

(d) a 3' region of the genomic TRAC gene;

and said means binds to said an endonuclease modified genomic TRAC gene and/or to off sites

Degenerated oligonucleotides for adapted PCR are provided here as a probe binding to the engineered TRAC gene, optionally to off sites and further amplification by pcr.

A degenerate primer is defined as: *"A mix of oligonucleotide sequences in which some positions contain a number of possible bases, giving a population of primers with similar sequences that cover all possible nucleotide combinations for a given protein sequence".*

In particular embodiments, the present invention provides means for detecting a TALEN modified endogenous αβ-TCR negative human cell, said means binding to at least 5, 6, 7, 8, 9, 10 bases of the following sequence ttgtcccacagATATC.

In particular embodiments, the present invention provides means for detecting an endonuclease modified endogenous αβ-TCR negative human cell comprising a probe wherein said probe binds to a sequence in the modified genomic TRAC gene, said probe binds to a sequence upstream the first endonuclease binding domain or said probe binds to a sequence upstream said endonuclease recognition domain, and/or to a sequence encoding a tag.

In the case of endonucleases (TALENs, meganucleases) comprising at least two separated domains or monomers, the first endonuclease binding domain or recognition domain is the first binding domain or recognition domain in 5' and binds the left sequence .

In particular embodiments, the present invention provides means for detecting a TALEN modified endogenous αβ-TCR negative human cell comprising a probe wherein said probe binds to a sequence in the modified genomic TRAC gene, upstream a TALEN binding domain or TALEN recognition domain, and/or to a sequence encoding a tag.

In particular embodiments, the present invention provides means for detecting a TALEN modified

endogenous αβ-TCR negative human cell comprising a probe wherein said probe binds to at least 10 bases of a sequence comprising **ttgtcccacagATATC, or ttgtcccacagATATCCAG**, in the modified genomic TRAC gene.

In particular embodiments, the present invention provides means for detecting an endonuclease-modified endogenous αβ-TCR negative human cell by polymerase chain reaction (pcr) and off sites modifications, preferably by guide sequence.

In particular embodiments, the present invention provides means for detecting an endonuclease-modified endogenous αβ-TCR negative human cell by deep sequencing and off sites modifications, preferably by guide sequence.

In particular embodiments, the present invention provides means comprising a sequence complementary to an inactivated genomic TCRA gene wherein an exogenous coding sequence was integrated using one or more endonucleases and/or a viral vector.

The present invention provides an endonuclease-modified endogenous αβ-TCR negative human cell as any one of the above comprising an inactivated genomic TCRA gene wherein an exogenous sequence coding a CAR was integrated into the genomic TCRA gene using a lentiviral vector or a AAV vector and said genomic disruptions are performed using a CRISPR/CAS9, meganuclease, a megaTAL or a TALEN endonuclease system.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell according to any one of the above wherein an exogenous sequence coding a CAR was integrated into the genomic TCRA gene using a lentiviral vector or a AAV vector introduced by electroporation or nucleofection.

The present invention provides a TRAC-specific endonuclease engineered human cell with an endonuclease specific off site pattern engineered using an AAV6 vector.

The present invention provides a TRAC exon 1-specific endonuclease engineered human cell with an endonuclease specific off site pattern, engineered using an AAV6 vector.

The present invention provides a TRAC exon 1-specific endonuclease engineered human cell with an endonuclease specific off site pattern, engineered using an AAV6/AAV2 vector.

The present invention provides a TRAC exon 1-specific TALEN that binds to at least 10 bases of the sequence ttgtcccacagATATC engineered human cell, engineered using an AAV6 vector and with a reduced specific off target pattern as compared to other TRAC exon 1-specific endonuclease engineered human cell.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell wherein an exogenous sequence coding a CAR was integrated into the genomic TCRA gene using an AAV6/2 vector.

The present invention provides a TALEN-modified endogenous αβ-TCR negative human cell wherein an exogenous sequence coding a CAR was integrated into the genomic TCRA gene using an AAV6/2 vector said vector was introduced by electroporation or by nucleofection.

The present invention provides a method for treating a patient in need thereof, the method comprising administering a cell according to any one of the preceding embodiments.

The present invention provides a kit comprising at least one TALEN that binds to the genomic TRAC gene, a TALEN that binds to an endogenous cytokine inducible SH2 -containing (CISH) gene and a TALEN that binds to an endogenous gene selected from adenosine A2a receptor (ADORA), CD276, V-set domain containing T cell activation inhibitor 1 (VTCN1), B and T lymphocyte associated (BTLA), cytotoxic T-lymphocyte -associated protein 4 (CTLA4), indoleamine 2,3-dioxygenase 1 (IDOl), killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 1 (KIR3DL1), lymphocyte -activation gene 3 (LAG3), programmed cell death 1 (PD-1), hepatitis A virus cellular receptor 2 (HAVCR2), V-domain immunoglobulin suppressor of T-cell activation (VISTA), natural killer cell receptor 2B4 (CD244), hypoxanthine phosphoribosyltransferase 1 (HPRT), adeno-associated virus integration site (AAVS SITE (E.G. AAVS 1, AAVS2, ETC.)), or chemokine (C-C motif) receptor 5 (gene/pseudogene) (CCR5).

The present invention provides a method for producing a TALEN-modified endogenous αβ-TCR negative human primary cell said method comprising:

(a) introducing into a human primary cell:

(i) a first nucleic acid sequence encoding an engineered nuclease; or an engineered nuclease protein;

wherein said engineered nuclease produces a cleavage at a recognition sequence within said human TCR alpha constant region gene; and

(b) a second nucleic acid sequence comprising an exogenous polynucleotide;

wherein the sequence of said exogenous polynucleotide is inserted into said human TCR alpha constant region gene at said cleavage site; and further wherein said genetically-modified primary cell has reduced cell-surface expression of the endogenous TCR when compared to an unmodified control primary cell.

The present invention provides a method as above wherein said exogenous polynucleotide comprises a nucleic acid sequence encoding a chimeric antigen receptor, wherein said chimeric antigen receptor comprises an extracellular ligand-binding domain and one or more intracellular signaling domains.

The present invention provides a method as above, wherein said exogenous polynucleotide comprises a first promoter sequence, such as a promoter which activity is conditional that drives expression of said exogenous polynucleotide.

The present invention provides a method as above, wherein at least said second nucleic acid sequence is introduced into said cell by contacting said cell with a recombinant adeno-associated virus (AAV6) vector comprising said second nucleic acid sequence.

The present invention provides a method as above, wherein said recombinant AAV vector is a self-complementary AAV vector.

The present invention provides a method as above, wherein said recombinant AAV vector derived at least in part from an AAV6.

The present invention provides a method as above, wherein said engineered nuclease is a meganuclease, a zinc-finger nuclease (ZFN), a transcription activator-like effector nuclease (TALEN), a CRISPR/Cas nuclease, or a megaTAL nuclease.

The present invention provides a method as above, wherein said engineered nuclease is a recombinant TALEN.

The present invention provides a method as above, wherein said recombinant TALEN comprises a first subunit and a second subunit, wherein said first subunit binds to a first recognition half-site, and wherein said second subunit binds to a second recognition half-site.

The present invention provides a method as above, wherein the first recognition half-site of the TALEN has the following sequence ttgtcccacagATATCCAG.

The present invention provides a method as above, wherein said engineered nuclease is a meganuclease.

The present invention provides a method as above, wherein said recombinant meganuclease recognizes and cleaves a recognition sequence within residues 93-208 of the wild- type human TCR alpha constant region, wherein said recombinant meganuclease comprises a first subunit and a second subunit, wherein said first subunit binds to a first recognition half-site of said recognition sequence and comprises a first hypervariable (HVR1) region, and wherein said second subunit binds to a second recognition half-site of said recognition sequence and comprises a second hypervariable (HVR2) region.

The present invention provides a method as above, wherein said meganuclease is a single-chain meganuclease comprising a linker, wherein said linker covalently joins said first subunit and said second subunit.

The present invention provides a means of detection of cells obtained according to any of one the method above wherein a meganuclease recognizing and cleaving a recognition sequence within residues 93-208 of the wild- type human TCR alpha constant region,is used.

In particular embodiments, the present invention provides a human cell or a population of human cells wherein the constant region of the genomic TCR gene (TRAC gene) comprises a genetic modification generated by a meganuclease recognizing and cleaving a recognition domain or sequence within residues 93-208 of the wild- type human TCR alpha constant region and affecting cell surface expression of the alpha beta TCR, said genomic TRAC gene further comprising from 5' to 3':

(a) a 5' region of said human genomic TRAC gene upstream a recognition domain for meganuclease recognizing and cleaving a recognition domain or sequence within residues 93-208 of the wild- type human TCR alpha constant region,

(b) a recognition domain for a meganuclease, within residues 93-208 of the wild- type human TCR alpha constant region,

(c) a gap or an insertion as compared to the wild type TRAC gene affecting the cell surface expression of the extracellular domain or transmembrane domain of the alpha beta TCR,

said insertion comprising an exogenous polynucleotide selected from a noncoding sequence, a stop codon, a sequence coding for a self-cleaving peptide in frame with the TRAC open reading frame, an IRES, a sequence coding a chimeric antigen receptor (CAR), a sequence coding a TCR, a sequence coding a protein conferring sensitivity to a drug, a sequence coding a protein conferring resistance to a drug, a termination sequence, a combination thereof,

(c') optionally a second megonuclease recognition domain, within residues 93-208 of the wild- type human TCR alpha constant region,

(d) a 3' region of the genomic TRAC gene,

In one embodiment, the present invention provides a mean of detecting a human cell produced using a meganuclease recognizing and cleaving a recognition domain or sequence within residues 93-208 of the wild- type human TCR alpha constant region.

In particular embodiments, the present invention provides a mean, means for detecting a human cell produced using a crispr /cas 9 system wherein said guide mRNA recognizes the following sequence in the TRAC exon 1 : GGGTGTCTATAGGTCTTGGGAC

Thus, in one aspect, the invention provides a means for detecting a Crispr/cas 9 -modified cell comprising in its genome a modified human TCR alpha constant region gene, wherein the modified human TCR alpha constant region gene comprises from 5' to 3': (a) a 5' region of the human TCR alpha constant region gene; (b) a PAM, (b') an exogenous polynucleotide; and (c) a 3' region of the human TCR alpha constant region gene.

The present invention provides therefore a means for detecting cells obtained according to any of one the method above.

The present invention provides a means of SEQ ID N° 5, SEQ ID N° 6, SEQ ID N° 7, a combination thereof or any degenerated means derived from SEQ ID N° 5, SEQ ID N° 6, SEQ ID N° 7, a combination thereof.

The present invention provides a means as above for detecting on site and/or off site cleavage by endonuclease, in particular as a step in a GUIDE SEQ method.

The present invention provides the means as above for detecting on site and/or off site cleavage by endonuclease.

The present invention provides means as above for detecting on site and/or off site cleavage by endonuclease of any one of SEQ ID N° 13 to 22, a combination thereof.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1. TCR KO, exogenous gene such as a chimeric antigen receptor (CAR), expressed

Figure 2. Endogenous TCR maintained, exogenous gene, such as a chimeric antigen receptor (CAR), co-expressed

Figure 3. Endogenous TCR inactivated, Recombinant TCR expressed

Figure 4. TCR KO, exogenous gene expressed (IRES)

Figure 5. Endogenous TCR maintained, exogenous gene co-expressed (IRES)

Figure 6. Endogenous TCR inactivated, Recombinant TCR expressed (IRES)

Figure 7. TCR KO, exogenous gene expressed, TALEN target site edited

Figure 8: Inactivation of CD52 gene and inactivation by insertion of a sequence coding a self cleaving peptide 2A, aCAR and a polyA terminator sequence into the TRAC gene.

DETAILED DESCRIPTION OF THE INVENTION

1.1 References and Definitions

The patent and scientific literature referred to herein establishes knowledge that is available to those of skill in the art. The issued patents, allowed applications, published foreign applications, and references, including GenBank database sequences, which are cited herein are hereby incorporated by reference to the same extent as if each was specifically and individually indicated to be incorporated by reference.

The present invention can be embodied in different forms and should not be construed as limited to the embodiments set forth herein. Rather, these embodiments are provided so that this disclosure will be thorough and complete, and will fully convey the scope of the invention to those skilled in the art. For example, features illustrated with respect to one embodiment can be incorporated into other

embodiments, and features illustrated with respect to a particular embodiment can be deleted from that embodiment. In addition, numerous variations and additions to the embodiments suggested herein will be apparent to those skilled in the art in light of the instant disclosure, which do not depart from the instant invention.

Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. The terminology used in the description of the invention herein is for the purpose of describing particular embodiments only and is not intended to be limiting of the invention.

All publications, patent applications, patents, and other references mentioned herein are incorporated by reference herein in their entirety.

As used herein, "a," "an," or "the" can mean one or more than one. For example, "a" cell can mean a single cell or a multiplicity of cells, an homogenous population of cells.

As used herein, unless specifically indicated otherwise, the word "or" is used in the inclusive sense of "and/or" and not the exclusive sense of "either/or."

As used herein, the term "TALEN" or “TALE-nucleases” refers to an endonuclease comprising a DNA-binding domain comprising 14-20 or 16-22 TAL domain repeats fused to any portion of the FokI nuclease domain (WO2011072246).

TALE-nucleases, are fusion protein of a TALE binding domain with a cleavage catalytic domain. These endonucleases have been successfully applied to primary immune cells, in particular T-cells from peripheral blood mononuclear cell (PBMC). Such TALE-nucleases, marketed under the name TALEN, are those currently used to simultaneously inactivate gene sequences in T-cells originating from donors, in particular to produce allogeneic therapeutic T-Cells in which the genes encoding TCR (T-cell receptor) and CD52 are disrupted. These cells can be endowed with chimeric antigen receptors (CAR) for treating cancer patients (US2013/0315884). TALE-nucleases are very specific reagents

because they need to bind DNA by pairs under obligatory heterodimeric form to obtain dimerization of the cleavage domain Fok-1. Left and right heterodimer members each recognizes a different nucleic sequence of about 14 to 20 bp, together spanning target sequences of 30 to 50 bp overall specificity.

Other endonuclease systems derived from homing endonucleases (ex: I-OnuI, or I-CreI), combined or not with TAL-nuclease (ex: MegaTAL) or zing-finger nucleases have also proven specificity, but to a lesser extend so far.

As used herein, the term "Compact TALEN" refers to an endonuclease comprising a DNA-binding domain with 16-22 TAL domain repeats fused in any orientation to any catalytically active portion of nuclease domain of the I-TevI homing endonuclease.

As used herein, the term "meganuclease" refers to an endonuclease that binds double-stranded DNA at a recognition sequence that is greater than 12 base pairs. Preferably, the recognition sequence for a meganuclease of the invention is 22 base pairs. A meganuclease can be an endonuclease that is derived from I-CreI, and can refer to an engineered variant of I-CreI that has been modified relative to natural I-CreI with respect to, for example, DNA-binding specificity, DNA cleavage activity, DNA-binding affinity, or dimerization properties. Methods for producing such modified variants of I-CreI are known in the art (e.g., WO 2007/047859). A meganuclease as used herein binds to double-stranded DNA as a heterodimer or as a "single-chain meganuclease" in which a pair of DNA-binding domains are joined into a single polypeptide using a peptide linker. The term "homing endonuclease" is synonymous with the term "meganuclease." Meganucleases of the invention are substantially non-toxic when expressed in cells, particularly in human T cells, such that cells can be transfected and maintained at 37°C without observing deleterious effects on cell viability or significant reductions in meganuclease cleavage activity when measured using the methods described herein.

As used herein, the term "single-chain meganuclease" refers to a polypeptide comprising a

pair of nuclease subunits joined by a linker. A single-chain meganuclease has the organization: N-terminal subunit - Linker - C-terminal subunit. The two meganuclease subunits will generally be non-identical in amino acid sequence and will recognize non-identical DNA sequences. Thus, single-chain meganucleases typically cleave pseudo-palindromic or non-palindromic recognition sequences. A single-chain meganuclease may be referred to as a "single-chain heterodimer" or "single-chain heterodimeric meganuclease" although it is not, in fact, dimeric. For clarity, unless otherwise specified, the term "meganuclease" can refer to a dimeric or single-chain meganuclease.

As used herein, the term "linker" can refer to an exogenous peptide sequence used to join two meganuclease subunits into a single polypeptide. A linker may have a sequence that is found in natural proteins, or may be an artificial sequence that is not found in any natural protein. A linker may be flexible and lacking in secondary structure or may have a propensity to form a specific three-dimensional structure under physiological conditions. A linker can include, without limitation, those encompassed by U.S. Patent No. 8,445,251.

As used herein, the term "CRISPR" (Clustered Regularly Interspaced Short palindromic Repeats) refers to ~~a caspase-based endonuclease comprising a caspase~~, such as Cas9, and a guide RNA that directs DNA cleavage of the caspase by hybridizing to a recognition site in the genomic DNA.

Other endonucleases reagents have been developed based on the components of the type II prokaryotic CRISPR (Clustered Regularly Interspaced Short palindromic Repeats) adaptive immune system of the bacteria S. pyogenes. This multi-component system referred to as RNA-guided nuclease system (Gasiunas, Barrangou et al. 2012; Jinek, Chylinski et al. 2012), involves members of Cas9 or Cpf1 endonuclease families coupled with a guide RNA molecules that have the ability to drive said nuclease to some specific genome sequences (Zetsche et al. (2015). Cpf1 is a single RNA-guided endonuclease that provides immunity in bacteria and can be adapted for genome editing in mammalian cells. Cell 163:759-771). Such programmable RNA-guided endonucleases are easy to produce because the cleavage specificity is determined by the sequence of the RNA guide, which can

be easily designed and cheaply produced. The specificity of CRISPR/Cas9 although stands on shorter sequences than TAL-nucleases of about 10 pb, which must be located near a particular motif (PAM) in the targeted genetic sequence.

As used herein, the term "megaTAL" refers to a single-chain nuclease comprising a transcription activator-like effector (TALE) DNA binding domain with an engineered, sequence-specific homing endonuclease.

As used herein, with respect to a protein, the term "recombinant" means having an altered amino acid sequence as a result of the application of genetic engineering techniques to nucleic acids that encode the protein, and cells or organisms that express the protein. With respect to a nucleic acid, the term "recombinant" means having an altered nucleic acid sequence as a result of the application of genetic engineering techniques. Genetic engineering techniques include, but are not limited to, PCR and DNA cloning technologies; transfection, transformation and other gene transfer technologies; homologous recombination; site-directed mutagenesis; and gene fusion. In accordance with this definition, a protein having an amino acid sequence identical to a naturally-occurring protein, but produced by cloning and expression in a heterologous host, is not considered recombinant.

The following documents are entirely incorporated herein by reference WO2017062451, WO2015057980, WO2017106528, in particular for a method of preparing a crispr or meganuclease - TRAC modified human cells.

As used herein, the term "wild-type" or “native “ refers to the most common naturally occurring allele (i.e. , polynucleotide sequence) in the allele population of the same type of gene, wherein a polypeptide encoded by the wild-type allele has its original functions. The term "wild-type" also refers a polypeptide encoded by a wild-type allele. Wild -type alleles (i. e. , polynucleotides) and polypeptides are distinguishable from mutant or variant alleles and polypeptides, which comprise one or more mutations and/or substitutions relative to the wild-type sequence(s). Whereas a wild-

type allele or polypeptide can confer a normal phenotype in an organism, a mutant or variant allele or polypeptide can, in some instances, confer an altered phenotype. Wild-type nucleases are distinguishable from recombinant or non-naturally-occurring nucleases.

As used herein, the term "modification" means any insertion, deletion or substitution of an amino acid residue in the sequence relative to a reference sequence (e.g. , a wild-type or a native or genomic sequence).

As used herein, the term "recognition sequence" or “recognition domain” refers to a DNA sequence that is bound and cleaved by an endonuclease. In the case of a meganuclease, a recognition sequence comprises a pair of inverted, 9 base pair "half sites" that are separated by four base pairs.

In the case of a single-chain meganuclease, the N-terminal domain of the protein contacts a first half-site and the C-terminal domain of the protein contacts a second half-site.

Cleavage by a meganuclease produces four base pair 3 ' "overhangs". "Overhangs", or "sticky ends" are short, single-stranded DNA segments that can be produced by endonuclease cleavage of a double -stranded DNA sequence. In the case of meganucleases and single-chain meganucleases derived from I-CreI, the overhang comprises bases 10-13 of the 22 bases pair recognition sequence.

In the case of a Compact TALEN, the recognition sequence comprises a first CNNNGN sequence that is recognized by the I-TevI domain, followed by a nonspecific spacer 4-16 base pairs in length, followed by a second sequence 16-22 bp in length that is recognized by the TAL-effector domain (this sequence typically has a 5 ' T base). Cleavage by a Compact TALEN produces two base pair 3 ' overhangs.

In the case of a CRISPR, the recognition sequence is the sequence, typically 16-24 basepairs, to which the guide RNA binds to direct Cas9 cleavage. Cleavage by a CRISPR produced blunt ends.

As used herein, the term "target site" or "target sequence" refers to a region of the chromosomal DNA of a cell comprising a recognition sequence for a nuclease.

As used herein, the term "DNA-binding affinity" or "binding affinity" means the tendency of a nuclease to non-covalently associate with a reference DNA molecule (e.g. , a recognition sequence or an arbitrary sequence). Binding affinity is measured by a dissociation constant, ¾ . As used herein, a nuclease has "altered" binding affinity if the Kd of the nuclease for a reference recognition sequence is increased or decreased by a statistically significant percent change relative to a reference nuclease.

As used herein, the term "homologous recombination" or "HR" refers to the natural, cellular process in which a double-stranded DNA-break is repaired using a homologous DNA sequence as the repair template (see, e.g., Cahill et al. (2006), Front. Biosci. 11 : 1958-1976). The homologous DNA sequence may be an endogenous chromosomal sequence or an exogenous nucleic acid that was delivered to the cell.

As used herein, the term "non-homologous end-joining" or "NHEJ" refers to the natural, cellular process in which a double-stranded DNA-break is repaired by the direct joining of two non-homologous DNA segments (see, e.g., Cahill et al. (2006), Front. Biosci. 1 1 : 1958- 1976). DNA repair by non-homologous end-joining is error-prone and frequently results in the untemplated addition or deletion of DNA sequences at the site of repair. In some instances, cleavage at a target recognition sequence results in NHEJ at a target recognition site. Nuclease-induced cleavage of a target site in the coding sequence of a gene followed by DNA repair by NHEJ can introduce mutations into the coding sequence, such as frameshift mutations, that disrupt gene function. Thus, engineered nucleases can be used to effectively knock-out a gene in a population of cells.

CAR

As used herein, a "chimeric antigen receptor" or "CAR" refers to an engineered receptor that confers or grafts specificity for an antigen onto an immune effector cell (e.g. , a human T cell). A chimeric

antigen receptor typically comprises an extracellular ligand-binding domain or moiety and an intracellular domain that comprises one or more stimulatory domains that transduce the signals necessary for T cell activation. In some embodiments, the extracellular ligand-binding domain or moiety can be in the form of single-chain variable fragments (scFvs) derived from a monoclonal antibody, which provide specificity for a particular epitope or antigen (e.g., an epitope or antigen preferentially present on the surface of a cancer cell or other disease-causing cell or particle). The extracellular ligand-binding domain can be specific for any antigen or epitope of interest.

In a particular embodiment, the ligand-binding domain is specific for CD22. In another particular embodiment, the ligand-binding domain is specific for CD123.

SCFV

In particular embodiments, the CAR encoded by the exogenous polynucleotide inserted into the TRAC gene comprises a scfv. The scfvs of the invention can be attached via a linker sequence.

HINGE

The extracellular domain can further comprise a hinge region between said extracellular ligand-binding domain and said transmembrane domain. The term “hinge region” used herein generally means any oligo- or polypeptide that functions to link the transmembrane domain to the extracellular ligand-binding domain. In particular, hinge region is used to provide more flexibility and accessibility for the extracellular ligand-binding domain. A hinge region may comprise up to 300 amino acids, preferably 10 to 100 amino acids and most preferably 10 to 50 amino acids. Hinge region may be derived from all or part of naturally occurring molecules, such as from all or part of the extracellular region of CD8, or CD4, or from all or part of an antibody constant region. Alternatively, the hinge region may be a synthetic sequence that corresponds to a naturally occurring hinge sequence, or may be an entirely synthetic hinge sequence. In a preferred embodiment said hinge domain comprises a part of a human CD8 alpha chain, FcRIIIα receptor or IgG1, respectively.

A Hinge from IgG4 or from PD1 is part of the present invention and disclosed in WO2016120216 and may be used in the construction of a CAR according to the invention.

A CAR according to the present invention is anchored into the membrane of the cell. Thus, such CAR further comprises a transmembrane domain. The distinguishing features of appropriate transmembrane domains comprise the ability to be expressed at the surface of a cell, preferably in the present invention an immune cell, in particular lymphocyte cells or Natural killer (NK) cells, and to interact together for directing cellular response of immune cell against a predefined target cell. The transmembrane domain can be derived either from a natural or from a synthetic source. The transmembrane domain can be derived from any membrane-bound or transmembrane protein. As non-limiting examples, the transmembrane polypeptide can be a subunit of the T-cell receptor such as α, β, or δ, polypeptide constituting CD3 complex, IL2 receptor p55 (α chain), p75 (β chain) or chain, subunit chain of Fc receptors, in particular Fc receptor III or CD proteins. Alternatively the transmembrane domain can be synthetic and can comprise predominantly hydrophobic residues such as leucine and valine.

MIMOTOPE – suicide switch

In the present invention, the extracellular domain of a chimeric antigen receptor may also comprise a monoclonal antibody epitope that can be recognized by a monoclonal antibody such as those described in WO2016120216. In a preferred embodiment? a CAR of the invention is a “QR3”, “QR2”, “QR1”, “Q”, “R”, “R2” or “R3” CAR with Q is an epitope recognized by Q ben10 antibody and R an epitope recognized by rituximab as described in *WO2016120216A1 which is incorporated herein by reference.*

The extracellular domain of a chimeric antigen receptor can also comprise an autoantigen (see, Payne et al. (2016), Science 353 (6295): 179-184), that can be recognized by an autoantigen-specific B cell receptor on B lymphocytes. The autoantibody allows directing T cells to specifically target and kill autoreactive B lymphocytes in antibody-mediated autoimmune diseases. Such CARs can be referred

to as chimeric autoantibody receptors (CAARs), and their use is encompassed by the invention.

one or several monoclonal antibody epitope(s) can be inserted into the scFv and/or the hinge of the CAR, and their use is encompassed by the invention for eliminating cells in vivo for example.

INTRACELLULAR DOMAIN

The signal transducing domain or intracellular signaling domain of a CAR according to the present invention is responsible for intracellular signaling following the binding of extracellular ligand binding domain to the target resulting in the activation of the immune cell and immune response (cytolytic activity against the target cell). In other words, the signal transducing domain is responsible for the activation of at least one of the normal effector functions of the immune cell in which the CAR is expressed. For example, the effector function of a T cell can be a cytolytic activity or helper activity including the secretion of cytokines. Thus, the term “signal transducing domain” refers to the portion of a protein which transduces the effector signal function and directs the cell to perform a specialized function.

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

The intracellular stimulatory domain can include one or more cytoplasmic signaling domains that transmit an activation signal to the immune effector cell following antigen binding. In one embodiment, a cytoplasmic signaling domains includes, a CD3-zeta intracellular stimulatory domain.

In another embodiment, the signaling transducing domain of the CAR of the invention consists in a CD3zeta signaling domain and excludes any sequence from human CD28 signaling domain.

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

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

1), CD2, CD7, LIGHT, NKG2C, B7-H3 and a ligand that specifically binds with CD83 and the like.

In one embodiment, the signal transduction domain of the CAR of the present invention comprises a part of co-stimulatory signal molecule consisting of fragment of 4-1BB (GenBank: AAA53133.). In another embodiment, the signal transduction domain of a CAR according to the present invention comprises no sequence from CD28 (NP_006130.1).

In one embodiment, all the embodiments of the present invention comprise no sequence from CD28 (NP_006130.1).

In one embodiment, the signal transduction domain of a CAR of the present invention comprises a part of co-stimulatory signal molecule 4-1BB (GenBank: AAA53133) and no sequence from CD28 (NP_006130.1).

In one embodiment, the signal transduction domain of a CAR of the present invention comprises a part of co-stimulatory signal molecule 4-1BB (GenBank: AAA53133) and CD3 zeta signaling domain.

In one embodiment, the signal transduction domain of a CAR of the present invention comprises a part of co-stimulatory signal molecule 4-1BB (GenBank: AAA53133) and CD3 zeta intracellular signaling domain and CD28 intracellular signaling domain.

The intracellular stimulatory domain can also include one or more intracellular co-stimulatory domains that transmit a proliferative and/or cell-survival signal after ligand binding. Such intracellular co-stimulatory domains can include, without limitation, a CD28 domain, an OX40 domain, or a combination thereof. A chimeric antigen receptor can further include additional structural elements, including a transmembrane domain that is attached to the extracellular ligand-binding domain via a hinge or spacer sequence.

MULTICHAIN CAR

The insertion in the TRAC gene may comprise a sequence encoding a multichain CAR are those

described in WO2014039523 A1.

EXOGENOUS TCR or recombinant exogenous TCR

As used herein, an "exogenous T cell receptor (TCR)" or "recombinant TCR" refers to a TCR whose sequence is introduced into the genome of an immune effector cell (e.g. , a human T cell) which does not express the endogenous TCR. Expression of an exogenous TCR in an immune effector cell can confer specificity for a specific and known epitope or antigen (e.g., an epitope or antigen preferentially present on the surface of a cancer cell or other disease-causing cell or particle). Such exogenous T cell receptors can comprise alpha and beta chains or, alternatively, may comprise gamma and delta chains. Exogenous TCRs useful in the invention may have specificity to any antigen or epitope of interest as those listed herein.

As used herein, the term "undetectable expression" of an antigen refers to a reduction in the expression at the cell surface, in a genetically-modified cell, to the level measured using a negative control cell that do not express said antigen.

Term "undetectable expression" of the T cell receptor at the cell surface of a genetically-modified cell corresponds to the level of the endogenous alpha and beta chains of the TCR in genetically-modified cell measured using a control cell that does not express the endogenous alpha and beta chains of the T cell receptor (and an Ab specific for the alpha beta TCR).

The term reduced can also refer to a reduction in the percentage of cells in a population of cells that express an endogenous polypeptide (i.e., an endogenous alpha and beta T cell receptor and a chimeric antigen receptor) at the cell surface when compared to a population of control cells.

Such a reduction may be up to 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, or up to 100% (undetectable). Accordingly, the term "reduced" encompasses both a partial knockdown and a complete knockdown (undetectable) of the endogenous T cell receptor, and undetectable cell surface expression of the endogenous alpha beta TCR.

As used herein with respect to both amino acid sequences and nucleic acid sequences, the terms "percent identity," "sequence identity," "percentage similarity," "sequence similarity" and the like refer to a measure of the degree of similarity of two sequences based upon an alignment of the sequences that maximizes similarity between aligned amino acid residues or nucleotides, and that is a function of the number of identical or similar residues or nucleotides, the number of total residues or nucleotides, and the presence and length of gaps in the sequence alignment. A variety of algorithms and computer programs are available for determining sequence similarity using standard parameters. As used herein, sequence similarity is measured using the BLASTp program for amino acid sequences and the BLASTn program for nucleic acid sequences, both of which are available through the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/), and are described in, for example, Altschul et al. (1990), J. Mol. Biol. 215:403-410; Gish and States (1993), Nature Genet. 3:266-272; Madden ei a/. (1996), Meth. Enzymol.266: \3 \-U\;Altschul et al. (1997), Nucleic Acids Res. 25:33 89-3402); Zhang et al. (2000), J. Comput. Biol. 7(l-2):203-14.

As used herein, percent similarity of two amino acid sequences is the score based upon the following parameters for the BLASTp algorithm: word size=3; gap opening penalty=-l 1; gap extension penalty=-l; and scoring matrix=BLOSUM62. As used herein, percent similarity of two nucleic acid sequences is the score based upon the following parameters for the BLASTn algorithm: word size=l 1; gap opening penalty=-5; gap extension penalty=-2; match reward=l; and mismatch penalty=-3.

As used herein with respect to modifications of two proteins or amino acid sequences, the term "corresponding to" is used to indicate that a specified modification in the first protein is a substitution of the same amino acid residue as in the modification in the second protein, and that the amino acid position of the modification in the first proteins corresponds to or aligns with the amino acid position of the modification in the second protein when the two proteins are subjected to standard sequence alignments (e.g. , using the BLASTp program). Thus, the modification of residue "X" to amino acid "A" in the first protein will correspond to the modification of residue "Y" to amino

acid "A" in the second protein if residues X and Y correspond to each other in a sequence alignment, and despite the fact that X and Y may be different numbers.

As used herein, the term "recognition half-site," "recognition sequence half-site," or simply "half-site" means a nucleic acid sequence in a double-stranded DNA molecule of the TRAC gene that is recognized by a monomer of a homodimeric or heterodimeric TALEN.

The first "recognition half-site," or "first recognition site" or "first recognition domain" means the nucleic acid sequence in a double-stranded DNA molecule recognized by the a monomer ((the left monomer)) located in 5' as compared to the second monomer ((the right monomer) of a TALEN

As used herein, the term "recognition half-site," "recognition sequence half-site," or simply "half-site" for a meganuclease means a nucleic acid sequence in a double-stranded DNA molecule that is recognized by a monomer of a homodimeric or heterodimeric meganuclease, or by one subunit of a single-chain meganuclease.

As used herein, the term "hypervariable region" refers to a localized sequence within a meganuclease monomer or subunit that comprises amino acids with relatively high variability. A hypervariable region can comprise about 50-60 contiguous residues, about 53-57 contiguous residues, or preferably about 56 residues.

A hypervariable region can comprise one or more residues that contact DNA bases in a recognition sequence and can be modified to alter base preference of the monomer or subunit. A hypervariable region can also comprise one or more residues that bind to the DNA backbone when the meganuclease associates with a double-stranded DNA recognition sequence. Such residues can be modified to alter the binding affinity of the meganuclease for the DNA backbone and the target recognition sequence. In different embodiments of the invention, a hypervariable region may comprise between 1-20 residues that exhibit variability and can be modified to influence base preference and/or DNA-binding affinity. In particular embodiments, a hypervariable region comprises

between about 15-18 residues that exhibit variability and can be modified to influence base preference and/or DNA-binding affinity

TCR ALPHA GENE Gene ID: 6955, as updated on 9-Jul-2017 (NCBI).

As used herein, the terms "T cell receptor alpha gene" refers to the human TCR alpha gene on the chromosome 14. from TRA in Genome Data Viewer Map ViewerLocation: 14q11.2.

T cell receptors recognize foreign antigens which have been processed as small peptides and bound to major histocompatibility complex (MHC) molecules at the surface of antigen presenting cells (APC). Each T cell receptor is a dimer consisting of one alpha and one beta chain or one delta and one gamma chain. In a single cell, the T cell receptor loci are rearranged and expressed in the order delta, gamma, beta, and alpha.

If both delta and gamma rearrangements produce functional chains, the cell expresses delta and gamma. If not, the cell proceeds to rearrange the beta and alpha loci. This region represents the germline organization of the T cell receptor alpha and delta loci. Both the alpha and delta loci include V (variable), J (joining), and C (constant) segments and the delta locus also includes diversity (D) segments. The delta locus is situated within the alpha locus, between the alpha V and J segments. During T cell development, the delta chain is synthesized by a recombination event at the DNA level joining a D segment with a J segment; a V segment is then joined to the D-J gene.

The alpha chain is synthesized by recombination joining a single V segment with a J segment. For both chains, the C segment is later joined by splicing at the RNA level. Recombination of many different V segments with several J segments provides a wide range of antigen recognition. Additional diversity is attained by junctional diversity, resulting from the random additional of nucleotides by terminal deoxynucleotidyltransferase. Five variable segments can be used in either alpha or delta chains and are described by TRAV/DV symbols. Several V and J segments of the alpha locus are known to be incapable of encoding a protein and are considered pseudogenes.

The constant region of the (T cell receptor alpha gene) TCRA or TRAC gene is identified by NCBI Gen ID NO. 28755. The regions of the TCR alpha gene targeted by the TALEN endonucleases of the invention correspond to a sequence that codes for an extracellular or intramembrane part of the T cell receptor alpha protein. The genetic modification (deletion, mutation or insertion) ultimately results in an alteration of cell surface expression of the alpha beta TCR.

In particular embodiments, the genetic modification alters cell surface expression of the delta gamma of the TCR.

In particular embodiments, the region(s) of the TCR alpha gene targeted by the nuclease is (are) as in WO2017062451 or as in WO2015057980 or as in WO2017106528.

In one embodiment, the genetic modification of the T cell receptor alpha constant region is located in a region corresponding to the c1, c2 or c3 region, preferably C1, of human T cell receptor alpha constant region, before the stop codon, and as defined in WO2017106528, preferably the genetic modification of the T cell receptor alpha constant region is located in a region corresponding to the c1 region of human T cell receptor alpha constant region.

In one embodiment, the genetic modification of the T cell receptor alpha constant region is located in a region corresponding to TTGTCCCACAGATATCCagaaccctgaccctgCCGTGTACCAGCTGAGAGA.

"T cell receptor alpha constant region gene" “TRAC gene” refers to the constant region of the human gene, in particular to the sequence identified by NCBI Gen ID NO. 28755. This is also referred as wild type (wt) TRAC gene and its sequence may slightly vary from one individual to another (slightly vary means 0.001% sequence change with no impact on the protein sequence (silent substitution deletion or insertion).

Genomic means that belongs to the genome (chromosomal DNA of the cells) and was not integrated into the genome as part of the experiments as a transgene or an exogenous polynucleotide performed in the present invention. In other words, the TRAC gene of the invention is not a

transgene, or an exogenous polynucleotide.

CELL

In one aspect, described herein is a cell (e.g., a eukaryotic cell such as a human cell including a lymphoid cell, a stem cell (e.g., iPSC, embryonic stem cell, MSC or HSC), or a progenitor cell) in which expression of a TCR gene is modulated.

In one aspect, described herein is a human cell (e.g., a eukaryotic cell such as a human cell including a lymphoid cell, a stem cell (e.g., iPSC, embryonic stem cell, MSC or HSC), or a progenitor cell) in which cell surface expression of a alpha beta TCR gene is inhibited by modification (mutation, deletion, or insertion, preferably an insertion) of exon cl, c2 and/or c3 of the TRAC gene.

In one aspect, described herein is a cell (e.g., a eukaryotic cell such as a human cell including a lymphoid cell, a stem cell (e.g., iPSC, embryonic stem cell, MSC or HSC), or a progenitor cell) in which expression of endogenous alpha beta TCR gene is modulated by insertion or an exogenous sequence into a TCR gene sequence coding for an extracellular domain of the TCR or a transmembrane domain so that cell surface expression of a functional alpha beta TCR can be compromised.

TAG

A tag is meant to be a sequence including a noncoding sequence or a coding sequence used as a marker or label and integrated into on-site (optionally in off sites) and can be detected.

ENGINEERING

The TRAC–deficient human cell of the invention may include further genomic modifications, for example an inactivated T-cell receptor beta gene, and inactivated PD1, CD52 and/or CTLA4 gene.

The TRAC–deficient human cell of the invention may include further genomic modifications, for example an inserted transgene or an inserted exogenous polynucleotide sequence such as a gene encoding a chimeric antigen receptor (CAR), a gene encoding a T-cell Receptor (TCR) and/or a gene

encoding an antibody, a transgene encoding a cytokine selected from IL-2, IL-7, IL-3, IL-12, IL-15, IL-17, IL-27, a protein conferring resistance to a drug, a protein conferring sensitivity to a drug, a combination thereof.

In a preferred embodiment, said transgene or polynucleotide is inserted in the TRAC gene.

PHARMACEUTICAL COMPOSITION

Pharmaceutical compositions comprising a pharmaceutically acceptable vehicle (or carrier) and any cell or cell population as described herein are also provided as well as methods of using the cells and pharmaceutical compositions in ex vivo therapies for the treatment of a disorder (e.g., a cancer) in a subject.

In the present invention described herein are cells in which the expression of a TCR gene is inactivated. In preferred embodiments, exon cl, c2 and/or c3 of a TCR gene is inactivated. In other embodiments, the activity of the endogenous TCR promotor is not inactivated and may be activated. The activation of the endogenous TCR promotor may be by an exogenous molecule (e.g., engineered transcription factor comprising a DNA-binding domain and a transcriptional activation domain) that binds to the TCR gene and activates TCR expression.

In some embodiments, cells are described that comprise a transiently expressed engineered nuclease to cause a knockout of a TRAC gene, knock-in of said TRAC gene, knock-in of said TRAC gene without a knockout of a TRAC gene, preferably knock-in of said TRAC gene with a knockout of a TRAC gene, more preferably knock-in of said TRAC gene with a knockout of a TRAC gene and expression of a CAR specific for an antigen expressed on a pathological cell.

Further, the human TRAC-deficient human cells of the invention described are cells wherein the expression of an endogenous TCR gene is reduced and TCR alpha beta expression at the cell surface is undetectable.

Further, the human TRAC-modified human cells of the invention described are cells wherein the expression of a known and selected TCR gene, which may be a known and selected alpha beta TCR gene, is maintained and the known TCR alpha beta expression at the cell surface is detectable.

Also provided here, cells wherein the expression of a TCR gene is reduced (as compared to a non engineered cells) and TCR alpha beta expression at the cell surface is undetectable (as compared to a positive control) and wherein the cells are further engineered to comprise a least one exogenous transgene and/or an additional knock out of at least one endogenous gene (e.g., beta 2 microglobulin (B2M) and/or immunological checkpoint gene such as PD1 and/or CTLA4) or combinations thereof.

The exogenous transgene may be integrated into a TCR gene (e.g., when the TCR gene is knocked out) and/or may be integrated into a non-TCR gene such as a safe harbor gene. In some cases, the exogenous transgene encodes an ACTR and/or a CAR. The transgene construct may be inserted by either HDR- or NITEJ- driven processes.

In some aspects, the cells with undetectable TCR expression comprise an exogenous TCR or an exogenous CAR. Cells of the invention further comprise a knockout of one or more check point inhibitor genes. In some embodiments, the check point inhibitor is PD1. In other embodiments, the check point inhibitor is CTLA4 or any of the immune check points disclosed in WO2014191128 selected from the list consisting of CTLA4, PPP2CA, PPP2CB, PTPN6, PTPN22, PDCD1 , LAG 3, HAVCR2, BTLA, CD160, TIGIT, CD96, CRTAM, LAIR1 , SIGLEC7, SIGLEC9, CD244, TNFRSF10B, TNFRSF10A, CASP8, CASP10, CASP3, CASP6, CASP7, FADD, FAS, TGFBRII, TGFRBRI, SMAD2, SMAD3, SMAD4, SMAD10, SKI, SKIL, TGIF1 , IL10RA, IL10RB, HMOX2, IL6R, IL6ST, EIF2AK4, CSK, PAG1 , SIT1 , FOXP3, PRDM1 , BATF, GUCY1A2, GUCY1A3, GUCY1 B2 and GUCY1 B3,

In some aspects, the cells with undetectable TCR expression comprise an exogenous TCR or an exogenous CAR and at least two inactivated genes selected from the group consisting of PD1 and TCR alpha, PD1 and TCR beta, CTLA-4 and TCR alpha, CTLA-4 and TCR beta, LAG 3 and TCR alpha, LAG 3 and TCR beta, Tim3 and TCR alpha, Tim3 and TCR beta, BTLA and TCR alpha, BTLA and TCR beta, BY55

and TCR alpha, BY55 and TCR beta, TIGIT and TCR alpha, TIGIT and TCR beta, B7H5 and TCR alpha, B7H5 and TCR beta, LAIR1 and TCR alpha, LAIR1 and TCR beta, SIGLEC10 and TCR alpha, SIGLEC10 and TCR beta, 2B4 and TCR alpha, 2B4 and TCR beta.

In further aspects, the TCR alpha KO cell of the invention comprises a PD1 knockout and a CTLA4 knockout. In some embodiments, another TCR gene inactivated is a gene encoding TCR β (TCRB). In some embodiments, this is achieved via targeted cleavage of the constant region of this gene (TCR β Constant region, or TRBC). In all embodiments, the TCR gene encoding TCR alpha (TCRA) is modified, and contain an insertion, a mutation and/or a deletion that affect (s) or not the cell surface of the TCRalpha beta. In further embodiments, insertion is achieved via targeted cleavage of the constant region of a TCR gene C1. In some embodiments, the TCR gene modified cells are further modified at the B2M gene, the HLA-A, -B, -C genes, or the TAP gene, or any combination thereof. In other embodiments, the regulator for HLA class II, CTLA, is also modified.

In further aspects, the TCR alpha KO cell of the invention comprises a CD25 KO with an insertion of a cytokine encoding sequence, preferably IL-2, IL-7, -12, 15, -17, -27, -27.

In a preferred embodiment, the TRAC gene modified human cells are further modified at the B2M gene, and/or the TAP gene, ant at the regulator for HLA class II, CTLA.

In a more preferred embodiment the TRAC gene KO human cells are further KO at the B2M gene, and/or the TAP gene, ant at the regulator for HLA class II, CTLA.

In certain embodiments, the cells described herein comprise a modification (e.g., deletion and/or insertion) to a TRAC gene (e.g., modification of exon cl, using a TALEN).

In certain embodiments, the modification comprises binding of an engineered Transcription Factor as described herein such that a TCRA gene expression is modulated, for example, repressed or activated. In other embodiments, the modification is a genetic modification (change of the native nucleotide sequence) at or near nuclease(s) binding (target) and/or cleavage site(s), including but not limited to,

modifications to sequences within 1-300 (or any number of base pairs therebetween) base pairs upstream, downstream and/or including 1 or more base pairs of the site(s) of cleavage and/or binding site; modifications within 1-100 base pairs (or any number of base pairs therebetween) of including and/or on either side of the binding and/or cleavage site(s); modifications within 1 to 50 base pairs (or any number of base pairs therebetween) including and/or on either side (e.g., 1 to 5, 1 to 10, 1 to 20 or more base pairs) of the binding and/or cleavage site(s); and/or modifications to one or more base pairs within the nuclease binding site and/or cleavage site.

In preferred embodiments, means for detecting a cell comprising Zinc-finger mediated TRAC designs as described in WO 2017106528, or in U. S. Patent Publication 20150132269.

Guide RNAs for the S. pyogenes CRISPR Cas9 system were also constructed to target the TCRA gene as in U.S. Publication No. 201500566705 or as in table 2 of WO2017106528.

The modified cell of the invention is a human cell such as lymphoid cell (e.g., a human T-cell), a human stem/progenitor cell (e.g., an induced human pluripotent stem cell (iPSC), a human embryonic stem cell (e.g., human ES), a human mesenchymal stem cell (MSC), or a human hematopoietic stem cell (HSC). The human stem cells may be totipotent or pluripotent (e.g., partially differentiated such as an HSC that is a pluripotent myeloid or lymphoid stem cell). In other embodiments, the invention provides methods for producing human cells that have a null genotype for TCR expression. Any of the modified human stem cells described herein (modified at the TCRA locus) may then be differentiated to generate a differentiated (in vivo or in vitro) cell descended from a stem cell as described herein with modified TCRA gene expression.

In another aspect, the cells, population of cells or pharmaceutical compositions, means of detection of said cells or of said pharmaceutical composition described herein can be used, for example, in the treatment or prevention or amelioration of a disorder. The methods typically comprise (a) cleaving or down regulating an endogenous TCR gene in an isolated human cell (e.g., progenitor, dedifferentiated or differentiated T-cell or lymphocyte) using a TALEN such that the TCR gene is inactivated or down

modulated, cell surface expression of the TCR alpha beta is undetectable and cell express a molecule to redirect its specificity and (b) introducing the cell into the subject, thereby treating or preventing the disorder.

In some embodiments, the gene encoding TCR β (TCRB) is inactivated or down modulated, as are the B2M gene, and the regulator for HLA class II gene, CIITA.

The methods typically comprise (a) cleaving or down regulating an endogenous TCR gene in an isolated cell (e.g., progenitor, dedifferentiated or differentiated T-cell or lymphocyte) using a nuclease (a TALEN) such that the TCR gene is modified such as inactivated (insertion stopping the expression of the TCR alpha protein) so that cell surface expression of the TCR alpha beta is undetectable and cell express a molecule (insertion in frame) to redirect its specificity, preferably a CAR and (b) detecting and identifying the nuclease TALEN-modified cells as compared to other nuclease modified cells using a CRISPR/Cas or engineered transcription factor (e.g. ZFN-TF, TALE-TF, Cfpl-TF or Cas9-TF) used to modify the TCR gene expression (c) identifying the (lack of) off sites as a signature and as a quality control of the engineered cells.

In some embodiments inactivation is achieved via targeted cleavage of the constant region of the beta gene (TCR β Constant region, or TRBC). In preferred embodiments, the gene encoding TCR alpha (TCRA) is modified such as comprises an insertion that inactivates the expression of TCR alpha beta at the cell surface.

In further preferred embodiments, the disorder is a cancer or an infectious disease. In further preferred embodiments inactivation is achieved via targeted cleavage of the constant region of this gene (TCR a Constant region, or abbreviated as TRAC). In some embodiments, additional genes are modulated (knocked-out), for example, B2M, PD1 and/or CTLA4 and/or one or more therapeutic transgenes are present in the cells (integrated via targeted integration such as nuclease-mediated integration using a vector).

The transcription factor(s) and/or nuclease(s) can be introduced into a cell or the surrounding culture media as mRNA, in protein form and/or as a DNA sequence encoding the nuclease(s). In certain embodiments, the isolated cell introduced into the subject further comprises additional genomic modification, for example, an integrated exogenous sequence (into the cleaved TCR gene or a different gene, for example a safe harbor gene or locus) and/or inactivation (e.g., nuclease-mediated) of additional genes, for example one or more HLA genes. The exogenous sequence or protein may be introduced via a vector (e.g. Ad, AAV, LV), or by using a technique such as electroporation. In some embodiments, the proteins are introduced into the cell by cell squeezing (see Kollmannsperger et al (2016) Nat Comm 7, 10372 doi: 10.1038/ncommsl0372).

Preferably, nucleases preferably mRNA encoding nucleases, even more preferably mRNA encoding TALEN are introduced into human primary cells by electroporation as described in US20130315884.

CELL THERAPY

In some aspects, the human engineered cells of the invention may be used for therapy, for example, for adoptive cell transfer, immunotherapy. In other embodiments, the cells for use in T cell transplant contain another gene modification of interest. In one aspect, the T cells contain an inserted chimeric antigen receptor (CAR) specific for a cancer marker. In a further aspect, the inserted CAR is specific for the CD22 marker characteristic of B cells, including B cell malignancies.

In one aspect, the T cells contain an inserted chimeric antigen receptor (CAR) specific for a cancer marker. In a further aspect, the inserted CAR is specific for the CD38 marker characteristic of B cells, including B cell malignancies and comprises a genomic inactivated CD38 gene.

In one aspect, the T cells contain an inserted chimeric antigen receptor (CAR) specific for a cancer marker. In a further aspect, the inserted CAR is specific for the CD123 marker characteristic of immune cells, including immune cell malignancies, AML, BPDCN.

In one aspect, the T cells contain an inserted chimeric antigen receptor (CAR) specific for a cancer B

cell marker. In a further aspect, the inserted CAR is specific for the CD22 marker characteristic of immune cells, including immune B cell malignancies.

Such cells would be useful in a therapeutic composition administered several times (repeated administration) for treating patients with match or partial match HLA, and so that unmatched HLA are never the same in the successive doses administered and so would be able to be used as an "off-the-shelf therapeutic for any patient in need thereof.

In another aspect, the TCR-modulated (modified) T cells contain an inserted Antibody-coupled T-cell Receptor (ACTR) donor sequence. In some embodiments, the ACTR donor sequence is inserted into a TCR gene to disrupt expression of that TCR gene following nuclease induced cleavage. In other embodiments, the donor sequence is inserted into a "safe harbor" locus, such as the AAVS 1, HPRT, albumin and CCR5 genes. In some embodiments, the ACTR sequence is inserted via targeted integration where the ACTR donor sequence comprises flanking homology arms that have homology to the sequence flanking the cleavage site of the engineered nuclease. In some embodiments, the ACTR donor sequence further comprises a promoter and/or other transcriptional regulatory sequences. In other embodiments, the ACTR donor sequence lacks a promoter. In some embodiments, the ACTR donor is inserted into a TCR β encoding gene (TCRB). In some embodiments insertion is achieved via targeted cleavage of the constant region of this gene (TCR β Constant region, or TRBC). In preferred embodiments, the ACTR donor is inserted into a TCR a encoding gene (TCRA). In further preferred embodiments insertion is achieved via targeted cleavage of the constant region of this gene (TCR a Constant region, abbreviated TRAC). In some embodiments, the donor is inserted into an exon sequence in TCRA, while in others, the donor is inserted into an intronic sequence in TCRA. In some embodiments, the TCR-modulated cells further comprise a CAR. In still further embodiments, the TCR-modulated cells are additionally modulated at an HLA gene or a checkpoint inhibitor gene.

Also provided are pharmaceutical compositions comprising the modified cells as described herein

(e.g., T cells or stem cells with inactivated TCR gene), or pharmaceutical compositions comprising one or more of the TCR gene binding molecules (e.g., engineered transcription factors and/or nucleases) and a pharmaceutically acceptable vehicle or excipient as described herein. In certain embodiments, the pharmaceutical compositions further comprise one or more pharmaceutically acceptable excipients. The modified cells, TCR gene binding molecules (or polynucleotides encoding these molecules) and/or pharmaceutical compositions comprising these cells or molecules are introduced into the subject via methods known in the art, e.g. through intravenous infusion, infusion into a specific vessel such as the hepatic artery, or through direct tissue injection (e.g. muscle). In some embodiments, the subject is an adult human with a disease or condition that can be treated or ameliorated with the composition. In other embodiments, the subject is a pediatric subject where the composition is administered to prevent, treat or ameliorate the disease or condition (e.g., cancer, graft versus host disease, etc.).

The terms "recombinant DNA construct," "recombinant construct," "expression cassette," "expression construct," "chimeric construct," "construct," and "recombinant DNA fragment" are used interchangeably herein and are single or double-stranded polynucleotides. A recombinant construct comprises an artificial combination of single or double-stranded polynucleotides, including, without limitation, regulatory and coding sequences that are not found together in nature. For example, a recombinant DNA construct may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source and arranged in a manner different than that found in nature. Such a construct may be used by itself or may be used in conjunction with a vector.

As used herein, a "vector" or "recombinant DNA vector" may be a construct that includes a replication system and sequences that are capable of transcription and translation of a polypeptide-encoding sequence in a given host cell. If a vector is used then the choice of vector is dependent upon the method that will be used to transform host cells as is well known to those skilled in the art.

Vectors can include, without limitation, plasmid vectors and recombinant AAV vectors, or any other vector known in that art suitable for delivering a gene encoding a meganuclease of the invention to a target cell. The skilled artisan is well aware of the genetic elements that must be present on the vector in order to successfully transform, select and propagate host cells comprising any of the isolated nucleotides or nucleic acid sequences of the invention.

As used herein, a "vector" can also refer to a viral vector. Viral vectors can include, without limitation, retroviral vectors, lentiviral vectors, adenoviral vectors, and adeno-associated viral vectors (AAV).

As used herein, a "vector" can also refer to adeno-associated viral vector, with AAV6 capsid protein and AAV2 sequences allowing guidance.

As used herein, a "polycistronic" mRNA refers to a single messenger RNA that comprises two or more coding sequences (i.e., cistrons) and encodes more than one protein. A polycistronic mRNA can comprise any element known in the art to allow for the translation of two or more genes from the same mRNA molecule including, but not limited to, an IRES element, a T2A element, a P2A element, an E2A element, and an F2A element.

As used herein, a "human T cell" or "T cell" refers to a T cell isolated from a human donor. Human T cells, and cells derived therefrom, include isolated T cells that have not been passaged in culture, T cells that have been passaged and maintained under cell culture conditions without immortalization, and T cells that have been immortalized and can be maintained under cell culture conditions indefinitely.

As used herein, a "control" or "control cell" refers to a cell that provides a reference point for measuring changes in genotype or phenotype of a genetically-modified cell. A control cell may comprise, for example: (a) a wild-type cell, i.e. , of the same genotype as the starting material for the genetic alteration that resulted in the genetically-modified cell; (b) a cell of the same genotype as the genetically-modified cell but that has been transformed with a null construct (i.e., with a construct

that has no known effect on the trait of interest); or, (c) a cell genetically identical to the genetically-modified cell but that is not exposed to conditions or stimuli or further genetic modifications that would induce expression of altered genotype or phenotype.

T cell expressing alpha beta TCR. T cell with a native TCR gene (eg as described in NCBI reference as above), that may include silent mutation(s).

THERAPEUTIC APPLICATIONS

2.1 Principle of the Invention

The present invention is based, in part, on the discovery that different engineered nucleases recognize and cleave recognition sequences found within the human TCR alpha gene, and depending on the nuclease used, off sites sequences. The sequence(s) cleaved (of sites and on sites) is (are) specific for the nuclease used and linked to the frequency and number of off target sites making the final product more or less reliable as a stable medicament. This is especially important to be provided due to the fact that a medicament must be stable in a host and resist the immune system that exerts constant pressure.

Moreover, according to the invention, an exogenous polynucleotide sequence can be inserted specifically into the TCR alpha constant region gene at the nuclease cleavage site, and may be used as a tag to detect engineered cells, even in cells with additional edited genes, such as CD52 or dCK KO gene. Insertion takes place for example by homologous recombination, such that a sequence of interest is concurrently expressed in the cell. Such exogenous sequences can be noncoding sequence and /or encode, for example, a chimeric antigen receptor, an exogenous TCR receptor, or any other polypeptide of interest. Ultimately the TRAC gene modification results in a specific sequence as compared to the wt TRAC non-engineered sequence.

The inventors provided human cells engineered for immunotherapy with no or reduced side effects (GVHD,...) with stable phenotypes and genotypes (that is to say with undetectable level of off sites)

comprising the following sequence:

AAGTAGCCCTGCATTTCAGGTTTCCTTGAGTGGCAGGCCAGGCCTGGCCGTGAACGTTCACTGAAATCATGGCC

TCTTGGCCAAGATTGATAGCTTGTGCCTGTCCCTGAGTCCCAGTCCATCACGAGCAGCTGGTTTCTAAGATGCTAT

TTCCCGTATAAAGCATGAGACCGTGACTTGCCAGCCCCACAGAGCCCCGCCCTTGTCCATCACTGGCATCTGGAC

TCCAGCCTGGGTTGGGGCAAAGAGGGAAATGAGATCATGTCCTAACCCTGATCCTCTTGTCCCACAGATATCCAG

TCCGGTGAGGGCAGAGGAAGTCTTCTAACATGCGGTGACGTGGAGGAGAATCCGGGCCCCGGATCCcodingse

quenceTCTAGAGGGCCCGTTTAAACCCGCTGATCAGCCTCGA*CTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTT*

GCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCA

TCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGG

*AAGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATG*ACTAGTGGCGAATTCCCGTGTACCAGCTGAGAG

ACTCTAAATCCAGTGACAAGTCTGTCTGCCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGA

TTCTGATGTGTATATCACAGACAAAACTGTGCTAGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGC

CTGGAGCAACAAATCTGACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGACACCTTCTTCCCC

AGCCCAGGTAAGGGCAGCTTTGGTGCCTTCGCAGGCTGTTTCCTTGCTTCAGGAA,

Preferably said insertion in the genomic TRAC comprises the following sequence:

AAGTAGCCCTGCATTTCAGGTTTCCTTGAGTGGCAGGCCAGGCCTGGCCGTGAACGTTCACTGAAATCATGGCC

TCTTGGCCAAGATTGATAGCTTGTGCCTGTCCCTGAGTCCCAGTCCATCACGAGCAGCTGGTTTCTAAGATGCTAT

TTCCCGTATAAAGCATGAGACCGTGACTTGCCAGCCCCACAGAGCCCCGCCCTTGTCCATCACTGGCATCTGGAC

TCCAGCCTGGGTTGGGGCAAAGAGGGAAATGAGATCATGTCCTAACCCTGATCCTCTTGTCCCACAGATATCCAG

TCCGGTGAGGGCAGAGGAAGTCTTCTAACATGCGGTGACGTGGAGGAGAATCCGGGCCCCGGATCCGCTCTGC

CCGTCACCGCTCTGCTGCTGCCACTGGCCCTGCTGCTGCACGCAGCAAGACCAGGAGGGGGAGGCAGCTGCCC

CTACAGCAACCCCAGCCTGTGCAGCGGAGGCGGCGGCAGCGGCGGAGGGGGTAGCCAGGTGCAGCTGCAGC

AGAGCGGCCCTGGCCTGGTGAAGCCAAGCCAGACACTGTCCCTGACCTGCGCCATCAGCGGCGATTCCGTGAG

CTCCAACTCCGCCGCCTGGAATTGGATCAGGCAGTCCCCTTCTCGGGGCCTGGAGTGGCTGGGAAGGACATACT

ATCGGTCTAAGTGGTACAACGATTATGCCGTGTCTGTGAAGAGCAGAATCACAATCAACCCTGACACCTCCAAGA

ATCAGTTCTCTCTGCAGCTGAATAGCGTGACACCAGAGGACACCGCCGTGTACTATTGCGCCAGGGAGGTGACC

GGCGACCTGGAGGATGCCTTTGACATCTGGGGCCAGGGCACAATGGTGACCGTGTCTAGCGGAGGAGGAGGAT

CCGGAGGAGGAGGATCTGGCGGCGGCGGCAGCGATATCCAGATGACACAGTCCCCATCCTCTCTGAGCGCCTCC

GTGGGCGACAGAGTGACAATCACCTGTAGGGCCTCCCAGACCATCTGGTCTTACCTGAACTGGTATCAGCAGAG

GCCCGGCAAGGCCCCTAATCTGCTGATCTACGCAGCAAGCTCCCTGCAGAGCGGAGTGCCATCCAGATTCTCTGG

CAGGGGCTCCGGCACAGACTTCACCCTGACCATCTCTAGCCTGCAGGCCGAGGACTTCGCCACCTACTATTGCCA

GCAGTCTTATAGCATCCCCCAGACATTTGGCCAGGGCACCAAGCTGGAGATCAAGGGAAGCGGAGGGGGAGG

CAGCTGCCCCTACAGCAACCCCAGCCTGTGCAGCGGAGGCGGCGGCAGCGAGCTGCCCACCCAGGGCACCTTC

TCCAACGTGTCCACCAACGTGAGCCCAGCCAAGCCCACCACCACCGCCTGTCCTTATTCCAATCCTTCCCTGTGTG

CTCCCACCACAACCCCAGCACCAAGGCCACCTACACCTGCACCAACCATCGCCTCTCAGCCCCTGAGCCTGAGAC

CTGAGGCATGTAGGCCAGCAGCAGGAGGAGCAGTCCATACAAGGGGTCTGGATTTTGCATGCGACATCTACATC

TGGGCACCTCTGGCAGGAACATGTGGCGTGCTCCTGCTCAGCCTGGTCATCACCCTGTACTGCAAGAGAGGCAG

GAAGAAGCTGCTGTATATCTTCAAGCAGCCCTTCATGCGCCCCGTGCAGACAACCCAGGAGGAGGATGGCTGCT

CCTGTAGGTTCCCAGAAGAGGAGGAGGGAGGATGTGAGCTGCGCGTGAAGTTTTCCCGGTCTGCCGACGCACC

TGCATACCAGCAGGGCCAGAACCAGCTGTATAACGAGCTGAATCTGGGCCGGAGAGAGGAGTACGATGTGCTG

GACAAGAGGCGCGGCAGAGATCCAGAGATGGGCGGCAAGCCCCGGAGAAAGAACCCTCAGGAGGGCCTGTA

CAATGAGCTGCAGAAGGATAAGATGGCCGAGGCCTATTCTGAGATCGGCATGAAGGGAGAGAGGCGCCGGGG

CAAGGGACACGACGGACTGTACCAGGGACTGAGCACAGCCACCAAGGATACCTATGACGCCCTGCATATGCAGG

CACTGCCTCCAAGGTGATCTAGAGGGCCCGTTTAAACCCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGC

CATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAAT

GAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGG

GGGAGGATTGGGAAGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATGACTAGTGGCGAATTCCCGTG

TACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTGCCTATTCACCGATTTTGATTCTCAAACAAATGTGT

CACAAAGTAAGGATTCTGATGTGTATATCACAGACAAAACTGTGCTAGACATGAGGTCTATGGACTTCAAGAGCA

ACAGTGCTGTGGCCTGGAGCAACAAATCTGACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAG

ACACCTTCTTCCCCAGCCCAGGTAAGGGCAGCTTTGGTGCCTTCGCAGGCTGTTTCCTTGCTTCAGGAA, (SEQ

ID N°9). or

AAGTAGCCCTGCATTTCAGGTTTCCTTGAGTGGCAGGCCAGGCCTGGCCGTGAACGTTCACTGAAATCATGGCC

TCTTGGCCAAGATTGATAGCTTGTGCCTGTCCCTGAGTCCCAGTCCATCACGAGCAGCTGGTTTCTAAGATGCTAT

TTCCCGTATAAAGCATGAGACCGTGACTTGCCAGCCCCACAGAGCCCCGCCCTTGTCCATCACTGGCATCTGGAC

TCCAGCCTGGGTTGGGGCAAAGAGGGAAATGAGATCATGTCCTAACCCTGATCCTCTTGTCCCACAGATATCCAG

TCCGGTGAGGGCAGAGGAAGTCTTCTAACATGCGGTGACGTGGAGGAGAATCCGGGCCCCGGATCCGCTCTGC

CCGTCACCGCTCTGCTGCTGCCACTGGCCCTGCTGCTGCACGCCGCCAGACCCGGCGGAGGAGGCTCTTGCCCC

TACAGCAACCCCAGCCTGTGCTCTGGCGGCGGCGGCAGCGGAGGCGGCGGCTCCCAGGTGCAGCTGCAGCAG

AGCGGCCCTGGCCTGGTGGAGCCAAGCCAGACACTGTCCCTGACCTGCGCCATCTCTGGCGACAGCGTGAGCTC

CAACAGCGCCGCATGGAATTGGATCAGGCAGTCCCCATCTCGGGGCCTGGAGTGGCTGGGCAGAACATACTATA

GGTCCACCTGGTACAACGACTATGCCGGCTCCGTGAAGTCTCGCATCACAATCAACCCCGATACCAGCAAGAATC

AGTTCTCCCTGCAGCTGACATCTGTGACCCCTGAGGACACAGCCGTGTACTATTGCACCAGAAGCAGGCACAATA

CATTTCGGGGAATGGACGTGTGGGGACAGGGCACACTGGTGACCGTGAGCGGAGGAGGAGGATCCGGCGGA

GGAGGCTCTGGCGGCGGCGGCAGCGACATCCAGCTGACCCAGTCCCCTTCTAGCCTGAGCGCCTCCGTGGGCG

ATAGAGTGACAATCACCTGTAGGGCCTCTCAGAGCATCTCCTCTTACCTGAACTGGTATCAGCAGAAGCCCGGCA

AGGCCCCTAAGCTGCTGATCTACGCAGCAAGCTCCCTGCAGTCTGGAGTGCCAAGCAGATTCTCCGGCTCTGGC

AGCGGCACCGACTTTACACTGACCATCTCTAGCCTGCAGCCTGAGGATTTCGCCACATACTATTGCCAGCAGTCCT

ATTCTACACCACTGACCTTTGGCGGCGGCACCAAGGTGGAGATCAAGGGAAGCGGCGGCGGCGGAAGTTGTCC

ATATTCAAACCCAAGTCTGTGCAGCGGCGGAGGAGGAAGCGAACTGCCTACTCAGGGAACCTTCAGCAACGTG

TCCACCAATGTGAGCCCAGCAAAGCCTACCACAACCGCATGCCCATACTCTAACCCCAGCCTGTGCACAACCACA

CCAGCACCCAGGCCCCCTACCCCTGCACCAACAATCGCCTCCCAGCCTCTGTCTCTGCGGCCAGAGGCCTGCAGA

CCCGCCGCCGGCGGAGCAGTGCACACACGGGGCCTGGACTTTGCCTGTGATATCTATATCTGGGCACCACTGGC

CGGAACATGTGGCGTGCTGCTGCTGTCACTGGTCATTACACTGTACTGTAAGCGAGGCCGGAAGAAACTGCTGTA

TATTTTCAAACAGCCCTTTATGAGACCTGTGCAGACTACCCAGGAGGAAGACGGCTGCAGCTGTAGGTTCCCCG

AGGAAGAGGAAGGCGGGTGTGAGCTGAGGGTCAAGTTTAGCCGCTCCGCAGATGCCCCTGCTTACCAGCAGG

GGCAGAATCAGCTGTATAACGAGCTGAATCTGGGACGGAGAGAGGAATACGACGTGCTGGATAAAAGGCGCGG

GAGAGACCCCGAAATGGGAGGCAAGCCACGACGGAAAAACCCCCAGGAGGGCCTGTACAATGAACTGCAGAA
GGACAAAATGGCAGAGGCCTATAGTGAAATCGGGATGAAGGGAGAGAGAAGGCGCGGCAAAGGGCACGATG
GCCTGTACCAGGGGCTGTCTACTGCCACCAAGGACACCTATGATGCTCTGCATATGCAGGCACTGCCTCCAAGGT
GATCTAGAGGGCCCGTTTAAACCCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCC
CTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCG
CATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAA
GACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATGACTAGTGGCGAATTCCCGTGTACCAGCTGAGAGAC
TCTAAATCCAGTGACAAGTCTGTCTGCCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTC
TGATGTGTATATCACAGACAAAACTGTGCTAGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTG
GAGCAACAAATCTGACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGACACCTTCTTCCCCAG
CCCAGGTAAGGGCAGCTTTGGTGCCTTCGCAGGCTGTTTCCTTGCTTCAGGAA (SEQ ID N°10).), or

AAGTAGCCCTGCATTTCAGGTTTCCTTGAGTGGCAGGCCAGGCCTGGCCGTGAACGTTCACTGAAATCATGGCC
TCTTGGCCAAGATTGATAGCTTGTGCCTGTCCCTGAGTCCCAGTCCATCACGAGCAGCTGGTTTCTAAGATGCTAT
TTCCCGTATAAAGCATGAGACCGTGACTTGCCAGCCCCACAGAGCCCCGCCCTTGTCCATCACTGGCATCTGGAC
TCCAGCCTGGGTTGGGGCAAAGAGGGAAATGAGATCATGTCCTAACCCTGATCCTCTTGTCCCACAGATATCCAG
TCCGGTGAGGGCAGAGGAAGTCTTCTAACATGCGGTGACGTGGAGGAGAATCCGGGCCCCGGATCCGCTCTGC
CCGTCACCGCTCTGCTGCTGCCACTGGCCCTGCTGCTGCACGCCGCCAGACCCGGCGGAGGAGGCTCTTGCCCC
TACAGCAACCCCAGCCTGTGCTCTGGCGGCGGCGGCAGCGGAGGCGGCGGCTCCCAGGTGCAGCTGCAGCAG
AGCGGCCCCGGCCTGGTGAAGCCTAGCCAGACACTGTCCCTGACCTGCGCAATCTCCGGCGACAGCGTGTCCGG
AAACAGGGCCACATGGAATTGGATCAGACAGTCTCCAAGCAGGGGCCTGGAGTGGCTGGGAAGGACCTACTAT
CGGTCCGCCTGGTACAACGACTATGCCGTGTCTGTGAAGGGCCGCATCACATTCAACCCAGATACCAGCAAGAAT
CAGTTTTCCCTGCAGCTGAATTCTGTGACACCCGAGGATACCGCCGTGTACTATTGCGCCAGAGGCGAGAGCGG
AGCAGCAGCAGACGCCTTCGATATCTGGGGCCAGGGCACCACAGTGACAGTGAGCGGAGGAGGAGGATCCGG

CGGAGGAGGCTCTGGCGGCGGCGGCAGCGACATCCAGCTGACCCAGAGCCCACCTTCCCTGTCTGCCAGCGTG

GGCGATCGCGTGACAATCACCTGTCGGGCCTCCCAGTCTATCAGCTCCTACCTGAACTGGTATCAGCAGAAGCCA

GGCAAGGCCCCCAAGCTGCTGATCTACGCAGCATCTAGCCTGCAGTCTGGAGTGCCAAGCAGATTCAGCGGATC

CGGATTCGGCACAGACTTTACACTGACCATCTCCTCTCTGCAGCCCGAGGATTTCGCCACCTACTATTGCCAGCAG

TCTTATAGCACACCTCAGACCTTTGGCCAGGGCACCAAGGTGGACATCAAGGGAAGTGGAGGAGGAGGAAGTT

GTCCCTACTCAAACCCATCTCTGTGCTCAGGAGGAGGAGGAAGTGAACTGCCTACTCAGGGAACATTCAGCAAC

GTGTCCACCAATGTGAGCCCAGCAAAGCCTACCACAACCGCATGCCCATACTCTAACCCCAGCCTGTGCACAACC

ACACCAGCACCCAGGCCCCCTACCCCTGCACCAACAATCGCCTCCCAGCCTCTGTCTCTGCGGCCAGAGGCCTGC

AGACCCGCCGCCGGCGGAGCAGTGCACACACGGGGCCTGGACTTTGCCTGTGATATCTATATCTGGGCACCACT

GGCCGGAACATGTGGCGTGCTGCTGCTGTCACTGGTCATTACACTGTACTGTAAGCGAGGCCGGAAGAAACTGC

TGTATATTTTCAAACAGCCCTTTATGAGACCTGTGCAGACTACCCAGGAGGAAGACGGCTGCAGCTGTAGGTTCC

CCGAGGAAGAGGAAGGCGGGTGTGAGCTGAGGGTCAAGTTTAGCCGCTCCGCAGATGCCCCTGCTTACCAGCA

GGGGCAGAATCAGCTGTATAACGAGCTGAATCTGGGACGGAGAGAGGAATACGACGTGCTGGATAAAAGGCGC

GGGAGAGACCCCGAAATGGGAGGCAAGCCACGACGGAAAAACCCCCAGGAGGGCCTGTACAATGAACTGCAG

AAGGACAAAATGGCAGAGGCCTATAGTGAAATCGGGATGAAGGGAGAGAGAAGGCGCGGCAAAGGGCACGAT

GGCCTGTACCAGGGGCTGTCTACTGCCACCAAGGACACCTATGATGCTCTGCATATGCAGGCACTGCCTCCAAGG

TGATCTAGAGGGCCCGTTTAAACCCGCTGATCAGCCTCGACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCC

CCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCCCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATC

GCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGGGTGGGGCAGGACAGCAAGGGGGAGGATTGGGA

AGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATGACTAGTGGCGAATTCCCGTGTACCAGCTGAGAGA

CTCTAAATCCAGTGACAAGTCTGTCTGCCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATT

CTGATGTGTATATCACAGACAAAACTGTGCTAGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCT

GGAGCAACAAATCTGACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGACACCTTCTTCCCCA

GCCCAGGTAAGGGCAGCTTTGGTGCCTTCGCAGGCTGTTTCCTTGCTTCAGGAA, ((SEQ ID N°11), or

AAGTAGCCCTGCATTTCAGGTTTCCTTGAGTGGCAGGCCAGGCCTGGCCGTGAACGTTCACTGAAATCATGGCC

TCTTGGCCAAGATTGATAGCTTGTGCCTGTCCCTGAGTCCCAGTCCATCACGAGCAGCTGGTTTCTAAGATGCTAT

TTCCCGTATAAAGCATGAGACCGTGACTTGCCAGCCCCACAGAGCCCCGCCCTTGTCCATCACTGGCATCTGGAC

TCCAGCCTGGGTTGGGGCAAAGAGGGAAATGAGATCATGTCCTAACCCTGATCCTCTTGTCCCACAGATATCCAG

TCCGGTGAGGGCAGAGGAAGTCTTCTAACATGCGGTGACGTGGAGGAGAATCCGGGCCCCGGATCCGCTCTGC

CCGTCACCGCTCTGCTGCTGCCTCTGGCCCTGCTGCTGCACGCAGCCAGACCAGGCGGAGGAGGCTCCTGCCCT

TACTCTAACCCAAGCCTGTGCTCCGGAGGAGGAGGATCCGGCGGAGGAGGCTCTGAGGTGAAGCTGGTGGAG

AGCGGAGGAGGCCTGGTGCAGCCTGGCGGCTCCCTGTCTCTGAGCTGCGCAGCATCCGGCTTCACCTTTACAGA

CTACTATATGTCTTGGGTGAGACAGCCCCCTGGCAAGGCCCTGGAGTGGCTGGCCCTGATCAGGTCCAAGGCCG

ATGGCTACACCACAGAGTATTCCGCCTCTGTGAAGGGCAGATTCACCCTGTCTAGGGACGATAGCCAGTCCATCC

TGTACCTGCAGATGAATGCACTGCGCCCCGAGGACAGCGCCACATACTATTGTGCCAGAGACGCCGCCTACTATT

CTTACTATAGCCCTGAGGGCGCTATGGACTACTGGGGCCAGGGCACCTCCGTGACAGTGAGCTCCGGAGGAGG

AGGAAGCGGAGGAGGAGGCTCCGGCGGCGGCGGCTCTATGGCCGACTATAAGGATATCGTGATGACCCAGAGC

CACAAGTTTATGTCTACAAGCGTGGGCGACCGCGTGAACATCACCTGCAAGGCCAGCCAGAATGTGGATTCCGC

CGTGGCCTGGTACCAGCAGAAGCCTGGCCAGAGCCCTAAGGCCCTGATCTATTCCGCCTCTTACCGGTATAGCGG

AGTGCCTGACCGCTTCACCGGAAGGGGATCCGGAACAGACTTCACCCTGACAATCTCTAGCGTGCAGGCCGAG

GATCTGGCCGTGTACTATTGTCAGCAGTACTATAGCACCCCCTGGACCTTCGGCGGAGGAACCAAGCTGGAGATC

AAGAGAGGATCTGGAGGAGGAGGAAGCTGCCCATACTCCAACCCCTCTCTGTGCAGCGGAGGAGGAGGATCTG

AGCTGCCAACCCAGGGCACATTTTCCAACGTGTCTACAAATGTGAGCCCAGCAAAGCCAACCACAACCGCATGC

CCTTATAGCAATCCATCCCTGTGCACAACCACACCTGCACCAAGACCACCAACCCCAGCACCTACAATCGCCTCTC

AGCCACTGAGCCTGCGCCCCGAGGCATGCCGGCCTGCAGCAGGCGGCGCCGTGCACACCAGGGGCCTGGACT

TCGCCTGCGATATCTACATCTGGGCACCTCTGGCAGGAACCTGTGGCGTGCTGCTGCTGAGCCTGGTCATCACCC

TGTACTGCAAGAGAGGCAGGAAGAAGCTGCTGTATATCTTCAAGCAGCCCTTTATGCGCCCTGTGCAGACCACAC

AGGAGGAGGACGGCTGCAGCTGTCGGTTCCCAGAAGAGGAGGAGGGCGGCTGTGAGCTGAGAGTGAAGTTT

AGCAGGTCCGCCGATGCACCAGCATACCAGCAGGGACAGAACCAGCTGTATAACGAGCTGAATCTGGGCCGGA

GAGAGGAGTACGACGTGCTGGATAAGAGGAGGGGAAGGGACCCCGAGATGGGAGGCAAGCCACGGAGAAA

GAACCCCCAGGAGGGCCTGTACAATGAGCTGCAGAAGGACAAGATGGCCGAGGCCTATTCCGAGATCGGCATG

AAGGGAGAGAGGCGCCGGGGCAAGGGACACGATGGCCTGTACCAGGGCCTGTCTACCGCCACAAAGGACACC

TATGATGCCCTGCATATGCAGGCACTGCCTCCAAGGTGATCTAGAGGGCCCGTTTAAACCCGCTGATCAGCCTCG
ACTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTC
CCACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGG
GGTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTAT
GACTAGTGGCGAATTCCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTGCCTATTCACCGATT
TTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGACAAAACTGTGCTAGACATGAG
GTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCAACAAATCTGACTTTGCATGTGCAAACGCCTTCAA
CAACAGCATTATTCCAGAAGACACCTTCTTCCCCAGCCCAGGTAAGGGCAGCTTTGGTGCCTTCGCAGGCTGTTT
CCTTGCTTCAGGAA (SEQ ID N°12) wherein underlined sequences are sequence of the genomic TRAC (homology arm), ***Italic bold*** sequence is a sequence coding for a 2A peptide, *Italic* is a sequence corresponding to a ply A termination sequence (BGH polyA).

In frame is a sequence coding for a CAR sequence, in particular for an anti-CD22 CAR, an anti-CD123 CAR sequence, preferably anti-CD22 CAR, anti -CD123 CAR.

The inventors also provided means for detecting and identifying said engineered cells (TALEN-modified endogenous αβ-TCR negative human primary cell). This is an essential step for making a medicament and useful to provide quality controlled cells.

Thus, the present invention allows for both the knockout of the endogenous alpha beta T cell receptor and the expression of an exogenous nucleic acid sequence (e.g., a chimeric antigen receptor or exogenous TCR) by targeting a single recognition site with a single engineered nuclease in one cell.

Accordingly, the present invention provides a homogenous population of CAR-expressing cells.

In particular embodiments where a sequence encoding a chimeric antigen receptor is inserted into the TCR alpha constant region gene, the invention provides a simplified method for producing an “allogeneic” T cell that expresses an antigen-specific CAR and has reduced TCR alpha beta expression, or complete knockout, of the endogenous TCR. Such cells can exhibit reduced or no induction of

graft-versus-host-disease (GVHD) when administered to an allogeneic subject and no rejection by T cells of the host. (HvGD).

TALEN for Recognizing and Cleaving Recognition Sequences in the T Cell Receptor Alpha Constant Region Gene and comparative nucleases doing the same

In particular embodiments, the invention can be practiced using a TALEN or a Compact TALEN.

It is known in the art that it is possible to use a site-specific nuclease to make a DNA break in the genome of a living cell, and that such a DNA break can result in permanent modification of the genome via mutagenic NHEJ repair or via homologous recombination with a transgenic DNA sequence. NHEJ can produce mutagenesis at the cleavage site, resulting in inactivation of the allele. NHEJ-associated mutagenesis may inactivate an allele via generation of early stop codons, frameshift mutations producing aberrant non-functional proteins, or could trigger mechanisms such as nonsense-mediated mRNA decay. The use of nucleases to induce mutagenesis via NHEJ can be used to target a specific mutation or a sequence present in a wild-type allele.

The use of nucleases to induce a double-strand break in a target locus is known to stimulate homologous recombination, particularly of transgenic DNA sequences flanked by sequences that are homologous to the genomic target. In this manner, exogenous nucleic acid sequences can be inserted into a target locus. Such exogenous nucleic acids can be non-coding sequence or encode, for example, a chimeric antigen receptor, an exogenous TCR, or any sequence or polypeptide of interest. For said polypeptide of interest to be expressed, said open reading frame or encoding sequence must be in frame with the TCR promotor or have its own promoter. In case the encoding sequence is in frame with the TCR promotor it has to comprise a sequence encoding a self-cleaving peptide such as a 2A peptide, for the subsequent polypeptide to be expressed on its own, by itself.

IRES

In one embodiment said polypeptide encoded by an exogenous sequence comprises an IRES.

An IRES means Internal ribosome entry sites and may be any IRES that allows the transcription and then translation of a coding sequence inserted in the gene of the invention.

gcccctctccctccccccccctaacgttactggccgaagccgcttggaataaggccggtgtgcgtttgtctatatgttattttccaccata ttgccgtcttttggcaatgtgagggcccggaaacctggccctgtcttcttgacgagcattcctaggggtctttcccctctcgccaaaggaa tgcaaggtctgttgaatgtcgtgaaggaagcagttcctctggaagcttcttgaagacaaacaacgtctgtagcgacccctttgcaggcagc ggaacccccacctggcgacaggtgcctctgcggccaaaagccacgtgtataagatacacctgcaaaggcggcacaaccccagtgc cacgttgtgagttggatagttgtggaaagagtcaaatggctctcctcaagcgtattcaacaaggggctgaaggatgcccagaaggtacc ccattgtatgggatctgatctggggcctcggtgcacatgctttacatgtgtttagtcgaggttaaaaaaacgtctaggccccccgaaccac ggggacgtggttttcctttgaaaaacacgatgataatatggccacaacc

In an advantageous embodiment the invention may be practiced using a TALEN.

In an advantageous embodiment the invention may be practiced using a TALEN encoded by ATGGGCGATCCTAAAAAGAAACGTAAGGTCATCGATATCGCCGATCTACGCACGCTCGGCTACAGCCAGCAGCAA CAGGAGAAGATCAAACCGAAGGTTCGTTCGACAGTGGCGCAGCACCACGAGGCACTGGTCGGCCACGGGTTTA CACACGCGCACATCGTTGCGTTAAGCCAACACCCGGCAGCGTTAGGGACCGTCGCTGTCAAGTATCAGGACATG ATCGCAGCGTTGCCAGAGGCGACACACGAAGCGATCGTTGGCGTCGGCAAACAGTGGTCCGGCGCACGCGCTC TGGAGGCCTTGCTCACGGTGGCGGGAGAGTTGAGAGGTCCACCGTTACAGTTGGACACAGGCCAACTTCTCAA GATTGCAAAACGTGGCGGCGTGACCGCAGTGGAGGCAGTGCATGCATGGCGCAATGCACTGACGGGTGCCCCG CTCAACTTGACCCCCCAGCAGGTGGTGGCCATCGCCAGCAATGGCGGTGGCAAGCAGGCGCTGGAGACGGTCC AGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCCCAGCAGGTGGTGGCCATCGCCAGCAATAAT GGTGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCC CAGCAGGTGGTGGCCATCGCCAGCAATGGCGGTGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCG GTGCTGTGCCAGGCCCACGGCTTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCCACGATGGCGGCAAGCAG

GCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCGGAGCAGGTGGTG

GCCATCGCCAGCCACGATGGCGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAG

GCCCACGGCTTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCCACGATGGCGGCAAGCAGGCGCTGGAGACG

GTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCA

ATATTGGTGGCAAGCAGGCGCTGGAGACGGTGCAGGCGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGAC

CCCGGAGCAGGTGGTGGCCATCGCCAGCCACGATGGCGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTT

GCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCAATATTGGTGGCAAG

CAGGCGCTGGAGACGGTGCAGGCGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCCCAGCAGGTG

GTGGCCATCGCCAGCAATAATGGTGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCC

AGGCCCACGGCTTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCAATATTGGTGGCAAGCAGGCGCTGGAGAC

GGTGCAGGCGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCCCAGCAGGTGGTGGCCATCGCCAGC

AATGGCGGTGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTG

ACCCCGGAGCAGGTGGTGGCCATCGCCAGCAATATTGGTGGCAAGCAGGCGCTGGAGACGGTGCAGGCGCTG

TTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCCCAGCAGGTGGTGGCCATCGCCAGCAATGGCGGTGGCA

AGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCGGAGCAGG

TGGTGGCCATCGCCAGCCACGATGGCGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTG

CCAGGCCCACGGCTTGACCCCTCAGCAGGTGGTGGCCATCGCCAGCAATGGCGGCGGCAGGCCGGCGCTGGA

GAGCATTGTTGCCCAGTTATCTCGCCCTGATCCGGCGTTGGCCGCGTTGACCAACGACCACCTCGTCGCCTTGGC

CTGCCTCGGCGGGCGTCCTGCGCTGGATGCAGTGAAAAAGGGATTGGGGGATCCTATCAGCCGTTCCCAGCTGG

TGAAGTCCGAGCTGGAGGAGAAGAAATCCGAGTTGAGGCACAAGCTGAAGTACGTGCCCCACGAGTACATCGA

GCTGATCGAGATCGCCCGGAACAGCACCCAGGACCGTATCCTGGAGATGAAGGTGATGGAGTTCTTCATGAAGG

TGTACGGCTACAGGGGCAAGCACCTGGGCGGCTCCAGGAAGCCCGACGGCGCCATCTACACCGTGGGCTCCCC

CATCGACTACGGCGTGATCGTGGACACCAAGGCCTACTCCGGCGGCTACAACCTGCCCATCGGCCAGGCCGACG

AAATGCAGAGGTACGTGGAGGAGAACCAGACCAGGAACAAGCACATCAACCCCAACGAGTGGTGGAAGGTGT

ACCCCTCCAGCGTGACCGAGTTCAAGTTCCTGTTCGTGTCCGGCCACTTCAAGGGCAACTACAAGGCCCAGCTG

ACCAGGCTGAACCACATCACCAACTGCAACGGCGCCGTGCTGTCCGTGGAGGAGCTCCTGATCGGCGGCGAGA

TGATCAAGGCCGGCACCCTGACCCTGGAGGAGGTGAGGAGGAAGTTCAACAACGGCGAGATCAACTTCGCGG
CCGACTGATAA (SEQ ID N°1), resulting for example in the sequence :

MGDPKKKRKVIDIADLRTLGYSQQQQEKIKPKVRSTVAQHHEALVGHGFTHAHIVALSQHPAALGTVAVKYQDMIA
ALPEATHEAIVGVGKQWSGARALEALLTVAGELRGPPLQLDTGQLLKIAKRGGVTAVEAVHAWRNALTGAPLNLTPQ
QVVAIASNGGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLTPQQVVAIASN
GGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALE
TVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQALLPVLC
QAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQALLPVLCQAHGLTPQ
QVVAIASNNGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQALLPVLCQAHGLTPQQVVAIASN
GGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQALLPVLCQAHGLTPQQVVAIASNGGGKQALE
TVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNGGGRPALESIVAQLSRP
DPALAALTNDHLVALACLGGRPALDAVKKGLGDPISRSQLVKSELEEKKSELRHKLKYVPHEYIELIEIARNSTQDRILEM
KVMEFFMKVYGYRGKHLGGSRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMQRYVEENQTRNKHINP
NEWWKVYPSSVTEFKFLFVSGHFKGNYKAQLTRLNHITNCNGAVLSVEELLIGGEMIKAGTLTLEEVRRKFNNGEINF
AAD (SEQ ID N°3)-

and

ATGGGCGATCCTAAAAAGAAACGTAAGGTCATCGATATCGCCGATCTACGCACGCTCGGCTACAGCCAGCAGCAA
CAGGAGAAGATCAAACCGAAGGTTCGTTCGACAGTGGCGCAGCACCACGAGGCACTGGTCGGCCACGGGTTTA
CACACGCGCACATCGTTGCGTTAAGCCAACACCCGGCAGCGTTAGGGACCGTCGCTGTCAAGTATCAGGACATG
ATCGCAGCGTTGCCAGAGGCGACACACGAAGCGATCGTTGGCGTCGGCAAACAGTGGTCCGGCGCACGCGCTC
TGGAGGCCTTGCTCACGGTGGCGGGAGAGTTGAGAGGTCCACCGTTACAGTTGGACACAGGCCAACTTCTCAA
GATTGCAAAACGTGGCGGCGTGACCGCAGTGGAGGCAGTGCATGCATGGCGCAATGCACTGACGGGTGCCCCG
CTCAACTTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCCACGATGGCGGCAAGCAGGCGCTGGAGACGGTCC
AGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCCCAGCAGGTGGTGGCCATCGCCAGCAATGG
CGGTGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCC

GGAGCAGGTGGTGGCCATCGCCAGCCACGATGGCGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCC

GGTGCTGTGCCAGGCCCACGGCTTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCAATATTGGTGGCAAGCAG

GCGCTGGAGACGGTGCAGGCGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCCCAGCAGGTGGTG

GCCATCGCCAGCAATAATGGTGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGG

CCCACGGCTTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCCACGATGGCGGCAAGCAGGCGCTGGAGACGG

TCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCCCAGCAGGTGGTGGCCATCGCCAGCAAT

GGCGGTGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACC

CCCCAGCAGGTGGTGGCCATCGCCAGCAATAATGGTGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGC

CGGTGCTGTGCCAGGCCCACGGCTTGACCCCCCAGCAGGTGGTGGCCATCGCCAGCAATAATGGTGGCAAGCA

GGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCCCAGCAGGTGGTG

GCCATCGCCAGCAATGGCGGTGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAG

GCCCACGGCTTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCAATATTGGTGGCAAGCAGGCGCTGGAGACG

GTGCAGGCGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCC

ACGATGGCGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGA

CCCCGGAGCAGGTGGTGGCCATCGCCAGCAATATTGGTGGCAAGCAGGCGCTGGAGACGGTGCAGGCGCTGTT

GCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCCACGATGGCGGCAA

GCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCCCAGCAGGTG

GTGGCCATCGCCAGCAATAATGGTGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCC

AGGCCCACGGCTTGACCCCTCAGCAGGTGGTGGCCATCGCCAGCAATGGCGGCGGCAGGCCGGCGCTGGAGA

GCATTGTTGCCCAGTTATCTCGCCCTGATCCGGCGTTGGCCGCGTTGACCAACGACCACCTCGTCGCCTTGGCCT

GCCTCGGCGGGCGTCCTGCGCTGGATGCAGTGAAAAAGGGATTGGGGGATCCTATCAGCCGTTCCCAGCTGGT

GAAGTCCGAGCTGGAGGAGAAGAAATCCGAGTTGAGGCACAAGCTGAAGTACGTGCCCCACGAGTACATCGAG

CTGATCGAGATCGCCCGGAACAGCACCCAGGACCGTATCCTGGAGATGAAGGTGATGGAGTTCTTCATGAAGGT

GTACGGCTACAGGGGCAAGCACCTGGGCGGCTCCAGGAAGCCCGACGGCGCCATCTACACCGTGGGCTCCCCC

ATCGACTACGGCGTGATCGTGGACACCAAGGCCTACTCCGGCGGCTACAACCTGCCCATCGGCCAGGCCGACGA

AATGCAGAGGTACGTGGAGGAGAACCAGACCAGGAACAAGCACATCAACCCCAACGAGTGGTGGAAGGTGTA

CCCCTCCAGCGTGACCGAGTTCAAGTTCCTGTTCGTGTCCGGCCACTTCAAGGGCAACTACAAGGCCCAGCTGA
CCAGGCTGAACCACATCACCAACTGCAACGGCGCCGTGCTGTCCGTGGAGGAGCTCCTGATCGGCGGCGAGAT
GATCAAGGCCGGCACCCTGACCCTGGAGGAGGTGAGGAGGAAGTTCAACAACGGCGAGATCAACTTCGCGGC
CGACTGATAA (SEQ ID N°2), encoding for example :

MGDPKKKRKVIDIADLRTLGYSQQQQEKIKPKVRSTVAQHHEALVGHGFTHAHIVALSQHPAALGTVAVKYQDMIA
ALPEATHEAIVGVGKQWSGARALEALLTVAGELRGPPLQLDTGQLLKIAKRGGVTAVEAVHAWRNALTGAPLNLTPE
QVVAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPEQVVAIASH
DGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQALLPVLCQAHGLTPQQVVAIASNNGGKQALE
TVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNGGGKQALETVQRLLPVL
CQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLT
PQQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQALLPVLCQAHGLTPEQVVAIAS
HDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQALLPVLCQAHGLTPEQVVAIASHDGGKQALE
TVQRLLPVLCQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNGGGRPALESIVAQLSRP
DPALAALTNDHLVALACLGGRPALDAVKKGLGDPISRSQLVKSELEEKKSELRHKLKYVPHEYIELIEIARNSTQDRILEM
KVMEFFMKVYGYRGKHLGGSRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMQRYVEENQTRNKHINP
NEWWKVYPSSVTEFKFLFVSGHFKGNYKAQLTRLNHITNCNGAVLSVEELLIGGEMIKAGTLTLEEVRRKFNNGEINF
AAD, (SEQ ID N° 4), For the right TALEN.

In an advantageous embodiment, the invention can be practiced using a TALEN recognizing the following sequence : TTGTCCCACAGATATCCagaaccctgaccctgCCGTGTACCAGCTGAGAGA.

Methods for making TALEN domains that bind to pre-determined DNA sites are known in the art, for example Reyon et al. (2012) Nat Biotechnol. 30:460-5.

An Improved method of the invention allows making endonucleases inducing below detection off target cleavage.

An Improved method of the invention allows making TALEN endonucleases inducing no or very

limited and silent off target cleavage.

In particular embodiments, the nucleases used to compare the invention are single-chain meganucleases. A single-chain meganuclease comprises an N-terminal subunit and a C-terminal subunit joined by a linker peptide. Each of the two domains recognizes half of the recognition sequence (i. e., a recognition half-site) and the site of DNA cleavage is at the middle of the recognition sequence near the interface of the two subunits. DNA strand breaks are offset by four base pairs such that DNA cleavage by a meganuclease generates a pair of four base pair, 3' single-strand overhangs as discloses in WO2017106528.

The present invention provides means for detecting such cells (on and off-sites dsDNA cut induced by said meganucleases).

Recombinant meganucleases used here comprise a first subunit, comprising a first hypervariable (HVR1) region, and a second subunit, comprising a second hypervariable (HVR2) region. Further, the first subunit binds to a first recognition half-site in the recognition sequence (e.g. , the TRC 1, TRC3, or TRC7 half-site), and the second subunit binds to a second recognition half-site in the recognition sequence (e.g. , the TRC2 , TRC4, or TRC8 half-site). In embodiments where the recombinant meganuclease is a single-chain meganuclease, the first and second subunits can be oriented such that the first subunit, which comprises the HVR1 region and binds the first half-site, is positioned as the N-terminal subunit, and the second subunit, which comprises the HVR2 region and binds the second half-site, is positioned as the C-terminal subunit. In alternative embodiments, the first and second subunits can be oriented such that the first subunit, which comprises the HVR1 region and binds the first half-site, is positioned as the C-terminal subunit, and the second subunit, which comprises the HVR2 region and binds the second half-site, is positioned as the N-terminal subunit. Comparative exemplary TRC 1-2 meganucleases, TRC 3-4 meganucleases and TRC 7-8 meganucleases are disclosed in WO2017106528.

A variety of different types of nuclease are useful for practicing the invention. In one embodiment,

the invention can be practiced using recombinant meganucleases. In another embodiment, the invention can be practiced using a CRISPR nuclease or CRISPR Nickase. Methods for making CRISPRs and CRISPR Nickases that recognize pre-determined DNA sites are known in the art, for example Ran, et al. (2013) Nat Protoc. 8:2281-308.

General Methods for Producing Genetically-Modified Cells

The invention provides methods for producing genetically-modified cells using engineered TALEN nucleases that recognize and cleave recognition sequences found within the human TCR alpha constant region gene.

In one embodiment, the invention provides methods for producing genetically-modified cells using engineered TALEN nucleases that recognize and cleave recognition sequences found within the human TCR alpha constant region gene, TTGTCCCACAGATATCCagaaccctgaccctgCCGTGTACCAGCTGAGAGA

In one embodiment, the invention provides methods for producing genetically-modified cells using engineered TALEN nucleases that recognize and cleave sequence having at least 80% identity with sequence recognition sequences found within the human TCR alpha constant region gene TTGTCCCACAGATATCCagaaccctgaccctgCCGTGTACCAGCTGAGAGA.

In one embodiment, the invention provides methods for producing genetically-modified cells using engineered TALEN nucleases that recognize and cleave sequence having at least 90% identity with sequence recognition sequences found within the human TCR alpha constant region gene TTGTCCCACAGATATCCagaaccctgaccctgCCGTGTACCAGCTGAGAGA.

In one embodiment, the invention provides methods for producing genetically-modified cells using engineered TALEN nucleases that recognize and cleave sequence having at least 91% identity with sequence recognition sequences found within the human TCR alpha constant region gene TTGTCCCACAGATATCCagaaccctgaccctgCCGTGTACCAGCTGAGAGA.

In one embodiment, the invention provides methods for producing genetically-modified cells using engineered TALEN nucleases that recognize and cleave sequence having at least 92% identity with sequence recognition sequences found within the human TCR alpha constant region gene TTGTCCCACAGATATCCagaaccctgaccctgCCGTGTACCAGCTGAGAGA.

In one embodiment, the invention provides methods for producing genetically-modified cells using engineered TALEN nucleases that recognize and cleave sequence having at least 93% identity with sequence recognition sequences found within the human TCR alpha constant region gene TTGTCCCACAGATATCCagaaccctgaccctgCCGTGTACCAGCTGAGAGA.

In one embodiment, the invention provides methods for producing genetically-modified cells using engineered TALEN nucleases that recognize and cleave sequence having at least 94% identity with sequence recognition sequences found within the human TCR alpha constant region gene TTGTCCCACAGATATCCagaaccctgaccctgCCGTGTACCAGCTGAGAGA.

In one embodiment, the invention provides methods for producing genetically-modified cells using engineered TALEN nucleases that recognize and cleave sequence having at least 95% identity with sequence recognition sequences found within the human TCR alpha constant region gene TTGTCCCACAGATATCCagaaccctgaccctgCCGTGTACCAGCTGAGAGA.

In one embodiment, the invention provides methods for producing genetically-modified cells using engineered TALEN nucleases that recognize and cleave sequence having at least 96% identity with sequence recognition sequences found within the human TCR alpha constant region gene (TTGTCCCACAGATATCCagaaccctgaccctgCCGTGTACCAGCTGAGAGA.

In one embodiment, the invention provides methods for producing genetically-modified cells using engineered TALEN nucleases that recognize and cleave sequence having at least 97% identity with sequence recognition sequences found within the human TCR alpha constant region gene TTGTCCCACAGATATCCagaaccctgaccctgCCGTGTACCAGCTGAGAGA.

In one embodiment, the invention provides methods for producing genetically-modified cells using engineered TALEN nucleases that recognize and cleave sequence having at least 98% identity with sequence recognition sequences found within the human TCR alpha constant region gene TTGTCCCACAGATATCCagaaccctgaccctgCCGTGTACCAGCTGAGAGA.

In one embodiment, the invention provides methods for producing genetically-modified cells using engineered TALEN nucleases that recognize and cleave sequence having at 99% identity with sequence recognition sequences found within the human TCR alpha constant region gene (TTGTCCCACAGATATCCagaaccctgaccctgCCGTGTACCAGCTGAGAGA.

In one embodiment, the invention provides methods for producing genetically-modified cells using engineered TALEN nucleases that recognize and cleave sequence having 100% identity with sequence recognition sequences found within the human TCR alpha constant region gene (TTGTCCCACAGATATCCagaaccctgaccctgCCGTGTACCAGCTGAGAGA.

Cleavage at such recognition sequences can allow for NHEJ at the cleavage site and disrupted expression of the human T cell receptor alpha chain subunit, leading to reduced expression and/or function of the T cell receptor at the cell surface. Additionally, cleavage at such recognition sequences can further allow for homologous recombination of exogenous nucleic acid sequences directly into the TCR alpha constant region gene.

RNA encoding nucleases

Engineered TALEN nucleases of the invention can be delivered into a cell in the form of a protein or, preferably, as a nucleic acid encoding the engineered nuclease. Such nucleic acid can be DNA (e.g., circular or linearized plasmid DNA or PCR products) or RNA or a combination of RNAs.

Such RNA may have various stability, various lengths and be delivered in various amounts.

For embodiments in which the engineered TALEN nuclease coding sequence is delivered in DNA form, it should be operably linked to a promoter to facilitate transcription of the nuclease, (TALEN or

meganuclease gene). Mammalian promoters suitable for the invention include constitutive promoters such as the cytomegalovirus early (CMV) promoter (Thomsen et al. (1984), Proc Natl Acad Sci USA. 81(3):659-63) or the SV40 early promoter (Benoist and Chambon (1981), Nature. 290(5804):304-10) as well as inducible promoters such as the tetracycline-inducible promoter (Dingermann ei a/. (1992), Mol Cell Biol. 12(9):4038-45).

In some embodiments, mRNA encoding the engineered nuclease is delivered to the cell because this reduces the likelihood that the gene encoding the engineered nuclease will integrate into the genome of the cell. Such mRNA encoding an engineered nuclease can be produced using methods known in the art such as in vitro transcription. In some embodiments, the mRNA is capped using 7-methyl-guanosine. In some embodiments, the mRNA may be polyadenylated. in other embodiments, the mRNA may have different lengths of poly A tail.

In particular embodiments, an mRNA encoding an engineered nuclease of the invention can be a polycistronic mRNA encoding two or more nucleases (or half) that are simultaneously expressed in the cell. A polycistronic mRNA can encode two or more nucleases of the invention that target different recognition sequences in the same target gene. Alternatively, a polycistronic mRNA can encode at least one nuclease described herein and at least one additional nuclease targeting a separate recognition sequence positioned in the same gene, or targeting a second recognition sequence positioned in a second gene such that cleavage sites are produced in both genes. A polycistronic mRNA can comprise any element known in the art to allow for the translation of two or more genes (/'. e. , cistrons) from the same mRNA molecule including, but not limited to, an IRES element, a T2A element, a P2A element, an E2A element, and an F2A element.

In particular embodiments, an mRNA encoding an engineered nuclease of the invention can be a polycistronic mRNA encoding two or more nucleases that are simultaneously expressed in the cell. A polycistronic mRNA can encode two or more nucleases of the invention that target different recognition sequences in the same target gene. Alternatively, a polycistronic mRNA can encode at

least one nuclease described herein and at least one additional nuclease targeting a separate recognition sequence positioned in the same gene, or targeting a second recognition sequence positioned in a second gene such that cleavage sites are produced in both genes. A polycistronic mRNA can comprise any element known in the art to allow for the translation of two or more genes (/'. e. , cistrons) from the same mRNA molecule including, but not limited to, an IRES element, a T2A element, a P2A element, an E2A element, and an F2A element.

In particular embodiments, an mRNA encoding an engineered nuclease of the invention can be a polycistronic mRNA encoding two or more nucleases that are simultaneously expressed in the cell. A polycistronic mRNA can encode two or more nucleases of the invention that target different recognition sequences in the same target gene. Alternatively, a polycistronic mRNA can encode at least one nuclease described herein and at least one additional nuclease targeting a separate recognition sequence positioned in the same gene, or targeting a second recognition sequence positioned in a second gene such that cleavage sites are produced in both genes. A polycistronic mRNA can comprise any element known in the art to allow for the translation of two or more genes (/'. e. , cistrons) from the same mRNA molecule including, but not limited to, an IRES element, a T2A element, a P2A element, an E2A element, and an F2A element.

In particular embodiments, an mRNA encoding an engineered nuclease of the invention can be a polycistronic mRNA encoding two or more nucleases that are simultaneously expressed in the cell. A polycistronic mRNA can encode two or more nucleases of the invention that target different recognition sequences in the same target gene. Alternatively, a polycistronic mRNA can encode at least one nuclease described herein and at least one additional nuclease targeting a separate recognition sequence positioned in the same gene, or targeting a second recognition sequence positioned in a second gene such that cleavage sites are produced in both genes. A polycistronic mRNA can comprise any element known in the art to allow for the translation of two or more genes (/'. e. , cistrons) from the same mRNA molecule including, but not limited to, an IRES element, a T2A

element, a P2A element, an E2A element, and an F2A element.

In particular embodiments, an mRNA encoding an engineered nuclease of the invention can be a polycistronic mRNA encoding two or more nucleases that are simultaneously expressed in the cell. A polycistronic mRNA can encode two or more nucleases of the invention that target different recognition sequences in the same target gene. Alternatively, a polycistronic mRNA can encode at least one nuclease described herein and at least one additional nuclease targeting a separate recognition sequence positioned in the same gene, or targeting a second recognition sequence positioned in a second gene such that cleavage sites are produced in both genes. A polycistronic mRNA can comprise any element known in the art to allow for the translation of two or more genes (/'. e. , cistrons) from the same mRNA molecule including, but not limited to, an IRES element, a T2A element, a P2A element, an E2A element, and an F2A element.

In particular embodiments, an mRNA encoding an engineered nuclease of the invention can be a polycistronic mRNA encoding two or more nucleases that are simultaneously expressed in the cell and no coding regulatory RNA. A polycistronic mRNA can encode two or more nucleases of the invention that target different recognition sequences in the same target gene and activates program(s) of T cell differentiation or dedifferentiation. Alternatively, a polycistronic mRNA can encode at least one nuclease described herein and at least one additional nuclease targeting a separate recognition sequence positioned in the same gene, or targeting a second recognition sequence positioned in a second gene such that cleavage sites are produced in both genes and a non coding ARN regulating the maturing state of the cell as described (Neilson .R. Genes Dev. 2007 Mar 1; 21(5): 578–589. doi:10.1101/gad.1522907).

A polycistronic mRNA comprising any element known in the art to allow for the translation of two or more genes (/'. e. , cistrons) from the same mRNA molecule including, but not limited to, an IRES element, a T2A element, a P2A element, an E2A element, and an F2A element.

Purified nuclease proteins can be delivered into cells to cleave genomic DNA, which allows for

homologous recombination or non-homologous end-joining at the cleavage site with a sequence of interest, by a variety of different mechanisms known in the art.

In some embodiments, engineered nuclease proteins, or DNA/mRNA encoding engineered nucleases, are coupled to a cell penetrating peptide or targeting ligand to facilitate cellular uptake. Examples of cell penetrating peptides known in the art include poly-arginine (Jearawiriyapaisarn, et al. (2008) Mol Ther. 16: 1624-9), TAT peptide from the HIV virus (Hudecz et al. (2005), Med. Res. Rev. 25: 679-736), MPG (Simeoni, et al. (2003) Nucleic Acids Res. 31 :2717-2724), Pep-1 (Deshayes et al. (2004) Biochemistry 43: 7698-7706, and HSV-1 VP-22 (Deshayes et al. (2005) Cell Mol Life Sci. 62: 1839-49. In an alternative embodiment, engineered nucleases, or DNA/mRNA encoding engineered nucleases, are coupled covalently or non-covalently to an antibody that recognizes a specific cell-surface receptor expressed on target cells such that the nuclease protein/DNA/mRNA binds to and is internalized by the target cells. Alternatively, engineered nuclease protein/DNA/mRNA can be coupled covalently or non-covalently to the natural ligand (or a portion of the natural ligand) for such a cell-surface receptor. (McCall, et al. (2014) Tissue Barriers. 2(4):e944449; Dinda, et al. (2013) Curr Pharm Biotechnol. 14: 1264-74; Kang, et al. (2014) Curr Pharm Biotechnol. 15(3):220-30; Qian et al. (2014) Expert Opin Drug Metab Toxicol. 10(11): 1491-508).

In some embodiments, engineered nuclease proteins, or DNA/mRNA encoding engineered nucleases, are coupled covalently or, preferably, non-covalently to a nanoparticle or encapsulated within such a nanoparticle using methods known in the art (Sharma, et al. (2014) Biomed Res Int. 2014). A nanoparticle is a nanoscale delivery system whose length scale is <1 μιη, preferably <100 nm. Such nanoparticles may be designed using a core composed of metal, lipid, polymer, or biological macromolecule, and multiple copies of the recombinant meganuclease proteins, mRNA, or DNA can be attached to or encapsulated with the nanoparticle core. This increases the copy number of the protein/mRNA/DNA that is delivered to each cell and, so, increases the intracellular expression of each engineered nuclease to maximize the likelihood that the target recognition sequences will be

cut. The surface of such nanoparticles may be further modified with polymers or lipids (e.g. , chitosan, cationic polymers, or cationic lipids) to form a core-shell nanoparticle whose surface confers additional functionalities to enhance cellular delivery and uptake of the payload (Jian et al. (2012) Biomaterials . 33(30): 7621-30). Nanoparticles may additionally be advantageously coupled to targeting molecules to direct the nanoparticle to the appropriate cell type and/or increase the likelihood of cellular uptake. Examples of such targeting molecules include antibodies specific for cell-surface receptors and the natural ligands (or portions of the natural ligands) for cell surface receptors.

In some embodiments, the engineered nucleases or DNA/mRNA encoding the engineered nucleases, are encapsulated within liposomes or complexed using cationic lipids (see, e.g. , Lipofectamine™, Life Technologies Corp., Carlsbad, CA; Zuris et al. (2015) Nat Biotechnol. 33 : 73-80; Mishra et al. (201 1) J Drug Deliv. 201 1 :863734). The liposome and lipoplex formulations can protect the payload from degradation, and facilitate cellular uptake and delivery efficiency through fusion with and/or disruption of the cellular membranes of the cells.

In some embodiments, engineered nuclease proteins, or DNA/mRNA encoding engineered nucleases, are encapsulated within polymeric scaffolds (e.g. , PLGA) or complexed using cationic polymers (e.g. , PEI, PLL) (Tamboli et al. (201 1) Ther Deliv. 2(4): 523-536).

In some embodiments, engineered nuclease proteins, or DNA/mRNA encoding engineered nucleases, are combined with amphiphilic molecules that self-assemble into micelles (Tong et al. (2007) J Gene Med. 9(11): 956-66). Polymeric micelles may include a micellar shell formed with a hydrophilic polymer (e.g. , polyethyleneglycol) that can prevent aggregation, mask charge interactions, and reduce nonspecific interactions outside of the cell.

In some embodiments, engineered nuclease proteins, (also called nuclease or endonuclease in the present invention) or DNA/mRNA encoding engineered nucleases, are formulated into an emulsion or a nanoemulsion (i.e. , having an average particle diameter of < lnm) for delivery to the cell. The term "emulsion" refers to, without limitation, any oil-in-water, water-in-oil, water-in-oil-in-water, or oil-in-

water-in-oil dispersions or droplets, including lipid structures that can form as a result of hydrophobic forces that drive apolar residues (e.g., long hydrocarbon chains) away from water and polar head groups toward water, when a water immiscible phase is mixed with an aqueous phase. These other lipid structures include, but are not limited to, unilamellar, paucilamellar, and multilamellar lipid vesicles, micelles, and lamellar phases. Emulsions are composed of an aqueous phase and a lipophilic phase (typically containing an oil and an organic solvent). Emulsions also frequently contain one or more surfactants. Nanoemulsion formulations are well known, e.g., as described in US Patent Application Nos. 2002/0045667 and 2004/0043041, and US Pat. Nos. 6,015,832, 6,506,803, 6,635,676, and 6,559,189, each of which is incorporated herein by reference in its entirety.

In some embodiments, engineered nuclease proteins, or DNA/mRNA encoding engineered nucleases, are covalently attached to, or non-covalently associated with, multifunctional polymer conjugates, DNA dendrimers, and polymeric dendrimers (Mastorakos et al. (2015) Nanoscale . 7(9): 3845-56; Cheng et al. (2008) J.Pharm Sci. 97(1): 123-43). The dendrimer generation can control the payload capacity and size, and can provide a high payload capacity. Moreover, display of multiple surface groups can be leveraged to improve stability and reduce nonspecific interactions.

In some embodiments, genes encoding an engineered nuclease are introduced into a cell using a viral vector. Such vectors are known in the art and include lentiviral vectors, adenoviral vectors, and adeno-associated virus (AAV) vectors (reviewed in Vannucci, et al. (2013 New Microbiol. 36: 1-22). Recombinant AAV vectors useful in the invention can be derived from any AAV that allows for transduction of the virus into the cell and insertion of the nuclease gene into the cell genome. In particular embodiments, recombinant AAV vectors encode AAV6 capsid protein, and structural elements from AAV2 allowing genetic material encapsidation in particules with AAV6 capsid protein.

Recombinant AAV vectors can also be self-complementary such that they do not require second-strand DNA synthesis in the host cell (McCarty, et al. (2001) Gene Ther. 8: 1248-54).

If the engineered TALEN nuclease genes are delivered in DNA form (e.g. plasmid) and/or via a viral

vector (e.g. AAV) they must be operably linked to a promoter. In some embodiments, this can be a viral promoter such as endogenous promoters from the viral vector (e.g. the LTR of a lentiviral vector) or the well-known cytomegalovirus- or SV40 virus-early promoters. In a preferred embodiment, nuclease genes are operably linked to a promoter that drives gene expression preferentially in the target cell (e.g. , a human T cell).

Similarly, a polynucleotide encoding a gene such as a CAR or a TCR may be delivered as a vector (e.g. lenti, AAV, preferably AAV, more preferably AAV6 and even more preferably AAV6 and AAV2 ITR)

The invention further provides for the introduction of an exogenous nucleic acid into the cell, such that the exogenous nucleic acid sequence is inserted into the TRAC constant region gene at a nuclease cleavage site. In some embodiments, the exogenous nucleic acid comprises a 5' homology arm and a 3' homology arm to promote recombination of the nucleic acid sequence into the cell genome at the nuclease cleavage site.

Exogenous nucleic acids or polynucleotides of the invention may be introduced into the cell by any of the means previously disclosed. In a particular embodiment, exogenous nucleic acids are introduced by way of a viral vector, such as a lentivirus, retrovirus, adenovirus, or preferably a recombinant AAV vector, more preferably a recombinant AAV6 vector, with AAV2 inverted terminal repeat (ITR). Recombinant AAV vectors useful for introducing an exogenous nucleic acid can have any serotype that allows for transduction of the virus into the cell and insertion of the exogenous nucleic acid sequence into the cell genome. In particular embodiments, the recombinant AAV vectors have a serotype AAV6. The recombinant AAV vectors can also be self-complementary such that they do not require second-strand DNA synthesis in the host cell.

In another particular embodiment, an exogenous nucleic acid can be introduced into the cell using a single-stranded DNA template. The single-stranded DNA can comprise the exogenous nucleic acid and, in preferred embodiments, can comprise 5' and 3' homology arms to promote insertion of the nucleic acid sequence into the nuclease cleavage site by homologous recombination. The single-

stranded DNA can further comprise a 5' AAV inverted terminal repeat (ITR) sequence 5' upstream of the 5' homology arm, and a 3' AAV2 ITR sequence 3' downstream of the 3' homology arm.

In another particular embodiment, genes encoding an endonuclease of the invention and/or an exogenous nucleic acid sequence of the invention (e;g. a CAR) can be introduced into the cell by transfection with a linearized DNA template. In some examples, a plasmid DNA encoding an endonuclease and/or an exogenous nucleic acid sequence can be digested by one or more restriction enzymes such that the circular plasmid DNA is linearized prior to transfection into the cell.

When delivered to a cell, an exogenous nucleic acid of the invention can be operably linked to any promoter suitable for expression of the encoded polypeptide in the cell, including those mammalian promoters and inducible promoters previously discussed. An exogenous nucleic acid of the invention can also be operably linked to a synthetic promoter. Synthetic promoters can include, without limitation, the JeT promoter (WO 2002/012514).

ACTIVATION

In examples where the genetically-modified cells of the invention are human T cells, or cells derived therefrom, such cells may require activation prior to introduction of a nuclease and/or an exogenous nucleic acid sequence. For example, T cells can be contacted with anti-CD3 and anti-CD28 antibodies that are soluble or conjugated to a support (i.e. , beads) for a period of time sufficient to activate the cells.

SUICIDE GENE

Genetically-modified cells of the invention can be further modified to express one or more inducible suicide genes, the induction of which provokes cell death and allows for selective destruction of the cells in vitro or in vivo. In some examples, a suicide gene can encode a cytotoxic polypeptide, a polypeptide that has the ability to convert a non-toxic prodrug into a cytotoxic drug, and/or a polypeptide that activates a cytotoxic gene pathway within the cell. That is, a suicide gene is a nucleic

acid that encodes a product that causes cell death by itself or in the presence of other compounds. A representative example of such a suicide gene is one that encodes thymidine kinase of herpes simplex virus. Additional examples are genes that encode thymidine kinase of varicella zoster virus and the bacterial gene cytosine deaminase that can convert 5-fluorocytosine to the highly toxic compound 5-fluorouracil. Suicide genes also include as non-limiting examples genes that encode caspase-9, caspase-8, or cytosine deaminase. In some examples, caspase-9 can be activated using a specific chemical inducer of dimerization (CID). A suicide gene can also encode a polypeptide that is expressed at the surface of the cell that makes the cells sensitive to therapeutic and/or cytotoxic monoclonal antibodies. In further examples, a suicide gene can encode recombinant antigenic polypeptide comprising an antigenic motif recognized by the anti-CD20 mAb Rituximab and an epitope that allows for selection of cells expressing the suicide gene. See, for example, the RQR8 polypeptide described in WO2013153391, which comprises two Rituximab-binding epitopes and a QBEndlO-binding epitope. For such a gene, Rituximab can be administered to a subject to induce cell depletion when needed.

In one embodiment two Rituximab-binding epitopes are inserted directly into the chimeric antigen receptor, such as in an anti- CD22 CAR, or an anti-CD123 CAR, or in an anti-CD30 CAR in the linker of the scfv and/or in the hinge linking the scfv to the transmembrane domain.

In one embodiment two Rituximab-binding epitopes and a QBEndlO-binding epitope are inserted directly into the chimeric antigen receptor, such as in an anti- CD22 CAR, or an anti-CD123 CAR, or in an anti-CD30 CAR, in the linker between the VH and the VL of the scfv and/or in the hinge linking the scfv to the transmembrane domain.

In some embodiments, the invention provides a pharmaceutical composition comprising a genetically-modified cell of the invention (a TALEN-modified endogenous $\alpha\beta$-TCR negative human primary cell wherein the constant region of the genomic TCR gene (TRAC gene) comprises a genetic modification generated by a TALEN and affecting cell surface expression of the alpha beta TCR, said

genomic TRAC gene comprising from 5' to 3':

(a) a 5' region of said human genomic TRAC gene upstream

(b) a recognition domain for a TALEN,

(c) a gap or an insertion as compared to the wild type TRAC gene affecting the cell surface expression of the extracellular domain or transmembrane domain of the alpha beta TCR,

said insertion comprising an exogenous polynucleotide selected from a noncoding sequence such as, a stop codon, an IRES, a sequence coding such as a sequence coding for a self-cleaving peptide in frame with the TRAC open reading frame, a sequence coding a chimeric antigen receptor (CAR), a sequence coding a TCR, a sequence coding a protein conferring sensitivity to a drug, a sequence coding a protein conferring resistance to a drug, a cytokine, a termination sequence, a combination thereof,

(c') optionally a second TALEN recognition domain,

(d) a 3' region of the genomic TRAC gene.), or a population of said genetically-modified cells of the invention, and a pharmaceutical carrier, and another medicament, such as a PNA for dCK KO cells, alemtuzumab for CD52 KO, glucocorticoid for a GR KO cell...

Such pharmaceutical compositions can be prepared in accordance with known techniques. See, e.g. ,Remington, The Science And Practice of Pharmacy (21s ed. 2005). In the manufacture of a pharmaceutical formulation according to the invention, cells are typically admixed with a pharmaceutically acceptable carrier and the resulting composition is administered to a subject. The carrier must, of course, be acceptable in the sense of being compatible with any other ingredients in the formulation and must not be deleterious to the subject. In some embodiments, pharmaceutical compositions of the invention can further comprise one or more additional agents useful in the treatment of a disease in the subject. In additional embodiments, where the genetically -modified cell is a genetically-modified human T cell (or a cell derived therefrom), pharmaceutical compositions of the invention

can further include biological molecules, such as cytokines (e.g. , IL-2, IL-7, IL- 15, and/or IL-21, IL-27), which promote in vivo cell proliferation and engraftment. Pharmaceutical compositions comprising genetically -modified cells of the invention can be administered in the same composition as an additional agent or biological molecule or, alternatively, can be co-administered in separate compositions.

Therapeutic applications

Pharmaceutical compositions of the invention can be useful for treating any disease state that can be targeted by adoptive immunotherapy. The disease that may be treated using the object of the present invention (engineered primary human cells expressing a CAR or a TCR) may or may not be characterized by cells or tissue expressing the target of the CAR expressed at the cells surface of the said objects.

In a particular embodiment, the pharmaceutical compositions of the invention are useful in the treatment of cancer. Such cancers can include, without limitation, carcinoma, lymphoma, sarcoma, blastomas, leukemia, cancers of B -cell origin, breast cancer, gastric cancer, neuroblastoma, osteosarcoma, lung cancer, melanoma, prostate cancer, colon cancer, renal cell carcinoma, ovarian cancer, rhabdomyo sarcoma, leukemia, and Hodgkin's lymphoma. In certain embodiments, cancers of B-cell origin include, without limitation, B-lineage acute lymphoblastic leukemia, B-cell chronic lymphocytic leukemia, and B-cell non-Hodgkin's lymphoma.

In particular embodiments, the object of the invention may be used as a function of the CAR expressed at the surface of the cell of the invention to treat a disease characterized in the expression of the CAR target.

Doses of cells genetically modified cells (engineered cells of the invention), useful for the treatment of cancer may range from 1 to 108 cells.

MEANS for DETECTING the cells of the invention

The present invention provides means for detecting engineered cells. Such means of the invention

encompasses any means capable of recognizing and detecting a modified TCR gene that underwent a deletion, a mutation, an insertion. Such means of the invention encompasses any means capable of recognizing and detecting a modified TCR gene that underwent a deletion, a mutation, an insertion of the TRAC gene and off sites, if any.

The means may be an oligonucleotide, a degenerated oligonucleotide, a probe, a DNA probe, a RNA probe, a labeled endonuclease, a labeled TALEN, a combination thereof.

The present invention provides a method for detecting engineered cells, in particular for detecting a wt TRAC gene, a TRAC gene with a deletion, a TRAC gene with an insertion of from 05.kb, 1kb, 2, kb, 3kb, 4kb to 5 kb insertion into the TRAC gene. Such method of the invention comprises means recognizing, binding to a wt TRAC gene, means recognizing, binding to a modified TRAC gene that underwent a deletion, a mutation, or an insertion.

The engineered TRAC gene may have kept an endonuclease binding site. Thus, in particular embodiment, a means of the present invention comprises a sequence that is or is complementary to the binding sequence of the endonuclease.

In particular embodiments, a means of the present invention comprises a polynucleotide sequence that binds to the 10 to 15 first nucleotides in 5' of the TRAC gene and another polynucleotide sequence binding to the 10 to 15 last nucleotides in 3' of the TRAC gene. The present invention provides a method for detecting a wt TRAC gene or a modified TRAC gene comprising a deletion, a mutation or an insertion.

The present invention provides a method for detecting a wt TRAC gene or a modified TRAC gene comprising a deletion, a mutation or an insertion comprising :

(i) Purifying the DNA of a TRAC-modified cell, and of a cell used as an appropriate control,

(ii) Incubating said DNA with means for detecting a modified TRAC gene of the invention or a

wt TRAC gene, resulting in a mixture;

(iii) Submitting the mixture to a pcr

(iv) Sequencing the pcr product.

In particular embodiments, a means of the present invention comprises a sequence that is complementary to the binding sequence of the TALEN, preferably complementary to TTGTCCCACAGATATCC, CCGTGTACCAGCTGAGAGA or comprising TTGTCCCACAGATATCC, CCGTGTACCAGCTGAGAGA.

In particular embodiments, a means of the present invention comprises a sequence that is or is complementary to the binding sequence of a meganuclease.

In particular embodiments, a means of the present invention comprises a sequence that is complementary to the binding sequence of a compact TALEN nuclease.

In particular embodiments, a means of the present invention comprises a sequence that is complementary to the binding sequence of a megaTAL nuclease.

In particular embodiments, a means of the present invention comprises a sequence that is complementary to the sequence to which the guide of the Crispr/Cas9 system binds to or comprise the guide used to engineer the TRAC gene.

In all cases, the means can comprise a sequence designed to recognize the sequence in the engineered TRAC gene just upstream the sequence recognized by the endonuclease (homology arm) that is or was recognized by the first monomer of the endonuclease (if the binding domain of the nuclease is cut, then the sequence just upstream subsists in the engineered TRAC gene).

As used herein, the term "recognition sequence" refers to a DNA sequence that is bound and cleaved by an endonuclease.

In the case of a TALEN, a recognition sequence comprises two sites, ie from 5’ to 3’: the left recognition sequence followed by the right recognition sequence, more preferably TTGTCCCACAGATATC and CCGTGTACCAGCTGAGA.

In the case of a meganuclease, a recognition sequence comprises a pair of inverted, 9 basepair "half sites" that are separated by four basepairs. In the case of a single-chain meganuclease, the N-terminal domain of the protein contacts a first half-site and the C-terminal domain of the protein contacts a second half-site. Cleavage by a meganuclease produces four basepair 3 ' "overhangs". "Overhangs", or "sticky ends" are short, single-stranded DNA segments that can be produced by endonuclease cleavage of a double -stranded DNA sequence. In the case of meganucleases and single-chain meganucleases derived from I-CreI, the overhang comprises bases 10-13 of the 22 basepair recognition sequence. In the case of a Compact TALEN, the recognition sequence comprises a first CNNNGN sequence that is recognized by the I-TevI domain, followed by a nonspecific spacer 4-16 basepairs in length, followed by a second sequence 16-22 bp in length that is recognized by the TAL-effector domain (this sequence typically has a 5 ' T base). Cleavage by a Compact TALEN produces two basepair 3 ' overhangs. In the case of a CRISPR, the recognition sequence is the sequence, typically 16-24 basepairs, to which the guide RNA binds to direct Cas9 cleavage. Cleavage by a CRISPR produced blunt ends.

As used herein, the term "target site" or "target sequence" or "on site" or "on site target" refers to a region of the chromosomal DNA of a cell comprising a recognition sequence for a nuclease, that is intended to be modified or edited (for introducing a mutation, a deletion or an insertion).

Off target site or off target sequence refers to a region of the chromosomal DNA of a cell comprising a secondary recognition sequence for a nuclease which is not the expected target sequence (intended to be edited) and may bind less specifically and/or with a lower affinity the tested TALEN.

Preferred TALEN of the invention :

As used herein, the term "DNA-binding affinity" or "binding affinity" means the tendency of a nuclease to non-covalently associate with a reference DNA molecule (e.g. , a recognition sequence or an arbitrary sequence). Binding affinity is measured by a dissociation constant, ¾ . As used herein, a nuclease has "altered" binding affinity if the Kd of the nuclease for a reference recognition sequence is increased or decreased by a statistically significant percent change relative to a reference nuclease.

In the present invention a TALEN may associate preferentially to the following sequence : 5'

TGATCCTCTTGTcccacagatatccagaaccctgaccctgcccgtgtaccagctgagaga

3'

Or from 5'to 3' : TTGTCCCACAGATATCAGAACCCTGACCCTGCCGTGTACCAGCTGAGA (SEQ ID N°8)

with TTGTCCCACAGATATC (first or left binding site, first or left recognition domain)

CCGTGTACCAGCTGAGA (second or right binding site – second or right recognition domain).

In a preferred embodiment, the means comprise a sequence that binds to a TAG inserted into the engineered TRAC gene and /or part of the TRAC gene upstream the recognition sequence of the nuclease.

The underlined sequence below corresponds to a possible sequence used as a base for designing a probe or a mean of the invention for the detection of an engineered cells.

DDDDDDDDDDDTTGTCCCACAGATATCAGAACCCTGACCCTGCCGTGTACCAGCTGAGADDDDDDDDDDD

DDDDDDDDDDD : sequence of the TRAC gene upstream (in 5' of the first recognition domain) or down stream the second recognition domain.

According to another embodiment, a probe or a mean according to the present invention may comprise part of a sequence of the TRAC gene and part of the first recognition domain of a TALEN.

According to another embodiment, a probe or a mean according to the present invention may comprise part of the first recognition domain of a TALEN or the entire first recognition domain of a TALEN.

According to another embodiment, a probe or a mean according to the present invention may comprise the first recognition domain of a TALEN.

According to another embodiment, a probe or a mean according to the present invention may comprise the first recognition domain of a TALEN part of the sequence of the integrated polynucleotide.

According to another embodiment, a probe or a mean according to the present invention may comprise a sequence or a domain that binds to the genome-integrated polynucleotide, to the sequence encoding a self-cleaving peptide, to the sequence coding an IRES, to the sequence coding a CAR, said sequence may be one of those as follows:

DDDDDDDDDDDTTGTCCCACAGATATCAGAACCCTGACCCTGCCGTGTACCAGCTGAGADDDDDDDDDDD,

DDDDDDDDDDDTTGTCCCACAGATATCAGAACCCTGACCCTGCCGTGTACCAGCTGAGADDDDDDDDDDD,

DDDDDDDDDDDTTGTCCCACAGATATCAGAACCCTGACCCTGCCGTGTACCAGCTGAGADDDDDDDDDDD,

DDDDDDDDDDDTTGTCCCACAGATATCAGAACCCTGACCCTGCCGTGTACCAGCTGAGADDDDDDDDDDD,

DDDDDDDDDDDTTGTCCCACAGATATC(XXX)nCCGTGTACCAGCTGAGADDDDDDDDDDD with (XXX)n is an integrated polynucleotide comprising a tag, said tag may be a non coding sequence, a sequence encoding protein such as a self-cleaving peptide, a CAR, D represents a base, successive Ds represent an homology with the genomic wt TRAC gene.

Means of the invention were designed based on the "recognition sequence" of the nucleases in WO2017062451 or disclosed in WO 2017106528 but also based on other recognition sequence identified herewith and for which the frequency of off site either calculated or calculated and

measured in silico would not be superior to those generated using the nucleases generated so far, preferably

The TALEN of the present invention have no detectable off site target, using any one of the method disclosed so far to detect said off target, including guide-seq method adapted to TALEN.

The TALEN of the present invention target and cleave an active gene in a human cell genome, (active means that the gene is expressed in a normal cell, a in normal adult cell, in a normal progenitor cell in a normal embryonic cell.

In a preferred embodiment, the TALEN of the present invention target and cleave a component of the TCR gene, more preferably a TCRalpha or a TCR beta, a TCR delta or a TCR gamma, even more preferably a TCR alpha and/or beta.

TALEN cleaving stop codon of TCR alpha or beta constant region.

TCR beta constant region has two possible genes (TRBC1 and TRBC2).

TALEN TRAC on Stop

hsTRAC_ex1

gatacgaacctaaactttcaaaacctgtcagtgattgggttccgaatcctcctcctgaaagtggccgggtttaatctgctcatgac-

tgattgggttccgaatcctcctcctgaaagtggccgggtttaatctgctcatgac-

gctgcggctgtggtccagcTGAggtgaggggccttgaagctggg

Four TALEN were identified

TCTGCTCATGACGCTGCGGCTGTGGTCCAGCTGAGGTGAGGGGCCTTGAA

TCTGCTCATGACGCTGC GGCTGTGGTCCAGCTG AGGTGAGGGGCCTTGAA

TGCTCATGACGCTGCGGCTGTGGTCCAGCTGAGGTGAGGGGCCTTGAA

TGCTCATGACGCTGCGG CTGTGGTCCAGCTG AGGTGAGGGGCCTTGAA

TGACGCTGCGGCTGTGGTCCAGCTGAGGTGAGGGGCCTTGAAGCTGGGA

TGACGCTGCGGCTGTGG TCCAGCTGAGGTGAG GGGCCTTGAAGCTGGGA

TGGTCCAGCTGAGGTGAGGGGCCTTGAAGCTGGGAGTGGGGTTTAGGGA

TGGTCCAGCTGAGGTGA GGGGCCTTGAAGCTG GGAGTGGGGTTTAGGGA

Best TALEN based on specificity results is TALEN 2

TGCTCATGACGCTGCGGCTGTGGTCCAGCTGAGGTGAGGGGCCTTGAA

TGCTCATGACGCTGCGG CTGTGGTCCAGCTG AGGTGAGGGGCCTTGAA

hsTRBC1_stop

taacccccaaaactttctcttctgcagGTCAAGAGAAAGGATTTCtgaAGGCAGCCCTG-

GAAGTGGAGTTAGGAGCTTCTAACCCGTCATGGTTTCAATA

Three TALEN were identified

TCTGCAGGTCAAGAGAAAGGATTTCTGAAGGCAGCCCTGGAAGTGGA

TCTGCAGGTCAAGAGAA AGGATTTCTGAAG GCAGCCCTGGAAGTGGA

TTTCTCTTCTGCAGGTCAAGAGAAAGGATTTCTGAAGGCAGCCCTGGAA

TTTCTCTTCTGCAGGTC AAGAGAAAGGATTTC TGAAGGCAGCCCTGGAA

TTCTGAAGGCAGCCCTGGAAGTGGAGTTAGGAGCTTCTAACCCGTCA

TTCTGAAGGCAGCCCTG GAAGTGGAGTTAG GAGCTTCTAACCCGTCA

Best TALEN identified based on specificity results is the TALEN

TTCTGAAGGCAGCCCTGGAAGTGGAGTTAGGAGCTTCTAACCCGTCA

TTCTGAAGGCAGCCCTG GAAGTGGAGTTAG GAGCTTCTAACCCGTCA

hsTRBC2_stop

tagcccctgaaaccctgaaaatgttctctcttccacagGTCAAGAGAAAGGAT-

TCCAGAGGCtagCTCCAAAACCATCCCAGGTCATTCTTCATCCTCACCCACTCCAAAAC-

CATCCCAGGTCATTCTTCATCC

Four TALEN were identified:

TGAAAATGTTCTCTCTTCCACAGGTCAAGAGAAAGGATTCCAGAGGCTA

TGAAAATGTTCTCTCTT CCACAGGTCAAGAGA AAGGATTCCAGAGGCTA

TTCTCTCTTCCACAGGTCAAGAGAAAGGATTCCAGAGGCTAGCTCCAAA

TTCTCTCTTCCACAGGT CAAGAGAAAGGATTC CAGAGGCTAGCTCCAAA

TTCTCTCTTCCACAGGTCAAGAGAAAGGATTCCAGAGGCTAGCTCCAAA

TCTCTCTTCCACAGGTC AAGAGAAAGGATTCC AGAGGCTAGCTCCAAAA

TAGCTCCAAAACCATCCCAGGTCATTCTTCATCCTCACCCACTCCAAAA

TAGCTCCAAAACCATCC CAGGTCATTCTTCAT CCTCACCCACTCCAAAA

Best TALEN based on specificity TALEN 2 (or 4)

TTCTCTCTTCCACAGGTCAAGAGAAAGGATTCCAGAGGCTAGCTCCAAA

TTCTCTCTTCCACAGGT CAAGAGAAAGGATTC CAGAGGCTAGCTCCAAA

In other embodiments, the TALEN of the present invention targets and cleaves any one of the gene disclosed in patent application 62/410,187 (KO/KI).

For the purpose of making a drug or medication administrable in human (universal TCR-negative CART cells _ or off the shelve) that would be stable once administered and wherein a frequency of off site cutting would be near zero, the present inventors analyzed systematically the frequency of off sites that would be generated when using TALEN of the invention and compared the data to those obtained with a meganuclease, aMegaTAL, a crispr/cas system, reported to target the same sequences (or successive sequences) of the human TRAC gene coding for extracellular or transmembrane domain of the TCRalpha beta protein (those which, when inactivated by deletion insertion, would result in a decrease in cell surface expression of functional TCR alpha beta).

To measure the stability of such cells an in vitro system was set up wherein cells were challenges everyday for weeks ‘at least 5) with immune cells and with cells expressing targeted antigen as described as in USP 62/537,435.

Blood sample from two healthy individuals (human) individuals that received "allogenic therapy" with the cells of the present invention were also analyzed four weeks after the first injection for the presence of cells in the blood.

A TAG means a element, preferably a genetic element such as a sequence that allows detecting an engineered human cell comprising it.

The TAG of the invention may comprise, an endonuclease recognition domain, a sequence or part of a sequence encoding a self-cleaving peptide, an IRES, and /or a sequence or part of a sequence just

downstream the sequence upstream the recognition sequence of an rare cutting endonuclease such as a TALEN, a CRISPR, a meganuclease, a megatal, a zinc finger.

Detection is achieved using a mean of detection, that may be a specific antibody, a oligonucleotide, preferably two nucleotides, an RNA, a DNA, a combination thereof.

According to the present invention, a means can comprise a sequence designed to recognize the tag inserted into the engineered TRAC gene and /or part of the TRAC gene in 5' of the insertion, part of the sequence inserted into the TRAC gene (CAR, exogenous TCR, gene making cells sensitive to a drug).

In one embodiment, the tag inserted into the TRAC gene may comprise a sequence encoding a self-cleaving peptide, and/ or another chosen sequence such as GAC TAC AAA GAC GAT GAC GAC AAG,

TAC CCA TAC GAT GTT CCA GAT TAC GCT, CAC CAC CAC CAC CAC CAC, GAA CAA AAA CTC ATC TCA GAA GAG GAT CTG, or any one of those disclosed in Costea PI, Lundeberg J, Akan P (2013) TagGD: Fast and Accurate Software for DNA Tag Generation and Demultiplexing. PLoS ONE 8(3): e57521. https://doi.org/10.1371/journal.pone.0057521.

A means for detecting any one of these tags, is part of the present invention.

In addition, the inventors have determined appropriate target sequences within the 3 exons of the TCR alpha, allowing to prevent TCR expression at the cell surface if cleaved and engineered by insertion of a CAR and off site above detection.

The invention provides therefore at least one means for detecting a TCR engineered cell of the invention comprising one of the following sequences : GAGAATCAAAATCGGTGAATAGG, TTCAAAACCTGTCAGTGATTGGG, TGTGCTAGACATGAGGTCTATGG, CGTCATGAGCAGATTAAACCCGG, TCAGGGTTCTGGATATCTGTGGG, GTCAGGGTTCTGGATATCTGTGG, TTCGGAACCCAATCACTGACAGG, TAAACCCGGCCACTTTCAGGAGG, AAAGTCAGATTTGTTGCTCCAGG, AACAAATGTGTCACAAAGTAAGG, TGGATTTAGAGTCTCTCAGCTGG, TAGGCAGACAGACTTGTCACTGG,

AGCTGGTACACGGCAGGGTCAGG, GCTGGTACACGGCAGGGTCAGGG, TCTCTCAGCTGGTACACGGCAGG, TTTCAAAACCTGTCAGTGATTGG, GATTAAACCCGGCCACTTTCAGG, CTCGACCAGCTTGACATCACAGG, AGAGTCTCTCAGCTGGTACACGG, CTCTCAGCTGGTACACGGCAGGG, AAGTTCCTGTGATGTCAAGCTGG, ATCCTCCTCCTGAAAGTGGCCGG, TGCTCATGACGCTGCGGCTGTGG, ACAAAACTGTGCTAGACATGAGG, ATTTGTTTGAGAATCAAAATCGG, CATCACAGGAACTTTCTAAAAGG,GTCGAGAAAAGCTTTGAAACAGG, CCACTTTCAGGAGGAGGATTCGG, CTGACAGGTTTTGAAAGTTTAGG, AGCTTTGAAACAGGTAAGACAGG, TGGAATAATGCTGTTGTTGAAGG, AGAGCAACAGTGCTGTGGCCTGG, CTGTGGTCCAGCTGAGGTGAGGG, CTGCGGCTGTGGTCCAGCTGAGG, TGTGGTCCAGCTGAGGTGAGGGG, CTTCTTCCCCAGCCCAGGTAAGG, ACACGGCAGGGTCAGGGTTCTGG, CTTCAAGAGCAACAGTGCTGTGG, CTGGGGAAGAAGGTGTCTTCTGG, TCCTCCTCCTGAAAGTGGCCGGG, TTAATCTGCTCATGACGCTGCGG, ACCCGGCCACTTTCAGGAGGAGG, TTCTTCCCCAGCCCAGGTAAGGG, CTTACCTGGGCTGGGGAAGAAGG, GACACCTTCTTCCCCAGCCCAGG, GCTGTGGTCCAGCTGAGGTGAGG, CCGAATCCTCCTCCTGAAAGTGG, a complementary sequence thereof; said mean may be associated for example for a mean binding to the CAR sequence of peptide 2A sequence, and allowing a PCR according to the present invention.

In other embodiments, the present invention also provides a mean for detecting engineered immune cells (cell were engineered using various endonucleases Crispr /Cas9,) comprising a sequence comprising at least one of the following sequences : AGAGTCTCTCAGCTGGTACA, GCACCAAAGCTGCCCTTACC, AAGTTCCTGTGATGTCAAGC , TTCGGAACCCAATCACTGAC, GATTAAACCCGGCCACTTTC, CGTCATGAGCAGATTAAACC, CTCAAGGTTCAGATCAGAAG, TAGGCAGACAGACTTGTCAC, AACAAATGTGTCACAAAGTA, CACCAAAGCTGCCCTTACCT, CTGACAGGTTTTGAAAGTTT, TTCAAAACCTGTCAGTGATT, CCGAATCCTCCTCCTGAAAG, CCACTTTCAGGAGGAGGATT, TAAACCCGGCCACTTTCAGG, TCTCAAACAAATGTGTCACAAAGTA, CTTACAATCTTGCAGATCTGGAATG, TTAATCTGCTCATGACGCTG, GGAGAAGAGGGGCAATGCAG, TCTTCTCCCTCTCCAAACAG, AGCAGCTTTCACCTCCTTGG, GTAGCAGCTTTCACCTCCTT, AGTTGGTGGCATTGCCGGGG, TCTGTGATATACACATCAGAATC, TCTGTGATATACACATCAGAATCC,

GAGTCTCTCAGCTGGTACACGGC, GAGTCTCTCAGCTGGTACACGGCA, ATTCTCAAACAAATGTGTCACAA, ATTCTCAAACAAATGTGTCACAAA, GTCTGTGATATACACATCAGAAT, GTCTGTGATATACACATCAGAATC, GAGAATCAAAATCGGTGAATAGG, TGTGCTAGACATGAGGTCTATGG , TCAGGGTTCTGGATATCTGTGGG, GTCAGGGTTCTGGATATCTGTGG, AAAGTCAGATTTGTTGCTCCAGG, AACAAATGTGTCACAAAGTAAGG, TGGATTTAGAGTCTCTCAGCTGG, TAGGCAGACAGACTTGTCACTGG, AGCTGGTACACGGCAGGGTCAGG, GCTGGTACACGGCAGGGTCAGGG, TCTCTCAGCTGGTACACGGCAGG, AGAGTCTCTCAGCTGGTACACGG, CTCTCAGCTGGTACACGGCAGGG, ACAAAACTGTGCTAGACATGAGG, ATTTGTTTGAGAATCAAAATCGG, TGGAATAATGCTGTTGTTGAAGG, AGAGCAACAGTGCTGTGGCCTGG, CTTCTTCCCCAGCCCAGGTAAGG, ACACGGCAGGGTCAGGGTTCTGG, CTTCAAGAGCAACAGTGCTGTGG, CTGGGGAAGAAGGTGTCTTCTGG, TTCTTCCCCAGCCCAGGTAAGGG, CTTACCTGGGCTGGGGAAGAAGG, GACACCTTCTTCCCCAGCCCAGG, TTCAAAACCTGTCAGTGATTGGG, CGTCATGAGCAGATTAAACCCGG, TTCGGAACCCAATCACTGACAGG, TAAACCCGGCCACTTTCAGGAGG, TTTCAAAACCTGTCAGTGATTGG, GATTAAACCCGGCCACTTTCAGG, CTCGACCAGCTTGACATCACAGG, AAGTTCCTGTGATGTCAAGCTGG ,ATCCTCCTCCTGAAAGTGGCCGG, TGCTCATGACGCTGCGGCTGTGG, CATCACAGGAACTTTCTAAAAGG, GTCGAGAAAAGCTTTGAAACAGG, CCACTTTCAGGAGGAGGATTCGG, CTGACAGGTTTTGAAAGTTTAGG, AGCTTTGAAACAGGTAAGACAGG, CTGTGGTCCAGCTGAGGTGAGGG, CTGCGGCTGTGGTCCAGCTGAGG, TGTGGTCCAGCTGAGGTGAGGGG, TCCTCCTCCTGAAAGTGGCCGGG, TTAATCTGCTCATGACGCTGCGG, ACCCGGCCACTTTCAGGAGGAGG, GCTGTGGTCCAGCTGAGGTGAGG, CCGAATCCTCCTCCTGAAAGTGG and complementary sequence of any one thereof.

EXAMPLES

This invention is further illustrated by the following examples, which should not be construed as limiting. Those skilled in the art will recognize, or be able to ascertain, using no more than routine experimentation, numerous equivalents to the specific substances and procedures described herein. Such equivalents are intended to be encompassed in the scope of the claims that follow the examples

below.

A list of bona fide off sites induced by a meganuclease, a Crispr/cas9, a Zn finger or a MegaTAL was established using the respective TRAC-specific endonucleases as previously described in *WO2014153470A2,* WO2017062451, WO2015057980, WO2017106528.

To establish these lists, different donors of different genetic background origin can be tested.
Said off sites sequences were identified using an adapted guide seq method.

Example 1: TALEN binding sequences (or recognition sequence) in the TRAC gene and sequences in engineered TRAC gene for insertion of a polynucleotide comprising a self-cleaving peptide, a CAR, and a termination signal (poly A).
Frame for the design of any means, probe, oligonuleotides of the invention for detecting alpha beta TCR deficient cells of the invention

TRAC exon1 (capital letter)

ttggccaagattgatagcttgtgcctgtccctgagtcccagtccatcacgagcagctggtttc-
taagatgctatttcccgtataaagcatgagaccgtgactt-
gccagccccacagagccccgcccttgtccatcactggcatctggactccagcctgggttgggg-
caaagagggaaatgagatcatgtcctaaccctgatcctcttgtcccacagA-
TATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAA**TCCAG-**
TGACAAGTCTGTCTGCCTATTCACCGATTTTGAT-
TCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGACAAAACTGTGCTA-
GACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAG-
CAACAAATCTGACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGACAC-

CTTCTTCCCCAGCCCAGGtaagggcagctttggtgccttcgcaggctgtttccttgcttcag-

gaatggccaggttctgcccagagctctggtcaatgatgtctaaaactcctctgattggtggtctcggccttatccattgccaccaaaaccctcttttt

actaagaaacagtgagccttgttctggcagtccagagaatgacacgggaaaaaagcagatgaa-

gagaaggtggcaggagagggcacgtggcccagcctcagtctctccaactgagttcctgcctgcctgcctttgctcagactgttt-

ttcctgcctgcctgcctttgctcagactgtttgcccccttactgctcttctaggcctcattctaagccccttctccaagtt-

gcccccttactgctcttctaggcctcattctaagccccttctccaagtt-

gcctctccttatttctccctgtctgccaaaaaatctttcccagctcactaagtcagtctcac-

gcagtcactcattaacccaccaatcactgattgtgccggcacatgaatgcac

Integration site preferred :

ttggccaagattgatagcttgtgcctgtccctgagtcccagtccatcacgagcagctggtttc-

taagatgctatttcccgtataaagcatgagaccgtgactt-

gccagccccacagagccccgcccttgtccatcactggcatctggactccagcctgggttgggg-

caaagagggaaatgagatcatgtcctaaccctgatcctcttgtcccacagATATCCAG

XXX CCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTGCCTATTCACCGATTTT-

GATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGA-

CAAAACTGTGCTAGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAG-

CAACAAATCTGACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGACAC-

CTTCTTCCCCAGCCCAGGtaagggcagctttggtgccttcgcaggctgtttccttgcttcag-

gaatggccaggttctgcccagagctctggtcaatgatgtctaaaactcctctgattggtggtctcggccttatccattgccaccaaaaccctcttttt

actaagaaacagtgagccttgttctggcagtccagagaatgacacgggaaaaaagcagatgaa-

gagaaggtggcaggagagggcacgtggcccagcctcagtctctccaactgagttcctgcctgcctgcctttgctcagactgttt-

ttcctgcctgcctgcctttgctcagactgtttgcccccttactgctcttctaggcctcattctaagccccttctccaagtt-

gcccccttactgctcttctaggcctcattctaagccccttctccaagtt-

gcctctccttatttctccctgtctgccaaaaaatctttcccagctcactaagtcagtctcac-

gcagtcactcattaacccaccaatcactgattgtgccggcacatgaatgcac

XXX represents a polynucleotide inserted.

Sequences detected after dsDNA cleavage (5')

Most preferred sequence in TRAC :

ttgtcccacagATATCCAG – 2A sequence in frame with TCR– CAR sequence – pA – right homology

alternatives:

ttgtcccacagATATCCAGAAC – 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCT – 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGAC – 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCT – 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCC – 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTG – 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTAC – 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAG – 2A sequence – CAR sequence – pA – right homology

Possible sequence in 3' : no restriction, underlined sequence may be absent :

Left homology -2A sequence – CAR sequence – pA – CCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAA

Left homology -2A sequence – CAR sequence – pA – CTGACCCTGCCGTGTACCAGCTGAGAGACTCTAA

Left homology -2A sequence – CAR sequence – pA –TGACCCTGCCGTGTACCAGCTGAGAGACTCTAA

Left homology -2A sequence – CAR sequence – pA –GACCCTGCCGTGTACCAGCTGAGAGACTCTAA

Left homology -2A sequence – CAR sequence – pA –ACCCTGCCGTGTACCAGCTGAGAGACTCTAA

Left homology -2A sequence – CAR sequence – pA –CCCTGCCGTGTACCAGCTGAGAGACTCTAA

Left homology -2A sequence – CAR sequence – pA –CCTGCCGTGTACCAGCTGAGAGACTCTAA

Left homology -2A sequence – CAR sequence – pA –CTGCCGTGTACCAGCTGAGAGACTCTAA

Left homology -2A sequence – CAR sequence – pA –TGCCGTGTACCAGCTGAGAGACTCTAA

Left homology -2A sequence – CAR sequence – pA –GCCGTGTACCAGCTGAGAGACTCTAA

Left homology -2A sequence – CAR sequence – pA –CCGTGTACCAGCTGAGAGACTCTAA

(most preferred sequence).

Left homology -2A sequence – CAR sequence – pA –CGTGTACCAGCTGAGAGACTCTAA

Left homology -2A sequence – CAR sequence – pA –GTGTACCAGCTGAGAGACTCTAA

Left homology -2A sequence – CAR sequence – pA –TGTACCAGCTGAGAGACTCTAA

Left homology -2A sequence – CAR sequence – pA –GTACCAGCTGAGAGACTCTAA

Left homology -2A sequence – CAR sequence – pA –TGTACCAGCTGAGAGACTCTAA

Left homology -2A sequence – CAR sequence – pA –GTACCAGCTGAGAGACTCTAA

Left homology -2A sequence – CAR sequence – pA –TACCAGCTGAGAGACTCTAA

Left homology -2A sequence – CAR sequence – pA –ACCAGCTGAGAGACTCTAA

Left homology -2A sequence – CAR sequence – pA –CCAGCTGAGAGACTCTAA

Left homology -2A sequence – CAR sequence – pA –CAGCTGAGAGACTCTAA

Left homology -2A sequence – CAR sequence – pA –AGCTGAGAGACTCTAA

Left homology -2A sequence – CAR sequence – pA –GCTGAGAGACTCTAA

Left homology -2A sequence – CAR sequence – pA –CTGAGAGACTCTAA

Left homology -2A sequence – CAR sequence – pA –TGAGAGACTCTAA

Left homology -2A sequence – CAR sequence – pA –GAGAGACTCTAA

Left homology -2A sequence – CAR sequence – pA –AGAGACTCTAA

Left homology -2A sequence – CAR sequence – pA –GAGACTCTAA

Left homology -2A sequence – CAR sequence – pA –AGACTCTAA

Left homology -2A sequence – CAR sequence – pA –GACTCTAA

OTHER TALEN studied in TRAC

In Exon1 :

TCCAGTGACAAGTCTGTctgcctattcaccgaTTTTGATTCTCAAACAA

Combination in 5' (with 2A sequence in frame with TRAC)

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAA 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCC 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGT 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGAC 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAG - 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCT - 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTC - - 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG C - 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG CCTA- 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG CCTATTC - 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG CCTATTCACC - 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG

CCTATTCACCGAT - 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG

CCTATTCACCGATTTT - 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG

CCTATTCACCGATTTTGAT - 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG

CCTATTCACCGATTTTGATTCT - 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG

CCTATTCACCGATTTTGATTCTCAA - 2A sequence – CAR sequence – pA – right homology

Possible combination in 3’

Left homology -2A sequence – CAR sequence – pA – TTCACCGATTTTGATTCTCAAACAAATGTGTCACAA

Left homology -2A sequence – CAR sequence – pA – TCACCGATTTTGATTCTCAAACAAATGTGTCACAA

Left homology -2A sequence – CAR sequence – pA – CACCGATTTTGATTCTCAAACAAATGTGTCACAA

Left homology -2A sequence – CAR sequence – pA – ACCGATTTTGATTCTCAAACAAATGTGTCACAA

Left homology -2A sequence – CAR sequence – pA – CCGATTTTGATTCTCAAACAAATGTGTCACAA

Left homology -2A sequence – CAR sequence – pA – CGATTTTGATTCTCAAACAAATGTGTCACAA

Left homology -2A sequence – CAR sequence – pA – GATTTTGATTCTCAAACAAATGTGTCACAA

Left homology -2A sequence – CAR sequence – pA – ATTTTGATTCTCAAACAAATGTGTCACAA

Left homology -2A sequence – CAR sequence – pA – TTTTGATTCTCAAACAAATGTGTCACAA

Left homology -2A sequence – CAR sequence – pA – TTTGATTCTCAAACAAATGTGTCACAA

Left homology -2A sequence – CAR sequence – pA – TTGATTCTCAAACAAATGTGTCACAA

Left homology -2A sequence – CAR sequence – pA – TGATTCTCAAACAAATGTGTCACAA

Left homology -2A sequence – CAR sequence – pA – GATTCTCAAACAAATGTGTCACAA

Left homology -2A sequence – CAR sequence – pA – ATTCTCAAACAAATGTGTCACAA

Left homology -2A sequence – CAR sequence – pA – TTCTCAAACAAATGTGTCACAA

Left homology -2A sequence – CAR sequence – pA – TCTCAAACAAATGTGTCACAA

Left homology -2A sequence – CAR sequence – pA – CTCAAACAAATGTGTCACAA

Left homology -2A sequence – CAR sequence – pA – TCAAACAAATGTGTCACAA

Left homology -2A sequence – CAR sequence – pA – CAAACAAATGTGTCACAA

Left homology -2A sequence – CAR sequence – pA – AAACAAATGTGTCACAA

Left homology -2A sequence – CAR sequence – pA – AACAAATGTGTCACAA

Left homology -2A sequence – CAR sequence – pA – ACAAATGTGTCACAA

Left homology -2A sequence – CAR sequence – pA – CAAATGTGTCACAA

Left homology -2A sequence – CAR sequence – pA – AAATGTGTCACAA

Left homology -2A sequence – CAR sequence – pA – AATGTGTCACAA

Left homology -2A sequence – CAR sequence – pA – ATGTGTCACAA

Left homology -2A sequence – CAR sequence – pA – TGTGTCACAA

Left homology -2A sequence – CAR sequence – pA – GTGTCACAA

Left homology -2A sequence – CAR sequence – pA – TGTCACAA

Left homology -2A sequence – CAR sequence – pA – GTCACAA

TRAC_T04 that binds to

TATATCACAGACAAAACtgtgctagacatgagGTCTATGGACTTCAAGA

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG

CCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTG - 2A sequence – CAR

sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG

CCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTAT - 2A sequence – CAR

sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG

CCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATC - 2A sequence –

CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG

CCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACA - 2A

sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG

CCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGAC- 2A

sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG

CCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGACAAA - 2A

sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG

CCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGACAAAACT -

2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG

CCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGACAAAACTG

TG - 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG

CCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGACAAAACTG

TGCTA - 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG

CCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGACAAAACTG

TGCTAGAC - 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG

CCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGACAAAACTG

TGCTAGACATG - 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG

CCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGACAAAACTG

TGCTAGACATGAGG - 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG

CCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGACAAAACTG

TGCTAGACATGAGGTCT - 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG

CCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGACAAAACTG

TGCTAGACATGAGGTCTATG - 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG

CCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGACAAAACTG

TGCTAGACATGAGGTCTATGGAC - 2A sequence – CAR sequence – pA – right homology

TRAC_T05 (underlined sequence): Talen binding site.

TGAGGTCTATGGACTTCaagagcaacagtgctGTGGCCTGGAGCAACAA

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG

CCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGACAAAACTG

TGCTAGACATGAGGTCTATGGAC - 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG

CCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGACAAAACTG

TGCTAGACATGAGGTCTATGGACTTC - 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG

CCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGACAAAACTG

TGCTAGACATGAGGTCTATGGACTTCAAG - 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG
CCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGACAAAACTG
TGCTAGACATGAGGTCTATGGACTTCAAGAGC - 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG
CCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGACAAAACTG
TGCTAGACATGAGGTCTATGGACTTCAAGAGCAAC - 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG
CCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGACAAAACTG
TGCTAGACATGAGGTCTATGGACTTCAAGAGCAACAGT - 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG
CCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGACAAAACTG
TGCTAGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCT - 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG
CCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGACAAAACTG
TGCTAGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTG - 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG
CCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGACAAAACTG
TGCTAGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCC - 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG
CCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGACAAAACTG
TGCTAGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGG- 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTG CCTATTCACCGATTTTGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGACAAAACTG TGCTAGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGC - 2A sequence – CAR sequence – pA – right homology

hsTRAC_T01

TATCCAGAACCCTGACCctgccgtgtaccagctGAGAGACTCTAAATCCA

ttgtcccacagATATCCAG – 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAAC - 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCT - 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGAC – 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCT – 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCC – 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTG – 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTAC – 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAG – 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTG – 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGA – 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGAC – 2A sequence – CAR sequence – pA – right homology

ttgtcccacagATATCCAGAACCCTGACCCTGCCGTGTACCAGCTGAGAGACTCT – 2A sequence – CAR

sequence – pA – right homology

Example of TALEN targeting Exon2

TRAC_T02: that binds to TTTAGAAAGTTCCTGTGatgtcaagctggtcgAGAAAAGCTTTGAAACA

Example 2: Production of UCART cells by inserting a CAR to the TRAC locus with a TALEN

Experimental protocol

To disrupt the TRAC locus and place a CAR (ex: CD22-specific m971 QR3 or CD123-specific K43 QR3) under its transcriptional control (TRAC-CAR), a TRAC TALEN targeting the first exon of TRAC locus and an adeno-associated virus (AAV) vector repair matrix encoding a self-cleaving T2A peptide followed by the CAR cDNA were prepared. PBMCs were thawed and activated using Transact human T activator CD3/CD28 beads. 3 days after their activation, T cells were passed to be transfected 4 hours later at the earliest. T cells were then transfected by electrotransfer of 1 µg of mRNA encoding TRAC TALEN per million cells using an AgilePulse system (Harvard Apparatus) into a 0.4 cm cuvette. Following electroporation, cells were immediately diluted in X-Vivo-15 media supplemented by 20 ng/ml IL-2 and 5% CTS™ Immune Cell SR at the concentration of $4x10^6$ cells/mL and incubated in 12 well-plates (500 µl per well) at 37°C in the presence of 5% CO2.

A Recombinant AAV6 donor vector comprising 300kb homology arms in 5' and 3' was added to the culture (1.5h after electroporation at the multiplicity of infection of $3x10^4$ vg/cell). Subsequently, edited cells were cultured overnight at 30°C in X-Vivo-15 media supplemented by 20 ng/ml IL-2 and 5% CTS™ Immune Cell SR and cultured back in the standard conditions starting from the day after (37°C, $1x10^6$ cells/mL, X-Vivo-15 media supplemented by 20 ng/ml IL-2 and 5% CTS™ Immune Cell SR). Cells were then expanded in the standard conditions and passed every 2 to 3 days. 10 days after transfection/transduction TRAC knock-out and CAR expression were assessed by flow cytometry.

Then, 10 days post-transduction, CAR^+ T cells cytolytic capacities towards antigen presenting cells were assessed in a flow-based cytotoxicity assay after a 4h or overnight coculture at 37°C in the presence of 5% CO2.

The target cells that were chosen for their level of expression in antigen (CD22 or CD123). CART and target cells were cocultured in X-Vivo-15 medium at effector (CAR^+):target ratios of 1:1, 2:1, 5:1 and 10:1 for the 4h experiment and 0.1:1, 0.2:1, 0.5:1 and 1:1. Culture medium is supplemented with 5% CTS™ Immune Cell SR. To distinguish positive (Daudi or Raji) and negative (SUP-T1) tumor cell lines, positive target cells were stained with CFSE while SUP-T1 were stained with the CellTrace violet proliferation marker. At the end of the coculture, cell viability was measured and the percentage of specific lysis was calculated after normalization to non-specific target cell lysis.

A simultaneous editing of the TRAC and CD52 genes or TRAC dCK genes results in TCR/CD52-deficient T cells, or TCR/dCK-deficient T cells which can be administered with, or following, alemtuzumab treatment or PNA treatment, respectively. Alemtuzumab mediates lymphodepletion/immunosuppression thereby promoting engraftment. The same protocol as described previously was used, with the only difference that T cells were transfected by electrotransfer of 1 µg of mRNA encoding TRAC TALEN and 1 µg of mRNA encoding CD52 TALEN or dCK TALEN per million cells.

In parallel, cells were transfected with different plasmids encoding the different CARs of interest and a control CAR (CAR1). Supernatants were harvested 48h later and concentrated by ultracentrifugation. Titration was performed using Jurkat cells transduced with different quantities (µl) of supernatant. 4 days later CAR expression was assessed by flow cytometry on viable $RQR8^+$ cells in combination with a live/dead cell marker and viral titers were determined.

TALEN-targeted CAR gene integration into the TRAC locus. rAAV6 contained a CAR cassette flanked by 1000 bp to 100 homology arms and the bottom panel the edited TRAC locus.

3 days after activation, T cells were transfected or not preferably by electrotransfer, at a dose of 1 µg of mRNA encoding TRAC TALEN per million cells. 1.5h later, rAAV6 donor vector was added or not to the culture at the multiplicity of infection of $3x10^4$ vg/cell. TCR and CAR expressions were assessed by flow cytometry on viable T cells using CD4, CD8, TCR$\alpha\beta$ mAb, CD22 or CD123 recombinant protein (full length) in combination with a live/dead cell marker.

The results show that the population of CAR^+ cells after rAAV transduction is more homogenous than after rLv transduction.

Using this 2-in-1 strategy of TCR KO and CAR KI, the integration of the CAR at the TRAC locus is highly efficient since the frequency of CAR^+ TCR^- cells reached more than 42%.

Total cells or CAR^+ T cells cytolytic capacities towards antigen presenting cells was assessed in a flow-based cytotoxicity assay. The cell viability was measured after 4h or after an overnight coculture with CAR T cells at effector/target ratios set at 10:1, 5:1, 2:1 and 1:1 or 1:1, 0.5:1, 0.2:1 and 0.1:1 respectively.

The results show that TRAC-CART cells produced after rAAV6 transduction are as cytotoxic in vitro as UCART cells produced after rLV transduction.

3 days after activation, T cells were transfected or not by electrotransfer of 1 µg of each mRNA encoding TRAC and CD52 TALEN per million cells. 1.5h later, rAAV6 donor vector was added or not to the culture at the multiplicity of infection of $3x10^4$ vg/cell. TCR, CD52 and CAR expressions were

assessed by flow cytometry on viable T cells using CD4, CD8, TCRαβ mAb, CD22 or CD123 recombinant protein (full length) in combination with a live/dead cell marker.

The results show that this 2-in-1 strategy of TCR KO and CAR KI can be extended to the use of more than one TALEN. As previously demonstrated, the integration of the CAR at the TRAC locus is highly efficient and specific since the frequency of CAR^+ TCR^- cells reached more than 47%. Importantly, no CAR expression was detected at the CD52 locus when T cells were transfected only with 1 µg of mRNA encoding CD52 TALEN. More than 80% of the population of CAR^+ T cells is knocked-out for both TCRαβ and CD52.

Characterization of TALEN, meganuclease, Zn finger engineered primary cell - TRAC Recognition Sequences - off site measurements.

Recombinant nucleases engineered to recognize recognition sequences of the human TRAC gene (all in the human T cell receptor alpha constant region) were previously disclosed in WO2017062451 Precision TRAC, WO2015057980 or in WO2017106528. The activity of said endonucleases results in a disablement of the extracellular part of the endogenous TCR alpha beta and ultimately to a down regulation of cell surface of the endogenous alpha beta TCR. Comparative Zn finger were those disclosed in US2011158957.

all nucleases were compared to the reference TALEN as disclosed in WO2013176915.

To determine whether nucleases could recognize and cleave their respective recognition sequences, primary cells isolated from leukapheresis were used.

Cells were engineered as below and cell surface expression of alpha beta TCR was measured as a read out.

In particular experiments, purified cell populations were used to test whether TRAC gene editing could be different in whole blood cells, purified naive T cells, purified memory T cells etc .

Means for detecting the engineered TRAC gene is a probe, oligonucleotides, oligonucleotides designed for PCR amplification of the DNA in the TRAC gene greater than 1 kb. A labelled (P32) may be prepared as an alternative.

Any PCR method may be adapted such as real time PCR, TaqMan one-step reverse transcription-quantitative PCR (qRT-PCR) targeting conserved regions of the TRAC gene upstream the recognition sequence and are part of the present invention.

The following sequence was amplified to quantify on target integration:

GCTGGGGTTTTGAAGAAGATCCTATTAAATAAAAGAATAAGCAGTATTATTAAGTAGCCCTGCATTTCAGGTTTCC

TTGAGTGGCAGGCCAGGCCTGGCCGTGAACGTTCACTGAAATCATGGCCTCTTGGCCAAGATTGATAGCTTGTG

CCTGTCCCTGAGTCCCAGTCCATCACGAGCAGCTGGTTTCTAAGATGCTATTTCCCGTATAAAGCATGAGACCGTG

ACTTGCCAGCCCCACAGAGCCCCGCCCTTGTCCATCACTGGCATCTGGACTCCAGCCTGGGTTGGGGCAAAGAG

GGAAATGAGATCATGTCCTAACCCTGATCCTCTTGTCCCACAGATATCCAGTCCGGTGAGGGCAGAGGAAGTCTT

CTAACATGCGGTGACGTGGAGGAGAATCCGGGCCCC

Using the following means:

Forward : GCTGGGGTTTTGAAGAAGATCC

Reverse : GACTTCCTCTGCCCTCACC

Probe : CCCTTGTCCATCACTGGCAT

Cells were harvested at different time points of their expansion.

Two steps of washes are performed in X-Vivo15 medium before one additional wash performed in the presence of 140U/ml of benzonase for 30 min at 37°C.

Two following washes are necessary to remove the excess of benzonase.

Cells are then dry pelleted and stored at -20°C.

Genomic DNA is extracted using the DNeasy Blood & Tissue Kit (QIAGEN) following manufacturer's guidelines.

gDNA concentration was assessed using the Quant-iT™ PicoGreen™ dsDNA Assay Kit (ThermoFisher

Scientific).

For qPCR assay, primers and probe (FAM) are designed to amplify a sequence of 373 bp, the forward primer being located in the TRAC locus outside of the left homology sequence and the reverse primer in the T2A sequence. A mix of primers and probe is prepared in TE buffer containing 300 nM of forward primer, 900 nM of reverse primer and 250 nM of probe (IDT Technologies).

In another preferred embodiment the mix of primers and probe is prepared in TE buffer containing 300 nM of forward primer, 900 nM of reverse primer and 220 nM of probe (IDT Technologies).

In another preferred embodiment, the mix of primers and probe is prepared in TE buffer containing 300 nM of forward primer, 900 nM of reverse primer and 220 nM of probe (IDT Technologies) and the optimal quantity of gDNA to use ranges from 100 to 200 ng (10 µl), preferably between 20 and 30ng (10 µl).

Target amplification is performed using the TaqMan Gene Expression Master Mix (Thermo Fisher 4369514) and run on a C1000 Touch™ Thermal Cycler (BioRad). Gene expression is normalized to RPP30 gene expression (ddPCR™ CNV Assay, Validated (HEX)) and the number of copies is calculated from the dilution (10^6 copies / µl down to 10^0 copies/µl) of a standard plasmid that includes the sequence of interest as previously described, extended in 5' by some nucleotides corresponding to the TRAC locus sequence.

To amplify a sequence according to the present invention, the concentration of primers/probe was adjusted and the annealing/extension step was increased by 2, such as from 45 sec to 1 min 30 sec.

Preferably, the annealing/extension step was increased from 45 sec to 1 min 30 sec and performed at 61.5°C.

On site and/or off sites cleavage determination by GUIDE SEQ for the following genes :

CD28 Talen, CD28 Talen, CD28 Crispr and PD1 TALEN, TRAC Talen (two conditions of stringency)

CS1, CS1 + TRAC Talen, CD52 + TRAC Talen, CS1 + TRAC Talen

Sequence of the dsODN :

GTTTAATTGAGTTGTCATATGTTAATAACGGTAT used for insertion in off sites and onsite in the presence or absence of

AAGTAGCCCTGCATTTCAGGTTTCCTTGAGTGGCAGGCCAGGCCTGGCCGTGAACGTTCACTGAAATCATGGCC

TCTTGGCCAAGATTGATAGCTTGTGCCTGTCCCTGAGTCCCAGTCCATCACGAGCAGCTGGTTTCTAAGATGCTAT

TTCCCGTATAAAGCATGAGACCGTGACTTGCCAGCCCCACAGAGCCCCGCCCTTGTCCATCACTGGCATCTGGAC

TCCAGCCTGGGTTGGGGCAAAGAGGGAAATGAGATCATGTCCTAACCCTGATCCTCTTGTCCCACAGATATCCAG

TCCGGTGAGGGCAGAGGAAGTCTTCTAACATGCGGTGACGTGGAGGAGAATCCGGGCCCCGGATCCGCTCTG

CCCGTCACCGCTCTGCTGCTGCCTCTGGCCCTGCTGCTGCACGCAGCCAGACCAGGCGGAGGAGGCTCCTGCCC

TTACTCTAACCCAAGCCTGTGCTCCGGAGGAGGAGGATCCGGCGGAGGAGGCTCTGAGGTGAAGCTGGTGGA

GAGCGGAGGAGGCCTGGTGCAGCCTGGCGGCTCCCTGTCTCTGAGCTGCGCAGCATCCGGCTTCACCTTTACAG

ACTACTATATGTCTTGGGTGAGACAGCCCCCTGGCAAGGCCCTGGAGTGGCTGGCCCTGATCAGGTCCAAGGCC

GATGGCTACACCACAGAGTATTCCGCCTCTGTGAAGGGCAGATTCACCCTGTCTAGGGACGATAGCCAGTCCATC

CTGTACCTGCAGATGAATGCACTGCGCCCCGAGGACAGCGCCACATACTATTGTGCCAGAGACGCCGCCTACTAT

TCTTACTATAGCCCTGAGGGCGCTATGGACTACTGGGGCCAGGGCACCTCCGTGACAGTGAGCTCCGGAGGAGG

AGGAAGCGGAGGAGGAGGCTCCGGCGGCGGCGGCTCTATGGCCGACTATAAGGATATCGTGATGACCCAGAGC

CACAAGTTTATGTCTACAAGCGTGGGCGACCGCGTGAACATCACCTGCAAGGCCAGCCAGAATGTGGATTCCGC

CGTGGCCTGGTACCAGCAGAAGCCTGGCCAGAGCCCTAAGGCCCTGATCTATTCCGCCTCTTACCGGTATAGCGG

AGTGCCTGACCGCTTCACCGGAAGGGGATCCGGAACAGACTTCACCCTGACAATCTCTAGCGTGCAGGCCGAG

GATCTGGCCGTGTACTATTGTCAGCAGTACTATAGCACCCCCTGGACCTTCGGCGGAGGAACCAAGCTGGAGATC

AAGAGAGGATCTGGAGGAGGAGGAAGCTGCCCATACTCCAACCCCTCTCTGTGCAGCGGAGGAGGAGGATCTG

AGCTGCCAACCCAGGGCACATTTTCCAACGTGTCTACAAATGTGAGCCCAGCAAAGCCAACCACAACCGCATGC

CCTTATAGCAATCCATCCCTGTGCACAACCACACCTGCACCAAGACCACCAACCCCAGCACCTACAATCGCCTCTC

AGCCACTGAGCCTGCGCCCCGAGGCATGCCGGCCTGCAGCAGGCGGCGCCGTGCACACCAGGGGCCTGGACT

TCGCCTGCGATATCTACATCTGGGCACCTCTGGCAGGAACCTGTGGCGTGCTGCTGCTGAGCCTGGTCATCACCC

TGTACTGCAAGAGAGGCAGGAAGAAGCTGCTGTATATCTTCAAGCAGCCCTTTATGCGCCCTGTGCAGACCACAC

AGGAGGAGGACGGCTGCAGCTGTCGGTTCCCAGAAGAGGAGGAGGGCGGCTGTGAGCTGAGAGTGAAGTTT

AGCAGGTCCGCCGATGCACCAGCATACCAGCAGGGACAGAACCAGCTGTATAACGAGCTGAATCTGGGCCGGA

GAGAGGAGTACGACGTGCTGGATAAGAGGAGGGGAAGGGACCCCGAGATGGGAGGCAAGCCACGGAGAAA

GAACCCCCAGGAGGGCCTGTACAATGAGCTGCAGAAGGACAAGATGGCCGAGGCCTATTCCGAGATCGGCATG

AAGGGAGAGAGGCGCCGGGGCAAGGGACACGATGGCCTGTACCAGGGCCTGTCTACCGCCACAAAGGACACC

TATGATGCCCTGCATATGCAGGCACTGCCTCCAAGGTGATCTAGAGGGCCCGTTTAAACCCGCTGATCAGCCTCG

A*CTGTGCCTTCTAGTTGCCAGCCATCTGTTGTTTGCCCCTCCCCCGTGCCTTCCTTGACCCTGGAAGGTGCCACTCC*

CACTGTCCTTTCCTAATAAAATGAGGAAATTGCATCGCATTGTCTGAGTAGGTGTCATTCTATTCTGGGGGGTGGG

GTGGGGCAGGACAGCAAGGGGGAGGATTGGGAAGACAATAGCAGGCATGCTGGGGATGCGGTGGGCTCTATG

ACTAGTGGCGAATTCCCGTGTACCAGCTGAGAGACTCTAAATCCAGTGACAAGTCTGTCTGCCTATTCACCGATTT

TGATTCTCAAACAAATGTGTCACAAAGTAAGGATTCTGATGTGTATATCACAGACAAAACTGTGCTAGACATGAGG

TCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCAACAAATCTGACTTTGCATGTGCAAACGCCTTCAAC

AACAGCATTATTCCAGAAGACACCTTCTTCCCCAGCCCAGGTAAGGGCAGCTTTGGTGCCTTCGCAGGCTGTTTC

CTTGCTTCAGGAA

Samples.

Primary T cells were electroporated either with a TRAC endonuclease or nothing plus 1000 pmol of a DS oligonucleotide.

Two samples of each condition were done, and the genomic DNA extracted. Each genomic DNA was

divided in 3 aliquots which were individually processed using the GUIDE-seq protocol.

The genomic DNA was extracted and submitted to standard GUIDE-seq procedure :

- shearing of the DNA by sonication.
- end repair to have blunt extremities.
- ligation of a Y-shape adaptor blunt on one side, and with overhangs on the other (so that it can ligate only on one side).
- successive PCRs between the DS oligo and the adaptor above : these can be done either using the sequence of the oligo ("plus" amplification that will amplify one side of the flanking region) or the reverse complement sequence of the oligo ("minus" amplification that will amplify the other side of the flanking region).
- each PCR product is then sequenced on both sides (R1 and R2)

All samples were amplified on both strands minus and plus, and sequenced in both extremities R1 and R2.

Nuclease :

The arms of the TALENs are the following (with T0 included)

- for TRAC : TRACL = TTGTCCCACAGATAT and TRACR = TCTCAGCTGGTACAC

Locations of the sites on the v37.1 of the genome :

- onsite TRAC : contig NT_026437.12, starting on position 4016436 (middle of spacer on 4016460)

Primers used for the PCR

The primers used on the side of the oligo were :

- for the plus strand, ATACCGTTATTAACATATGACA (SEQ ID N°13) for the first PCR, and then CATATGACAACTCAATTAAAC (SEQ ID N°14) for the second PCR :

- ATACCGTTATTAACATATGACAACTCAATTAAAC (SEQ ID N°15) oligo (rev compl)
- ATACCGTTATTAACATATGACA 1st PCR (SEQ ID N°16)
- CATATGACAACTCAATTAAAC 2nd PCR (SEQ ID N°17)

- for the minus strand, GTTTAATTGAGTTGTCATATGTTAATAAC (SEQ ID N°18) for the first PCR, and then TTGAGTTGTCATATGTTAATAACGGTA (SEQ ID N°19) for the second PCR :
- GTTTAATTGAGTTGTCATATGTTAATAACGGTAT (SEQ ID N°20) oligo
- GTTTAATTGAGTTGTCATATGTTAATAAC 1st PCR (SEQ ID N°21)
- TTGAGTTGTCATATGTTAATAACGGTA 2nd PCR (SEQ ID N°22.

Structure of the Product

The structure of the waited products should be the following, with the DS oligonucleotide being in uppercase, the primers that should remain after the PCRs in lowercase, and the unknown genomic sequence represented by NNNNNNNNNN :

- for "plus" PCRs : R1 -> aatgatacggcgaccaccgagatctacactctttccctacacgacgctcttccgatct-NNNNNNNNNNGTTTAATTGAGTTGTCATATG-tatcaccgactgcccatagagaggactccagtcactaaggcgaatctcgtatgccgtcttctgcttg <- R2
- for "minus" PCRs : R2 -> caagcagaagacggcatacgagattcgccttagtgactggagtcctctctatgggcag-tcggtgat-TTGAGTTGTCATATGTTAATAACGGTATNNNNNNNNNNNN-agatcggaagagcgtcgtgtaggaaagagtgtagatctcggtggtcgccgtatcatt <- R1

Sequence data processing:

Reads were first tested for the presence of the expected primer :

- for minus_R2 reads, the presence of the sequence TGAGTTGTCATATGTTAATAACGGTAT which is the sequence of the second primer without the first T was checked,

- for plus_R2 reads, the presence of the sequence CATATGACAACTCAATTAAAC which is the sequence of the second PCR primer was checked.

when the R2 read had not this sequence, R2 was eliminated. Remaining reads were then successively trimmed in 3' and 5' for adaptor presence with a minimum required overlap length of 20 bp and remaining sequences shorter than 10 bp were discarded. The counts are represented with :

- the initial number of reads
- the number of filtered reads (for the required oligo)
- the final number of trimmed reads once short trimmed sequences are discarded.

A GUIDE-Seq read count or GUIDE-Seq score for a given site in the TRAC gene by a given endonuclease, represents a quantitative measurement of the cleavage efficiency of that sequence by an RNA or protein guided nuclease. Preferably the RNA or protein guided nuclease of the invention affecting the TRAC gene have a score near zero (undetectable).

The results indicated on site cleavage for 4 TALENs tested affecting the TRAC gene and cell surface expression of alpha beta TCR. Offsite cleavages were below detection in TALEN edited primary cells using the guide-seq analysis adapted to TALEN.

At 48, 72, 96 hours post-transfection, cells were evaluated by flow cytometry to determine the percentage of TCR alpha beta-positive and negative cells as compared to the appropriate control. All meganucleases and crispr tested were found to produce TCR-negative cells.

Insertion of an anti-CD22 or anti-CD123 chimeric antigen receptor sequence was confirmed by pcr and sequencing of the TCR alpha constant region gene.

Cell-surface expression of the chimeric antigen receptor confirmed by flow cytometry, using an anti-Fab or rituximab. Knockout of the endogenous T cell receptor at the cell surface was verified by flow cytometry as previously described.

These studies demonstrated that TALEN, meganucleases and Crispr can recognize and cleave the TRAC gene in T cells obtained from a human donor. Further, these studies demonstrated that NHEJ occurs at the cleavage site, as evidenced by the appearance of indels in 3' of the insertion. Moreover, TRAC TALEN were shown to reduce cell- surface expression of the T cell receptor in human T cells obtained from a donor, even in the presence of a CD52 TALEN in cells with a CAR inserted into the genomic TRAC gene.

These studies demonstrate that AAV vectors can be used in conjunction with recombinant TALENs or Crispr to incorporate specifically an exogenous nucleic acid sequence into a cleavage site in the TCR alpha constant region via homologous recombination without insertion in other sites (CD52, dCK).

Example 3

TALEN®-mediated double targeted integration of IL-15 and CAR encoding matrices in T-cells

This example describes methods to improve the therapeutic outcome of CAR T-cell therapies by integrating an IL-15/soluble IL-15 receptor alpha heterodimer (IL15/sIL15rα) expression cassette under the control of the endogenous T-cell promoters regulating PD1 and CD25 genes. Because both genes are known to be upregulated upon tumor engagement by CAR T-cells, they could be hijacked to re-express IL- IL15/sIL15rα only in vicinity of a tumor. This method would reduce the potential side effects of IL15/sIL15rα systemic secretion while maintaining its capacity to reduced activation induced T-cell death (AICD), promote T-cell survival, enhance T-cell antitumor activity and to reverse T-cell anergy.

The method developed to integrate IL15/sIL15rα at PD1 and CD25 loci consisted in generating a double-strand break at both loci using TALEN in the presence of a DNA repair matrix vectorized by AAV6. This matrix consists of two homology arms embedding IL15/sIL15rα coding regions separated by a 2A cis acting elements and regulatory elements (stop codon and polyA sequences). Depending on the locus targeted and its involvement in T-cell activity, the targeted endogenous gene could be inactivated or not via specific matrix design.

When CD25 gene was considered as targeted locus, the insertion matrix was designed to knock-in (KI) IL15/sIL15rα without inactivating CD25 because the protein product of this gene is essential for T-cell function. In contrary, because PD1 is involved in T-cell inhibition/exhaustion of T-cells, the insertion matrix was designed to prevent its expression while enabling the expression and secretion of IL15/sIL15rα.

To illustrate this approach and demonstrate the feasibility of multiple targeted insertion in primary T-cells, three different matrices were designed. The first one named CARm was designed to insert an anti-CD22 CAR cDNA at the TRAC locus in the presence of TRAC TALEN. The second one, IL-15_CD25m that was designed to integrate IL15, sIL15rα and the surface marker named ΔLNGFR cDNAs separated by 2A cis-acting elements just before the stop codon of CD25 endogenous coding sequence using CD25 TALEN®. The third one, IL-15_PD1m, contained the same expression cassette and was designed to integrate in the middle of the PD1 open reading frame using PD1 TALEN. The three matrices contained an additional 2A cis-acting element located upstream expression cassettes to enable co-expression of IL15/sIL15rα and CAR with the endogenous gene targeted.

We first assessed the efficiency of double targeted insertion in T-cells by transducing them with one of the AAV6 encoding IL15/sIL15rα matrices along with the one encoding the CAR and subsequently transfected the corresponding TALEN®. AAV6-assisted vectorization of matrices in the presence of mRNA encoding TRAC TALEN and PD1 or CD25 TALEN enabled expression of the anti CD22 CAR more than 46% of engineered T-cells.

To determine the extent of IL15m integration at CD25 and PD1 locus, engineered T-cells were activated with either antiCD3/CD28 coated beads or with CD22 expressing Raji tumor cells. 2 days post activation, cells were recovered and analyzed by FACS using LNGFR expression as IL15/sIL15rα secretion surrogate. Our results showed that antiCD3/CD28 coated beads induced expression of ΔLNGFR by T-cells containing IL-15m_CD25 or IL-15m_PD1, independently of the presence of the anti CD22 CAR. Tumor cells however, only induced expression of ΔLNGFR by T-cell treated by both CARm and IL-15m. This indicated that expression of ΔLNGFR could be specifically induced through tumor

cell engagement by the CAR.

As expected the endogenous CD25 gene was still expressed in activated treated T-cells while PD1 expression was strongly impaired.

To verify that expression of ΔLNGFR correlated with secretion of IL15 in the media, T-cells expressing the anti-CD22 CAR and ΔLNGFR were incubated in the presence of CD22 expressing Raji tumor cells (E:T ratio = 1:1) for a total of 10 days. Supernatant were recovered at day 2, 4, 7 and 10 and the presence of IL15 was quantified by ELISA assay. Our results showed that IL15 was secreted in the media only by T-cells that were co-treated by both CARm and IL15m matrices along with their corresponding TALEN. T-cell treated with either one of these matrices were unable to secrete any significant level of IL15 with respect to resting T-cells.

To assess whether the level of secreted IL-15 could impact CAR T-cell activity, CAR T-cell were cocultured in the presence of tumor cells at E:T ratio of 5:1 for 4 days. Their antitumor activity was challenged everyday by pelleting and resuspended them in a culture media lacking IL-2 and containing fresh tumor cells. Antitumor activity of CAR T-cell was monitored everyday by measuring the luminescence of the remaining Raji tumor cells expressing luciferase. Our results showed that CAR T-cells co-expressing IL-15 had a higher antitumor activity than those lacking IL15 at all time points considered.

Thus, together our results showed a simultaneous targeted insertions of CAR and IL15 cDNA at TRAC and CD25 or PD1 loci. This double targeted insertion led to robust expression of an antiCD22 CAR and to the secretion of IL15 in the media. Levels of secreted IL15 were sufficient to enhance the activity of CAR T-cells.

The present invention encompasses a TALEN-modified endogenous αβ-TCR negative human primary cell wherein the constant region of the genomic TCR gene (TRAC gene) comprises a genetic modification generated by a TALEN and affecting cell surface expression of the endogenous alpha

beta TCR, said genomic TRAC gene comprising from 5' to 3':

(a) a 5' region of said human genomic TRAC gene upstream,

(b) a recognition domain for a half TALEN,

(c) a gap or an insertion as compared to the wild type TRAC gene affecting the cell surface expression of the extracellular domain or transmembrane domain of the alpha beta TCR,

said insertion comprising an exogenous polynucleotide selected from a noncoding sequence such as, a stop codon, an IRES, a coding sequence such as a sequence coding for a self-cleaving peptide in frame with the TRAC open reading frame, a sequence coding a chimeric antigen receptor (CAR), a sequence coding a TCR, a sequence coding a protein conferring sensitivity to a drug, a sequence coding a protein conferring resistance to a drug, a cytokine, a termination sequence, a combination thereof,

(c') optionally a) a recognition domain for another half TALEN,

(d) a 3' region of the genomic TRAC gene

further comprising another inactivated genomic gene , such as any one of the following encoding genes: a gene encoding interleukin 3, interleukin 2, chemokine (C-C motif) ligand 4, interleukin 21, glycoprotein 49 A, nuclear receptor subfamily 4, group A, member 3, leukocyte immunoglobulin-like receptor, subfamily B, member 4, CD200 antigen, cyclin-dependent kinase inhibitor 1A (P21), granzyme C, nuclear receptor subfamily 4, group A, member 2, cytokine inducible SH2-containing protein, chemokine (C-C motif) receptor 8, ladinin, cellular retinoic acid binding protein II, granzyme B, T-box 21, programmed cell death 1, pleckstrin, checkpoint kinase 1, SLAM family member 7, zinc finger and BTB domain containing 32, T cell immunoreceptor with Ig and ITIM domains, lymphocyte-activation gene 3, granzyme A, WEE 1 homolog 1 (S. pombe), interleukin 12 receptor, beta 2, chemokine (C-C motif) receptor 5, early endosome antigen 1, and denticleless homolog (Drosophila).

The present invention encompasses a TALEN-modified endogenous αβ-TCR negative human primary cell wherein the constant region of the genomic TCR gene (TRAC gene) comprises a genetic modification generated by a TALEN and affecting cell surface expression of the endogenous alpha beta TCR, said genomic TRAC gene comprising from 5' to 3':

(a) a 5' region of said human genomic TRAC gene upstream,

(b) a recognition domain for a half TALEN,

(c) a gap or an insertion as compared to the wild type TRAC gene affecting the cell surface expression of the extracellular domain or transmembrane domain of the alpha beta TCR,

said insertion comprising an exogenous polynucleotide selected from a noncoding sequence such as, a stop codon, an IRES, a coding sequence such as a sequence coding for a self-cleaving peptide in frame with the TRAC open reading frame, a sequence coding a chimeric antigen receptor (CAR), a sequence coding a TCR, a sequence coding a protein conferring sensitivity to a drug, a sequence coding a protein conferring resistance to a drug, a cytokine, a termination sequence, a combination thereof,

(c') optionally a) a recognition domain for another half TALEN,

(d) a 3' region of the genomic TRAC gene

further comprising another inactivated genomic gene , such as any one of the following encoding genes: CXCL13, NFRSF1B, RGS2, TIGIT, CD27, NFRSF9, SLA, NF19A, NPP5F, XCL2, HLA-DMA, FAM3C, QCRC1, WARS, EIF3L, KCNK5, TMBIM6, CD200, C3H7A, SH2D1A, ATP1B3, YO7A, THADA, PARK7, EGR2, FDFT1, CRTAM and IFI16.

In one particular embodiment the second inactivated gene is comprising an insertion wherein said insertion comprises a cytokine, in particular IL-3, IL-7, IL-12 and IL-15.

CLAIMS

1. A method for producing an endonuclease-modified endogenous αβ-TCR negative human cell, said method comprising:

(a) introducing into a human cell:

(i) a first nucleic acid sequence encoding an engineered nuclease; or an engineered nuclease protein;

wherein said engineered nuclease produces a cleavage at a recognition sequence within said human TCR alpha constant region gene; said cleavage resulting in an inhibition of cell surface expression of the αβ-TCR to undetectable level;

(ii) a second nucleic acid sequence comprising an exogenous polynucleotide comprising a nucleic acid sequence encoding a self-cleaving peptide in frame with the genomic TRAC coding sequence and a nucleic acid sequence encoding a chimeric antigen receptor (CAR) or a recombinant TCR,

wherein the sequence of said exogenous polynucleotide is inserted into said genomic human TCR alpha constant region gene at said cleavage site; wherein said self-cleaving peptide is selected from a 2A peptide, a 2A like peptide, a P2A peptide, a E2A peptide and a F2A peptide, and further wherein said genetically-modified cell has reduced cell-surface expression of the endogenous TCR when compared to an unmodified control cell.

2. The method according to claim 1, wherein said self-cleaving peptide is a 2A peptide.

3. The method according to claim 2, wherein the 2A peptide is of sequence GSGEGRGSLLTCGDVEENPGP, GSGATNFSLLKQAGDVEENPGP or GSGQCTNYALLKLAGDVESNNPGP.

4. The method according to any one of claims 1 to 3, said method comprising:

detecting endonuclease(s)-induced on site and off site insertions, preferably by PCR and/or by guide sequence.

5. The method of any one of claims 1 to 4, wherein said engineered nuclease is a meganuclease, a zinc-finger nuclease (ZFN), a transcription activator-like effector nuclease (TALEN), a CRISPR/Cas nuclease, or a megaTAL nuclease.

6. The method of claims 1 to 5, wherein said engineered nuclease is a TALEN.

7. The method of claims 1 to 6, wherein said exogenous polynucleotide comprises a nucleic acid sequence encoding a chimeric antigen receptor.

8. The method of any one of claims 1 to 7 wherein said chimeric antigen receptor comprises an extracellular ligand-binding domain and one or more intracellular signaling domains.

9. The method of any one of claims 1 to 8, wherein at least said second nucleic acid sequence is introduced into said cell by contacting said cell with a recombinant adeno-associated virus (AAV6) vector comprising said second nucleic acid sequence.

10. The method of claim 9, wherein said recombinant AAV vector is a self-complementary AAV vector, preferably derived at least in part from an AAV6.

11. The method of claim 6, wherein said TALE-Nuclease recognizes a sequence ttgtcccacagATATC in the wild- type human TCR alpha constant region.

12. The method of claim 5, wherein said endonuclease is a Zing finger that recognizes from 5' to 3' TGCTGTGGCCTGGAGCAAC and GACTTTGCATGTGCA and cleaves within AAATCT, a Crispr/Cas9 that recognizes a complementary sequence to any one of the following sequences: AGAGTCTCTCAGCTGGTACA, GCACCAAAGCTGCCCTTACC, AAGTTCCTGTGATGTCAAGC, TTCGGAACCCAATCACTGAC, GATTAAACCCGGCCACTTTC, CGTCATGAGCAGATTAAACC, CTCAAGGTTCAGATCAGAAG, TAGGCAGACAGACTTGTCAC, AACAAATGTGTCACAAAGTA, CACCAAAGCTGCCCTTACCT, CTGACAGGTTTTGAAAGTTT, TTCAAAACCTGTCAGTGATT, CCGAATCCTCCTCCTGAAAG, CCACTTTCAGGAGGAGGATT, TAAACCCGGCCACTTTCAGG, TCTCAAACAAATGTGTCACAAAGTA, CTTACAATCTTGCAGATCTGGAATG, TTAATCTGCTCATGACGCTG, GGAGAAGAGGGGCAATGCAG, TCTTCTCCCTCTCCAAACAG, AGCAGCTTTCACCTCCTTGG,

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13. The method of claim 12, wherein said meganuclease is a single-chain meganuclease comprising a linker, wherein said linker covalently joins said first subunit and said second subunit.

14. A TALEN-modified endogenous αβ-TCR negative human primary cell wherein the constant region of the genomic TCR gene (TRAC gene) comprises a genetic modification generated by a TALEN and affecting cell surface expression of the endogenous αβ-TCR, said genomic TRAC gene comprising from 5' to 3':

(a) a 5' region of said human genomic TRAC gene,

(b) a first recognition domain for said TALEN,

(c) an insertion as compared to the wild-type TRAC gene affecting the cell surface expression of the extracellular domain or transmembrane domain of the αβ-TCR, said insertion comprising an exogenous polynucleotide comprising a sequence coding for a self-cleaving peptide in frame with the TRAC open reading frame, a sequence coding a chimeric antigen receptor or recombinant TCR, and a termination sequence,

(c') optionally a second TALEN recognition domain, and

(d) a 3' region of the genomic TRAC gene;

wherein said self-cleaving peptide is selected from a 2A peptide, a 2A like peptide, a P2A peptide, a E2A peptide and a F2A peptide.

15. The TALEN-modified endogenous αβ-TCR negative human cell according to claim 14, wherein said self-cleaving peptide is a 2A peptide.

16. The TALEN-modified endogenous αβ-TCR negative human cell according to claim 15, wherein the 2A peptide is of sequence GSGEGRGSLLTCGDVEENPGP, GSGATNFSLLKQAGDVEENPGP or GSGQCTNYALLKLAGDVESNPGP.

17. The TALEN-modified endogenous αβ-TCR negative human cell according to any one of claims 14 to 16, wherein said first recognition domain comprises the sequence ttgtcccacagATATC.

18. The TALEN-modified endogenous αβ-TCR negative human cell, according to any one of claims 14 to 17, wherein said CAR or recombinant TCR comprises an antigen binding domain, wherein said antigen binding domain binds to an antigen associated with a disease, and wherein said antigen is a tumor antigen selected from a group consisting of: CD19 molecule (CD19); membrane spanning 4-domains A1 (MS4A1 also known as CD20); CD22 molecule (CD22); CD24 molecule (CD24); CD248 molecule (CD248); CD276 molecule (CD276 or B7H3); CD33 molecule (CD33); CD38 molecule (CD38); CD44v6; CD70 molecule (CD70); CD72; CD79a; CD79b; interleukin 3 receptor subunit alpha (IL3RA also known as CD123); TNF receptor superfamily member 8 (TNFRSF8 also known as CD30); KIT proto-oncogene receptor tyrosine kinase (CD117); V-set pre-B cell surrogate light chain 1 (VPREB1 or CD179a); adhesion G protein-coupled receptor E5 (ADGRE5 or CD97); TNF receptor superfamily member 17 (TNFRSF17 also known as BCMA); SLAM family member 7 (SLAMF7 also known as CS1); L1 cell adhesion molecule (L1CAM); C-type lectin domain family 12 member A (CLEC12A also known as CLL-1); tumor-specific variant of the epidermal growth factor receptor (EGFRvIII); thyroid stimulating hormone receptor (TSHR); Fms related tyrosine kinase 3 (FLT3); ganglioside GD3 (GD3); Tn antigen (Tn Ag); lymphocyte antigen 6 family member G6D (LY6G6D); Delta like canonical Notch ligand 3 (DLL3); Interleukin- 13 receptor subunit alpha-2 (IL-13RA2); Interleukin 11 receptor subunit alpha (IL11RA); mesothelin (MSLN); Receptor tyrosine kinase like orphan receptor 1 (ROR1); Prostate stem cell antigen (PSCA); erb-b2 receptor tyrosine kinase 2 (ERBB2 or Her2/neu); Protease Serine 21 (PRSS21); Kinase insert domain receptor (KDR also known as VEGFR2); Lewis y antigen (LewisY); Solute carrier family 39 member 6 (SLC39A6); Fibroblast activation protein alpha (FAP); Hsp70 family chaperone (HSP70); Platelet-derived growth factor receptor beta (PDGFR-beta); Cholinergic receptor nicotinic alpha 2 subunit (CHRNA2); Stage-Specific Embryonic Antigen-4 (SSEA-4); Mucin 1, cell surface associated (MUC1); mucin 16, cell surface associated (MUC16); claudin 18 (CLDN18); claudin 6 (CLDN6); Epidermal Growth Factor Receptor (EGFR); Preferentially expressed antigen in melanoma (PRAME); Neural Cell Adhesion Molecule (NCAM); ADAM metallopeptidase domain 10 (ADAM10); Folate receptor 1 (FOLR1); Folate receptor beta (FOLR2); Carbonic Anhydrase IX (CA9); Proteasome subunit beta 9 (PSMB9 or

LMP2); Ephrin receptor A2 (EphA2); Tetraspanin 10 (TSPAN10); Fucosyl GM1 (Fuc-GM1); sialyl Lewis adhesion molecule (sLe); TGS5; high molecular weight- melanoma-associated antigen (HMWMAA); o-acetyl- GD2 ganglioside (OAcGD2); tumor endothelial marker 7-related (TEM7R); G protein-coupled receptor class C group 5, member D (GPRC5D); chromosome X open reading frame 61 (CXORF61); ALK receptor tyrosine kinase (ALK); Polysialic acid; Placenta-specific 1 (PLAC1); hexasaccharide portion of globoH glycoceramide (GloboH); NY-BR-1 antigen; uroplakin 2 (UPK2); Hepatitis A virus cellular receptor 1 (HAVCR1); adrenoceptor beta 3 (ADRB3); pannexin 3 (PANX3); G protein-coupled receptor 20 (GPR20); lymphocyte antigen 6 family member K (LY6K); olfactory receptor family 51 subfamily E member 2 (OR51E2); TCR Gamma Alternate Reading Frame Protein (TARP); Wilms tumor protein (WT1); ETV6-AML1 fusion protein due to 12; 21 chromosomal translocation (ETV6-AML1); sperm autoantigenic protein 17 (SPA17); X Antigen Family, Member 1E (XAGE1E); TEK receptor tyrosine kinase (Tie2); melanoma cancer testis antigen- 1 (MAD-CT-1); melanoma cancer testis antigen-2 (MAD-CT-2); Fos-related antigen 1; p53 mutant; human Telomerase reverse transcriptase (hTERT); sarcoma translocation breakpoints; melanoma inhibitor of apoptosis (ML-IAP); ERG (transmembrane protease, serine 2 (TMPRSS2) ETS fusion gene); N- Acetyl glucosaminyl-transferase V (NA17); paired box protein Pax-3 (PAX3); Androgen receptor; Cyclin B 1; v-myc avian myelocytomatosis viral oncogene neuroblastoma derived homolog (MYCN); Ras Homolog Family Member C (RhoC); Cytochrome P450 1B 1 (CYP1B 1); CCCTC-Binding Factor (Zinc Finger Protein)-Like (BORIS); Squamous Cell Carcinoma Antigen Recognized By T Cells 3 (SART3); Paired box protein Pax-5 (PAX5); proacrosin binding protein sp32 (OY-TES 1); lymphocyte-specific protein tyrosine kinase (LCK); A kinase anchor protein 4 (AKAP-4); synovial sarcoma, X breakpoint 2 (SSX2); Leukocyte- associated immunoglobulin-like receptor 1 (LAIR1); Fc fragment of IgA receptor (FCAR); Leukocyte immunoglobulin-like receptor subfamily A member 2 (LILRA2); CD300 molecule-like family member f (CD300LF); bone marrow stromal cell antigen 2 (BST2); EGF-like module-containing mucin-like hormone receptor- like 2 (EMR2); lymphocyte antigen 75 (LY75); Glypican-3 (GPC3); Fc receptor-like 5 (FCRL5); immunoglobulin lambda-like polypeptide 1 (IGLL1), and Heat shock protein 70 (HSP70).

19. The TALEN-modified endogenous αβ-TCR negative human cell according to any one of claims 14 to 17, wherein said recombinant TCR comprises an antigen binding domain, wherein said antigen binding domain binds to a tumor antigen associated with a disease, and said tumor antigen is selected from a group consisting of PCTA-l/Galectin 8, CD171, TAG72, CEA, EPCAM, PSCA, PRSS21, PDGFR-beta, Prostase, PAP, ELF2M, Ephrin B2, IGF-I receptor, CAIX, ,gp100, bcr-abl, tyrosinase, GM3, NY-ESO-1, LAGE-la, MAGE-A1, legumain, HPV E6,E7, MAGE Al, prostein, survivin and telomerase, PCTA-l/Galectin 8, MelanA/MARTl, Ras mutant, TRP-2, RAGE-1, RU1, RU2, and intestinal carboxyl esterase.

20. The TALEN-modified endogenous αβ-TCR negative human cell according to any one of claims 14 to 17, wherein the CAR comprises an antigen binding domain that binds to a tumor antigen associated with a disease, and said tumor antigen is selected from a group consisting of: CD22, CD123, CS-1, CLL-1, CD38, HSP70, MUC-1, CD30, FAP, HER2, CD79a and CD79b.

21. The TALEN-modified endogenous αβ-TCR negative human cell according to any one of claims 14 to 17, wherein said exogenous polynucleotide comprises a sequence encoding anti-CD22 CAR of SEQ ID NO:9, SEQ ID NO:10 or SEQ ID NO:11.

22. The TALEN-modified endogenous αβ-TCR negative human cell, according to any one of claims 14 to 17, wherein said exogenous polynucleotide comprises a sequence encoding anti-CD123 CAR of SEQ ID NO:12.

CELLECTIS

Patent Attorneys for the Applicant/Nominated Person

SPRUSON & FERGUSON

TCR KO, Exogenous gene inserted

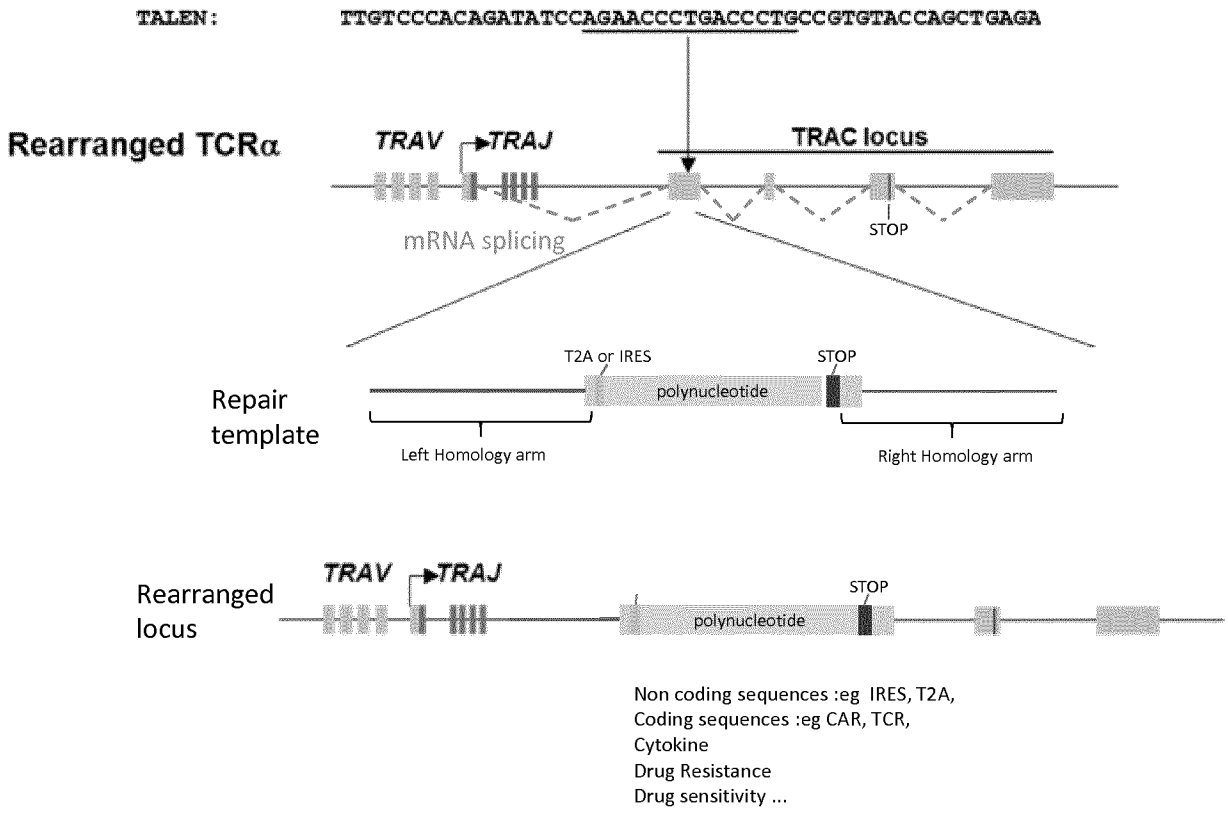

Figure 1

Endogenous TCR maintained, Exogenous gene co-expressed

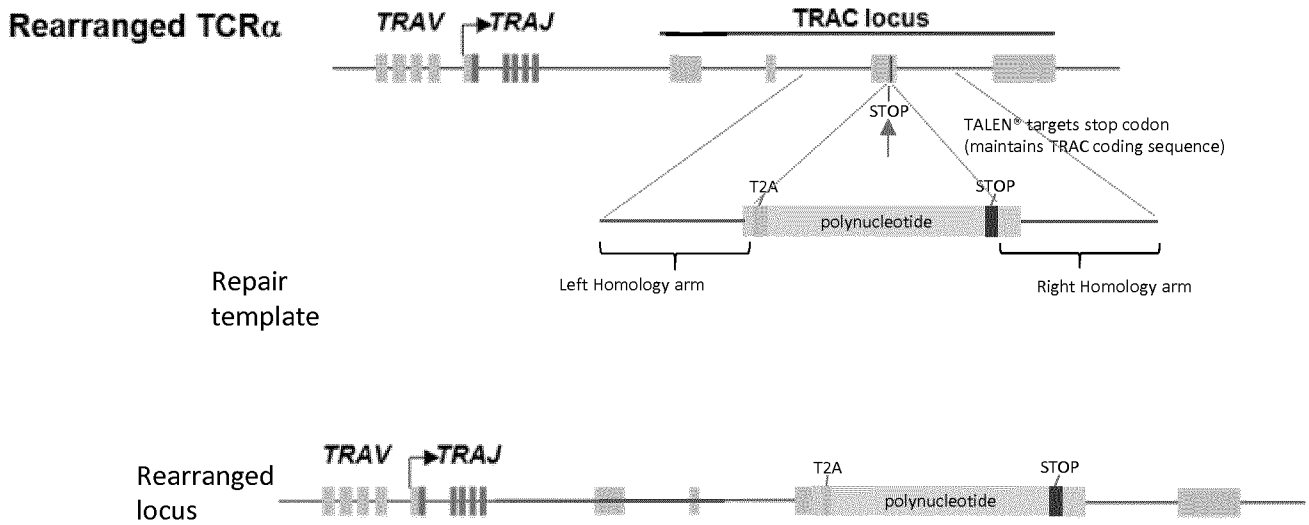

Figure 2

Endogenous TCR inactivated,
Recombinant TCR expressed

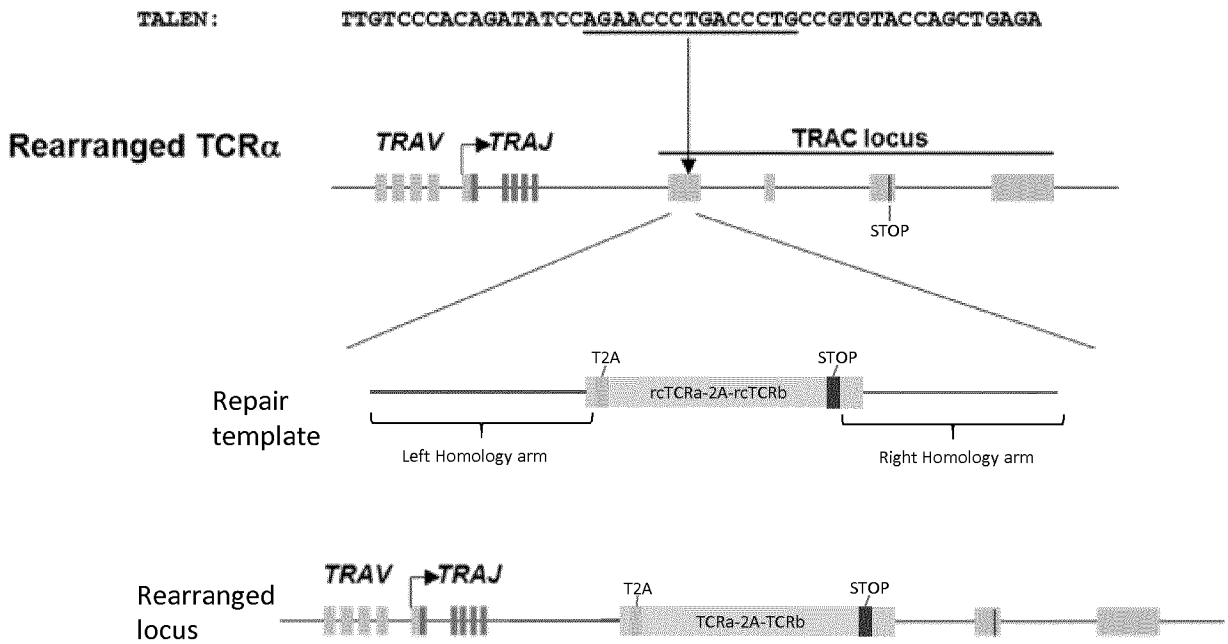

Figure 3

TCR KO, Exogenous gene expressed (IRES)

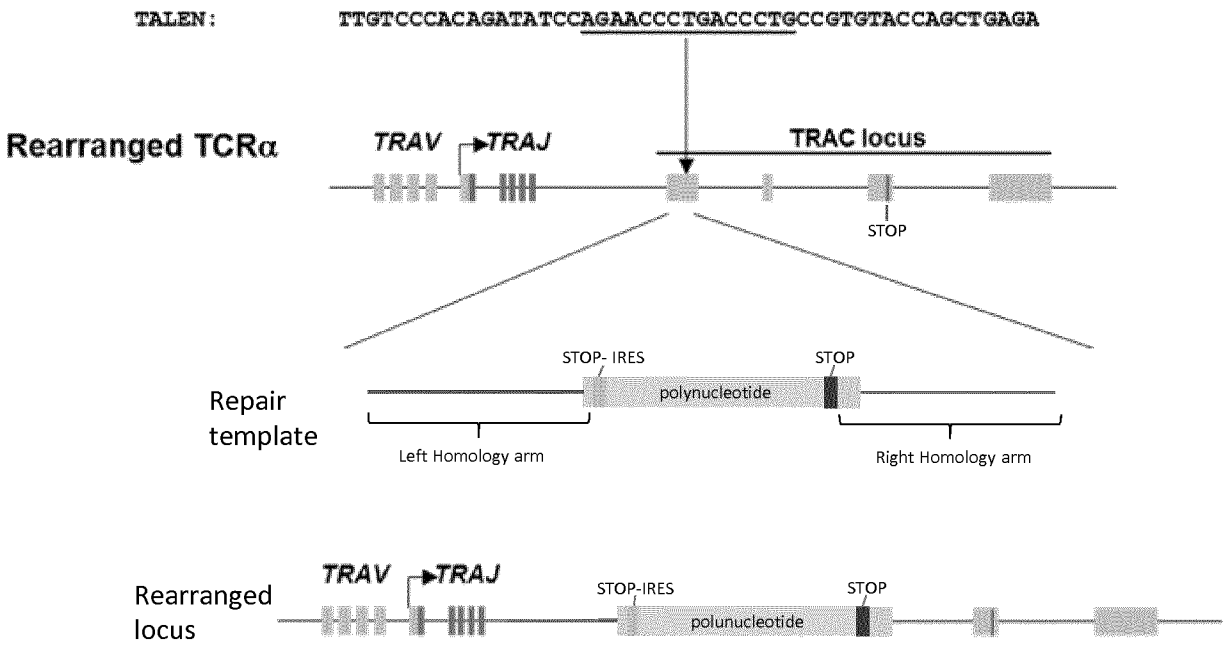

Figure 4

Endogenous TCR maintained, exogenous gene co-expressed (IRES)

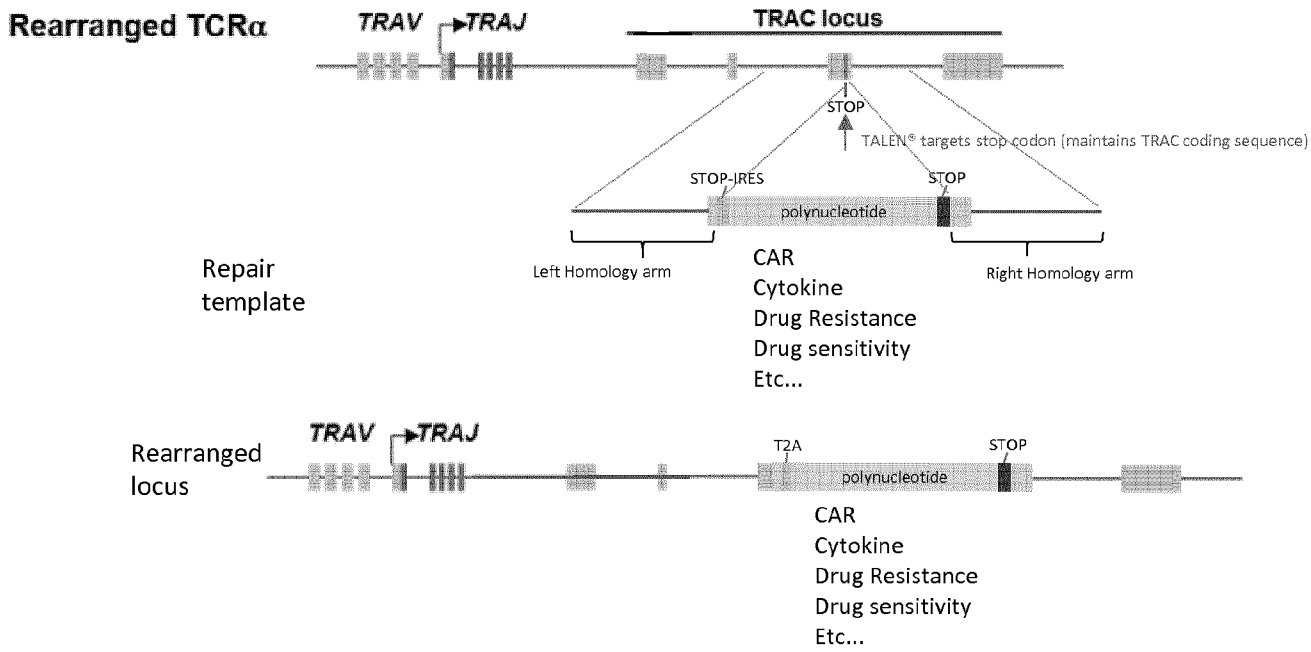

Figure 5

Endogenous TCR inactivated,
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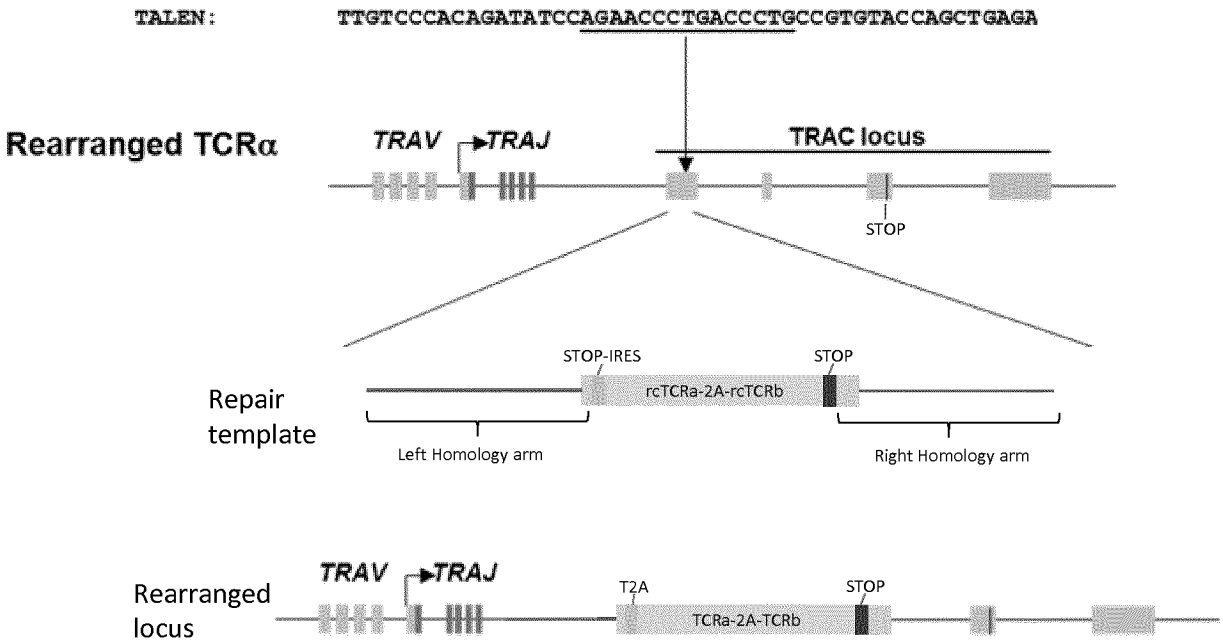

Figure 6

TCR KO, Exogenous gene expressed,
TALEN target site edited

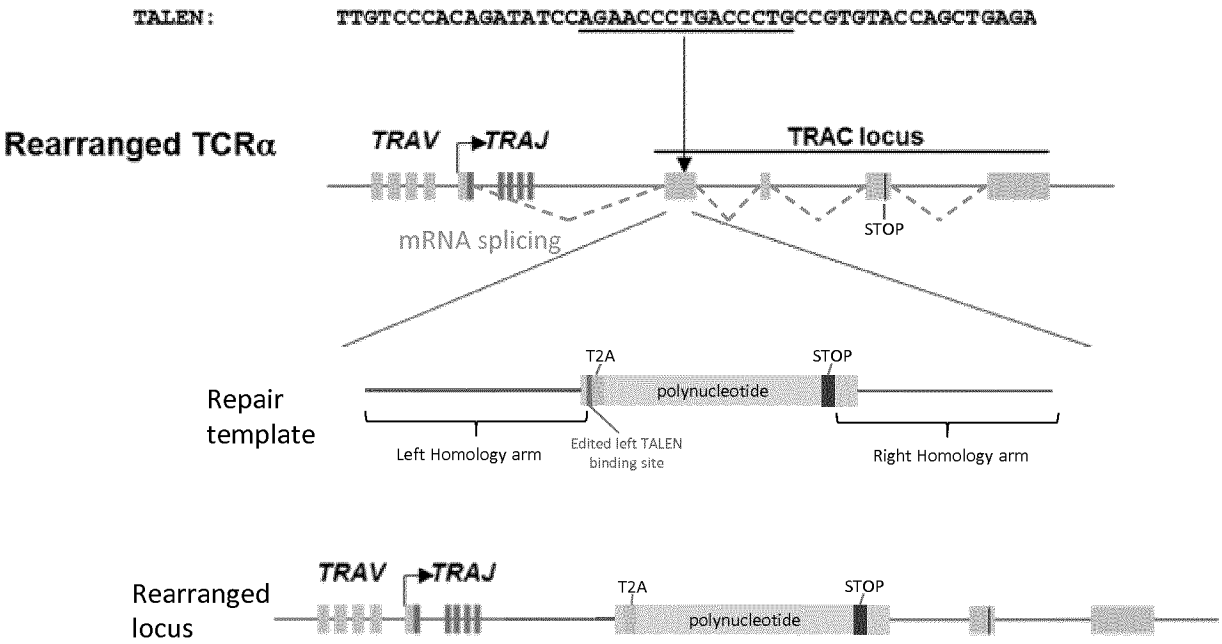

Figure 7

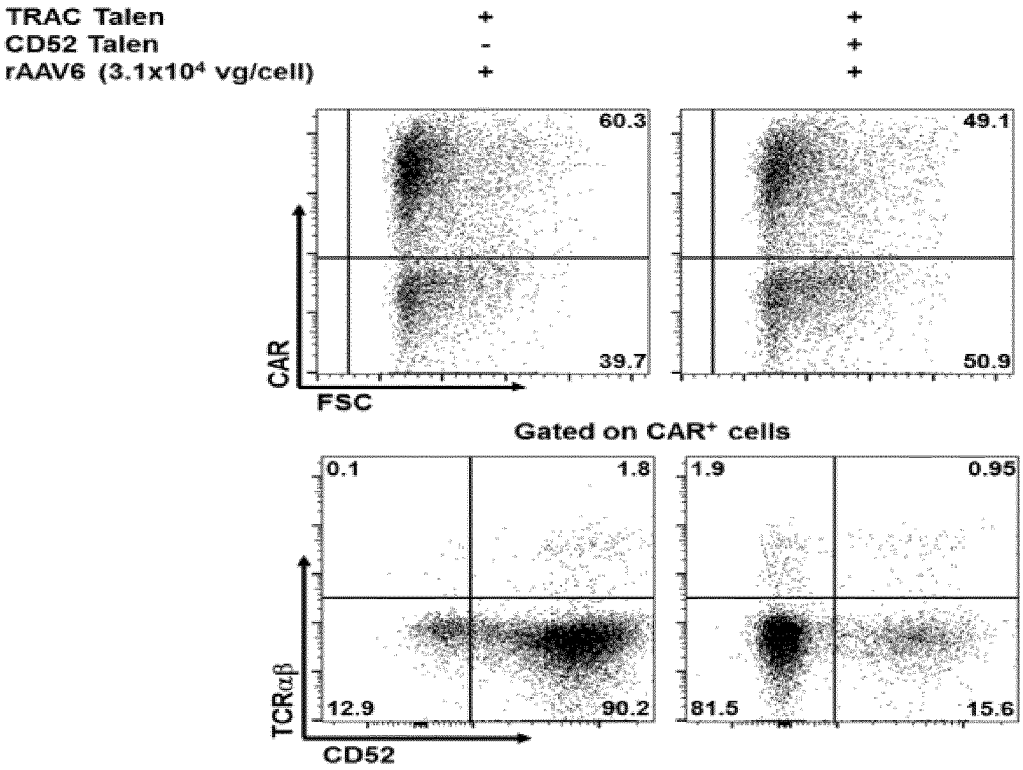
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Figure 8

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atcaccaact gcaacggcgc cgtgctgtcc gtggaggagc tcctgatcgg cggcgagatg   2700
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<210> 3

<211> 925
<212> PRT
<213> artificial sequence

<220>
<223> TALEN TRAC_T01-L1

<400> 3

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1               5                   10                  15


Arg Thr Leu Gly Tyr Ser Gln Gln Gln Gln Glu Lys Ile Lys Pro Lys
            20                  25                  30


Val Arg Ser Thr Val Ala Gln His His Glu Ala Leu Val Gly His Gly
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        35                  40                  45

Phe Thr His Ala His Ile Val Ala Leu Ser Gln His Pro Ala Ala Leu
    50                  55                  60

Gly Thr Val Ala Val Lys Tyr Gln Asp Met Ile Ala Ala Leu Pro Glu
65                  70                  75                  80

Ala Thr His Glu Ala Ile Val Gly Val Gly Lys Gln Trp Ser Gly Ala
                85                  90                  95

Arg Ala Leu Glu Ala Leu Leu Thr Val Ala Gly Glu Leu Arg Gly Pro
            100                 105                 110

Pro Leu Gln Leu Asp Thr Gly Gln Leu Leu Lys Ile Ala Lys Arg Gly
        115                 120                 125

Gly Val Thr Ala Val Glu Ala Val His Ala Trp Arg Asn Ala Leu Thr
    130                 135                 140

Gly Ala Pro Leu Asn Leu Thr Pro Gln Gln Val Val Ala Ile Ala Ser
145                 150                 155                 160

Asn Gly Gly Gly Lys Gln Ala Leu Glu Thr Val Gln Arg Leu Leu Pro
                165                 170                 175

Val Leu Cys Gln Ala His Gly Leu Thr Pro Gln Gln Val Val Ala Ile
            180                 185                 190

Ala Ser Asn Asn Gly Gly Lys Gln Ala Leu Glu Thr Val Gln Arg Leu
        195                 200                 205

Leu Pro Val Leu Cys Gln Ala His Gly Leu Thr Pro Gln Gln Val Val
    210                 215                 220

Ala Ile Ala Ser Asn Gly Gly Gly Lys Gln Ala Leu Glu Thr Val Gln
225                 230                 235                 240

Arg Leu Leu Pro Val Leu Cys Gln Ala His Gly Leu Thr Pro Glu Gln
```

```
                245                 250                 255

Val Val Ala Ile Ala Ser His Asp Gly Gly Lys Gln Ala Leu Glu Thr
            260                 265                 270

Val Gln Arg Leu Leu Pro Val Leu Cys Gln Ala His Gly Leu Thr Pro
        275                 280                 285

Glu Gln Val Val Ala Ile Ala Ser His Asp Gly Gly Lys Gln Ala Leu
    290                 295                 300

Glu Thr Val Gln Arg Leu Leu Pro Val Leu Cys Gln Ala His Gly Leu
305                 310                 315                 320

Thr Pro Glu Gln Val Val Ala Ile Ala Ser His Asp Gly Gly Lys Gln
                325                 330                 335

Ala Leu Glu Thr Val Gln Arg Leu Leu Pro Val Leu Cys Gln Ala His
            340                 345                 350

Gly Leu Thr Pro Glu Gln Val Val Ala Ile Ala Ser Asn Ile Gly Gly
        355                 360                 365

Lys Gln Ala Leu Glu Thr Val Gln Ala Leu Leu Pro Val Leu Cys Gln
    370                 375                 380

Ala His Gly Leu Thr Pro Glu Gln Val Val Ala Ile Ala Ser His Asp
385                 390                 395                 400

Gly Gly Lys Gln Ala Leu Glu Thr Val Gln Arg Leu Leu Pro Val Leu
                405                 410                 415

Cys Gln Ala His Gly Leu Thr Pro Glu Gln Val Val Ala Ile Ala Ser
            420                 425                 430

Asn Ile Gly Gly Lys Gln Ala Leu Glu Thr Val Gln Ala Leu Leu Pro
        435                 440                 445

Val Leu Cys Gln Ala His Gly Leu Thr Pro Gln Gln Val Val Ala Ile
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    450                 455                 460

Ala Ser Asn Asn Gly Gly Lys Gln Ala Leu Glu Thr Val Gln Arg Leu
465                 470                 475                 480

Leu Pro Val Leu Cys Gln Ala His Gly Leu Thr Pro Glu Gln Val Val
                485                 490                 495

Ala Ile Ala Ser Asn Ile Gly Gly Lys Gln Ala Leu Glu Thr Val Gln
            500                 505                 510

Ala Leu Leu Pro Val Leu Cys Gln Ala His Gly Leu Thr Pro Gln Gln
        515                 520                 525

Val Val Ala Ile Ala Ser Asn Gly Gly Gly Lys Gln Ala Leu Glu Thr
    530                 535                 540

Val Gln Arg Leu Leu Pro Val Leu Cys Gln Ala His Gly Leu Thr Pro
545                 550                 555                 560

Glu Gln Val Val Ala Ile Ala Ser Asn Ile Gly Gly Lys Gln Ala Leu
                565                 570                 575

Glu Thr Val Gln Ala Leu Leu Pro Val Leu Cys Gln Ala His Gly Leu
            580                 585                 590

Thr Pro Gln Gln Val Val Ala Ile Ala Ser Asn Gly Gly Gly Lys Gln
        595                 600                 605

Ala Leu Glu Thr Val Gln Arg Leu Leu Pro Val Leu Cys Gln Ala His
    610                 615                 620

Gly Leu Thr Pro Glu Gln Val Val Ala Ile Ala Ser His Asp Gly Gly
625                 630                 635                 640

Lys Gln Ala Leu Glu Thr Val Gln Arg Leu Leu Pro Val Leu Cys Gln
                645                 650                 655

Ala His Gly Leu Thr Pro Gln Gln Val Val Ala Ile Ala Ser Asn Gly
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            660                 665                 670

Gly Gly Arg Pro Ala Leu Glu Ser Ile Val Ala Gln Leu Ser Arg Pro
        675                 680                 685

Asp Pro Ala Leu Ala Ala Leu Thr Asn Asp His Leu Val Ala Leu Ala
    690                 695                 700

Cys Leu Gly Gly Arg Pro Ala Leu Asp Ala Val Lys Lys Gly Leu Gly
705                 710                 715                 720

Asp Pro Ile Ser Arg Ser Gln Leu Val Lys Ser Glu Leu Glu Glu Lys
                725                 730                 735

Lys Ser Glu Leu Arg His Lys Leu Lys Tyr Val Pro His Glu Tyr Ile
            740                 745                 750

Glu Leu Ile Glu Ile Ala Arg Asn Ser Thr Gln Asp Arg Ile Leu Glu
        755                 760                 765

Met Lys Val Met Glu Phe Phe Met Lys Val Tyr Gly Tyr Arg Gly Lys
    770                 775                 780

His Leu Gly Gly Ser Arg Lys Pro Asp Gly Ala Ile Tyr Thr Val Gly
785                 790                 795                 800

Ser Pro Ile Asp Tyr Gly Val Ile Val Asp Thr Lys Ala Tyr Ser Gly
                805                 810                 815

Gly Tyr Asn Leu Pro Ile Gly Gln Ala Asp Glu Met Gln Arg Tyr Val
            820                 825                 830

Glu Glu Asn Gln Thr Arg Asn Lys His Ile Asn Pro Asn Glu Trp Trp
        835                 840                 845

Lys Val Tyr Pro Ser Ser Val Thr Glu Phe Lys Phe Leu Phe Val Ser
    850                 855                 860

Gly His Phe Lys Gly Asn Tyr Lys Ala Gln Leu Thr Arg Leu Asn His
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865 870 875 880

Ile Thr Asn Cys Asn Gly Ala Val Leu Ser Val Glu Glu Leu Leu Ile
885 890 895

Gly Gly Glu Met Ile Lys Ala Gly Thr Leu Thr Leu Glu Glu Val Arg
900 905 910

Arg Lys Phe Asn Asn Gly Glu Ile Asn Phe Ala Ala Asp
915 920 925

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<211> 925
<212> PRT
<213> artificial sequence

<220>
<223> TALEN TRAC_T01-R1

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Arg Thr Leu Gly Tyr Ser Gln Gln Gln Gln Glu Lys Ile Lys Pro Lys
20 25 30

Val Arg Ser Thr Val Ala Gln His His Glu Ala Leu Val Gly His Gly
35 40 45

Phe Thr His Ala His Ile Val Ala Leu Ser Gln His Pro Ala Ala Leu
50 55 60

Gly Thr Val Ala Val Lys Tyr Gln Asp Met Ile Ala Ala Leu Pro Glu
65 70 75 80

Ala Thr His Glu Ala Ile Val Gly Val Gly Lys Gln Trp Ser Gly Ala
85 90 95

Arg Ala Leu Glu Ala Leu Leu Thr Val Ala Gly Glu Leu Arg Gly Pro
100 105 110

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Pro Leu Gln Leu Asp Thr Gly Gln Leu Leu Lys Ile Ala Lys Arg Gly
        115                 120                 125

Gly Val Thr Ala Val Glu Ala Val His Ala Trp Arg Asn Ala Leu Thr
    130                 135                 140

Gly Ala Pro Leu Asn Leu Thr Pro Glu Gln Val Val Ala Ile Ala Ser
145                 150                 155                 160

His Asp Gly Gly Lys Gln Ala Leu Glu Thr Val Gln Arg Leu Leu Pro
                165                 170                 175

Val Leu Cys Gln Ala His Gly Leu Thr Pro Gln Gln Val Val Ala Ile
            180                 185                 190

Ala Ser Asn Gly Gly Gly Lys Gln Ala Leu Glu Thr Val Gln Arg Leu
        195                 200                 205

Leu Pro Val Leu Cys Gln Ala His Gly Leu Thr Pro Glu Gln Val Val
    210                 215                 220

Ala Ile Ala Ser His Asp Gly Gly Lys Gln Ala Leu Glu Thr Val Gln
225                 230                 235                 240

Arg Leu Leu Pro Val Leu Cys Gln Ala His Gly Leu Thr Pro Glu Gln
                245                 250                 255

Val Val Ala Ile Ala Ser Asn Ile Gly Gly Lys Gln Ala Leu Glu Thr
            260                 265                 270

Val Gln Ala Leu Leu Pro Val Leu Cys Gln Ala His Gly Leu Thr Pro
        275                 280                 285

Gln Gln Val Val Ala Ile Ala Ser Asn Asn Gly Gly Lys Gln Ala Leu
    290                 295                 300

Glu Thr Val Gln Arg Leu Leu Pro Val Leu Cys Gln Ala His Gly Leu
305                 310                 315                 320
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Thr Pro Glu Gln Val Val Ala Ile Ala Ser His Asp Gly Gly Lys Gln
                325                 330                 335

Ala Leu Glu Thr Val Gln Arg Leu Leu Pro Val Leu Cys Gln Ala His
            340                 345                 350

Gly Leu Thr Pro Gln Gln Val Val Ala Ile Ala Ser Asn Gly Gly Gly
        355                 360                 365

Lys Gln Ala Leu Glu Thr Val Gln Arg Leu Leu Pro Val Leu Cys Gln
    370                 375                 380

Ala His Gly Leu Thr Pro Gln Gln Val Val Ala Ile Ala Ser Asn Asn
385                 390                 395                 400

Gly Gly Lys Gln Ala Leu Glu Thr Val Gln Arg Leu Leu Pro Val Leu
                405                 410                 415

Cys Gln Ala His Gly Leu Thr Pro Gln Gln Val Val Ala Ile Ala Ser
            420                 425                 430

Asn Asn Gly Gly Lys Gln Ala Leu Glu Thr Val Gln Arg Leu Leu Pro
        435                 440                 445

Val Leu Cys Gln Ala His Gly Leu Thr Pro Gln Gln Val Val Ala Ile
    450                 455                 460

Ala Ser Asn Gly Gly Gly Lys Gln Ala Leu Glu Thr Val Gln Arg Leu
465                 470                 475                 480

Leu Pro Val Leu Cys Gln Ala His Gly Leu Thr Pro Glu Gln Val Val
                485                 490                 495

Ala Ile Ala Ser Asn Ile Gly Gly Lys Gln Ala Leu Glu Thr Val Gln
            500                 505                 510

Ala Leu Leu Pro Val Leu Cys Gln Ala His Gly Leu Thr Pro Glu Gln
        515                 520                 525
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Val Val Ala Ile Ala Ser His Asp Gly Gly Lys Gln Ala Leu Glu Thr
    530                 535                 540

Val Gln Arg Leu Leu Pro Val Leu Cys Gln Ala His Gly Leu Thr Pro
545                 550                 555                 560

Glu Gln Val Val Ala Ile Ala Ser Asn Ile Gly Gly Lys Gln Ala Leu
                565                 570                 575

Glu Thr Val Gln Ala Leu Leu Pro Val Leu Cys Gln Ala His Gly Leu
            580                 585                 590

Thr Pro Glu Gln Val Val Ala Ile Ala Ser His Asp Gly Gly Lys Gln
        595                 600                 605

Ala Leu Glu Thr Val Gln Arg Leu Leu Pro Val Leu Cys Gln Ala His
    610                 615                 620

Gly Leu Thr Pro Gln Gln Val Val Ala Ile Ala Ser Asn Asn Gly Gly
625                 630                 635                 640

Lys Gln Ala Leu Glu Thr Val Gln Arg Leu Leu Pro Val Leu Cys Gln
                645                 650                 655

Ala His Gly Leu Thr Pro Gln Gln Val Val Ala Ile Ala Ser Asn Gly
            660                 665                 670

Gly Gly Arg Pro Ala Leu Glu Ser Ile Val Ala Gln Leu Ser Arg Pro
        675                 680                 685

Asp Pro Ala Leu Ala Ala Leu Thr Asn Asp His Leu Val Ala Leu Ala
    690                 695                 700

Cys Leu Gly Gly Arg Pro Ala Leu Asp Ala Val Lys Lys Gly Leu Gly
705                 710                 715                 720

Asp Pro Ile Ser Arg Ser Gln Leu Val Lys Ser Glu Leu Glu Glu Lys
                725                 730                 735
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Lys Ser Glu Leu Arg His Lys Leu Lys Tyr Val Pro His Glu Tyr Ile
            740                 745                 750

Glu Leu Ile Glu Ile Ala Arg Asn Ser Thr Gln Asp Arg Ile Leu Glu
        755                 760                 765

Met Lys Val Met Glu Phe Phe Met Lys Val Tyr Gly Tyr Arg Gly Lys
    770                 775                 780

His Leu Gly Gly Ser Arg Lys Pro Asp Gly Ala Ile Tyr Thr Val Gly
785                 790                 795                 800

Ser Pro Ile Asp Tyr Gly Val Ile Val Asp Thr Lys Ala Tyr Ser Gly
                805                 810                 815

Gly Tyr Asn Leu Pro Ile Gly Gln Ala Asp Glu Met Gln Arg Tyr Val
            820                 825                 830

Glu Glu Asn Gln Thr Arg Asn Lys His Ile Asn Pro Asn Glu Trp Trp
        835                 840                 845

Lys Val Tyr Pro Ser Ser Val Thr Glu Phe Lys Phe Leu Phe Val Ser
    850                 855                 860

Gly His Phe Lys Gly Asn Tyr Lys Ala Gln Leu Thr Arg Leu Asn His
865                 870                 875                 880

Ile Thr Asn Cys Asn Gly Ala Val Leu Ser Val Glu Glu Leu Leu Ile
                885                 890                 895

Gly Gly Glu Met Ile Lys Ala Gly Thr Leu Thr Leu Glu Glu Val Arg
            900                 905                 910

Arg Lys Phe Asn Asn Gly Glu Ile Asn Phe Ala Ala Asp
        915                 920                 925
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<211> 22

<212> DNA
<213> artificial sequence

<220>
<223> FORWARD

<400> 5
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<210> 6

<211> 19
<212> DNA
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<220>
<223> REVERSE

<400> 6
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<210> 7

<211> 20
<212> DNA
<213> artificial sequence

<220>
<223> PROBE

<400> 7
cccttgtcca tcactggcat 20

<210> 8

<211> 411
<212> DNA
<213> artificial sequence

<220>
<223> SEQUENCE IN TRAC

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tggcctcttg gccaagattg atagcttgtg cctgtccctg agtcccagtc catcacgagc 180
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cagagccccg cccttgtcca tcactggcat ctggactcca gcctgggttg gggcaaagag 300
ggaaatgaga tcatgtccta accctgatcc tcttgtccca cagatatcca gtccggtgag 360

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<211> 2658
<212> DNA
<213> artificial sequence

<220>
<223> SEQUENCE IN TRAC ANTI-CD22 CAR

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aaccctgaca cctccaagaa tcagttctct ctgcagctga atagcgtgac accagaggac 780
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<211> 2646
<212> DNA
<213> artificial sequence

<220>
<223> SEQUENCE IN TRAC ANTI-CD22 B-B7 QR3

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<211> 2649
<212> DNA
<213> artificial sequence

<220>
<223> SEQUENCE IN TRAC ANTI-CD22 A-D4 QR3

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ccagccccac agagccccgc ccttgtccat cactggcatc tggactccag cctgggttgg 240
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tccggtgagg gcagaggaag tcttctaaca tgcggtgacg tggaggagaa tccgggcccc 360
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