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(54) Title: SEQUENTIAL GENE EDITING IN PRIMARY IMMUNE CELLS

(57) Abstract: The invention pertains to the field of adaptive cell immunotherapy. It aims at reducing the occurrence of translocations and cell deaths when several specific endonuclease reagents are used altogether to genetically modify primary immune cells at different genetic loci. The method of the invention allows to yield safer immune primary cells harboring several genetic modifications, such as triple or quadruple gene inactivated cells, from populations or sub-populations of cells originating from a single donor or patient, for their subsequent use in therapeutic treatments.

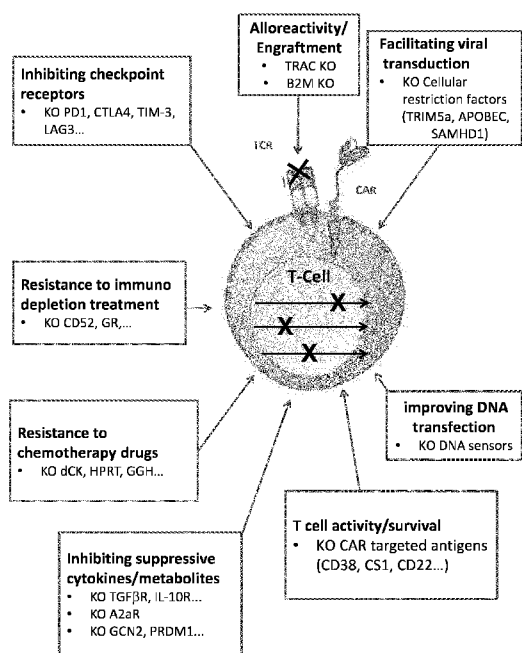


Figure 1

WO 2018/007263 A1

SEQUENTIAL GENE EDITING IN PRIMARY IMMUNE CELLS**Field of the invention**

The invention pertains to the field of adaptive cell immunotherapy. It aims at reducing
5 the occurrence of translocations and cell deaths when several specific endonuclease
reagents are used altogether to genetically modify primary immune cells at different genetic
loci. The method of the invention allows to yield safer immune primary cells harboring
several genetic modifications, such as triple or quadruple gene inactivated cells, from
populations or sub-populations of cells originating from a single donor or patient, for their
10 subsequent use in therapeutic treatments.

Background of the invention

The potential of gene editing in various therapies has long been envisioned by the
applicant (WO2004067753), especially in the field of cell therapy, where immune cells can be
15 genetically modified *ex-vivo* and then reintroduced into patients, as already described, for
instance, in US 8921332.

Since the emergence of the first programmable sequence-specific reagents by the
turn of the century, initially referred to as Meganucleases [Smith et al. (2006) A combinatorial
approach to create artificial homing endonucleases cleaving chosen sequences. *Nucl. Acids*
20 *Res.* 34 (22):e149.], endonucleases reagents have rapidly evolved, offering improved
specificity, safety and reliability. In particular, TALE-nucleases (WO2011072246), which are
fusions of a TALE binding domain with a cleavage catalytic domain have been successfully
applied to primary immune cells, in particular T-cells from peripheral blood mononuclear cells
(PBMC). Such TALE-nucleases, marketed under the name TALEN[®], are currently used to
25 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)
or recombinant TCR for treating cancer patients (US2013/0315884). TALE-nucleases are
very specific reagents because they need to bind DNA by pairs under obligatory
30 heterodimeric form to obtain dimerization of the cleavage domain Fok-1. Left and right
heterodimer members each recognizes a different nucleic sequences of about 14 to 20 bp,
together spanning target sequences of 30 to 50 bp overall specificity.

More recently, further 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) Cas9–crRNA ribonucleoprotein complex mediates specific DNA cleavage for adaptive immunity in bacteria; PNAS 109(39):E2579–E2586]; Doudna, J. Charpentier E. (2014) The new frontier of genome engineering with CRISPR-Cas9 *Science* 346 (6213):1258096], involves members of Cas9 or Cpf1 [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] endonuclease families coupled with a guide RNA molecules that have the ability to drive said nuclease to some specific genome sequences. 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.

Other endonuclease systems derived from homing endonucleases (ex: I-OnuI, or I-CreI), combined or not with TAL-nuclease (ex: MegaTAL) or zinc-finger nucleases have also proven specificity, but with less efficiency so far.

Various proofs of concept of the efficiency and safety of the above specific endonuclease reagents have been reported in human cells *in-vitro* or *ex-vivo*, but the co-delivery into the same cells of sequence specific reagents acting on different loci has still to be carefully considered as a potential factor of off-site mutations, large genomic deletions and translocations inherent to the DNA repair mechanisms (Poirot et al. (2015) Multiplex Genome-Edited T-cell Manufacturing Platform for “Off-the-Shelf” Adoptive T-cell Immunotherapies *Cancer Res.* 75: 3853-64).

In parallel, novel specificities have been conferred to immune cells through the genetic transfer of transgenic T-cell receptors or so-called chimeric antigen receptors (CARs) (Jena et al. (2010) Redirecting T-cell specificity by introducing a tumor-specific chimeric antigen receptor. *Blood.* 116:1035-1044). CARs are recombinant receptors comprising a targeting moiety that is associated with one or more signaling domains in a single fusion molecule. In general, the binding moiety of a CAR consists of an antigen-binding domain of a single-chain antibody (scFv), comprising the light and heavy variable fragments of a monoclonal antibody joined by a flexible linker. Binding moieties based on receptor or ligand domains have also been used successfully. The signaling domains for first generation CARs are derived from the cytoplasmic region of the CD3zeta or the Fc receptor gamma chains. First generation CARs have been shown to successfully redirect T cell cytotoxicity, however, they failed to provide prolonged expansion and anti-tumor activity *in vivo*. Signaling domains

from co-stimulatory molecules including CD28, OX-40 (CD134), ICOS and 4-1BB (CD137) have been added alone (second generation) or in combination (third generation) to enhance survival and increase proliferation of CAR modified T cells. CARs, as well as the expression of recombinant TCRs, have successfully allowed T cells to be redirected against antigens expressed by tumor cells from various malignancies including lymphomas and solid tumors.

Recently engineered T-cells disrupted in their T-cell receptor (TCR) using TALE-nucleases, endowed with chimeric antigen receptor (CAR) targeting CD19 malignant antigen, referred to as “UCART19” product, have shown therapeutic potential in at least two infants who had refractory leukemia (Leukaemia success heralds wave of gene-editing therapies (2015) *Nature* 527:146–147). To obtain such UCART19 cells, the TALE-nuclease was transiently expressed into the cells upon electroporation of capped mRNA to operate TCR gene disruption, whereas a cassette encoding the chimeric antigen receptor (CAR CD19) was introduced randomly into the genome using a retroviral vector.

In this later approach, the steps of gene inactivation and of expressing the chimeric antigen receptor are independently performed after inducing activation of the T-Cell “*ex-vivo*”.

However, engineering primary immune cells is not without any consequences on the growth/physiology of such cells. In particular one major challenge is to avoid cells exhaustion/anergy that significantly reduces their immune reaction and life span. This is more likely to happen when the cells are artificially activated ahead of their infusion into the patient. It is also the case when a cell is endowed with a CAR that is too reactive.

The introduction of the polynucleotides expressing recombinant receptors into those cells, through an independent step of viral transduction, also has an impact on the overall production process.

The inventors have explored safer means for *ex-vivo* delivery of endonucleases reagents into primary cells with the requirements that said cells (1) are modified at different genetic loci, (2) not bearing too many translocations, (3) produced in sufficient number to enable treating at least a hundred patients, and (4) produced in a limited time frame of less than 30 days to avoid exhaustion of the cells. They came up with the invention as described herein, where sequential gene editing is performed instead of multiplexing gene editing. Surprisingly, this occurred to be less destructive to the cells, resulting into higher quality primary immune cells.

This invention paves the way to standard and affordable adoptive immune cell therapy treatments.

Summary of the invention

The present invention is drawn to a method of sequential gene editing aiming to improve the genetic modification of primary human cells, especially immune cells originating from individual donors or patients.

5 Primary immune cells, in particular T-cells or NK cells, have a limited life span, and although they can be expanded and activated ex-vivo by methods known in the art [Rasmussen A.M. et al. (2010) Ex-vivo expansion protocol for human tumor specific T cells for adoptive T cell therapy. *Journal of Immunological Methods* 355:52–60], their immune reactivity tends to reduce over time. They can also get exhausted from the moment they are collected from a donor to the moment they are reintroduced *in-vivo* to track down and
10 eliminate malignant or infected cells into the patient.

Gene editing techniques using nucleotide sequence-specific reagents, such as rare-cutting endonucleases, have become the state of the art for the introduction of genetic modifications into primary cells. However, when such endonucleases are used to cleave target sequences simultaneously at different loci, the risk of inter- or intra-chromosomal
15 translocations increases significantly, which concur to a higher risk of unwanted genetic recombination or off-site mutations.

To overcome this drawback and minimize adverse genome effects, the inventors have applied a safer approach, where gene editing is sequentially applied, in particular through several rounds of electroporation. To their surprise, sequential gene editing resulted
20 into cells of higher quality and even into an increase of the yield of the engineered cells modified at different loci, as compared to multiplexing gene editing (i.e. gene editing performed simultaneously at different loci).

The present invention thus primarily concerns a method comprising one or several steps of:

- 25
- Providing at least one primary immune cell from a culture or a blood sample, such as from peripheral blood mononuclear cells (PBMCs);
 - Subjecting said cell to a step of gene editing, where a first set of sequence-specific reagent(s) is introduced into said cell;
 - Cultivating said cell to enable said first sequence-specific reagent to stably
30 modifying its genome at a first locus,
 - Subjecting said cell to at least a second gene editing step to introduce at least a second set of sequence-specific reagent(s) into said cell, and optionally
 - Cultivating said cell to enable said second sequence-specific reagent to stably modifying its genome at said second locus.

According to a preferred embodiment, the first, second and any subsequent sequence-specific reagents are introduced into said cell by electroporation, so that the method of the invention comprises:

5 a) Subjecting the immune cells to a first electroporation to introduce at least a first sequence-specific reagent into said immune cell;

b) cultivating said immune cell to enable said first sequence-specific reagent to modify its genome at a first locus,

c) submitting said cell to at least a second electroporation to introduce at least a second sequence-specific reagent into said cell, and optionally

10 d) cultivating said immune cell to enable said second sequence-specific reagent to modify its genome at said second locus.

Several strategies may be applied when applying the sequential gene editing of the present invention to obtain immune cells with higher recovery, better activation, persistence or therapeutic efficiency. As an example, the first electroporation step can be performed in view of editing or modifying a gene, in such a way that the cell will better expand or get more permissive to the subsequent modification steps.

As another example, the first step of gene editing can be performed on a receptor or surface protein, such as TCR. The TCR negative cells obtained by this first step can be purified by removal of the cells remaining TCR positive, such that said TCR negative cells can be cultivated and then subjected to a second step of gene editing, for instance to make them resistant to a chemotherapy drug. The resulting population of cells in which the second gene editing has been achieved, can then be enriched in TCR negative drug resistant cells by culture in a medium containing said chemotherapy drug.

Several examples are developed herein showing that, although deemed more destructive, the successive gene editing steps surprisingly contribute to improve the yield and therapeutic potential of the engineered immune cells.

The invention is drawn to the methods, but also to the new gene edited cells obtainable by these methods, especially new triple and quadruple gene inactivated immune cells, as well as the population of cells resulting thereof, which are useful for the preparation of therapeutic compositions.

The present invention may be further summarized by the following items:

1) A method for introducing genetic modifications at different loci of a primary immune cell, comprising the sequential steps of:

35 a) subjecting said primary immune cell to a first electroporation step to introduce at least a first sequence-specific reagent into said immune cell;

b) cultivating said primary immune cell thereby enabling said first sequence-specific reagent to modify its genome at a first locus,

c) subjecting said primary immune to at least a second electroporation step to introduce at least a second sequence-specific reagent into said cell,

5 d) cultivating and expanding said primary immune thereby enabling said second sequence-specific reagent to modify its genome at said second locus.

2) The method according to item 1, wherein the primary immune cell is cultivated in step b) from 12 to 72 hours, preferably from 24 to 48 hours.

10 3) The method according to item 1, wherein a purification step is performed between step b) and c) relying on a product resulting from the expression or the deletion of the gene that is modified at least at said first locus.

15 4) The method according to any one of items 1 to 3, wherein steps a) to d) are performed within 240 hours, preferably within 120 hours, more preferably within 96 hours, even more preferably within 72 hours.

20 5) The method according to any one of items 1 to 4, wherein said method comprises at least one further step of submitting said primary immune cell to a third electroporation step to introduce at least a third sequence-specific reagent into said cell.

25 6) The method according to item 1, wherein said first and/or second sequence-specific reagent is a polynucleotide or polypeptide encoding a rare-cutting endonuclease, a subunit thereof, or a conjugate of both a polynucleotide and a polypeptide.

30 7) The method according to item 2, wherein said first and/or second sequence-specific reagent is a polynucleotide or polypeptide encoding a rare-cutting endonuclease selected from programmable RNA or DNA guided endonuclease, TALEN, ZFN or a homing endonuclease.

8) The method according to item 3, wherein said first and/or second sequence-specific reagent is a conjugate of RNA guide and a Cas9 or Cpf1 polypeptide.

35 9) The method according to item 1, wherein said first and/or second sequence-specific reagent is an interference RNA (RNAi) or a polynucleotide encoding same.

- 10) The method according to item 1, wherein a transduction step is introduced between b) and c) with a retroviral or lentiviral vector.
- 5 11) The method according to item 10, wherein said transduction step involves an integrative lentiviral or retroviral vector for stable expression of a transgene.
- 12) The method according to item 11, wherein said transgene encodes a Chimeric Antigen Receptor (CAR).
- 10 13) The method according to item 10, wherein said transduction step involves a non-integrative viral vector.
- 14) The method according to item 13, wherein said non integrative viral vector is used as a
15 template for homologous recombination or NHEJ integration of said transgene into the immune cell's genome.
- 15) The method according to item 10, wherein said first sequence-specific reagent is acting on a genomic sequence that facilitates the transduction step.
- 20 16) The method according to any one of items 1 to 15, wherein said first sequence-specific reagent is acting on a genomic sequence that facilitates the genetic modification of step d).
- 25 17) The method according to any one of items 1 to 16, wherein step b) is performed below about 35 °C, preferably at about 30 °C.
- 18) The method according to any one of items 1 to 17, wherein said immune cell is a T-cell.
- 30 19) The method according to item 18, comprising a preliminary step of activating the primary T-cell by signal transduction.
- 20) The method according to item 18 or 19, wherein said first sequence-specific reagent permanently reduces or prevents expression of TCR by the primary T-cell.

- 21) The method according to any one of items 1 to 20, wherein said first or second sequence-specific reagent permanently reduces or prevents expression of at least one gene encoding an immune checkpoint.
- 5 22) The method according to item 21, wherein said at least one gene encoding an immune checkpoint is selected from PD1, CTLA4, PPP2CA, PPP2CB, PTPN6, PTPN22, PDCD1, LAG3, HAVCR2, BTLA, CD160, TIGIT, CD96, CRTAM, LAIR1, SIGLEC7, SIGLEC9, CD244, TNFRSF10B, TNFRSF10A, CASP8, CASP10, CASP3, CASP6, CASP7, FADD, FAS, TGFBR2, TGFBR1, SMAD2, SMAD3, SMAD4, SMAD10, SKI, SKIL, TGIF1, IL10RA, IL10RB, HMOX2, IL6R, IL6ST, EIF2AK4, CSK, PAG1, SIT1, 10 FOXP3, PRDM1, BATF, GUCY1A2, GUCY1A3, GUCY1B2, GUCY1B3.
- 23) The method according to any one of items 1 to 20, wherein said first or second sequence-specific reagent permanently confers resistance of said primary immune cell 15 against drugs or immune depleting agents.
- 24) The method according to item 23, wherein said resistance is conferred by inactivating a gene expressing CD52, dCK, GGH or HPRT.
- 20 25) The method according to any one of items 1 to 24, wherein a final step of purification is performed relying on a at least one product resulting from the expression or the deletion of one gene that is modified at said first and/or second and/or third locus.
- 26) A population of primary TCR negative T-cells resulting from a single donor obtainable 25 according to the method according to any one of items 1 to 25, comprising at least two subpopulations of T-cells selected from:
- TCR negative and PD1 negative,
 - TCR negative and CD52 negative,
 - TCR negative and CTLA4 negative,
 - 30 - TCR negative and dCK negative,
 - TCR negative and GGH negative,
 - TCR negative and HPRT negative, and
 - TCR negative and β 2m negative.

27) A population of primary TCR negative T-cells originating from a single donor, wherein at least 20 %, preferably 30 %, more preferably 50 % of the cells in said population have been modified using sequence-specific reagents in at least three different loci.

5 28) A pharmaceutical composition comprising a population of primary T cells according to any one of items 26 or 27.

Brief description of the figures and Tables:

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Figure 1: Examples of genes and cell functions which can be sequentially modified by gene editing according to the method of the present invention to produce engineered allogeneic primary immune T-cells. Arrows within the cell represents the various genetic loci that can be inactivated by the sequence specific nuclease reagent introduced into the T-cells.

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Figure 2: Rationale for the sequential gene editing according to the present invention versus the multiplexing gene editing from the prior art in terms of yielding primary immune cells stacking mutations at three different loci, such as genes encoding TCR, PD1 and DCK. The method of the present invention ends up with a population of immune cells where at least 80% of the cells are triple mutants. By contrast, with the same reagent efficiency, simultaneous gene editing amounts about 50 % of triple mutants. Upon cell expansion, this proportion should not much increase in the population, if not be decreasing.

20

Figure 3: Schematic representation of one embodiment of the method according to the invention, where a viral transduction step is performed between two electroporation gene editing steps. The viral transduction is preferably performed after a cell sorting step where the cells modified by the first gene editing modifications are purified. This cell sorting step reduces the overall number of cells, thereby reducing the number of viral particles to be used for the viral transduction. This can be advantageous, for instance, when TCR is first inactivated, to follow-up with stable viral transduction and expression of the CAR. Subsequent gene editing step can then be carried out to make the cells resistant to a drug. In such a situation, culturing or expanding the cells in a medium containing the drug allows the selection of the cells that are both TCR negative and drug resistant in view of their therapeutic use.

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Figure 4: Schematic representation of one embodiment of the method of the invention, wherein a cell sorting step is performed between the two electroporation gene editing steps.

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Figure 5: Schematic representation of one embodiment of the method of the invention, wherein a cell sorting step plus a viral transduction is performed between the two electroporation gene editing steps.

Figure 6: Schematic representation of one embodiment of the method according to the invention, where a culture step in a selective medium is performed between two electroporation gene editing steps, to select the cells that have been made resistant to a compound as per the first gene modification. This culture step increases the number of cells that are eventually modified at multiple loci after the second gene editing step. This approach can be applied to produce drug resistant CAR positive T-cells, that are further gene edited to be more active (inactivation of a locus inhibiting T-cell activation/cytotoxicity, such as PD1 and/or CTLA4), especially in a context of an autologous treatment where the T-cells (eg: Tumor Infiltrating Lymphocytes (TIL)) are collected from a patient, engineered and re-infused to said patient.

Figure 7: Schematic representation of one embodiment of the method according to the invention, where the first gene editing step is performed in a gene coding or regulating the expression of a surface antigen and the second gene editing step is performed in a gene coding or regulating the expression of a product that is not a surface antigen. A cell separation step is performed between the two gene editing steps to enrich the cells allowed to pass to the second gene editing step. Optionally, the second gene editing step is followed by a culture step in a selective medium to favor/select expansion of the cells bearing the second gene editing.

Figure 8: Schematic representation of one embodiment wherein a cell sorting based on immune cells subtypes, for instance CD4+ and CD8+ cells is performed after the first gene editing step (ex: TCR inactivation) and before the second gene editing step. As per this embodiment, a different gene editing can be applied to the different subtypes respectively, such as for instance the inactivation of FOXP3 in CD4+ cells and the inactivation of PD1 in CD8+ cells. The gene edited cells from the separate batches (ex: CD8+ and CD4+) can be respectively mixed at a predetermined ratio (ex: 1 to 1) to produce more active therapeutic compositions.

Figure 9: Diagrams displaying the % number of cells TCR negative, CD52 negative and both TCR and CD52 negative after simultaneous gene editing (TALEN CD52 and TCR together) (see Example 1). Controls are untransfected cells, which results are represented the left columns of the diagrams

Figure 10: T-cell growth curve observed after the different electroporation strategies: simultaneous and sequential according to the invention (6, 20 and 40h interval between the two CD52 and TCR gene editing steps – see example 1).

Figure 11: Frequency of In-deletions (Indels) for 5 off-site targets (OFS1, OFS2, OFS3, OFS4 and OFS5) and TRAC and PD-1 on-site targets after simultaneous or sequential electroporation of TRAC and PD-1 TALEN[®]. Red stars highlight the abrogation of off-site 3 (OFS3) target cleavage by the method according to the invention (see example 2).

Figure 12: Diagrams showing the growth of the engineered cells populations over time from Day 5 (D5) post thawing to Day 15 (D15) with respect to the different gene editing strategies detailed in Example 3 and Table 2.

Figure 13: Schematic representation of workflow for the generation of triple KO CAR T cells, as carried out in example 4.

Figure 14: Diagram displaying the triple KO efficacy (% number TCR/B2M and PD1 negative cells), in two different donors using indicated doses of mRNA encoding TRAC, β 2m and PD-1 TALEN[®]s that were electroporated either simultaneously (Sim.) or sequentially (Seq.), resulting into [TCR]^{neg}[β 2m]^{neg}[PD1]^{neg} therapeutically effective number of cells originating from donors, as explained in Example 4.

Figure 15: Diagrams showing cytotoxic activity on Raji cells at different effector to target ratios (E:T) of CD22 CAR-T cells that were either untransfected with TALEN[®] reagents (WT: black) or TALEN[®] transfected sequentially (dark grey) or TALEN[®] transfected simultaneously (light grey).

Table 1: List of genes involved into immune cells inhibitory pathways

Table 2: gene editing efficiency of various sequential gene editing strategies according to the invention as presented in Example 3 (percentage of gene edited cells based on numbers of CD38, TCR and/or CD52 negative cells; D4, D5 and D6 are the number of days after thawing frozen primary cells).

Table 3: Sequence of TALEN[®] used in the examples.

Table 4: Selection of antigen markers of various cancers found to be expressed on the surface of T-cells. The inactivation of the genes encoding these antigen markers is proposed as part of one of one of the gene editing steps according to the invention, especially when the engineered immune cells are endowed with chimeric antigen receptors targeting these very antigens.

Detailed description of the invention

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

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

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

In a general aspect, the present invention relates to methods to perform genome modification in multiple loci in primary cells through sequential electroporation steps, spaced by cell culture, sorting and/or expansion phase(s).

In particular, these methods comprise the steps of:

a) subjecting the primary immune cell to a first electroporation to introduce at least a first sequence-specific reagent into said immune cell;

b) cultivating said primary immune cell thereby enabling said first sequence-specific reagent to modify its genome at a first locus,

5 c) subjecting said primary immune to at least a second electroporation to introduce at least a second sequence-specific reagent into said cell,

d) cultivating and expanding said primary immune thereby enabling said second sequence-specific reagent to modify its genome at said second locus.

By "primary cell" or "primary cells" are intended cells taken directly from living tissue
10 (e.g. biopsy material) and established for growth in vitro for a limited amount of time, meaning that they can undergo a limited number of population doublings. Primary cells are opposed to continuous tumorigenic or artificially immortalized cell lines. Non-limiting examples of such cell lines are CHO-K1 cells; HEK293 cells; Caco2 cells; U2-OS cells; NIH 3T3 cells; NSO cells; SP2 cells; CHO-S cells; DG44 cells; K-562 cells, U-937 cells; MRC5
15 cells; IMR90 cells; Jurkat cells; HepG2 cells; HeLa cells; HT-1080 cells; HCT-116 cells; Hu-h7 cells; Huvec cells; Molt 4 cells. Primary cells are generally used in cell therapy as they are deemed more functional and less tumorigenic.

In general, primary immune cells are provided from donors or patients through a variety of methods known in the art, as for instance by leukapheresis techniques as reviewed
20 by Schwartz J. et al. (Guidelines on the use of therapeutic apheresis in clinical practice-evidence-based approach from the Writing Committee of the American Society for Apheresis: the sixth special issue (2013) *J Clin Apher.* 28(3):145-284). The primary immune cells according to the present invention can also be differentiated from stem cells, such as cord blood stem cells, progenitor cells, bone marrow stem cells, hematopoietic stem cells
25 (HSC) and induced pluripotent stem cells (iPS).

By "immune cell" is meant a cell of hematopoietic origin functionally involved in the initiation and/or execution of innate and/or adaptative immune response, such as typically CD3 or CD4 positive cells. The immune cell according to the present invention can be a dendritic cell, killer dendritic cell, a mast cell, a NK-cell, a B-cell or a T-cell selected from the
30 group consisting of inflammatory T-lymphocytes, cytotoxic T-lymphocytes, regulatory T-lymphocytes or helper T-lymphocytes. Cells can be obtained from a number of non-limiting sources, including peripheral blood mononuclear cells, bone marrow, lymph node tissue, cord blood, thymus tissue, tissue from a site of infection, ascites, pleural effusion, spleen tissue, and from tumors, such as tumor infiltrating lymphocytes. In some embodiments, said
35 immune cell can be derived from a healthy donor, from a patient diagnosed with cancer or

from a patient diagnosed with an infection. In another embodiment, said cell is part of a mixed population of immune cells which present different phenotypic characteristics, such as comprising CD4, CD8 and CD56 positive cells.

By “endonuclease reagent” is meant a nucleic acid molecule that contributes to an endonuclease catalytic reaction in the target cell, itself or as a subunit of a complex, preferably leading to the cleavage of a nucleic acid sequence target. The endonuclease reagents of the invention are generally sequence-specific reagents, meaning that they can induce DNA cleavage in the cells at predetermined loci, referred to by extension as “gene targets”. The nucleic acid sequence which is recognized by the sequence specific reagents is referred to as “target sequence”. Said target sequence is usually selected to be rare or unique in the cell’s genome, and more extensively in the human genome, as can be determined using software and data available from human genome databases, such as <http://www.ensembl.org/index.html>.

“Rare-cutting endonucleases” are sequence-specific endonuclease reagents of choice, insofar as their recognition sequences generally range from 10 to 50 successive base pairs, preferably from 12 to 30 bp, and more preferably from 14 to 20 bp.

According to a preferred aspect of the invention, the endonuclease reagent is transiently expressed into the cells, meaning that said reagent is not supposed to integrate into the genome or persist over a long period of time, such as be the case of RNA, more particularly mRNA, proteins or complexes mixing proteins and nucleic acids (eg: Ribonucleoproteins). In general, 80% the endonuclease reagent is degraded by 30 hours, preferably by 24, more preferably by 20 hours after transfection.

According to a preferred aspect of the invention, said endonuclease reagent is a nucleic acid encoding an “engineered” or “programmable” rare-cutting endonuclease, such as a homing endonuclease as described for instance by Arnould S., et al. (WO2004067736), a zing finger nuclease (ZFN) as described, for instance, by Urnov F., et al. (Highly efficient endogenous human gene correction using designed zinc-finger nucleases (2005) *Nature* 435:646-651), a TALE-Nuclease as described, for instance, by Mussolino et al. (A novel TALE nuclease scaffold enables high genome editing activity in combination with low toxicity (2011) *Nucl. Acids Res.* 39(21):9283-9293), or a MegaTAL nuclease as described, for instance by Boissel et al. (MegaTALs: a rare-cleaving nuclease architecture for therapeutic genome engineering (2013) *Nucleic Acids Research* 42 (4):2591-2601).

According to the invention, the endonuclease reagent is preferentially under RNA form to allow transient endonuclease activity of said reagent into the target cell and make the entire capsule biodegradable *in-vivo*. Even more preferably, the endonuclease reagent is under the form of a mRNA for the expression of the rare cutting endonuclease into the cells.

The endonuclease under mRNA form is preferably synthesized with a cap to enhance its stability according to techniques well known in the art, as described, for instance, by Kore A.L., et al. (Locked nucleic acid (LNA)-modified dinucleotide mRNA cap analogue: synthesis, enzymatic incorporation, and utilization (2009) *J Am Chem Soc.* 131(18):6364-5).

5 Due to their higher specificity, TALE-nuclease have proven to be particularly appropriate for therapeutic applications, especially under heterodimeric forms – i.e. working by pairs with a “right” monomer (also referred to as “5” or “forward”) and ‘left” monomer (also referred to as “3” or “reverse”) as reported for instance by Mussolino et al. (TALEN[®] facilitate targeted genome editing in human cells with high specificity and low cytotoxicity (2014) *Nucl. Acids Res.* 42(10): 6762-6773).

10 According to another embodiment, the endonuclease reagent is a RNA-guide to be used in conjunction with a RNA guided endonuclease, such as Cas9 or Cpf1, as per, inter alia, the teaching by Doudna, J., and Chapentier, E., (The new frontier of genome engineering with CRISPR-Cas9 (2014) *Science* 346 (6213):1077), which is incorporated
15 herein by reference.

However, because engineered rare-cutting endonuclease are sequence specific unique reagents, it cannot be excluded that, in some instances, they can promote or induce some chromosomal rearrangements, especially when several gene loci need to be cleaved in the same cell.

20 Rearrangements are more prompt to happen, for instance, when multiple cleavage sites get simultaneously cut on the same chromosome or when pseudo cleavage sites appear from the unexpected combinations of heterodimers not initially designed to work together.

The inventors have more particularly sought for lowering this risk without reducing the
25 yield of the engineered cells when treated with any of the above endonuclease reagents (i.e. maintaining high gene KO efficacy and high cellular viability).

Sequential steps to produce batches of engineered primary immune cells of therapeutic grade

30

The present invention thus provides a method allowing stacking gene editing in mammalian cells, while preventing undesirable genome deletions or translocations.

By “gene editing” is meant, throughout the present specification, any methods by which a genomic sequence is modified by insertion, deletion or replacement at a selected
35 locus by using at least an enzyme that cleaves phosphodiester bond within a polynucleotide chain.

The method of the present invention can be associated with other methods involving physical or genetic transformations, such as a viral transduction or transfection using nanoparticles, in particular for transient expression of exogenous genetic sequences.

The invention can be applied to human immune cells, in particular activated T-cells by alternating transfection (preferably by electroporation) and culture steps. Examples of such cycles as delineated below as examples:

- (TNx -> h -> TNx) x n
- (TNx/TNx -> h -> TNx) x n
- (TNx -> h -> TNx/TNx) x n
- 10 - (TNx/TNx -> h -> TNx/TNx) x n

where TNx is a Transfection (T) of a specific endonuclease reagent N, ($x \geq 1$)

h an interval of time in hours, and ($h \geq 1$)

n is the number of subsequent transfection ($n \geq 1$)

The above cycle may be combined with each other depending on the number of the different endonuclease reagents to be used sequentially.

Preferably, each gene editing step targets one locus at a time.

On another hand, the use of sequence-specific endonuclease reagents can be combined with other types of cell transformation not involving sequence specific endonuclease reagents, such as a retroviral transduction. This is of particular interest, for instance, for the production of primary immune cells expressing a recombinant receptors, such as a chimeric antigen receptor (CAR) or a recombinant TCR. Such chimeric antigen receptors are generally encoded by exogenous sequences which are introduced into cells by means of viral vectors, in particular lentiviral vectors. It is therefore advantageous to combine the sequential gene editing steps of the present invention with viral transduction steps, such as illustrated in figures 3, 5, 6 and 7.

Example of combinations of sequential gene editing steps and transduction steps are delineated below:

- Transduction -> h -> (TNx -> h -> TNx) x n ;
- Transduction -> h -> (TNx/TNx -> h -> TNx) x n ;
- 30 - Transduction -> h -> (TNx -> h -> TNx/TNx) x n ;
- Transduction -> h -> (TNx/TNx -> h -> TNx/TNx) x n ;
- (TNx -> h -> TNx) x n -> h -> Transduction) x n ;
- (TNx/TNx -> h -> TNx) x n -> h -> Transduction) x n ;
- (TNx -> h -> TNx/TNx) x n -> h -> Transduction ;

- (TNx/TNx -> h -> TNx/TNx) x n -> h -> Transduction ;
- (TNx -> h -> Transduction -> h -> TNx) x n ;
- (TNx/TNx -> h -> Transduction -> h -> TNx) x n ;
- (TNx -> h -> Transduction -> h -> TNx/TNx) x n ;
- 5 - (TNx/TNx -> h -> Transduction -> h -> TNx/TNx) x n ;

The transduction step may also take place prior to a gene editing step, when, for instance, vector introduces a template DNA into the primary cell to be integrated at a locus during the subsequent gene editing step. The sequence-specific endonuclease reagent is then introduced into the cell to promote the integration of said exogenous DNA at said locus.

10 One preferred aspect is a method of the present invention comprising a first gene editing step, followed by a transduction step involving an AAV vector comprising an exogenous sequence to be integrated at a predetermined locus, prior to a second gene editing step wherein the exogenous DNA comprised in the AAV vector is integrated at said predetermined locus.

15 According to one aspect of the invention, the sequential gene editing method may combine single gene editing steps (TNx) with multiplexing gene editing steps (TNx/TNx). During the multiplexing gene editing step, different endonuclease reagents may be used at different/multiple loci together. Endonucleases have different types of cleavage signatures: some of them can create "blunt ends", such as CRISPR, 5' "cohesive ends" such as zing
20 Finger nucleases or TALE-nucleases or 3' "cohesive ends" by using homing endonucleases. Combinations of endonuclease reagents can be made based on the type of cleavage sought. For instance, cohesive ends are better suited for integration of exogenous DNA than blunt ends for instance. One preferred aspect of the invention is the combined use of endonuclease reagents creating different types of cleavage signatures to reduce the
25 deletions or translocations occurring at/or between the different gene-edited loci.

According to a preferred embodiment of the invention, the primary immune cells are cultivated for an interval of time (h) as referred to above that is more than 10 hours, preferably from 12 to 72 hours and more preferably from 12 to 48 hours.

30 According to a particular embodiment of the invention, a purification step can be performed between general step b) and c), as also illustrated in figures 4, 7 and 13.

This purification step can be performed for the sake of purity by any standard method known in the art. In the present case, the purification step can help to select the cells which have undergone the gene editing achieved in step a). The purification can thus rely on the product resulting from the first gene editing reaction, such as a product resulting from the
35 modification or insertion of a genetic sequence at said first locus, or the absence of a gene

product in case of a deletion of such genetic sequence. In a preferred embodiment, the first gene editing step may help the expression of receptors or membrane proteins, which make the immune cells more receptive to the second gene editing step. The cells can also become more receptive to viral vectors transduction if genes involved into viral transformation are modified prior to the second gene editing.

The genes that can be targeted as part of the first gene editing step, can be genes the modification of which will facilitate viral transduction or the realization of the second gene editing step or of any subsequent transduction or transfection step. Such genes can be, for instance, genes encoding cell restriction factors, such as TRIM5a (Uniprot Q9C035), APOBEC protein family (apolipoprotein B mRNA editing enzyme), and SAMHD1 (Uniprot Q9Y3Z3). By "cell restriction factors" is meant molecules that directly and dominantly cause a significant decrease in viral infectivity. TRIM5a, for instance, is a protein known to mediate/inhibit lentiviral, such as HIV, entry into immune cells (Stremlau M, et al. "Specific recognition and accelerated uncoating of retroviral capsids by the TRIM5alpha restriction factor" (2006) *PNAS* 103(14): 5514–9). The inactivation of TRIM5a, SAMHD1 or proteins of APOBEC family as part of one of the gene editing steps of the present invention can increase the infectivity of primary cells to viral vectors during subsequent transduction steps.

According to preferred embodiments, the invention provides that steps a) to d) of the general method are performed within 240 hours, preferably within 120 hours, more preferably within 96 hours, even more preferably within 72 hours. This limited period of time allows better recovery of the primary immune cells and limits their exhaustion. Limited exhaustion can be controlled at different steps during the process by one skilled in the art by using specific exhaustion markers such as reviewed by Wherry, J.A. (T cell exhaustion (2011) *Nature Immunology* 12:492–499).

According to a preferred embodiment of the invention, the transfection step T, such as steps a) and c) in the general method previously described, as well as any further steps TN (where N is the number of gene editing steps) are performed by electroporation.

Surprisingly, successive electroporation steps were found to be less destructive and/or less genotoxic for the primary cells than any other methods performing multiplex gene editing where various endonuclease reagents are transfected in one shot.

The method according to the present invention can comprise at least one further step of submitting the primary immune cell to a third electroporation to introduce at least a third sequence-specific reagent into said cell.

Such electroporation steps are typically performed in closed chambers comprising parallel plate electrodes producing a pulse electric field between said parallel plate electrodes greater than 100 volts/cm and less than 5,000 volts/cm, substantially uniform

throughout the treatment volume such as described in WO/2004/083379, which is incorporated by reference, especially from page 23, line 25 to page 29, line 11. One such electroporation chamber preferably has a geometric factor (cm^{-1}) defined by the quotient of the electrode gap squared (cm^2) divided by the chamber volume (cm^3), wherein the
5 geometric factor is less than or equal to 0.1 cm^{-1} , wherein the suspension of the cells and the sequence-specific reagent is in a medium which is adjusted such that the medium has conductivity in a range spanning 0.01 to 1.0 milliSiemens. In general, the suspension of cells undergoes one or more pulsed electric fields. With the method, the treatment volume of the suspension is scalable, and the time of treatment of the cells in the chamber is substantially
10 uniform.

According to a preferred embodiment of the invention, the sequence specific reagent is under the form of nucleic acids, more preferably under DNA or RNA form. Under such forms, the nucleic acids can either code for a polypeptide, typically a rare cutting endonuclease a subunit thereof, or a conjugate of both a polynucleotide and a polypeptide.
15 The reagents can also be under the form RNA or DNA guides directing guided endonucleases such as Cas9 or Cpf1 (RNA-guided endonucleases) or Argonaute (DNA-guided endonucleases) as recently respectively described by Zetsche, B. et al. (Cpf1 Is a Single RNA-Guided Endonuclease of a Class 2 CRISPR-Cas System (2015) *Cell* 163(3): 759–771) and by Gao F. et al. (DNA-guided genome editing using the *Natronobacterium gregoryi* Argonaute (2016) *Nature Biotech*). According to a most preferred embodiment, the sequence-specific reagent is under mRNA form and encodes a rare-cutting endonuclease, selected from programmable RNA or DNA guided endonuclease, TALE-nuclease, Zing-finger nuclease (ZFN), a megaTAL or a homing endonuclease.
20

According to another aspect of the invention said first and/or second sequence-specific reagent is an interference RNA (RNAi) or a polynucleotide encoding same.
25

As previously mentioned, a transduction step can be introduced between steps b) and c) of the general method, especially an integrative lentiviral or retroviral vector for stable expression of a transgene. This is particularly adapted to express chimeric antigen receptors (CAR) at the surface of the primary immune cells modified as the present invention. Such
30 methods for CAR expression in allogeneic primary cells are described for instance in WO2013176915. Non-integrative viral vectors can also be used, as also described by the applicant in WO2015028683. Non integrative viral vector can be used, inter alia, as a template or donor DNA for homologous recombination or NHEJ integration of transgene into the immune cell's genome as part of the second gene editing step as per the present
35 invention.

The steps of the present method are generally performed at mammalian cells physiological temperature (37°C for human cells), but certain steps of the method may be performed at non-physiological temperatures, between 30 and 37°C, or even lower between 25 and 35°C, during a limited period of time, from 30 minutes to 12 hours, preferably from 1 to 10 hours, more preferably from 1 to 5, and even more preferably from 30 minutes to 2 hours. For instance, it has been observed that performing the electroporation steps at lower temperature, e.g. below about 35°C, such as at about 30°C, favors transfection efficiency.

As further illustrated in the present specification, the present method aims particularly at producing immune cells, preferably primary immune cells, genetically modified at multiple loci, especially by gene editing, for their subsequent use in cell therapy.

Such immune cells are generally endowed with recombinant receptors, such as CAR or recombinant TCR, which confer them higher specificity toward malignant or infected cells. These recombinant receptors are generally encoded by exogenous polynucleotides which are introduced into the cell using viral vectors as per one of the transduction steps referred to previously.

The CARs expressed by these cells specifically target antigen markers at the surface of malignant or infected cells, which further help said immune cells to destroy these cells *in-vivo* as reviewed by Sadelain M. et al. ["The basic principles of chimeric antigen receptor design" (2013) *Cancer Discov.* 3(4):388-98].

In general, CAR polypeptides comprise an antigen binding domain, a transmembrane domain, and an intracellular domain comprising a costimulatory domain and/or a primary signaling domain, wherein said antigen binding domain binds to the tumor antigen associated with the disease.

Many CARs have been described in the art, which can be used to carry out the present method, which can bind tumor antigen as diverse as one selected from: 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 (GPC5D); chromosome X open reading frame 61 (CXORF61); ALK receptor tyrosine kinase (ALK); Polysialic acid; Placenta-specific 1 (PLAC1); hexasaccharide portion of globoH glycosceramide (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); and immunoglobulin lambda-like polypeptide 1 (IGLL1).

More preferred CARs according to the present invention are those described in the examples, which more preferably comprise an extracellular binding domain directed against one antigen selected from CD19, CD22, CD33, 5T4, ROR1, CD38, CD52, CD123, CS1, BCMA, Flt3, CD70, EGFRvIII, WT1, HSP-70 and CCL1. Even more preferred are CARs directed against CD22, CD38, 5T4, CD123, CS1, HSP-70 and CCL1. Such CARs have preferably one structure as described in WO2016120216.

Immune cells can also express recombinant T-Cell receptors. T cells recognise MHC-peptide conjugates on target cells through the paired α and β chains of the TCR. This pairing confers the antigen specificity of the immune cell. One gene therapy approach has involved the molecular cloning of the TCR genes known to be specific for an antigen of choice. These chains are then introduced into T cells usually by means of a retroviral vector in a similar way as with CAR. Consequently, expression of the cloned TCR α and TCR β genes endows the transduced immune cells with a functional specificity determined by the pairing of these new genes. Because TCRs recognize processed peptides presented on MHC, targeted antigens can be derived from the entire protein composition of the tumor cells, including intracellular proteins, whereas CARs are generally designed to recognize molecules expressed on the surface of target cells. This quality also allows TCRs to target a large number of non-surface antigens of virally infected cells and tumors associated with viral infection, such as hepatitis-associated hepatocellular carcinoma, papilloma virus-associated cervical cancer, and Epstein-Barr virus-related malignancies (Spear, T. et al. (2016). Strategies to genetically engineer T cells for cancer immunotherapy. *Cancer Immunology Immunotherapy*: 65(6):631–649).

Preferred recombinant TCR to be used in the present invention are those directed against antigen specific of cancer cells, such as MART-1, MAGE-1, MAGE-2, MAGE-3 MAGE-12, BAGE, GAGE, NY-ESO-1, or overexpressed in cancer cells, such as a-Fetoprotein, Telomerase catalytic protein, G-250, MUC-1, CarcinoEmbryonic antigen (CEA), p53, Her-2/Neu and WT1 [Rosenberg S.A., (2001) Progress in human tumour immunology and immunotherapy *Nature*. 411(6835):380-4].

The exogenous polynucleotide sequence encoding the recombinant receptors are generally introduced by a transduction step, which can be performed in the course of the present invention as shown for instance in Figures 4, 7 and 13 by using a viral vector, such as a lentiviral vector.

5 Alternatively, or as part of a gene editing step, AAV vectors can be used as a DNA template for gene targeted insertion of said polynucleotide sequence encoding a recombinant receptor at a desired locus by NHEJ or homologous recombination.

The insertion locus may be selected to disrupt an endogenous gene present at this locus, such a gene encoding a component of TCR or $\beta 2m$ as previously described.

10 Also said exogenous polynucleotide sequence can be integrated at a locus preferably encoding TCR, HLA, $\beta 2m$, HLA, PD1 or CTLA4, as part of the editing steps of the present invention.

In particular, the inventors have significantly improved the rate of gene targeted insertion into human cells by using AAV vectors from the AAV6 family.

15 According to a preferred embodiment, the method of the invention can therefore comprise a step consisting in:

- transducing into said cell an AAV vector comprising said exogenous nucleic acid sequence and sequences homologous to the targeted endogenous DNA sequence, and optionally,
- 20 - Inducing the expression of a sequence specific endonuclease reagent to cleave said endogenous sequence at the locus of insertion.

The obtained insertion of the exogenous nucleic acid sequence may then result into the introduction of genetic material, correction or replacement of the endogenous sequence, more preferably "in frame" with respect to the endogenous gene sequences at that locus.

25 • **Alloreactivity and/or engraftment of the immune cells:**

The method according to the invention is particularly adapted to prepare primary immune cells for allogeneic therapeutic use. By "allogeneic therapeutic use" is meant that the cells originate from a donor in view of being infused into patients having a different haplotype. Indeed, the present invention provides with an efficient method for obtaining
30 primary cells, which can be gene edited in various gene loci involved into host-graft interaction and recognition. Other loci may also be edited in view of improving the activity, the survival or the life-time of the engineered primary cells. Such engineered immune cells are preferably primary T cells.

Figure 1 maps the main cell functions that can be modified by gene editing according to the present invention to improve the efficiency of the engineered immune cells. Any gene inactivation listed under each function can be combined with another to obtain a synergistic effect on the overall therapeutic potency of the immune cells.

5 The present method is particularly useful for developing engineered non-alloreactive T-cells for immunotherapy and more specifically to methods for increasing the persistence and/or the engraftment of allogeneic immune cells by proceeding with at least one step of inactivation, preferably permanently, of a gene implicated in the self/non-self recognition, using preferably specific rare-cutting endonuclease.

10 According to a preferred aspect of the invention, one of the gene editing steps aims to reduce host versus graft disease (GVHD) reaction or immune rejection upon introduction of the allogeneic cells into the recipient patient. For instance, one of the sequence-specific reagents used in the method can reduce or prevent the expression of TCR in primary T-cells, such as the genes encoding TCR-alpha or TCR-beta.

15 As another preferred aspect, one gene editing step is to reduce or prevent the expression of the β 2m protein and/or another protein involved in its regulation such as C2TA (Uniprot P33076) or in MHC recognition, such as HLA proteins. This permits the engineered immune cells to be less alloreactive when infused into patients.

20 Most preferred, is the gene editing of both TCR and β 2m as part of the sequential gene editing method of the present invention into T-cells or precursor cells thereof, which method can comprise the steps of introducing an exogenous polynucleotide encoding recombinant receptors, such as CARs or recombinant TCR previously mentioned, and more preferably at the TCRalpha or TCRbeta locus.

25 • **Inhibiting checkpoint receptors and immune cells inhibitory pathways:**

 According to a preferred aspect of the invention, one of the gene editing steps, aims to disrupt the expression of a protein involved in immune cells inhibitory pathways, in particular those referred to in the literature as "immune checkpoint" (Pardoll, D.M. (2012) The blockade of immune checkpoints in cancer immunotherapy, *Nature Reviews Cancer*, 12:252-30 264). In the sense of the present invention, "immune cells inhibitory pathways" means any gene expression in immune cells that leads to a reduction of the cytotoxic activity of the lymphocytes towards malignant or infected cells. This can be for instance a gene involved into the expression of FOXP3, which is known to drive the activity of Tregs upon T cells (moderating T-cell activity).

35 "Immune checkpoints" are molecules in the immune system that either turn up a signal (co-stimulatory molecules) or turn down a signal of activation of an immune cell. As

per the present invention, immune checkpoints more particularly designate surface proteins involved in the ligand–receptor interactions between T cells and antigen-presenting cells (APCs) that regulate the T cell response to antigen (which is mediated by peptide–major histocompatibility complex (MHC) molecule complexes that are recognized by the T cell receptor (TCR)). These interactions can occur at the initiation of T cell responses in lymph nodes (where the major APCs are dendritic cells) or in peripheral tissues or tumours (where effector responses are regulated). One important family of membrane-bound ligands that bind both co-stimulatory and inhibitory receptors is the B7 family. All of the B7 family members and their known ligands belong to the immunoglobulin superfamily. Many of the receptors for more recently identified B7 family members have not yet been identified. Tumour necrosis factor (TNF) family members that bind to cognate TNF receptor family molecules represent a second family of regulatory ligand–receptor pairs. These receptors predominantly deliver co-stimulatory signals when engaged by their cognate ligands. Another major category of signals that regulate the activation of T cells comes from soluble cytokines in the microenvironment. In other cases, activated T cells upregulate ligands, such as CD40L, that engage cognate receptors on APCs. A2aR, adenosine A2a receptor; B7RP1, B7-related protein 1; BTLA, B and T lymphocyte attenuator; GAL9, galectin 9; HVEM, herpesvirus entry mediator; ICOS, inducible T cell co-stimulator; IL, interleukin; KIR, killer cell immunoglobulin-like receptor; LAG3, lymphocyte activation gene 3; PD1, programmed cell death protein 1; PDL, PD1 ligand; TGF β , transforming growth factor- β ; TIM3, T cell membrane protein 3.

Examples of further genes, which expression could be reduced or suppressed to turn-up activation in the engineered immune cells according the present invention are listed in Table 1.

For instance, one of the sequence-specific reagents used in the method can reduce or prevent the expression by the immune cell of at least one protein selected from 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), TGFBR2 (Uniprot P37173), TGFBR1 (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),
 5 GUCY1A2 (Uniprot P33402), GUCY1A3 (Uniprot Q02108), GUCY1B2 (Uniprot Q8BXH3) and GUCY1B3 (Uniprot Q02153). The gene editing introduced in the genes encoding the above proteins is preferably combined with an inactivation of TCR in CAR T cells.

Preference is given to inactivation of PD1 and CTLA4, in combination with TCR.

To improve the efficiency of the engineered cells according to the present
 10 invention, the steps of the present method using sequence-specific endonuclease reagents, can be followed by a step of contacting said engineered immune cells with at least one non-endogenous immunosuppressive polypeptide, such as a PD-L1 ligand and/or CTLA-4 Ig.

Table 1: List of genes involved into immune cells inhibitory pathways

Pathway		Genes that can be inactivated In the pathway	
Co-inhibitory receptors	CTLA4 (CD152)	CTLA4, PPP2CA, PPP2CB, PTPN6, PTPN22	
	PDCD1 (PD-1, CD279)	PDCD1	
	CD223 (lag3)	LAG3	
	HAVCR2 (tim3)	HAVCR2	
	BTLA(cd272)	BTLA	
	CD160(by55)	CD160	
	IgSF family		TIGIT
			CD96
			CRTAM
	LAIR1(cd305)	LAIR1	
	SIGLECs		SIGLEC7
		SIGLEC9	
CD244(2b4)	CD244		
Death receptors	TRAIL	TNFRSF10B, TNFRSF10A, CASP8, CASP10, CASP3, CASP6, CASP7	
	FAS	FADD, FAS	
Cytokine signalling	TGF-beta signaling	TGFBRII, TGFBRI, SMAD2, SMAD3, SMAD4, SMAD10, SKI, SKIL, TGIF1	
	IL10 signalling	IL10RA, IL10RB, HMOX2	
	IL6 signalling	IL6R, IL6ST	
Prevention of TCR signalling		CSK, PAG1	
		SIT1	
Induced Treg	induced Treg	FOXP3	

Transcription factors controlling exhaustion	transcription factors controlling exhaustion	PRDM1
		BATF
Hypoxia mediated tolerance	iNOS induced guanylated cyclase	GUCY1A2, GUCY1A3, GUCY1B2, GUCY1B3

Preference is given to the production of immune cells combining gene editing, into at least the genes encoding:

- 5 - TCR, PD1 and LAG3;
- TCR, PD1 and FOXP3;
- TCR, CTLA4 and LAG3;
- TCR, CTLA4 and FOXP3;

And even more preferably to the production of immune cells combining gene editing steps into at least the genes encoding:

- 10 - TCR, β 2m and PD1
- TCR, β 2m and CTLA4
- TCR, β 2m and LAG3
- TCR, β 2m and FOXP3

15 preferably by inhibiting or inactivating the expression of these proteins.

• **Inhibiting suppressive cytokines/metabolites**

According to another aspect of the invention, the gene editing step concerns genes encoding or positively regulating suppressive cytokines or metabolites or receptors thereof, in particular TGFbeta (Uniprot P01137), IL10R (Uniprot Q13651 and/or Q08334), A2aR (Uniprot P29274), GCN2 (Uniprot P15442) and PRDM1 (Uniprot O75626).

Preference is given to the production of immune cells combining gene editing, into at least the genes encoding:

- TCR, PD1 and TGFbeta;
- TCR, CTLA4 and TGFbeta;
- 25 - TCR, PD1 and IL10R;
- TCR, CTLA4 and IL10R;
- TCR, PD1 and TGFbeta;
- TCR, CTLA4 and TGFbeta;
- TCR, PD1 and GCN2;
- 30 - TCR, CTLA4 and GCN2;
- TCR, PD1 and A2aR;
- TCR, CTLA4 and A2aR;

- TCR, PD1 and PRDM1;
- TCR, CTLA4 and PRDM1;

preferably by inhibiting or inactivating the expression of these proteins.

5 • **Resistance to chemotherapy drugs**

As a preferred embodiment of the present method, one gene editing step is performed into a locus responsible for the sensitivity of the immune cells to compounds used in standard of care treatments for cancer or infection, such as drugs purine nucleotide analogs (PNA) or 6-Mercaptopurine (6MP) and 6 thio-guanine (6TG) commonly used in
10 chemotherapy. Reducing or inactivating the genes involved into the mode of action of such compounds (referred to as “drug sensitizing genes”) improves the resistance of the immune cells to same.

Examples of drug sensitizing gene are those encoding DCK (Uniprot P27707) with respect to the activity of PNA, such a clorofarabine et fludarabine, HPRT (Uniprot P00492)
15 with respect to the activity of purine antimetabolites such as 6MP and 6TG, and GGH (Uniprot Q92820) with respect to the activity of antifolate drugs, in particular methotrexate.

According to another aspect, resistance to drugs can be conferred immune cells by overexpressing a drug resistance gene as an additional optional step of the present method of sequential gene editing. Expression of variant alleles of several genes such as
20 dihydrofolate reductase (DHFR)(Uniprot P00374), inosine monophosphate dehydrogenase 2 (IMPDH2)(Uniprot P12268), calcineurin (Uniprot Q96LZ3, P63098 P48454, P16298 and Q08209) or methylguanine transferase (MGMT) (Uniprot P16455) have been identified to confer drug resistance to a cell according to the invention.

According to another aspect of the present invention, the engineering immune cells
25 are made resistant to drugs purine nucleotide analogs (PNA) chemotherapy drugs, such a clorofarabine et fludarabine, as part of the gene editing step. This enables the cells to be used after or in combination with conventional anti-cancer chemotherapies.

While, according to the present invention, the first gene editing step is preferably performed on a locus encoding or regulating a surface antigen, so that sorting of the
30 engineered cells can be carried out based on the presence/absence of said surface antigen, the second or ultimate gene editing step can be one conferring resistance of the cells to a compound, preferably a chemotherapy drug or an immune suppressive agent. By doing so, the double or triple gene edited cells can be selected and enriched by a culture step that

takes place after the second or ultimate gene editing step. Also, the present method provides a first gene editing step into at least one gene encoding a T-Cell Receptor (TCR) component, in particular TCRalpha (Uniprot P01848) and TCRbeta (Uniprot P01850) and sequentially a second gene editing step into a gene expressing DCK, HPRT or GGH, to confer respectively resistance to PNA compounds, purine antimetabolites and antifolate compounds.

As a result, significant populations of triple gene edited cells can be obtained for therapeutic treatments, said cells having loci modified to reduce or inactivate the expression of:

- TCR; β 2m; DCK;
- TCR; PD1; DCK;
- TCR, CTLA4, DCK;
- TCR, LAG3, DCK;

- **Resistance to immune-suppressive treatments**

According to another aspect of the present invention, the engineering immune cells are made resistant to immune-depletion treatments, such as those involving glucocorticoids or antibodies directed against immune cells surface proteins. As an example, the antibody Alemtuzumab is used to deplete CD52 positive immune cells as in many pre-cancer treatments.

Also the method of the invention can comprise a gene editing step with respect to the genes encoding or regulating the expression of CD52 (Uniprot P31358) and/or GR (Glucocorticoids receptor also referred to as NR3C1 - Uniprot P04150), optionally in combination with a gene editing step leading to a reduction of the inactivation of the TCR. This approach was previously described by Poirot, L. et al. (Multiplex Genome-Edited T-cell Manufacturing Platform for "Off-the-Shelf" Adoptive T-cell Immunotherapies (2013) *Cancer Res.* 75:3853), but as part of a method where the different loci were simultaneously gene edited.

Preferred engineered immune cells are those triple or quadruple gene edited cells detailed herein, in which CD52 and or GR are additionally inactivated.

- **Improving CAR positive immune cell activity and survival**

As previously stated, the present method allows introducing successive gene editing modifications into immune primary cells in a time frame that limits the impact of the gene

editing steps on the subsequent expansion of these cells i.e. without reducing significantly production yields.

As shown in the examples, the present invention solves the problem of producing immune cells that express recombinant receptors, such as chimeric antigen receptors (CAR), which are triply gene edited. Representative examples of such cells obtainable according to the invention display the following phenotypes:

- [CAR CS1]^{pos}[β2m]^{neg}[TCR]^{pos}[PD1]^{neg};
- [CAR CD38]^{pos}[β2m]^{neg} [TCR]^{neg} [PD1]^{neg};
- [CAR CD70]^{pos}[β2m]^{neg} [TCR]^{neg} [PD1]^{neg};
- 10 - [CAR CD22]^{pos}[β2m]^{neg}[TCR]^{neg}[PD1]^{neg};
- [CAR ROR1]^{pos}[β2m]^{neg}[TCR]^{pos}[PD1]^{neg};
- [CAR CD123]^{pos}[β2m]^{neg} [TCR]^{neg} [PD1]^{neg};
- [CAR CD19]^{pos}[β2m]^{neg} [TCR]^{neg} [PD1]^{neg};
- [CAR CD33]^{pos}[β2m]^{neg}[TCR]^{neg}[PD1]^{neg};
- 15 - [CAR 5T4]^{pos}[β2m]^{neg}[TCR]^{pos}[PD1]^{neg};
- [CAR BCMA]^{pos}[β2m]^{neg} [TCR]^{neg} [PD1]^{neg};
- [CAR Flt3]^{pos}[β2m]^{neg} [TCR]^{neg} [PD1]^{neg};
- [CAR EGFRvIII]^{pos}[β2m]^{neg}[TCR]^{neg}[PD1]^{neg};
- [CAR WT1]^{pos}[β2m]^{neg}[TCR]^{pos}[PD1]^{neg};
- 20 - [CAR HSP70]^{pos}[β2m]^{neg} [TCR]^{neg} [PD1]^{neg};
- [CAR CLL1]^{pos}[β2m]^{neg} [TCR]^{neg} [PD1]^{neg};
- [CAR CS1]^{pos}[β2m]^{neg}[TCR]^{pos}[CTLA4]^{neg};
- [CAR CD38]^{pos}[β2m]^{neg} [TCR]^{neg} [CTLA4]^{neg};
- [CAR CD70]^{pos}[β2m]^{neg} [TCR]^{neg} [CTLA4]^{neg};
- 25 - [CAR CD22]^{pos}[β2m]^{neg}[TCR]^{neg}[CTLA4]^{neg};
- [CAR ROR1]^{pos}[β2m]^{neg}[TCR]^{pos}[CTLA4]^{neg};
- [CAR CD123]^{pos}[β2m]^{neg} [TCR]^{neg} [CTLA4]^{neg};
- [CAR CD19]^{pos}[β2m]^{neg} [TCR]^{neg} [CTLA4]^{neg};
- [CAR CD33]^{pos}[β2m]^{neg}[TCR]^{neg}[CTLA4]^{neg};

- [CAR 5T4]^{pos}[β2m]^{neg}[TCR]^{pos}[CTLA4]^{neg};
- [CAR BCMA]^{pos}[β2m]^{neg}[TCR]^{neg}[CTLA4]^{neg};
- [CAR Flt3]^{pos}[β2m]^{neg}[TCR]^{neg}[CTLA4]^{neg};
- [CAR EGFRvIII]^{pos}[β2m]^{neg}[TCR]^{neg}[CTLA4]^{neg};
- 5 - [CAR WT1]^{pos}[β2m]^{neg}[TCR]^{pos}[CTLA4]^{neg};
- [CAR HSP70]^{pos}[β2m]^{neg}[TCR]^{neg}[CTLA4]^{neg}; and
- [CAR CLL1]^{pos}[β2m]^{neg}[TCR]^{neg}[CTLA4]^{neg};

[TCR]^{neg} designate cells in which the expression of a component of the T-Cell receptor, such as TCRbeta or TCRalpha, has been reduced or impaired.

10 One preferred aspect of the present invention further concerns the problem of immune cells that express chimeric antigen receptors (CAR), which target surface molecules that are also present at the surface of said very immune cells. Such cells are typically noted:

- [anti X CAR]positive (+ or pos)[X]positive (+ or pos),

where X can be, for instance any of the antigen listed in table 4.

15 Negative impact has been observed, for instance with respect to T-cells expressing antigens CS1, CD38 or CD22 endowed with CARs targeting same: [anti-CS1 CAR]^{pos}, [CS1]^{pos}, [anti-CD38 CAR]^{pos} [CD38]^{pos} or [anti-CD70 CAR]^{pos} [CD70]^{pos}. The CAR positive primary immune cells can attack each other resulting into immune cell depletion. This is observed even when such cells were TCR negative [TCR]^{neg}

20 The present invention provides with a technical solution to this problem by providing a method, wherein gene editing steps are sequentially performed as outlined below:

- a first gene editing step is performed to inactivate the expression of the surface molecule X;

25 - a CAR is expressed targeting said surface molecule X, preferably by viral transduction; and

- a second gene editing step is performed to inactivate the expression of TCR;

- optionally, a third gene editing step to inactivate the expression of an immune checkpoint gene, such as PD1 or CTLA4.

30 The method results into a population of engineered [antigen X CAR]^{pos}[antigen X]^{neg}[TCR]^{neg} immune cells. Preferred engineered immune primary cells are triple gene edited cells, such as the following ones:

- [CAR CS1]^{pos}[CS1]^{neg}[TCR]^{pos}[PD1]^{neg};
- [CAR CD38]^{pos}[CD38]^{neg}[TCR]^{neg}[PD1]^{neg};

- [CAR CD70]^{pos}[CD70]^{neg}[TCR]^{neg}[PD1]^{neg};
- [CAR CD22]^{pos}[CD22]^{neg}[TCR]^{neg}[PD1]^{neg};
- [CAR CS1]^{pos}[CS1]^{neg}[TCR]^{neg}[CTLA4]^{neg};
- [CAR CD38]^{pos}[CD38]^{neg}[TCR]^{neg}[CTLA4]^{neg};
- 5 - [CAR CD70]^{pos}[CD70]^{neg}[TCR]^{neg}[CTLA4]^{neg};
- [CAR CD22]^{pos}[CD22]^{neg}[TCR]^{neg}[CTLA4]^{neg};
- [CAR CS1]^{pos}[CS1]^{neg}[TCR]^{neg}[β2m]^{neg};
- [CAR CD38]^{pos}[CD38]^{neg}[TCR]^{neg}[β2m]^{neg};
- [CAR CD70]^{pos}[CD70]^{neg}[TCR]^{neg}[β2m]^{neg};
- 10 - [CAR CD22]^{pos}[CD22]^{neg}[TCR]^{neg}[β2m]^{neg};

Activation and expansion of T cells

Whether prior to or after genetic modification, the immune cells according to the present invention can be activated or expanded, even if they can activate or proliferate independently of antigen binding mechanisms. T-cells, in particular, can be activated and expanded using methods as described, for example, in U.S. Patents 6,352,694; 6,534,055; 6,905,680; 6,692,964; 5,858,358; 6,887,466; 6,905,681; 7,144,575; 7,067,318; 7,172,869; 7,232,566; 7,175,843; 5,883,223; 6,905,874; 6,797,514; 6,867,041; and U.S. Patent Application Publication No. 20060121005. T cells can be expanded *in vitro* or *in vivo*. T cells are generally expanded by contact with an agent that stimulates a CD3 TCR complex and a co-stimulatory molecule on the surface of the T cells to create an activation signal for the T-cell. For example, chemicals such as calcium ionophore A23187, phorbol 12-myristate 13-acetate (PMA), or mitogenic lectins like phytohemagglutinin (PHA) can be used to create an activation signal for the T-cell.

As non-limiting examples, T cell populations may be stimulated *in vitro* such as by contact with an anti-CD3 antibody, or antigen-binding fragment thereof, or an anti-CD2 antibody immobilized on a surface, or by contact with a protein kinase C activator (e.g., bryostatin) in conjunction with a calcium ionophore. For co-stimulation of an accessory molecule on the surface of the T cells, a ligand that binds the accessory molecule is used. For example, a population of T cells can be contacted with an anti-CD3 antibody and an anti-CD28 antibody, under conditions appropriate for stimulating proliferation of the T cells. Conditions appropriate for T cell culture include an appropriate media (e.g.,

Minimal Essential Media or RPMI Media 1640 or, X-vivo 5, (Lonza)) that may contain factors necessary for proliferation and viability, including serum (e.g., fetal bovine or human serum), interleukin-2 (IL-2), insulin, IFN- γ , IL-4, IL-7, GM-CSF, IL-10, IL-12, IL-15, TGF β , and TNF- or any other additives for the growth of cells known to the skilled artisan.

5 Other additives for the growth of cells include, but are not limited to, surfactant, plasmanate, and reducing agents such as N-acetyl-cysteine and 2-mercaptoethanol. Media can include RPMI 1640, A1M-V, DMEM, MEM, α -MEM, F-12, X-Vivo 1, and X-Vivo 20, Optimizer, with added amino acids, sodium pyruvate, and vitamins, either serum-free or supplemented with an appropriate amount of serum (or plasma) or a defined set of hormones, and/or an
10 amount of cytokine(s) sufficient for the growth and expansion of T cells. Antibiotics, e.g., penicillin and streptomycin, are included only in experimental cultures, not in cultures of cells that are to be infused into a subject. The target cells are maintained under conditions necessary to support growth, for example, an appropriate temperature (e.g., 37° C) and atmosphere (e.g., air plus 5% CO₂). T-cells that have been exposed to varied stimulation
15 times may exhibit different characteristics

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

Therapeutic compositions and applications

20 The method of the present invention described above allows producing engineered primary immune cells within a limited time frame of about 15 to 30 days, preferably between 15 and 20 days, and most preferably between 18 and 20 days so that they keep their full immune therapeutic potential, especially with respect to their cytotoxic activity.

These cells can form or be members of populations of cells, which preferably originate
25 from a single donor or patient. These populations of cells can be expanded under closed culture recipients to comply with highest manufacturing practices requirements and can be frozen prior to infusion into a patient, thereby providing "off the shelf" or "ready to use" therapeutic compositions.

As per the present invention, a significant number of cells originating from the same
30 Leukapheresis can be obtained, which is critical to obtain sufficient doses for treating a patient. Although variations between populations of cells originating from various donors may be observed, the number of immune cells procured by a leukapheresis is generally about from 10⁸ to 10¹⁰ cells of PBMC. PBMC comprises several types of cells: granulocytes, monocytes and lymphocytes, among which from 30 to 60 % of T-cells, which generally
35 represents between 10⁸ to 10⁹ of primary T-cells from one donor. The method of the present

invention generally ends up with a population of engineered cells that reaches generally more than about 10^8 T-cells, more generally more than about 10^9 T-cells, even more generally more than about 10^{10} T-cells, and usually more than 10^{11} T-cells. In general, the T-cells are gene edited at least at two different loci.

5 Such compositions or populations of cells can therefore be used as a medicament; especially for treating cancer, particularly for the treatment of lymphoma, but also for solid tumors such as melanomas, neuroblastomas, gliomas or carcinomas such as lung, breast, colon, prostate or ovary tumors in a patient in need thereof.

10 The invention is more particularly drawn to populations of primary TCR negative T-cells originating from a single donor, wherein at least 20 %, preferably 30 %, more preferably 50 % of the cells in said population have been modified using sequence-specific reagents in at least two, preferably three different loci.

In another aspect, the present invention relies on methods for treating patients in need thereof, said method comprising at least one of the following steps:

- 15 (a) Determining specific antigen markers present at the surface of patients tumors biopsies;
- (b) providing a population of engineered primary immune cells engineered by one of the methods of the present invention previously described expressing a CAR directed against said specific antigen markers;
- 20 (c) Administrating said engineered population of engineered primary immune cells to said patient,

Generally, said populations of cells mainly comprises CD4 and CD8 positive immune cells, such as T-cells, which can undergo robust *in vivo* T cell expansion and can persist for an extended amount of time *in-vitro* and *in-vivo*.

25 The treatments involving the engineered primary immune cells according to the present invention can be ameliorating, curative or prophylactic. It may be either part of an autologous immunotherapy or part of an allogenic immunotherapy treatment. By autologous, it is meant that cells, cell line or population of cells used for treating patients are originating from said patient or from a Human Leucocyte Antigen (HLA) compatible donor. By allogeneic
30 is meant that the cells or population of cells used for treating patients are not originating from said patient but from a donor.

In another embodiment, said isolated cell according to the invention or cell line derived from said isolated cell can be used for the treatment of liquid tumors, and preferably of T-cell acute lymphoblastic leukemia.

Adult tumors/cancers and pediatric tumors/cancers are also included.

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

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

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

The administration of the cells or population of cells can consist of the administration
20 of 10^4 - 10^9 cells per kg body weight, preferably 10^5 to 10^6 cells/kg body weight including all integer values of cell numbers within those ranges. The present invention thus can provide more than 10, generally more than 50, more generally more than 100 and usually more than 1000 doses comprising between 10^6 to 10^8 gene edited cells originating from a single donor's or patient's sampling.

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

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

In certain embodiments of the present invention, cells are administered to a patient in conjunction with (e.g., before, simultaneously or following) any number of relevant treatment modalities, including but not limited to treatment with agents such as antiviral therapy, 5 cidofovir and interleukin-2, Cytarabine (also known as ARA-C) or natalizimab treatment for MS patients or efalizimab treatment for psoriasis patients or other treatments for PML patients. In further embodiments, the T cells of the invention may be used in combination 10 with chemotherapy, radiation, immunosuppressive agents, such as cyclosporin, azathioprine, methotrexate, mycophenolate, and FK506, antibodies, or other immunoablative agents such as CAMPATH, anti-CD3 antibodies or other antibody therapies, cytoxin, fludarabine, cyclosporin, FK506, rapamycin, mycophenolic acid, steroids, FR901228, cytokines, and irradiation. These drugs inhibit either the calcium dependent phosphatase calcineurin 15 (cyclosporine and FK506) or inhibit the p70S6 kinase that is important for growth factor induced signaling (rapamycin) (Henderson, Naya et al. 1991; Liu, Albers et al. 1992; Bierer, Hollander et al. 1993). In a further embodiment, the cell compositions of the present invention are administered to a patient in conjunction with (e.g., before, simultaneously or following) bone marrow transplantation, T cell ablative therapy using either 20 chemotherapy agents such as, fludarabine, external-beam radiation therapy (XRT), cyclophosphamide, or antibodies such as OKT3 or CAMPATH, In another embodiment, the cell compositions of the present invention are administered following B-cell ablative therapy such as agents that react with CD20, e.g., Rituxan. For example, in one embodiment, subjects may undergo standard treatment with high dose chemotherapy followed by 25 peripheral blood stem cell transplantation. In certain embodiments, following the transplant, subjects receive an infusion of the expanded immune cells of the present invention. In an additional embodiment, expanded cells are administered before or following surgery.

Combination therapy involving at least two sub-populations of T cells.

30

The present invention encompasses a whole range of double, triple or quadruple gene edited cells now available for therapeutic use, including any of those illustrated herein, which could not be obtained by the prior art methods. Especially, those cells are engineered with a reduced risk of unwanted recombination or translocation at the different gene edited 35 loci, making them safer for therapeutic use.

As a further advantage of the present method of sequential gene editing is also the possibility to create subpopulations of primary immune cells from an initial population originating from a single donor or patient, which subpopulations are gene edited at different loci.

5 As an example, the primary immune cells from the donor or patient can be made less-alloreactive by performing a first gene editing step into a TCR gene or any gene implicated in the self/non-selfrecognition, and then after an expansion step, the population can be split into two subpopulations, which respectively undergo a second gene editing step that will target distinct loci in said subpopulations. Typically, CD4+ positive and CD8+ positive immune cells
10 can be (see figure 8) treated separately before being pooled together at a desired ratio to increase potency of the therapeutic compositions. This method will result into subpopulations of engineered primary immune cells that will not display exactly the same properties. Accordingly, the present invention is also drawn to compositions of populations of primary TCR negative T-cells resulting from a single donor comprising at least two subpopulations of
15 T-cells, said subpopulations comprising, for instance different gene edited immune checkpoint genes. Such sub-populations of cells can be selected, for instance, from:

- TCR negative and PD1 negative,
- TCR negative and CD52 negative,
- TCR negative and CTLA4 negative,
- 20 - TCR negative and dCK negative,
- TCR negative and GR negative,
- TCR negative and GGH negative,
- TCR negative and HPRT negative,
- TCR negative and β 2m negative.

25 The resulting cells can be optionally transformed to express chimeric antigen receptor to provide allogeneic CAR T Cells with various specificities, in particular as part of subpopulations expressing chimeric receptors respectively directed to different surface molecules.

Such sub-populations can be used separately or in combination with each other into
30 compositions for therapeutic treatments, in the same way as previously described with a single population of cells.

Other definitions

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

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

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

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

- Chimeric antigen receptor (CAR) is a term that encompasses molecules which combine an extracellular binding domain against a component present on the target cell, for example an antibody-based specificity for a desired antigen (e.g. , tumor antigen) with a T cell receptor-activating intracellular domain to generate a chimeric protein that exhibits a specific anti-target cellular immune activity. Generally, CAR consists of an extracellular single chain antibody (scFv), comprising the light (V_L) and the heavy (V_H) variable fragment of a target antigen specific monoclonal antibody joined by a flexible linker, fused to the intracellular signaling domain of the T cell antigen receptor complex zeta chain and have the ability, when expressed in immune effector cells, to redirect antigen recognition based on the monoclonal antibody's specificity. CAR can be single-chain or multi-chain as described in WO2014039523. Binding domain other than scFv can also be used for predefined targeting

of lymphocytes, such as camelid or shark (VNAR) single-domain antibody fragments or receptor ligands like a vascular endothelial growth factor polypeptide, an integrin-binding peptide, heregulin or an IL-13 mutein, antibody binding domains, antibody hypervariable loops or CDRs as non-limiting examples.

5 - “Recombinant TCR” are artificial polypeptide constructs consisting preferably of a single amino acid strand, which like native heterodimeric TCRs bind to MHC-peptide complexes. Recombinant TCRs are preferably single-chain polypeptides, such as described by Stone J.D., et al. [A novel T cell receptor single-chain signaling complex mediates antigen-specific T cell activity and tumor control (2014) *Cancer Immunol. Immunother.*
10 63(11):1163-76], Such single chain TCRs generally comprise:

- an α segment constituted by a human TCR α chain variable region sequence fused to the N terminus of a human TCR α chain constant region extracellular sequence,

- a β segment constituted by a human TCR β chain variable region sequence fused to the N terminus of a human TCR β chain constant region extracellular sequence, and

15 - a linker sequence linking the C terminus of the α segment to the N terminus of the β segment, or vice versa, the constant region extracellular sequences of the α and β segments being linked by a disulfide bond,

the length of the linker sequence and the position of the disulfide bond being such that the variable region sequences of the α and β segments are mutually orientated substantially as
20 in native $\alpha\beta$ T cell receptors.

- The term “endonuclease” refers to any wild-type or variant enzyme capable of catalyzing the hydrolysis (cleavage) of bonds between nucleic acids within a DNA or RNA molecule, preferably a DNA molecule. Endonucleases do not cleave the DNA or RNA molecule irrespective of its sequence, but recognize and cleave the DNA or RNA molecule at
25 specific polynucleotide sequences, further referred to as “target sequences” or “target sites”. Endonucleases can be classified as rare-cutting endonucleases when having typically a polynucleotide recognition site greater than 10 base pairs (bp) in length, more preferably of 14-55 bp. Rare-cutting endonucleases significantly increase homologous recombination by inducing DNA double-strand breaks (DSBs) at a defined locus thereby allowing gene repair
30 or gene insertion therapies (Pingoud, A. and G. H. Silva (2007). Precision genome surgery. *Nat. Biotechnol.* 25(7): 743-4.).

- by “DNA target”, “DNA target sequence”, “target DNA sequence”, “nucleic acid target sequence”, “target sequence”, or “processing site” is intended a polynucleotide sequence that can be targeted and processed by a rare-cutting endonuclease according to

the present invention. These terms refer to a specific DNA location, preferably a genomic location in a cell, but also a portion of genetic material that can exist independently to the main body of genetic material such as plasmids, episomes, virus, transposons or in organelles such as mitochondria as non-limiting example. As non-limiting examples of RNA
5 guided target sequences, are those genome sequences that can hybridize the guide RNA which directs the RNA guided endonuclease to a desired locus.

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

- by "variant" is intended a catalytically active mutant of an endonuclease reagent according to the present invention.

- the term "locus" is the specific physical location of a DNA sequence (e.g. of a gene)
15 into a genome. The term "locus" can refer to the specific physical location of a rare-cutting endonuclease target sequence on a chromosome or on an infection agent's genome sequence. Such a locus can comprise a target sequence that is recognized and/or cleaved by a sequence-specific endonuclease according to the invention. It is understood that the
20 locus of interest of the present invention can not only qualify a nucleic acid sequence that exists in the main body of genetic material (i.e. in a chromosome) of a cell but also a portion of genetic material that can exist independently to said main body of genetic material such as plasmids, episomes, virus, transposons or in organelles such as mitochondria as non-limiting examples.

- The term "cleavage" refers to the breakage of the covalent backbone of a
25 polynucleotide. Cleavage can be initiated by a variety of methods including, but not limited to, enzymatic or chemical hydrolysis of a phosphodiester bond. Both single-stranded cleavage and double-stranded cleavage are possible, and double-stranded cleavage can occur as a result of two distinct single-stranded cleavage events. Double stranded DNA, RNA, or
30 DNA/RNA hybrid cleavage can result in the production of either blunt ends or staggered ends.

-"identity" refers to sequence identity between two nucleic acid molecules or polypeptides. Identity can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the compared sequence is

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

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

- The above written description of the invention provides a manner and process of making and using it such that any person skilled in this art is enabled to make and use the same, this enablement being provided in particular for the subject matter of the appended claims, which make up a part of the original description.

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

Having generally described this invention, a further understanding can be obtained by reference to certain specific examples, which are provided herein for purposes of illustration only, and are not intended to limit the scope of the claimed invention.

EXAMPLES

5 **Example 1: Simultaneous vs. sequential electroporation of TRAC and CD52 TALE-nucleases into T cells.**

In order to analyze the impact of sequential electroporation of TALE-nuclease reagents on the overall survival of T-cells and gene knock-out efficiency, we have submitted
10 activated primary human T cells from a single donor sample to electroporation by using TALEN[®] reagents (Cellestis, Paris, France) specific for TRAC gene (TCR alpha chain) according to the following experimental procedure. The amino acid sequences of the various TALEN[®] heterodimers used in this experiment are given in Table 3):

Briefly, frozen human PBMCs (AllCells) were thawed and activated using anti CD3
15 and anti CD28 antibodies-coated beads (Dynabeads, Life Technologies, Carlsbad, California, United States) for 3 days. After magnetic beads removal (Day 4), 5x10⁶ activated T cells were transfected with 10µg of both mRNA encoding TALEN either simultaneously or sequentially with a 6 hour, 20 hour or 40 hour intervals by using AgilePulse electroporator (BTX Instrument Division, Harvard Apparatus, Inc., Holliston, MA 01746-1388) protocols.
20 Electroporated T cells were plated back in tissue culture vessels in Xvivo hematopoietic medium (Lonza, CH-4002 Basel, Switzerland) supplemented with 5% human AB serum and rIL2 (100 UI/ml) for a total of 12 days. Cells were passaged every 2 or 3 days for numeration and media renewal.

Surface expression of TCR and CD52 protein was measured by cytometry using
25 specific monoclonal antibodies and a Macsquant cytometer (Miltenyi) at D10 post activation. Furthermore, T cells growth was monitored from D4 to D12, by Trypan blue exclusion cell numeration.

On figure 9 are presented the percentage of TCR+, CD52+, double positive TCR/CD52+ and double negative TCR/CD52- cells measured 6 days after electroporation
30 according to the electroporation schedule. The data show that 70 to 80% of TCR KO and 65 to 75% of CD52 KO is achievable whatever the electroporation conditions, sequential or simultaneous. Furthermore, the percentage of double negative cells is comparable for all the tested conditions. This validates the finding that sequential electroporation does not reduce the rate of gene editing success.

35 Having shown that sequential electroporation does not affect the efficiency of TALE-nuclease mediated gene knock out, we sought to see if cellular growth was impaired when

cells are electroporated twice within 24 or 48h intervals, by monitoring electroporation conditions, cell and growth.

Data shown in figure 10 indicated that cellular growth was similar for all tested conditions with an advantage for the T cells sequentially transfected with a 40h interval.

5 Altogether, these data demonstrated that sequential electroporation does not affect KO efficiency and cellular growth.

Example 2: Simultaneous or sequential electroporation of mRNA encoding TRAC and PD1 TALE-nucleases into T cells.

10 Simultaneous electroporation of two TALE-Nuclease heterodimers was found to be highly efficient for performing simultaneous gene knock-out in Poirot et al. (Multiplex Genome-Edited T-cell Manufacturing Platform for “Off-the-Shelf” Adoptive T-cell Immunotherapies (2015) *Cancer Res.* 75: 3853-64). However, unwanted events such as off-site cleavage or translocation were found to occur in certain instances. For instance, co-
15 transfection of mRNA encoding TRAC TALEN[®] and PD1 TALEN[®] leads to the occurring of off-site cleavage activity due to the pairing of the TRAC TALEN left arm and PD1 TALEN left arm. Frequency of off-site cleavage (as well as on-site cleavage) is defined by the frequency of mutated sequences (either nucleotide deletion or addition) that are generated after endonucleases cleavage and unfaithful religation of broken ends. The amino acid sequences
20 used in this experiment encoding the various TALEN[®] heterodimers for TRAC (SEQ ID NO.1 and 2) and PD1 (SEQ ID NO. 5 and 6) are given in Table 3)

Potential off-site hits can be identified *in silico* with an algorithm according to several parameters including mainly TALE-nuclease DNA binding sequences, number of mismatches and position of those, length of the spacer between the 2 binding domains.
25 According to this computer search, numerous potential off-site targets have been identified in the human genome for TRAC and PD1 TALEN[®] combination. The 15 first target sequences with the highest score have been verified experimentally by PCR and Deep Sequencing. One out of the 15, “Off-Site 3”, has been found to be a true off-site target since about 1% of mutagenesis events (or Insertion/deletion, InDels events) was observed when T cell were
30 transfected simultaneously with TRAC and PD1 TALEN[®].

Since the data showed in example 1, demonstrated that sequential electroporation of TALEN did not affect KO efficiency and cell expansion, we sought to determine whether this sequential transfection strategy could abolish cleavage into the “Off-Site 3”.

Briefly, frozen human PBMCs (AllCells) were thawed and activated using anti CD3 and anti CD28 antibodies-coated beads (Dynabeads, LifeTech) for 3 days. 5×10^6 activated T cells were transfected with 10 μ g of both TALEN mRNA either simultaneously or sequentially with a 6 hour, 20 hour or 40 hour intervals by using AgilePulse electroporator (BTX Instrument Division, Harvard Apparatus, Inc., Holliston, MA 01746-1388) protocols. Electroporated T cells were plated back in tissue culture vessels in Xvivo (Lonza) media supplemented with 5% human AB serum and rIL2 (100 UI/ml) for a total of 6 days. Cells were passaged every 2 or 3 days for numeration and media renewal. At the end of the 6 day period, genomic DNA was extracted from transfected T cells and subjected to PCR amplification using specific primers allowing the amplification of TRAC and PD1 "On-Site" genomic targets and 5 "Off-Site" targets including "Off-Site 3". PCR products are then purified and modified for subsequent deep sequencing analysis using the Illumina technology. About 80,000 reads for each samples were computed.

Data are presented on figure 11. As previously observed, the simultaneous transfection of TRAC and PD-1 TALEN[®] into T-cells induces a high level of indels at TRAC and PD-1 on-site targets respectively. About 1% of indels is also observed at the "Off-Site 3" target whereas no significant mutagenesis event is observed for the other predicted off-site targets OF1, OF2, OF4 and OF5. Sequential transfection of TRAC TALEN first and PD-1 TALEN 24h or 40h later has no or minor impact on the level of mutagenesis at on site TRAC and On site PD-1. However, we observed a significant decrease of the % of indels for the OF3 at 24h (0.4%) and 40h (0,04%), These data indicate that sequential electroporation of TALEN allows the abrogation of unwanted events such as off-site cleavage without affecting KO efficiency.

Example 3: Simultaneous or sequential electroporation of CD38, TRAC and CD52 TALEN into T cells.

In order to analyze the impact of sequential TALEN electroporation on overall survival of T cells and gene knock-out efficiency, we have transfected activated human T cells with TALENs specific for TRAC gene (TCR alpha chain), CD52 and CD38 (Amino acid sequences are given in Table 3) according to the following experimental procedure:

Briefly, frozen human PBMCs (AllCells) are thawed and activated using anti CD3 and anti CD28 antibodies-coated beads (TransAct, Miltenyi) for 3 days. At day 4 post thawing, 10×10^6 activated T cells are transfected with 10 μ g of both TALEN mRNA either

simultaneously or sequentially with a 24 hour (Day 5) or 48 hour (Day 6) intervals using Collectis proprietary AgilPulse electroporator and protocols. The following conditions have been tested and compared in terms of KO efficiency and cell growth.

5 **Table 2:** gene editing efficiency of the sequential gene editing strategies

	Transfection Day post thawing			% cells in the population			
	D4	D5	D6	[CD38] ⁻	[TCRαβ] ⁻	[CD52] ⁻	[CD38] ⁻ [TCRαβ] ⁻ [CD52] ⁻
A	CD38/TRAC/CD52			66.1	67.8	57	35.2
B	CD38	TRAC/CD52		63.2	64.6	64.6	27.1
C	CD38		TRAC/CD52	63.8	68	60.6	36.7
D	CD38/TRAC	CD52		61.6	73.8	50.5	27.6
E	CD38/TRAC		CD52	67.5	72.9	69.4	40.2
F	CD38/CD52	TRAC		65.7	69.4	63.2	34.7
G	CD38/CD52		TRAC	65.6	73.7	61.9	35.7
H	CD38	TRAC	CD52	61.2	69.8	58.3	28.3
I	CD38	CD52	TRAC	59.2	73.4	56.9	28.4
	Untransfected						0,1

Electroporated T cells are plated back in tissue culture vessels in Xvivo (Lonza) media supplemented with 5% human AB serum and rIL2 (100 UI/ml) for a total of 15 days. Cells are passaged every 2 or 3 days for numeration and media renewal.

Surface expression of TCR, CD38 and CD52 protein is measured by cytometry using specific antibodies and a Canto10 cytometer (Becton-Dickinson) at D13 post thawing. Furthermore, T cells growth is monitored from Day 5 to Day15 by Trypan blue exclusion cell numeration.

Table 2 presents the percentage of single negative T cells (CD38⁻, TCR⁻ and CD52⁻) and the percentage of triple negative T cells (CD38⁻TCR⁻CD52⁻), according to the electroporation scheduling. These data show that 65 to 74% of TCR KO, 57 to 70% of CD52

KO, thus comparable among conditions. The best results is obtained when CD38 and TRAC TALEN are transfected 48h prior to CD52 TALEN transfection (Table 2, row E and figure 12, row E). The best results are obtained when CD38 and TRAC TALEN are transfected 48h prior to CD52 TALEN transfection, as for TCR and CD52 (Table 4, row E and Figure 12, row E). However, all tested conditions show little variation in the % of triple negative T cells ranging from 27% to 40%. Furthermore, the best percentage of triple knock out efficiency is obtained with sequential electroporation of CD38 and TRAC TALEN 48h prior to CD52 TALEN.

Having validated that sequential electroporation does not affect the efficacy of TALEN mediated gene knock out, we sought to see if cellular growth is impaired when cells are subjected to 2 electroporation shocks within 24 or 48h intervals. According to electroporation conditions, cell growth is measured from day 5 to Day 13 post-thawing (Figure 12).

At day 15, the best growth rate is observed when the 3 TALEN are electroporated simultaneously. T cell growth curves for all sequential electroporation conditions are comparable with best results for conditions where a 48 h interval is performed between the two electroporation shocks (conditions C, E and G of Table 4 and Figure 12).

These data indicate that cellular growth is similar for all tested sequential conditions with an advantage for T cells sequentially transfected with a 48h interval.

Altogether, these data demonstrate that sequential electroporation does not affect KO efficiency and cellular growth.

Example 4: Generation of triple KO (TCR/PD-1/B2M) CAR CD22 T cells.

T-cells were cultured from PBMC and activated as described in Example 1, in order to produce [TCR]^{neg}[PD1]^{neg}[B2M]^{neg} therapeutic immune cells endowed with a CAR directed against CD22 antigen.

Furthermore, in order to analyze the impact of sequential TALEN electroporation on gene knock-out efficiency and triple KO CAR T cells activity, the cells were edited either simultaneously or sequentially with TALEN specific for TRAC gene (SEQ ID NO. 1 and 2), PD-1 (SEQ ID NO. 9 and 6) and B2M (beta-2-microglobulin, SEQ ID NO. 10 and 11). The sequential protocol is illustrated in Figure 13. In both cases, T-cells were transduced with lentiviral particles for the expression of CAR targeting the antigen CD22 (SEQ ID NO. 12).

TALEN mRNA were generated from linearized plasmid DNA encoding each TALEN arm of interest. An in vitro RNA synthesis kit for RNA generation was used (Invitrogen #AMB1345-5). RNA was purified using the Qiagen RNeasy Kit (#74106) and eluted into T solution from BTX (47-0002).

5 Frozen human PBMCs from two different donors are thawed at 2×10^6 cells per ml on day prior activation and transduction step, in complete X-Vivo media (X-VIVO 15, Lonza#04-418Q; 5% Human serum AB, Gemini #100-318; 20ng/mL IL-2, Miltenyi#130-097-743;). One day post thawing, cells are transduced as described in (Poirot et al. (2015) Multiplex Genome-Edited T-cell Manufacturing Platform for "Off-the-Shelf" Adoptive T-cell
10 Immunotherapies *Cancer Res.* 75: 3853-64) with lentiviral particles allowing the expression of a Chimeric Antigen Receptor targeting CD22 antigen containing a mimotope sequence (highlighted in bold in SEQ ID NO. 12). Cells are further activated the same day using anti CD3 and anti CD28 antibodies-coated beads (TransAct, Miltenyi) according to manufacturer's protocol for 4 days.

15 At day 5 post thawing, T cells were electroporated with a dose response of mRNAs encoding TRAC TALEN (10 μ g), PD-1 TALEN (from 30 μ g to 70 μ g) and B2M TALEN (from 30 μ g to 70 μ g) either simultaneously or sequentially with a 48 hour intervals using Celectis proprietary AgilPulse electroporator and protocols. After each electroporation step cells were incubated for 15minutes at 30°C and then incubated at 37°C. Thirteen days post thawing
20 positive T-cells were analyzed for triple KO efficacy by first re-stimulating a portion of T cells with TransACT to induce PD-1 expression. Two days later, re-stimulated cells were labeled with antibodies at a 1:50 dilution of each antibody for 15 minutes at 4°C (Miltenyi; TCR#130-091-236, HLA-ABC#130-101-467, PD-1#130-099-878). For all the different donors tested sequential editing provide the best triple KO efficacy ranging from 20 up to 40% (Figure 14).

25 Triple KO T-cells were then enriched using a biotin and column based negative purification system for TCR and B2M dKO cells (Miltenyi; biotin-TCR #130-098-219, bitoin-HLA-ABC#130-101-463, Biotin beads #130-090-485, MS columns#130-042-201). Under this purification scheme, only TCR and B2M positive cells bind the MS column, and the TCR/B2M dKO cells of interest are enriched in the flowthrough fraction with 97% or greater
30 purity. Triple KO CAR-T cells enriched for TCR/B2M dKO were further incubated for an additional two days before assessing CAR-T cells activity. On day 15, T cells were analyzed for CD22 CAR cytotoxicity by co-culturing T cells with CD22 expressing Raji-Luciferase+ targets at effector to target ratios of 30:1, 15:1, 5:1, and 1:1 for 5 hours before luminescence was quantified using the ONE Glo luminescence kit (Promega). Figure 15 demonstrates that

triple KO CD22 CAR T were as active as their wild type counter part (non gene edited T-cells endowed with the same CAR CD22)

Table 3: Sequence of TALEN[®] used in experiments.

5

Sequence name	Ref. sequence	Amino acid sequence of TALEN [®] used in experiments
TRAC TALEN Left	SEQ ID NO.1	MGDPKKKRKRVIDIADLRTLGYSSQQQEKIKPKVRSTVAQHHEALVGHGF THAHIVALSQHPAALGTAVVKYQDMIAALPEATHEAIVGVGKQWSGAR ALEALLTVAGELRGPPLQLDTGQLLKIARGGVTAVEAVHAWRNALTGA PLNLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLTPQQVVAIASN NNGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNNGGKQALETVQRLL PVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVV AIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETV QRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQALLPVLCQAHGLTPE QVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQAL ETVQALLPVLCQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAH GLTPEQVVAIASNIGGKQALETVQALLPVLCQAHGLTPQQVVAIASNNG GKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQALLPVLC QAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLTPEQVVAIA SHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNNGGGRPALESIVAQ LSRPDPALAALNDHLVALACLGGRPALDAVKKGLGDPISRSQLVKSELEE KKSELRHKLKYPHEYELIEIARNSTQDRILEMKVMEFFMKVYGYRGKHL GGSRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMQRYVEEN QTRNKHINPNEWVKVYPSVTEFKFLVSGHFKGNYKAQLTRLNHITNC NGAVLSVEELLIGGEMIKAGTLTLEEVRKFNNGEINFAAD
TRAC TALEN Right	SEQ ID NO.2	MGDPKKKRKRVIDIADLRTLGYSSQQQEKIKPKVRSTVAQHHEALVGHGF THAHIVALSQHPAALGTAVVKYQDMIAALPEATHEAIVGVGKQWSGAR ALEALLTVAGELRGPPLQLDTGQLLKIARGGVTAVEAVHAWRNALTGA PLNLTPQQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIASN GGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLP VLCQAHGLTPEQVVAIASNIGGKQALETVQALLPVLCQAHGLTPQQVVA IASNNGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQ RLLPVLCQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLTPQ QVVAIASNNGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNNGGKQA LETVQRLLPVLCQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAH GLTPEQVVAIASNIGGKQALETVQALLPVLCQAHGLTPEQVVAIASHDG GKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQALLPVLC QAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIAS NNGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNNGGGRPALESIVAQ SRPDPALAALNDHLVALACLGGRPALDAVKKGLGDPISRSQLVKSELEE KKSELRHKLKYPHEYELIEIARNSTQDRILEMKVMEFFMKVYGYRGKHL GGSRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMQRYVEEN QTRNKHINPNEWVKVYPSVTEFKFLVSGHFKGNYKAQLTRLNHITNC NGAVLSVEELLIGGEMIKAGTLTLEEVRKFNNGEINFAAD

<p>CD52 TALEN Left</p>	<p>SEQ ID NO.3</p>	<p>MGDPKKKRKVIDIADLRTLGYSSQQQEKIKPKVRSTVAQHHEALVGHGF THAHIVALSQHPAALGTAVVKYQDMIAALPEATHEAIVGVGKQWSGAR ALEALLTVAGELRGPPLQLDTGQLLKIARGGVTAVEAVHAWRNALTGA PLNLTPQQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPEQVVAIASH DGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLP VLCQAHGLTPQQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPEQVV AIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETV QRLLPVLCQAHGLTPQQVVAIASNGGGKQALETVQRLLPVLCQAHGLTP EQVVAIASNIGGGKQALETVQALLPVLCQAHGLTPEQVVAIASHDGGKQA LETVQRLLPVLCQAHGLTPQQVVAIASNGGGKQALETVQRLLPVLCQAH GLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIG GKQALETVQALLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLC QAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIA SNIGGGKQALETVQALLPVLCQAHGLTPQQVVAIASNGGGKQALETVQRLL LSRPDPAALAALNDHLVALACLGGRPALDAVKKGLGDPISRSQVLKSELEE KKSELRHKLKYPHEYIELIEIARNSTQDRILEMKVMEFFMKVYGYRGKHL GGSRKPDAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMQRYVEEN QTRNKHINPNEWVKVYPSVTEFKFLFVSGHFKGNYKAQLTRLNHITNC NGAVLSVEELLIGGEMIKAGTLTLEEVRKFNNGEINFAAD</p>
<p>CD52 TALEN Right</p>	<p>SEQ ID NO.4</p>	<p>MGDPKKKRKVIDIADLRTLGYSSQQQEKIKPKVRSTVAQHHEALVGHGF THAHIVALSQHPAALGTAVVKYQDMIAALPEATHEAIVGVGKQWSGAR ALEALLTVAGELRGPPLQLDTGQLLKIARGGVTAVEAVHAWRNALTGA PLNLTPQQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPQQVVAIASN NGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLP VLCQAHGLTPQQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPEQVV AIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNGGGKQALET VQRLLPVLCQAHGLTPQQVVAIASNGGGKQALETVQRLLPVLCQAHGLT PEQVVAIASNIGGGKQALETVQALLPVLCQAHGLTPEQVVAIASHDGGKQ ALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQA HGLTPQQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPQQVVAIASN NGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNGGGKQALETVQRLL PVLCQAHGLTPEQVVAIASNIGGGKQALETVQALLPVLCQAHGLTPEQVV AIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNGGGKQALETV VAQLSRPDPAALAALNDHLVALACLGGRPALDAVKKGLGDPISRSQVLKS ELEEKSELRHKLKYPHEYIELIEIARNSTQDRILEMKVMEFFMKVYGYR GKHLGGSRKPDAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMQRY VEENQTRNKHINPNEWVKVYPSVTEFKFLFVSGHFKGNYKAQLTRLN HITNCNGAVLSVEELLIGGEMIKAGTLTLEEVRKFNNGEINFAAD</p>
<p>PD-1 TALEN Left</p>	<p>SEQ ID NO.5</p>	<p>MGDPKKKRKVIDIADLRTLGYSSQQQEKIKPKVRSTVAQHHEALVGHGF THAHIVALSQHPAALGTAVVKYQDMIAALPEATHEAIVGVGKQWSGAR ALEALLTVAGELRGPPLQLDTGQLLKIARGGVTAVEAVHAWRNALTGA PLNLTPQQVVAIASNGGGKQALETVQALLPVLCQAHGLTPEQVVAIASH DGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLP VLCQAHGLTPQQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPEQVV AIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNGGGKQALET VQRLLPVLCQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGL TPQQVVAIASNGGGKQALETVQRLLPVLCQAHGLTPQQVVAIASGRGG KQALETVQRLLPVLCQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLC QAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLTPQQVVAIA SNNGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQR</p>

		LLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQALLPVLCQAHGLTPQQVVAIASNNGGRRPALE SIVAQLSRPDPALAALTNDHLVALACLGGRRPALDAVKKGLGDPISRSQLV KSELEKKSELRHKLKYVPHEYIELIEIARNSTQDRILEMKVMEFFMKVYGY RGKHLGGSRRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMQR YVEENQTRNKHINPNEWVKVYSSVTEFKFLFVSGHFKGNYKAQLTRLN HITNCNGAVLSVEELLIGGEMIKAGTLTLEEVRRKFNNGEINFAAD
PD-1 TALEN Right	SEQ ID NO.6	MGDPKKRKRVIDIADLRTLGYSSQQQEKIKPKVRSTVAQHHEALVGHGF THAHIVALSQHPAALGTAVVKYQDMIAALPEATHEAIVGVGKQWSGAR ALEALLTVAGELRGPPQLDGTGQLLKIARGGVTAVEAVHAWRNALTGA PLNLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIASN GGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLP VLCQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLTPQQVV AIASNNGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNNGGKQALET VQRLLPVLCQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGL TPEQVVAIASNIGGKQALETVQALLPVLCQAHGLTPQQVVAIASNNGGK QALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQ AHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLTPQQVVAIAS NNGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNNGGKQALETVQRLLP VLCQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLTPEQVV AIAASHDGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNNGGRRPALES I VAQLSRPDPALAALTNDHLVALACLGGRRPALDAVKKGLGDPISRSQLVKS ELEEKKSELRHKLKYVPHEYIELIEIARNSTQDRILEMKVMEFFMKVYGYR GKHLGGSRRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMQR YVEENQTRNKHINPNEWVKVYSSVTEFKFLFVSGHFKGNYKAQLTRLN HITNCNGAVLSVEELLIGGEMIKAGTLTLEEVRRKFNNGEINFAAD
CD38 TALEN Left	SEQ ID NO.7	MGDPKKRKRVIDIADLRTLGYSSQQQEKIKPKVRSTVAQHHEALVGHGF THAHIVALSQHPAALGTAVVKYQDMIAALPEATHEAIVGVGKQWSGAR ALEALLTVAGELRGPPQLDGTGQLLKIARGGVTAVEAVHAWRNALTGA PLNLTPEQVVAIASNNGGKQALETVQRLLPVLCQAHGLTPEQVVAIASH DGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNNGGKQALETVQRLL PVLCQAHGLTPEQVVAIASNIGGKQALETVQALLPVLCQAHGLTPQQVV AIASNNGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNNGGKQALET VQRLLPVLCQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLT PEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQ ALETVQALLPVLCQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQA HGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHD GGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPV LCQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLTPQQVVA IASNNGGKQALETVQRLLPVLCQAHGLTPQQVVAIASNNGGRRPALESIV AQLSRPDPALAALTNDHLVALACLGGRRPALDAVKKGLGDPISRSQLVKS ELEEKKSELRHKLKYVPHEYIELIEIARNSTQDRILEMKVMEFFMKVYGYR GKHLGGSRRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMQR YVEENQTRNKHINPNEWVKVYSSVTEFKFLFVSGHFKGNYKAQLTRLNHI TNCNGAVLSVEELLIGGEMIKAGTLTLEEVRRKFNNGEINFAAD
CD38 TALEN Right	SEQ ID NO.8	MGDPKKRKRVIDIADLRTLGYSSQQQEKIKPKVRSTVAQHHEALVGHGF THAHIVALSQHPAALGTAVVKYQDMIAALPEATHEAIVGVGKQWSGAR ALEALLTVAGELRGPPQLDGTGQLLKIARGGVTAVEAVHAWRNALTGA PLNLTPEQVVAIASNIGGKQALETVQALLPVLCQAHGLTPQQVVAIASN

		<p> NGGKQALETVQRLLPVLCAHGLTPEQVVAIASNIGGKQALETVQALLP VLCQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCAHGLTPEQVV AIASNIGGKQALETVQALLPVLCAHGLTPQQVVAIASNNGGKQALETV QRLLPVLCAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCAHGLTP EQVVAIASHDGGKQALETVQRLLPVLCAHGLTPQQVVAIASNNGGKQ ALETVQRLLPVLCAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCA HGLTPEQVVAIASHDGGKQALETVQRLLPVLCAHGLTPEQVVAIASNI GGKQALETVQALLPVLCAHGLTPQQVVAIASNNGGKQALETVQRLLP VLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCAHGLTPEQVV AIASNIGGKQALETVQALLPVLCAHGLTPQQVVAIASNNGGGRPALESIV AQLSRPDPALAALTNHDLVALACLGGRPALDAVKKGLGDPISRSQLVKSE LEEKKSELRHKLKYPHEYIELIEIARNSTQDRILEMKVMEFFMKVYGYRG KHLGGSRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMQRYV EENQTRNKHINPNEWVKVYSSVTEFKFLFVSGHFKGNYKAQLTRLNHI TNCNGAVLSVEELLIGGEMIKAGTLTLEEVRKFNNGEINFAAD </p>
<p> PD1 TALEN Left 2 </p>	<p> SEQ ID NO.9 </p>	<p> MGDPKKKRVIDIADLRTLGYSSQQQEKIKPKVSRSTVAQHHEALVGHGF THAHIVALSQHPAALGTAVVKYQDMIAALPEATHEAIVGVGKQWSGAR ALEALLTVAGELRGPPLQLDTGQLLKIARGGVTAVEAVHAWRNALTGA PLNLTPQVVAIASKLGKQALETVQALLPVLCAHGLTPEQVVAIASHD GGKQALETVQRLLPVLCAHGLTPEQVVAIASHDGGKQALETVQRLLPV LCQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCAHGLTPEQVVAI ASHDGGKQALETVQRLLPVLCAHGLTPQQVVAIASNNGGKQALETVQ RLLPVLCAHGLTPQQVVAIASYKGGKQALETVQRLLPVLCAHGLTPQ QVVAIASNNGGKQALETVQRLLPVLCAHGLTPQQVVAIASNNGGKQA LETVQRLLPVLCAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCAH GLTPQQVVAIASNNGGKQALETVQRLLPVLCAHGLTPQQVVAIASNN GGKQALETVQRLLPVLCAHGLTPEQVVAIASHDGGKQALETVQRLLPV LCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCAHGLTPEQVVAI ASNIGGKQALETVQALLPVLCAHGLTPQQVVAIASNNGGGRPALESIVA QLSRPDPALAALTNHDLVALACLGGRPALDAVKKGLGDPISRSQLVKSEL EEKKSELRHKLKYPHEYIELIEIARNSTQDRILEMKVMEFFMKVYGYRGK HLGGSRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMQRYVE ENQTRNKHINPNEWVKVYSSVTEFKFLFVSGHFKGNYKAQLTRLNHIT NCNGAVLSVEELLIGGEMIKAGTLTLEEVRKFNNGEINFAAD </p>
<p> B2M TALEN left </p>	<p> SEQ ID NO.10 </p>	<p> MGDPKKKRVIDIADLRTLGYSSQQQEKIKPKVSRSTVAQHHEALVGHGF THAHIVALSQHPAALGTAVVKYQDMIAALPEATHEAIVGVGKQWSGAR ALEALLTVAGELRGPPLQLDTGQLLKIARGGVTAVEAVHAWRNALTGA PLNLTPQQVVAIASNNGGKQALETVQRLLPVLCAHGLTPQQVVAIASN NNGKQALETVQRLLPVLCAHGLTPEQVVAIASHDGGKQALETVQRLLP VLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCAHGLTPQQVV AIASNNGGKQALETVQRLLPVLCAHGLTPQQVVAIASNNGGKQALET VQRLLPVLCAHGLTPEQVVAIASNIGGKQALETVQALLPVLCAHGLTP QQVVAIASNNGGKQALETVQRLLPVLCAHGLTPEQVVAIASHDGGKQ ALETVQRLLPVLCAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCA HGLTPQQVVAIASNNGGKQALETVQRLLPVLCAHGLTPQQVVAIASN GGGKQALETVQRLLPVLCAHGLTPQQVVAIASNNGGKQALETVQRLL PVLCAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCAHGLTPQQV VAIASNNGGKQALETVQRLLPVLCAHGLTPQQVVAIASNNGGGRPALES IVAQLSRPDPALAALTNHDLVALACLGGRPALDAVKKGLGDPISRSQLVK SELEEKSELRHKLKYPHEYIELIEIARNSTQDRILEMKVMEFFMKVYGYR </p>

		GKHLGGSRRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMQRY VEENQTRNKHINPNEWVKVYSSVTEFKFLFVSGHFHFKGNYKAQLTRLN HITNCNGAVLSVEELLIGGEMIKAGTLTLEEVRKFNNGEINFAAD
B2M TALEN right	SEQ ID NO.11	MGDPKKKRKVIDIADLRTLGYSSQQQEKIKPKVRSTVAQHHEALVGHGF THAHIVALSQHPAALGTVAVKYQDMIAALPEATHEAIVGVGKQWSGAR ALEALLTVAGELRGPPLQLDTGQLLKIARGGVTAVEAVHAWRNALTGA PLNLTPEQVVAIASNIGGKQALETVQALLPVLCQAHGLTPQQVVAIASN NGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLP VLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPQQVV AIASNGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHDGGKQALETV QRLLPVLCQAHGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTP EQVVAIASNIGGKQALETVQALLPVLCQAHGLTPQQVVAIASNNGGKQ ALETVQRLLPVLCQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQA HGLTPEQVVAIASHDGGKQALETVQRLLPVLCQAHGLTPEQVVAIASHD GGKQALETVQRLLPVLCQAHGLTPEQVVAIASNIGGKQALETVQALLPV CQAHGLTPQQVVAIASNNGGKQALETVQRLLPVLCQAHGLTPEQVVAI ASNIGGKQALETVQALLPVLCQAHGLTPQQVVAIASNGGGRPALESIVA QLSRPDPALAALTNDHLVALACLGGRPALDAVKKGLGDPISRSQLVKSEL EEKKSELRHKLKYPHEYELIEIARNSTQDRILEMKVMEFFMKVYGYRGK HLGGSRRKPDGAIYTVGSPIDYGVIVDTKAYSGGYNLPIGQADEMQRYVE ENQTRNKHINPNEWVKVYSSVTEFKFLFVSGHFHFKGNYKAQLTRLNHIT NCNGAVLSVEELLIGGEMIKAGTLTLEEVRKFNNGEINFAAD
CD22 CAR	SEQ ID NO.12	MALPVTALLLPLALLLHAARPGGGGSCPYSNPSLCSGGGGSGGGGSQV QLQQSGPGLVKPSQTLSLTCAISGDSVSSNSAAWNWIRQSPSRGLEWL GRTYRYSKYNDYAVSVKSRITINPDTSKNQFSLQLNSVTPEDTAVYYCA REVTGDLEDAFDIWGQGTMTVTVSSGGGGSGGGGSGGGGSDIQMTQS PSSLSASVGDRTITCRASQTIWSYLNWYQQRPGKAPNLLIYAASSLQSG VPSRFSGRGSGTDFTLTISLQAEDFATYYCQSYSIPQTFGQGTKLEIKSD PGSGGGGSCPYSNPSLCSGGGGSELPTQGTFSNVSTNVSPAKPTTTACP YSNPSLCAPTTTTAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDF ACDIYIWAPLAGTCGVLLLSLVITLYCRRGRKLLYIFKQPFMRPVQTTQE EDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQGQNQLYNELNLGRREE YDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQDKMAEAYSEIGMKG ERRRGKGDGLYQGLSTATKDYDALHMQALPPRE

Table 4: Cluster of differentiation (CD) antigen markers of various cancers found to be expressed on the surface of T-cells

Antigen	Other Names	Structure	main Distribution	Function
CD1a	T6	IgSF, MHC-like	cortical thymocytes, Langerhans cells, DC	antigen presentation, with beta2m
CD1b	T6	IgSF, MHC-like	cortical thymocytes, Langerhans cells, DC	antigen presentation, with beta2m
CD1c	T6	IgSF, MHC-like	cortical thymocytes, Langerhans cells, DC, B subset	antigen presentation, with beta2m
CD1d		IgSF, MHC-like	intestinal epith, B subset, monolow, DC	antigen presentation, with beta2m
CD3 gamma, CD3 delta	T3	IgSF	T, thymocyte subset	with TCR, TCR surface expression / signal transduction
CD3 epsilon	T3	IgSF	T, thymocyte subset	with TCR, TCR surface expression / signal transduction
CD4	T4	IgSF	thymocyte subset, T subset, mono, mac	MHC class II coreceptor, HIV receptor, T cell differentiation / activation
CD5	T1, Tp67	Scavenger R SF	thymocytes, T, B subset, B-CLL	CD72 receptor, TCR or BCR signaling, T-B interaction
CD7		IgSF	hematopoietic progenitors, thymocytes, T, NK	T costimulation
CD8a	T8, Leu-2	IgSF	thymocyte subset, T subset, NK	MHC class I coreceptor, receptor for some mutated HIV-1, T cell differentiation / activation
CD8b		IgSF	thymocyte subset, T subset	
CD9	p24, MRP-1	TM4SF	pre-B, eosinophils, basophils, platelets, Tact	cellular adhesion and migration
CD10	CALLA, NEP, gp100	type II TM	B precursors, T precursors, neutrophils	zinc-binding metalloproteinase, B cell development
CD11a	LFA-1, integrin alphaL	Integrin family	lymph, gran, mono, mac	CD11a / CD18 receptor for ICAM-1, -2,-3, intercellular adhesion, T costimulation

CD11b	Mac-1, integrin alphaM	Integrin family	myeloid cells, NK	binds CD54, ECM, iC3b
CD11c	p150, 95, CR4, integrin alphaX	Integrin family	DC, myeloid cells, NK, B, T subset	binds CD54, fibrinogen and iC3b
CD13	Aminopeptidase N, APN	type II TM	myeloid cells	zinc-binding metalloproteinase, antigen processing, receptor for corona virus strains
CD14	LPS-R	GPI-linked	mono, mac, Langerhans cells, granlow	receptor for LPS/LBP, LPS recognition
CD15	Lewis-x, Lex	CHO	neutrophils, eosinophils, mono	adhesion
CD16a	FcgammaRIIIA	IgSF	neutrophils, mac, NK	component of low affinity Fc receptor, phagocytosis and ADCC
CD16b	FcgammaRIIIB	IgSF	neutrophils	component of low affinity Fc receptor, phagocytosis and ADCC
CD20	B1, Bp35	TM4SF	B, T subset	B cell activation
CD21	C3DR, CR2, EBV-R	CCRSF	B, FDC, T subset	complement C3d and EBV receptor, complex with CD19 and CD81, BCR coreceptor
CD22	BL-CAM, Siglec-2	IgSF, sialoadhesins	B	adhesion, B-mono, B-T interactions
CD23	FcepsilonRII	C-type lectin	B, activated mac, eosinophils, FDC, platelets	CD19-CD21-CD81 receptor, IgE low affinity receptor, signal transduction
CD24	BA-1	GPI-linked	thymocytes, erythrocytes, peripheral lymph, myeloid	binds P-selectin
CD25	Tac, p55	type I TM	Tact, Bact, lymph progenitors	IL-2Ralpha, with IL-2Rbeta and gamma to form high affinity complex
CD31	PECAM-1	IgSF	mono, platelets, gran, endoth, lymph subset	CD38 receptor, adhesion
CD33	p67, Siglec-3	IgSF, sialoadhesins	myeloid progenitors, mono, gran, DC, mast cells, Tact	adhesion
CD37		TM4SF	B, Tlow, granlow	signal transduction
CD38	T10		variable levels on majority of hematopoietic cells, high expression on plasma cells, B and Tact	ecto-ADP-ribosyl cyclase, cell activation
CD40		TNFRSF	B, mono, mac, FDC, endoth, T subset	CD154 receptor, B differentiation /

CD43	Leukosialin, sialophorin	Sialomucin, type I TM	leukocytes, except resting B, platelets	costimulation, isotype-switching, rescues B cells from apoptosis
CD44	H-CAM, Pgp-1	hyaladherin family	hematopoietic and non-hematopoietic cells, except platelets, hepatocytes, testis	inhibition of T cell interaction, CD54R, adhesion
CD45	LCA, T200, B220		hematopoietic cells, multiple isoforms from alternative splicing	binds hyaluronic acid, adhesion
CD45RA			B, T subset(naive), mono	tyrosine phosphatase, enhanced TCR & BCR signals
CD45RB			T subset, B, mono, mac, gran	exon A isoforms of CD45
CD45RO			Tact, memory T, B subset, mono, mac, gran	exon B isoforms of CD45
CD46	MCP	CCRSF	nucleated cells	isoform of CD45 lacking A, B, C exons
CD47	IAP	IgSF	hematopoietic cells, epith, endoth, fibroblasts, other tissues	membrane cofactor protein, binds C3b & C4b allowing degradation by Factor I, measles virus receptor
CD48	Blast-1	IgSF	broad, all leukocytes	leukocyte adhesion, migration, activation
CD52	CAMPATH-1		thymocytes, T, B (not plasma cells), mono, mac	cell adhesion
CD53		TM4SF	leukocytes, DC, osteoblasts, osteoclasts	signal transduction
CD55	DAF	GPI-linked	hematopoietic, endoth	binds C3b, complement regulation
CD56	NCAM	IgSF	NK, T subset, neurons, some large granular lymphocyte leukemias, myeloid leukemias	adhesion
CD57	HNK-1, Leu-7		NK subset, T subset	
CD58	LFA-3	IgSF	hematopoietic, non-hematopoietic cells	CD2 receptor, adhesion
CD59	Protectin, MAC-inhibitor	GPI-linked	hematopoietic, non-hematopoietic cells	binds complement C8 and C9, blocks assembly of membrane attack complex
CD60a	GD3	CHO	T subset, platelets, thymic epith, astrocytes	costimulation
CD63	LIMP, LAMP-3	TM4SF	activated platelets, mono, mac	lysosomal membrane protein, moves to cell surface after activation
CD68	Macrosialin,	Sialomucin	intracellularly in mono, mac, neutrophils, basophils, large lymph, mast cells, DC,	

	gp110		myeloid progenitors, liver	
CD69	AIM	C-type lectin	Tact, B, NK and gran, thymocytes, platelets, Langerhans cells	signal transduction
CD70	Ki-24	TNFSF	Bact and Tact	CD27 ligand, T and B cell costimulation
CD74	li, invariant chain	B, mac, mono, Langerhans cells, DC, Tact		MHC class II traffic and function
CD79a	Iga	IgSF	B	component of BCR, BCR surface expression and signal transduction
CD79b	Igb	IgSF	B	component of BCR, BCR surface expression and signal transduction
CD81	TAPA-1	TM4SF	T, B, NK, thymocytes, DC, endoth, fibroblast, neuroblastomas, melanomas	complex with CD19 & CD21, signaling, T costimulation
CD82	R2	TM4SF	leukocytes	signal transduction
CD83	HB15	IgSF	Bact and Tact, DC, Langerhans cells	
CDw84			mono, platelets, B, T subset, mac subset	
CD86	B70, B7-2	IgSF	mono, DC, Bact and Tact	binds to CD28, CD152, T costimulation
CD87	UPA-R	GPI-linked	gran, mono, NK, Tact, endoth, fibroblasts	urokinase plasminogen activator receptor, inflammatory cell invasion, metastasis
CD90	Thy-1	IgSF, GPI-linked	CD34+ hematopoietic subset, neurons	hematopoietic stem cell and neuron differentiation
CD94	KP43	C-type lectin	NK, T subset	complex with NKG2, inhibits NK function
CD95	Apo-1, Fas	TNFRSF	lymph (high upon activation), mono, neutrophils	FasL (CD178) receptor, apoptosis
CD96	TACTILE	IgSF	NK, Tact	adhesion of activated T and NK
CD97		TM7SF	Bact and Tact, mono, gran	
CD98	4F2		T, B, NK, gran, all human cell lines	cellular activation
CD99	MIC2, E2		leukocytes	T cell activation, adhesion
CD100			hematopoietic cells except immature bone marrow cells, RBC and platelets	cell adhesion, cellular activation
CD103	HML-1, alpha6,	Integrin family	intraepithelial lymph, lymph subset, activated	with integrin beta7, binds E-cadherin,

	integrin alphaE		lymph	lymph homing/retention
CD107a	LAMP-1		activated platelets, T, endoth, metastatic tumors	a lysosomal membrane protein
CD107b	LAMP-2		activated platelets, T, endoth, metastatic tumors	a lysosomal membrane protein
CD109			Tact and platelets, CD34+ subset, endoth	
CD123	IL-3R	CRSF	lymph subset, basophils, hematopoietic progenitors, mac, DC, megakaryocytes	IL-3Ralpha, with CDw131
CD146	MUC18, S-endo	IgSF	endoth, melanomas, FDC, Tact	adhesion
CD154	CD40L, gp39, TRAP	TNFSF	Tact	CD40 ligand, B and DC costimulation
CD158a	p58.1	IgSF, KIR family	NK subset, T subset	inhibition of NK cell cytolytic activity, MHC class-I specific NK receptor
CD158b	p58.2	IgSF, KIR family	NK subset, T subset	inhibition of NK cell cytolytic activity, MHC class-I specific NK receptor
CD163	130kD	Scavenger receptor SF	mono, mac	
CD164	MGC-24	epith, mono, lymphlow, bone marrow stromal cells, CD34+ erythroid progenitors	hematopoietic progenitor cell-stromal cell interaction	
CD168	RHAMM		mono, T subset, thymocyte subset, intracellularly in breast cancer cells	adhesion, tumor migration, metastasis
CD171	L1	IgSF	CNS, PNS, glial cells, mono, T subset, B, DC, several human tumor cells	kidney morphogenesis, lymph node architecture, T costimulation, neurohistogenesis, homotypic interaction, binds CD9, CD24, CD56, CD142, CD166, integrins
CD177	NB1		neutrophil subset	
CD178	FasL, CD95L	TNFSF	Tact, testis	CD95 ligand, apoptosis, immune privilege, soluble form in serum
CD180	RP-105	LRRF, TLR	B subset, mono, DC	B cell activation, LPS signaling, with

		family			MD-1
CD182	CXCR2, IL-8RB	GPCR1 family	neutrophils, basophils, NK, T subset, mono		binding of IL-8 induces chemotaxis of neutrophils
CD185	CXCR5, BLR1	GPCR1 family	mature B and Burkitt Lymphoma cells		with chemokine BLC, possible regulatory function in Burkitt Lymphomagenesis and/or B differentiation, activation of mature B
CD191	CCR1, MIP-1alphaR, RANTES-R	GPCR1 family	T, mono, stem cell subset		binds C-C type chemokines and transduces signal by increasing intracellular calcium ion levels
CD193	CCR3, CKR3	GPCR1 family	eosinophils, lower expression in neutrophils and mono, T subset		binds eotaxin, eotaxin-3, MCP-3, MCP-4, RANTES & MIP-1 delta, alternative coreceptor with CD4 for HIV-1 infection.
CD196	CCR6, LARC receptor, DRY6	GPCR1 family	T subset, B, DC subset		binds MIP-3alpha/LARC
CD197	CCR7		T subset, DC Subset		6Ckine and MIP-2beta receptor
CD200	OX-2		thymocytes, endoth, B, Tact		inhibition of immune response
CD209	DC-SIGN		DC subset		ICAM-3 receptor, HIV-1 binding protein
CD227	MUC1, EMA	Mucin family, type I TM	epith, stem cell subset, FDC, mono, B subset, some myelomas		adhesion, signaling, binds CD169, CD54, & selectins
CD231	TALLA-1, A15	TM4SF	T leukemias, neuroblastomas, brain neurons		marker for T cell acute lymphoblastic leukemia
CD246	ALK, Ki-1		anaplastic T cell leukemias, small intestine, testis, brain, not on normal lymph		brain development, implicated in ALK lymphomas
CD254	TRANCE, RANKL, OPGL	TNFSF	lymph node & BM stroma Tact		binds OPG and RANK, osteoclast differentiation, enhances DC to stimulate naive-T proliferation
CD263	TRAIL-R3, DcR1, LIT		peripheral blood lymphocytes		receptor for TRAIL but lacks death domain
CD272	BTLA	IgSF	Tact, B, remains on Th1		HVEM receptor, inhibitory response
CD273	B7DC, PD-L2, PDCD1L2	IgSF	DC subset, mono, mac		PD-1 receptor, costimulation or suppression of T proliferation

CD276	B7-H3	B7 Family, ASV	in vitro cultured DC and mono, Tact, mammary tissue	costimulation, T activation
CD277	BT3.1, butyrophilin SF3A1, BTF5	B7/BT family, ASV	T, B, NK, mono, DC, endoth, CD34+ cells, tumor cell lines	T activation
CD279	PD1, SLEB2		Tact and Bact	B7-H1 & B7-DC receptor, autoimmune disease and peripheral tolerance
CD298	Na+/K+-ATPase beta3 subunit		broad	transport sodium & potassium ions across membrane
CD300a	CMRF35H, IRC1, IRp60	IgSF, ASV	NK, mono, neutrophils, T and B subset and lymphocytic cell lines, AML	unknown
CD300c	CMRF35A, LIR	IgSF	mono, neutrophils, monocytic cell lines, B & T subsets	unknown
CD304	BDC4, neuropilin 1	semaphorin family	neurons, CD4+/CD25+ Treg, DC, endothelial and tumor cells	interacts with VEGF165 & semaphorins, co-receptor with plexin, axonal guidance, angiogenesis, cell survival, migration
CD305	LAIR1	IgSF, ASV	NK, B, T, mono	inhibitory receptor on NK and T cells
CD314	NKG2D, KLR	Type II lectin-like receptor	NK, CD8+ activated, NK1.1+ T, some myeloid cells	binds MHC class I, MICA, MICB, Rae1 & ULBP4, activates cytotoxicity and cytokine production, costimulation
CD317	BST2, HM1.24	Type II	B, T, NK, mono, DC, fibroblast cell line, myeloma	pre-B cell growth, overexpressed in multiple myeloma
CD319	CS1, CRACC, SLAMF7	SLAM receptor family	B Cells, Dendritic Cells, NK, NKT	multiple myeloma

CLAIMS

- 1) A method for introducing genetic modifications at different loci of a primary immune cell, comprising the sequential steps of:
 - a) subjecting said primary immune cell to a first electroporation step to introduce at least a first sequence-specific reagent into said immune cell;
 - b) cultivating said primary immune cell thereby enabling said first sequence-specific reagent to modify its genome at a first locus,
 - c) subjecting said primary immune to at least a second electroporation step to introduce at least a second sequence-specific reagent into said cell,
 - d) cultivating and expanding said primary immune thereby enabling said second sequence-specific reagent to modify its genome at said second locus.
- 2) The method according to claim 1, wherein the primary immune cell is cultivated in step b) from 12 to 72 hours, preferably from 24 to 48 hours.
- 3) The method according to claim 1, wherein a purification step is performed between step b) and c) relying on a product resulting from the expression or the deletion of the gene that is modified at least at said first locus.
- 4) The method according to any one of claims 1 to 3, wherein steps a) to d) are performed within 240 hours, preferably within 120 hours, more preferably within 96 hours, even more preferably within 72 hours.
- 5) The method according to any one of claims 1 to 4, wherein said method comprises at least one further step of submitting said primary immune cell to a third electroporation step to introduce at least a third sequence-specific reagent into said cell.
- 6) The method according to claim 1, wherein said first and/or second sequence-specific reagent is a polynucleotide or polypeptide encoding a rare-cutting endonuclease, a subunit thereof, or a conjugate of both a polynucleotide and a polypeptide.
- 7) The method according to claim 2, wherein said first and/or second sequence-specific reagent is a polynucleotide or polypeptide encoding a rare-cutting endonuclease selected from programmable RNA or DNA guided endonuclease, TALEN, ZFN, megaTAL or a homing endonuclease.
- 8) The method according to claim 3, wherein said first and/or second sequence-specific reagent is a conjugate of RNA guide and a Cas9 or Cpf1 polypeptide.
- 9) The method according to claim 1, wherein said first and/or second sequence-specific reagent is an interference RNA (RNAi) or a polynucleotide encoding same.

- 10) The method according to claim 1, wherein a transduction step is introduced between b) and c) with a viral vector.
- 11) The method according to claim 10, wherein said transduction step involves an integrative lentiviral or retroviral vector for stable expression of a transgene.
- 12) The method according to claim 11, wherein said transgene encodes a Chimeric Antigen Receptor (CAR).
- 13) The method according to claim 10, wherein said transduction step involves a non-integrative viral vector.
- 14) The method according to claim 13, wherein said non integrative viral vector is used as a template for homologous recombination or NHEJ integration of said transgene into the immune cell's genome.
- 15) The method according to claim 10, wherein said first sequence-specific reagent is acting on a genomic sequence that facilitates the transduction step.
- 16) The method according to any one of claims 1 to 15, wherein said first sequence-specific reagent is acting on a genomic sequence that facilitates the genetic modification of step d).
- 17) The method according to any one of claims 1 to 16, wherein step b) is performed below 35 °C, preferably at about 30 °C.
- 18) The method according to any one of claims 1 to 17, wherein said immune cell is a T-cell.
- 19) The method according to claim 18, comprising a preliminary step of activating the primary T-cell by signal transduction.
- 20) The method according to claim 18 or 19, wherein said first sequence-specific reagent permanently reduces or prevents expression of TCR by the primary T-cell.
- 21) The method according to any one of claims 1 to 20, wherein said first or second sequence-specific reagent permanently reduces or prevents expression of at least one gene encoding an immune checkpoint.
- 22) The method according to claim 21, wherein said at least one gene encoding an immune checkpoint is selected from PD1, CTLA4, PPP2CA, PPP2CB, PTPN6, PTPN22, PDCD1, LAG3, HAVCR2, BTLA, CD160, TIGIT, CD96, CRTAM, LAIR1, SIGLEC7, SIGLEC9, CD244, TNFRSF10B, TNFRSF10A, CASP8, CASP10, CASP3, CASP6, CASP7, FADD, FAS, TGFBR2, TGFBR1, SMAD2, SMAD3, SMAD4, SMAD10, SKI, SKIL, TGIF1, IL10RA, IL10RB, HMOX2, IL6R, IL6ST, EIF2AK4, CSK, PAG1, SIT1, FOXP3, PRDM1, BATF, GUCY1A2, GUCY1A3, GUCY1B2, GUCY1B3.

- 23) The method according to any one of claims 1 to 20, wherein said first or second sequence-specific reagent permanently confers resistance of said primary immune cell against drugs or immune depleting agents.
- 24) The method according to claim 23, wherein said resistance is conferred by inactivating a gene expressing CD52, dCK, GGH or HPRT.
- 25) The method according to any one of claims 1 to 24, wherein a final step of purification is performed relying on a at least one product resulting from the expression or the deletion of one gene that is modified at said first and/or second and/or third locus.
- 26) A population of primary TCR negative T-cells resulting from a single donor obtainable according to the method according to any one of claims 1 to 25, comprising at least two subpopulations of T-cells selected from:
 - TCR negative and PD1 negative,
 - TCR negative and CD52 negative,
 - TCR negative and CTLA4 negative,
 - TCR negative and dCK negative,
 - TCR negative and GGH negative,
 - TCR negative and HPRT negative, and
 - TCR negative and β 2m negative.
- 27) A population of primary TCR negative T-cells originating from a single donor, wherein at least 20 %, preferably 30 %, more preferably 50 % of the cells in said population have been modified using sequence-specific reagents in at least three different loci.
- 28) A pharmaceutical composition comprising a population of primary T cells according to any one of claims 26 or 27.

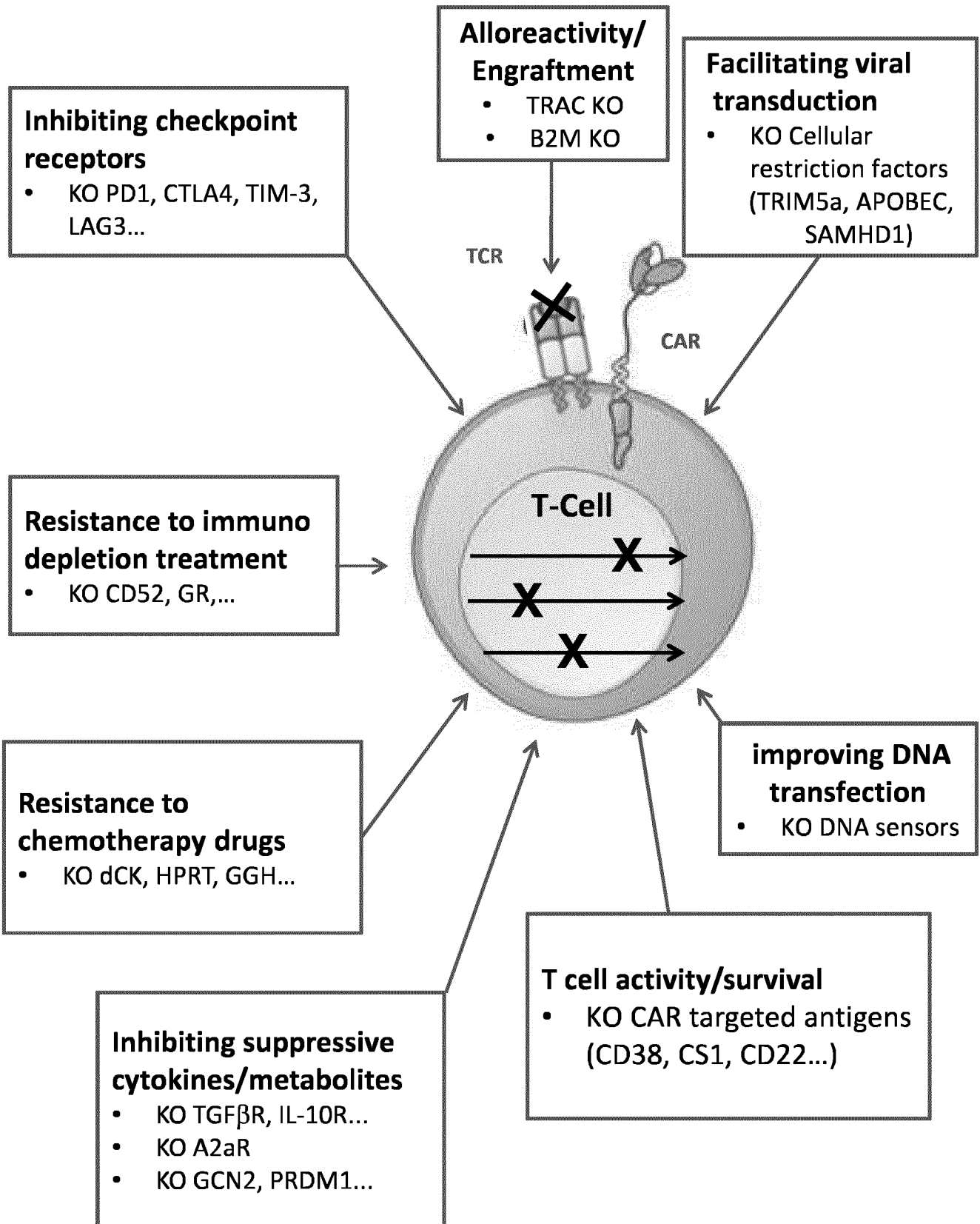
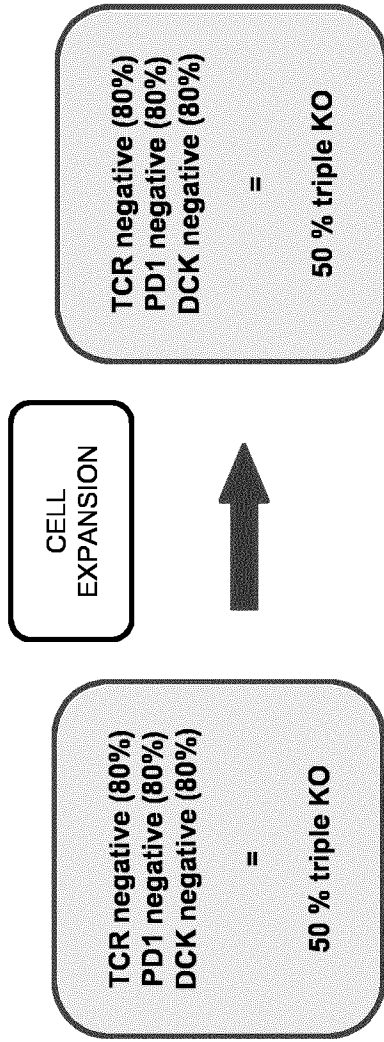


Figure 1

MULTIPLExING GENE EDITING



SEQUENTIAL GENE EDITING

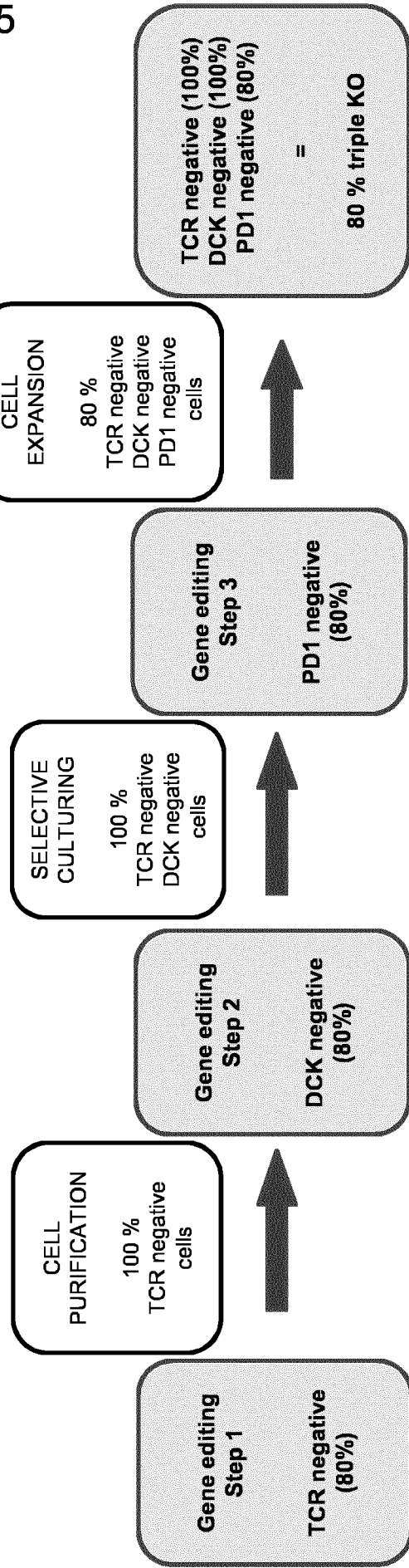


Figure 2

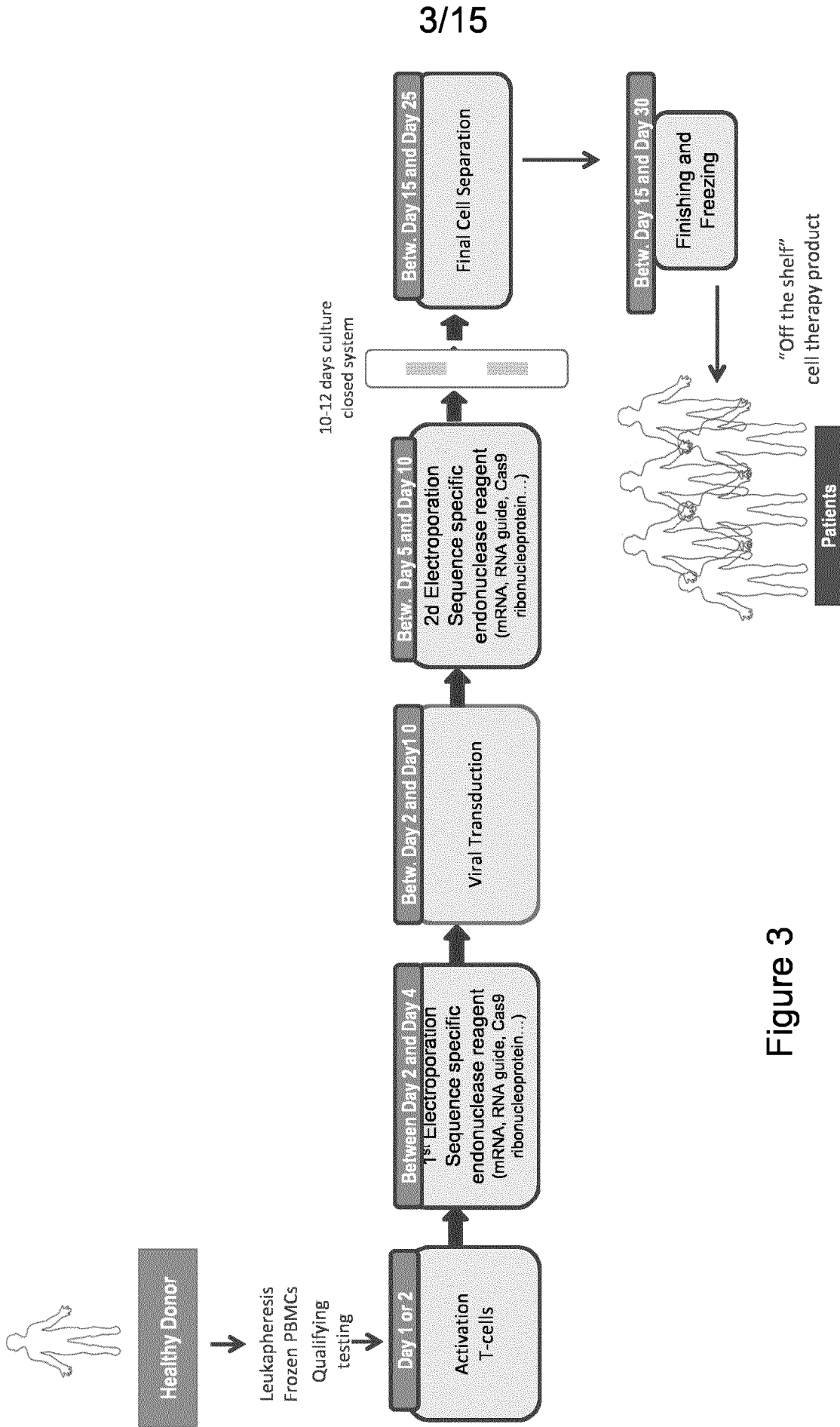


Figure 3

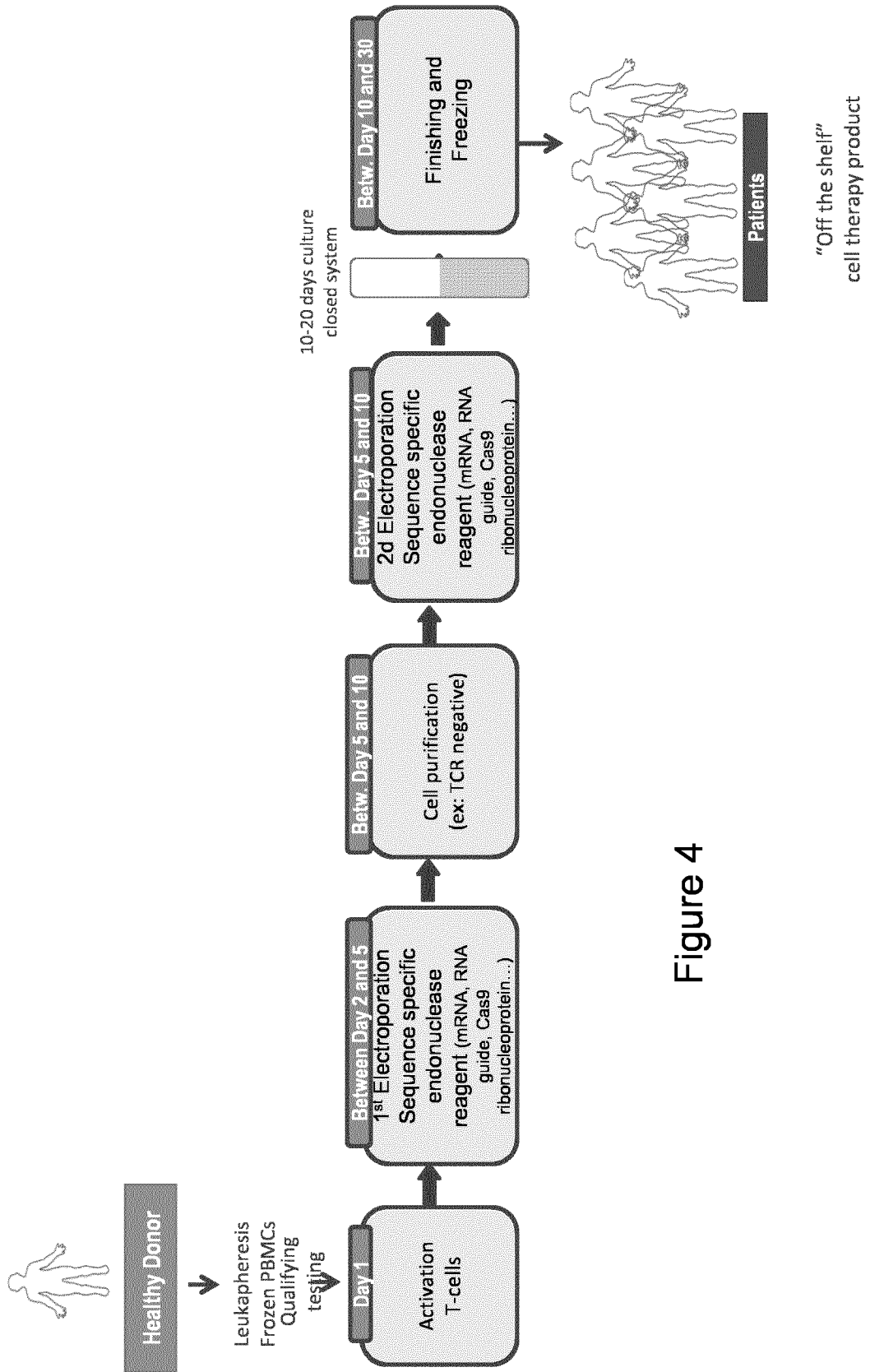


Figure 4

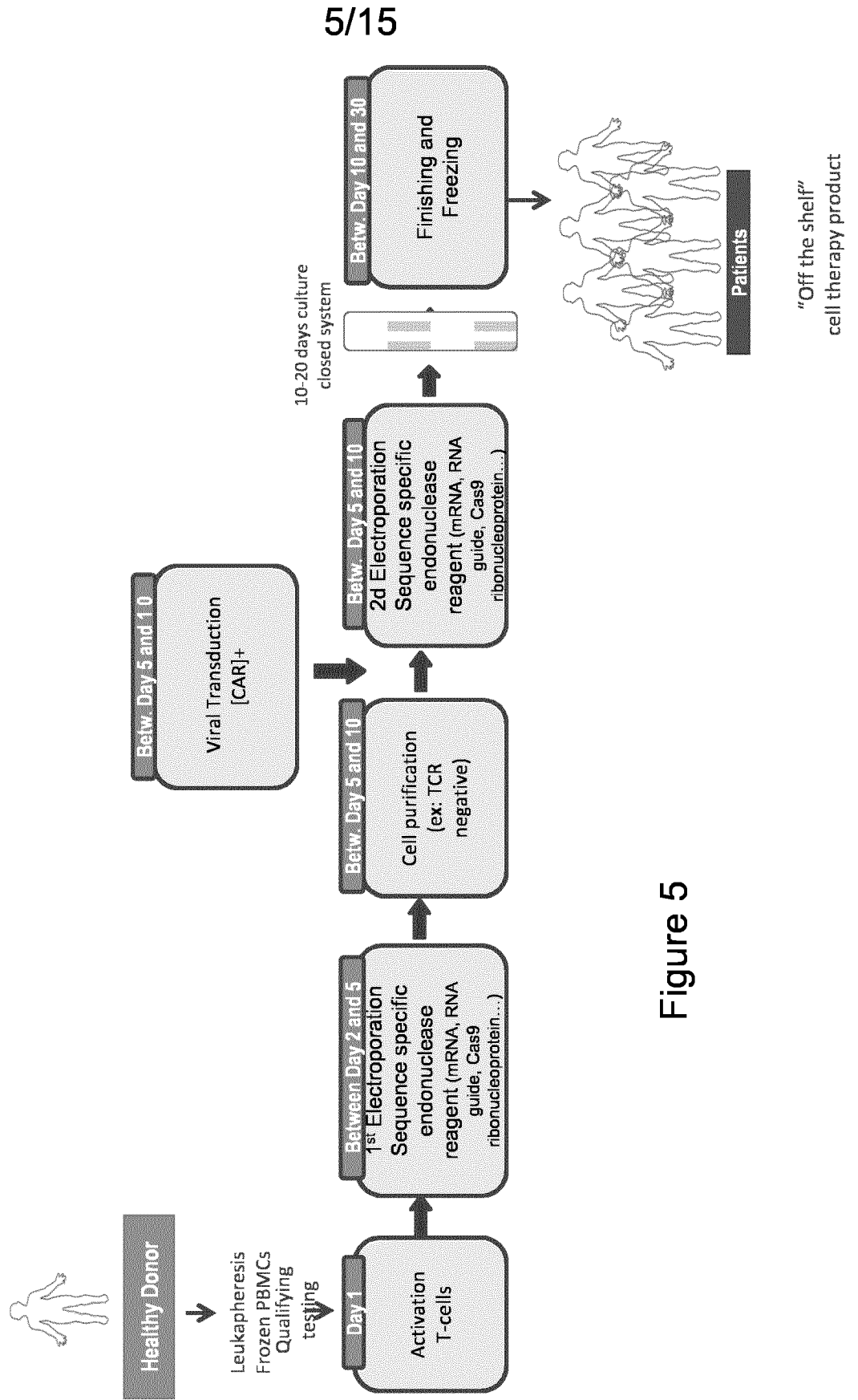


Figure 5

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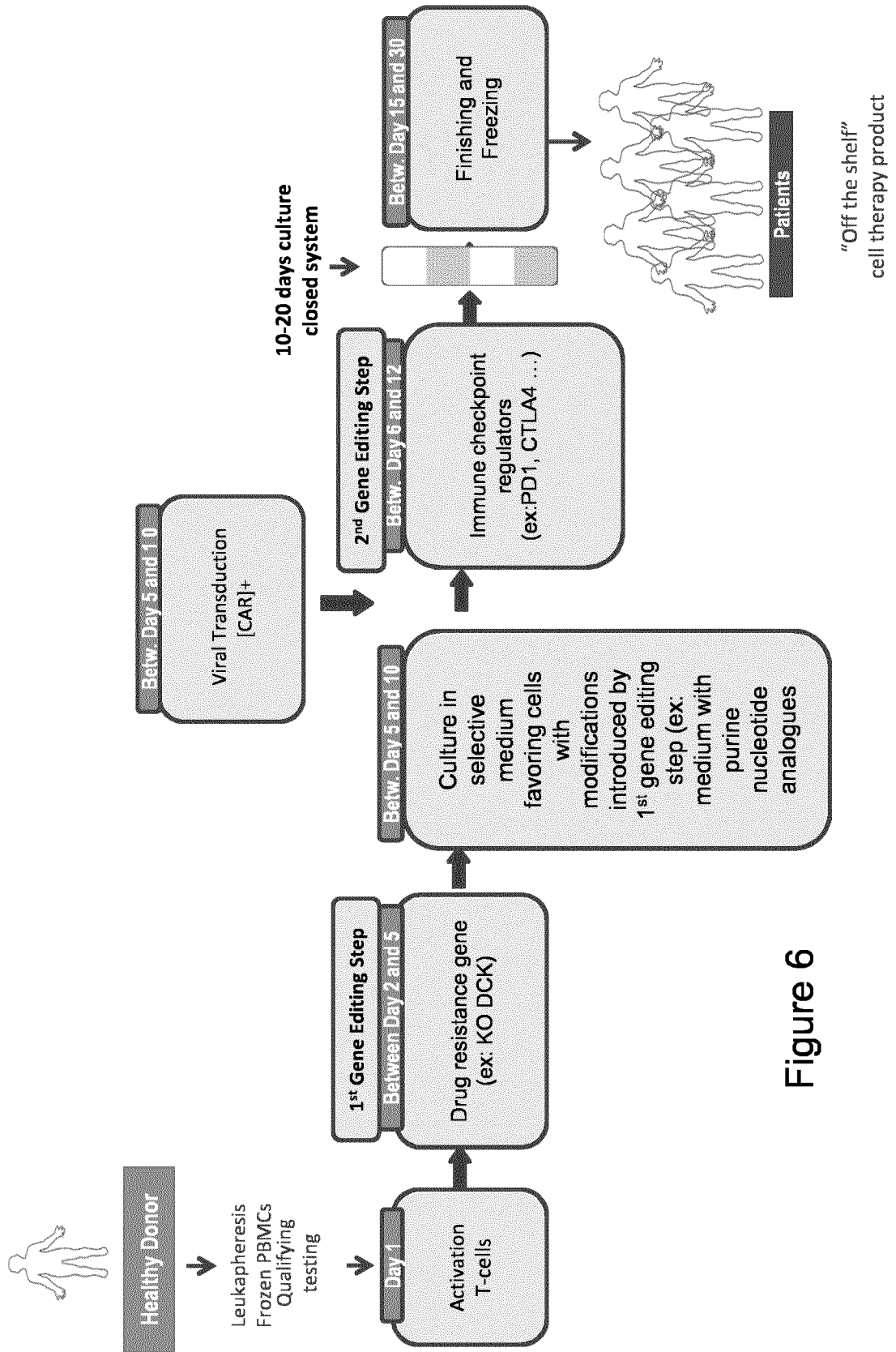


Figure 6

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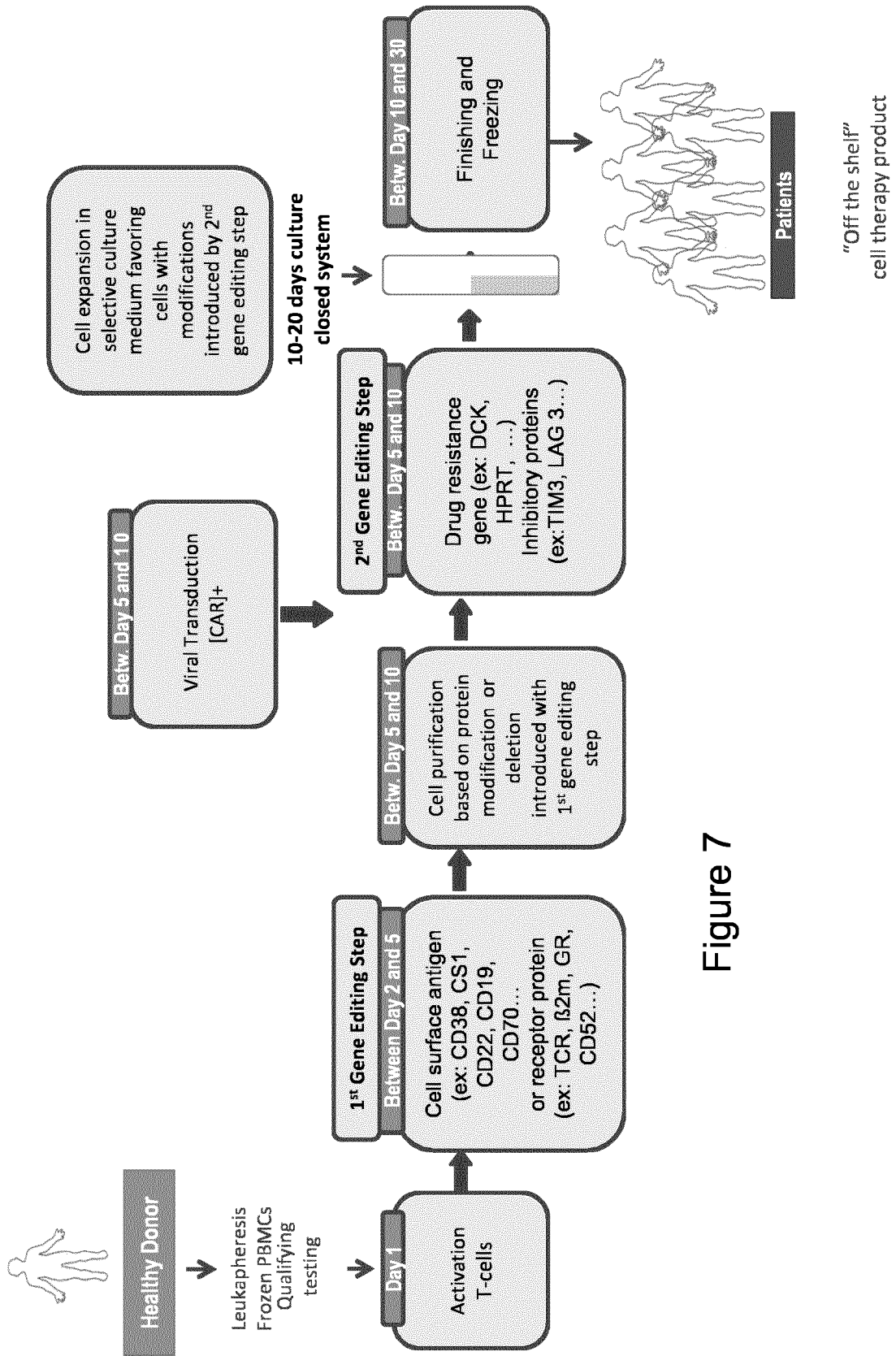


Figure 7

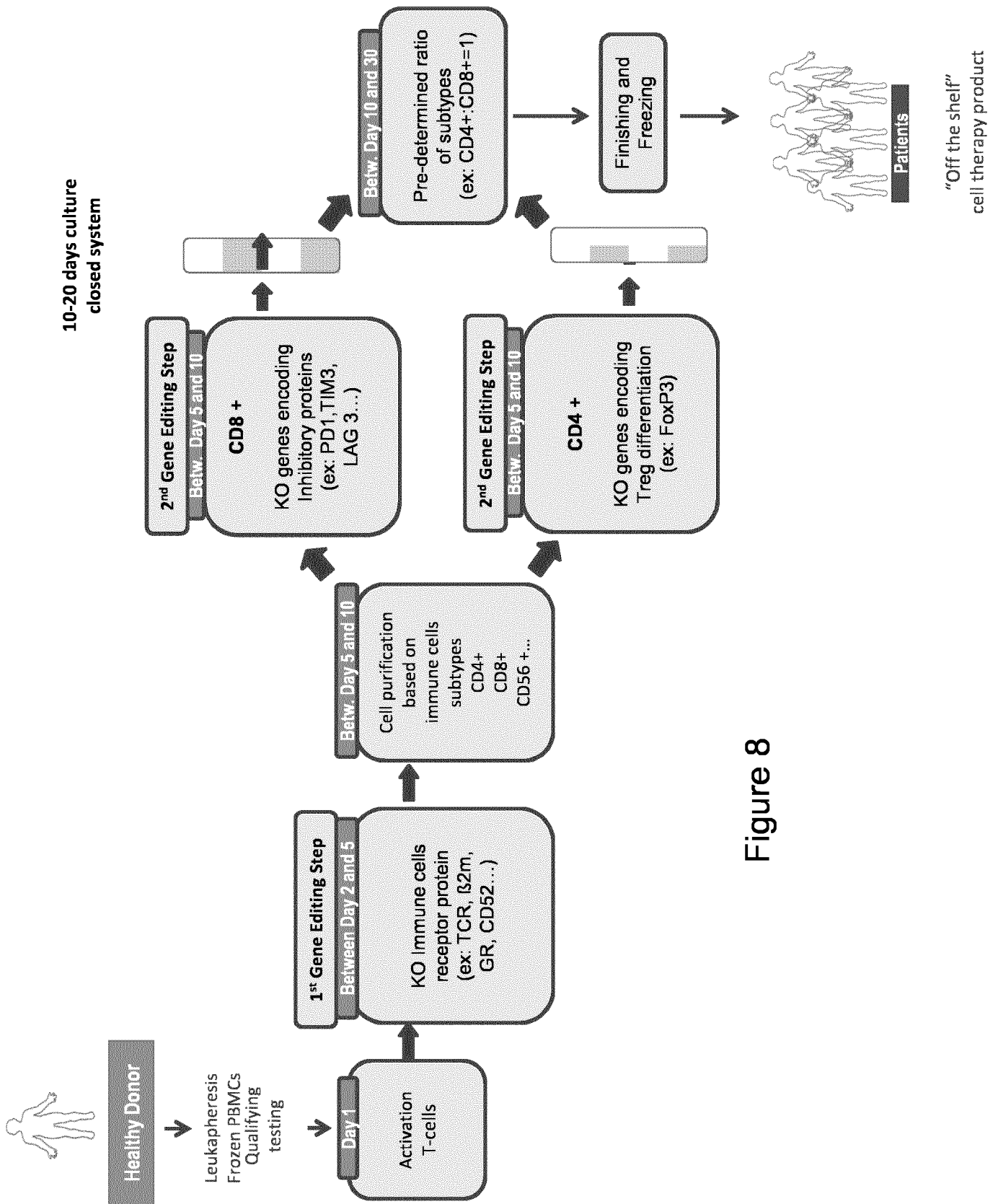


Figure 8

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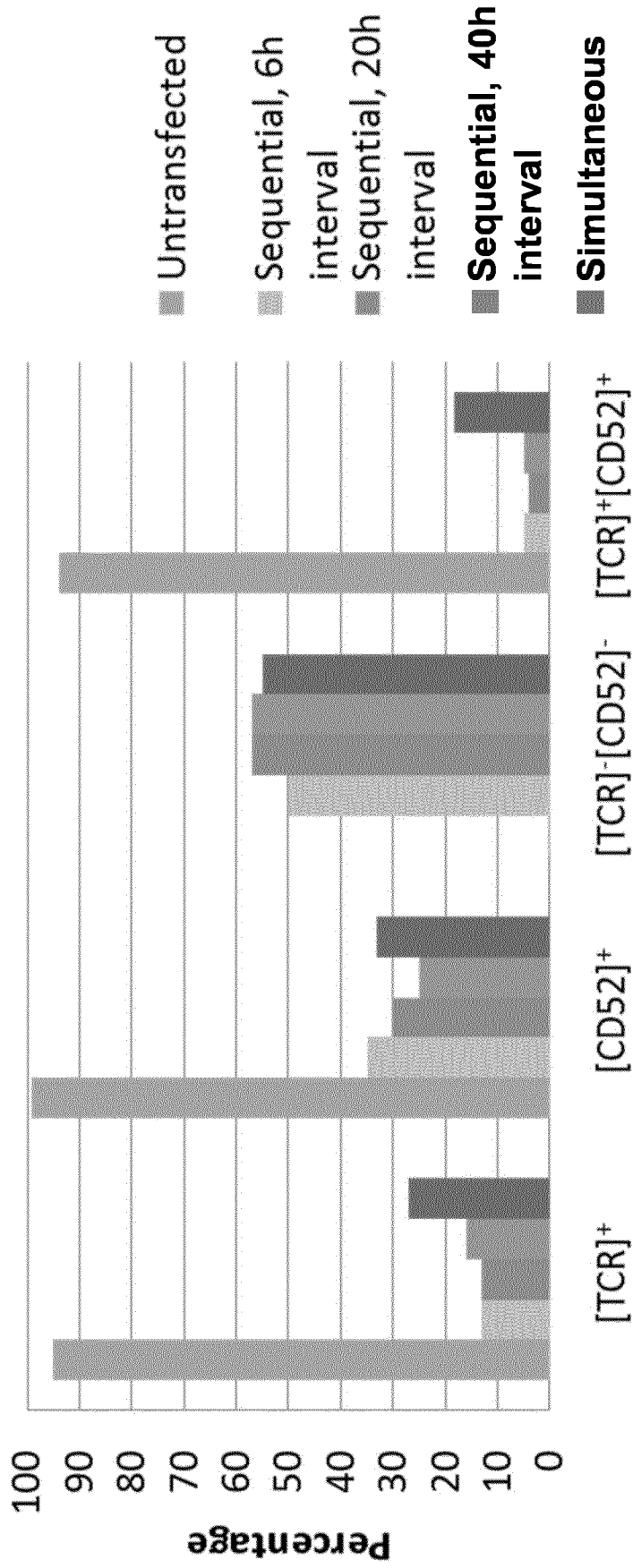


Figure 9

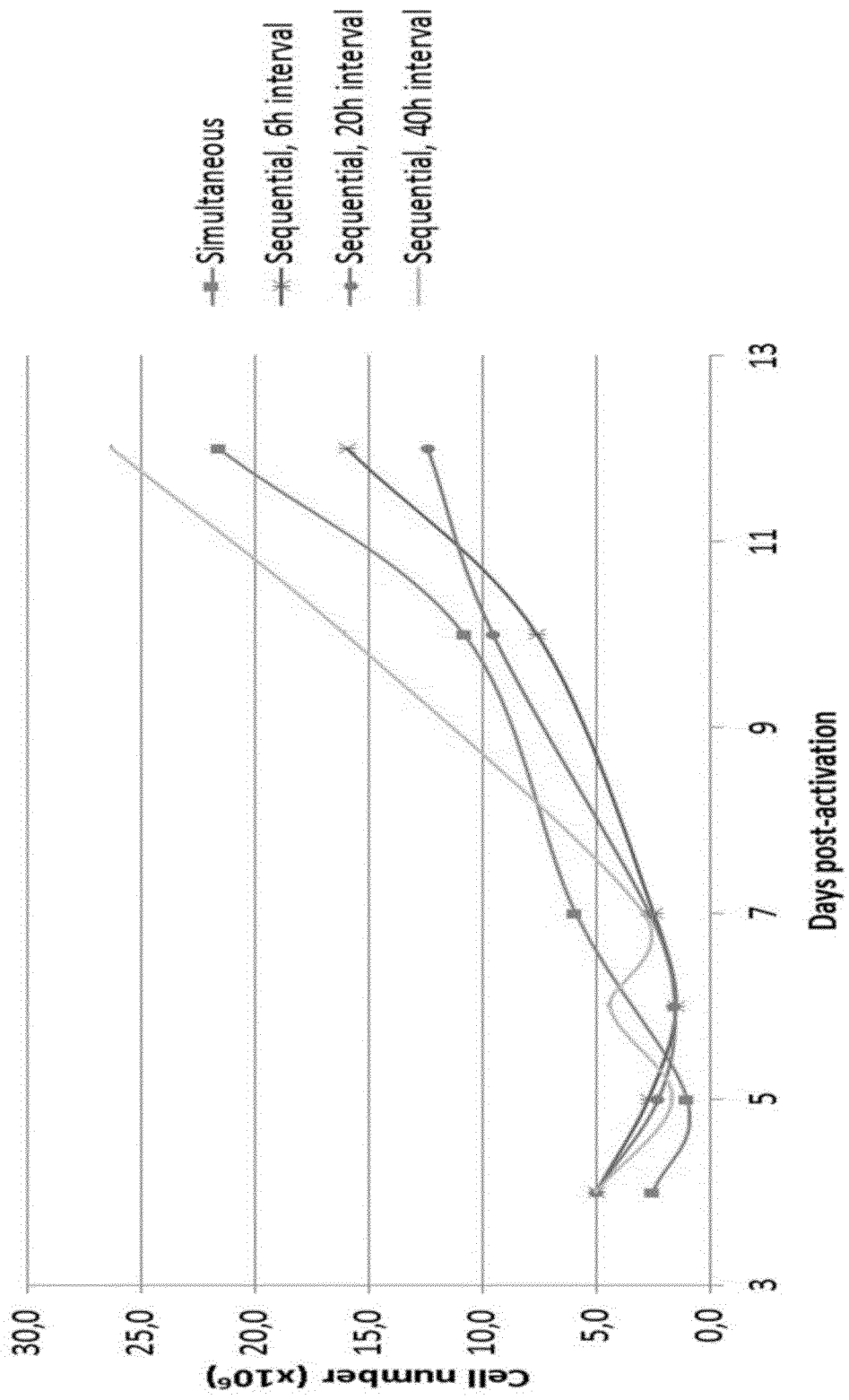


Figure 10

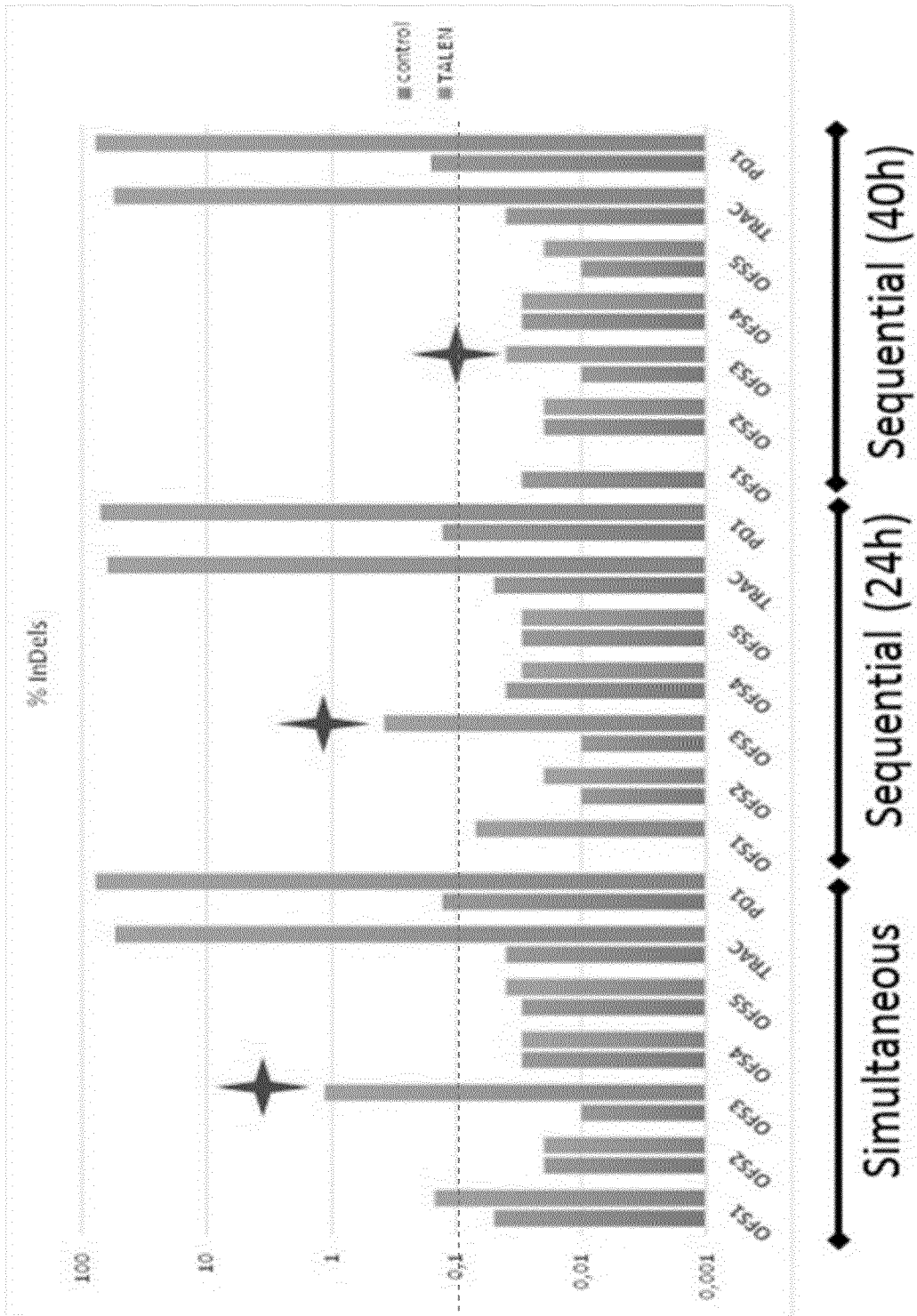


Figure 11

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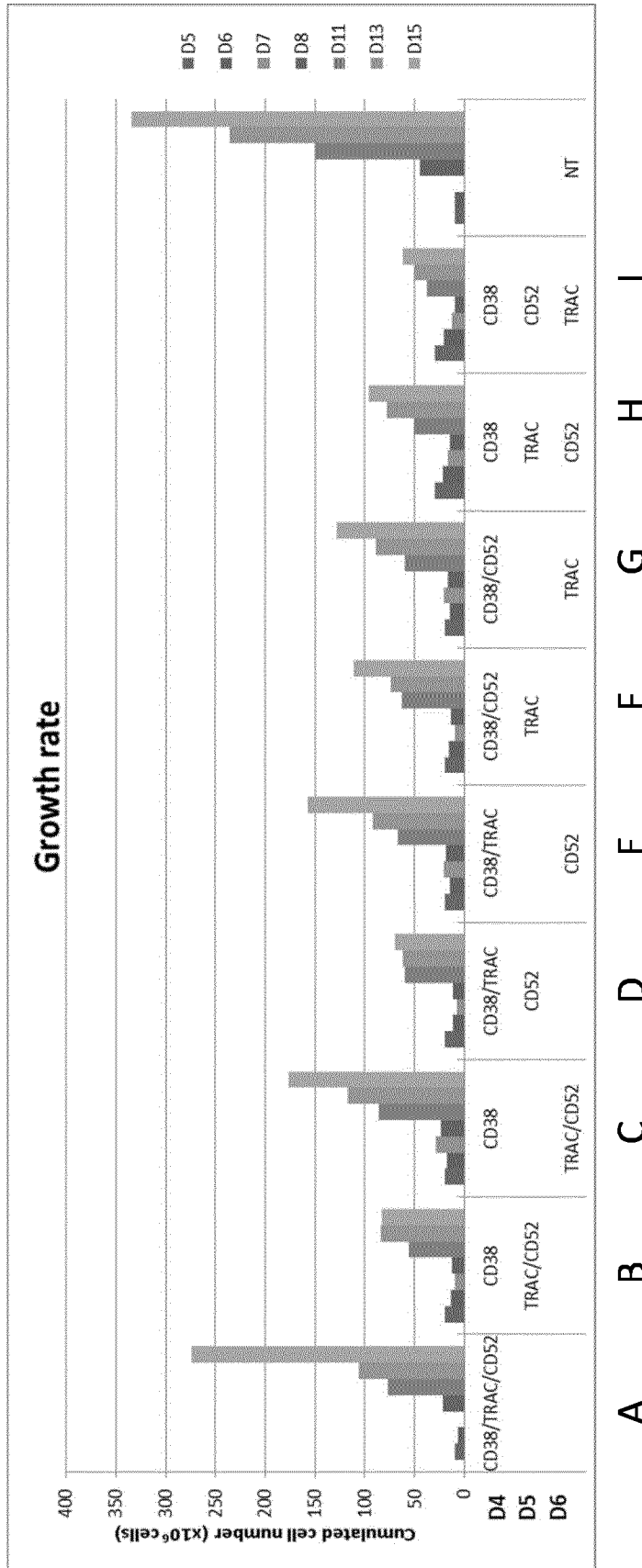


Figure 12

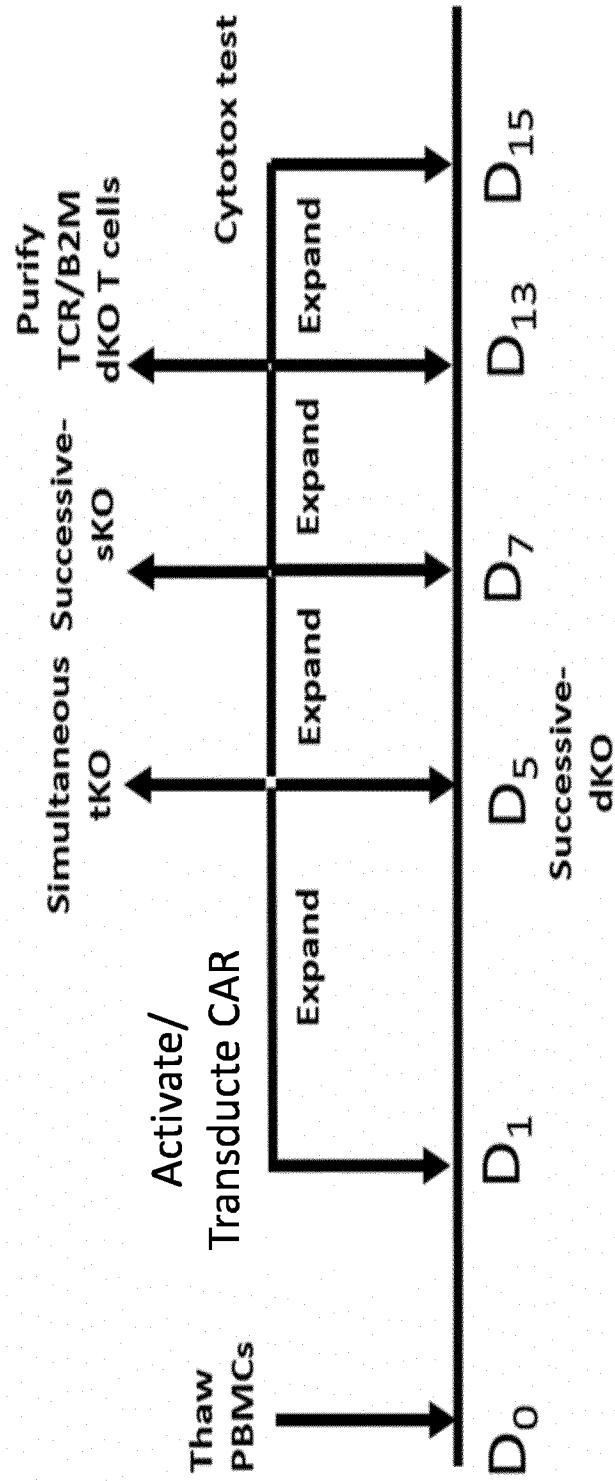


Figure 13

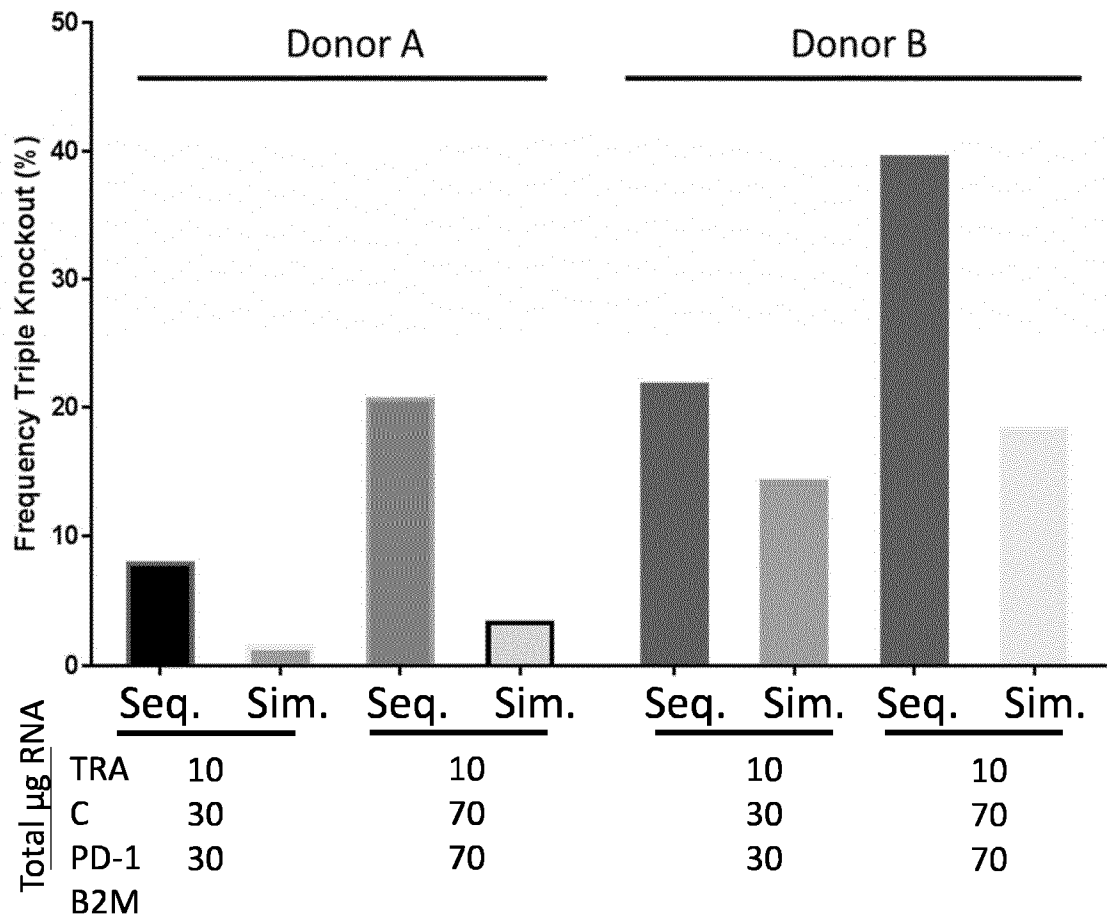


Figure 14

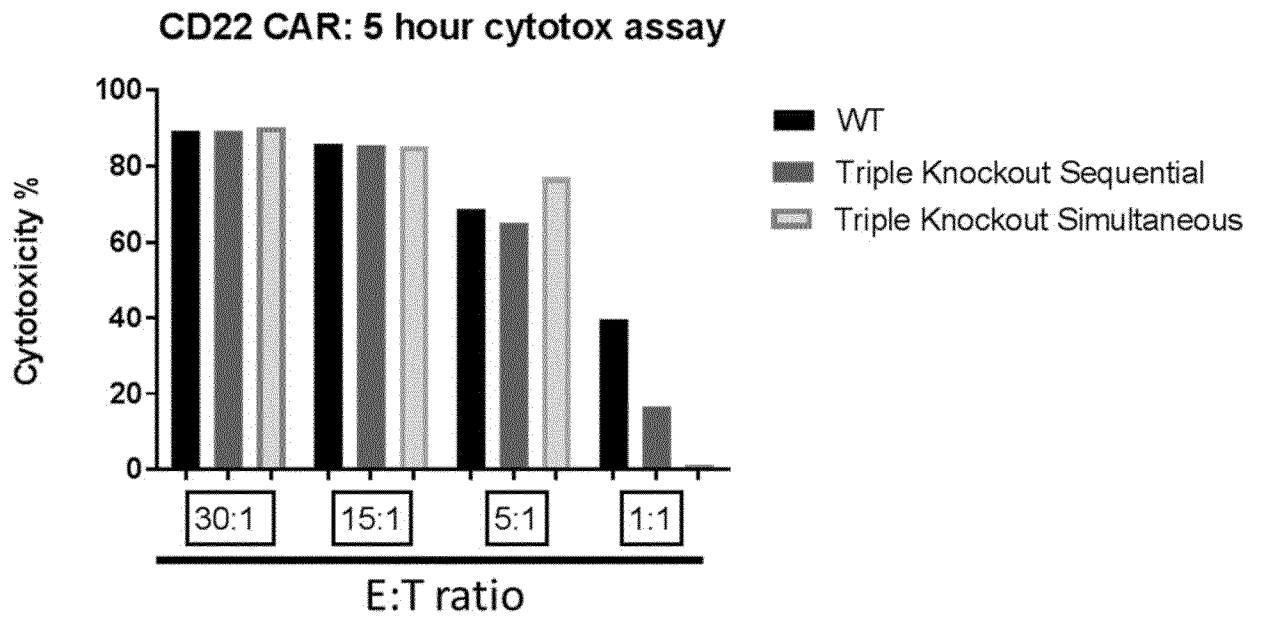


Figure 15

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2017/066355

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/725 C12N5/0783 C12N9/22 C12N15/63
ADD.
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED
Minimum documentation searched (classification system followed by classification symbols)
C07K C12N

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	B BERDIEN ET AL: "TALEN-mediated editing of endogenous T-cell receptors facilitates efficient reprogramming of T lymphocytes by lentiviral gene transfer", GENE THERAPY, vol. 21, no. 6, 27 March 2014 (2014-03-27), pages 539-548, XP055138166, ISSN: 0969-7128, DOI: 10.1038/gt.2014.26	1,6,7, 10,11, 17,18, 20,25, 27,28
Y	the whole document figure 5	1-28
X	WO 2015/075195 A1 (COLLECTIS [FR]) 28 May 2015 (2015-05-28)	1,6,7, 17-25
Y	page 12, line 25 - line 29; claims 1-32	1-28
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Further documents are listed in the continuation of Box C.

See patent family annex.

* Special categories of cited documents :

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- "E" earlier application or patent but published on or after the international filing date
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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

- "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- "X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- "Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
- "&" document member of the same patent family

Date of the actual completion of the international search 11 September 2017	Date of mailing of the international search report 20/09/2017
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Authorized officer Offermann, Stefanie

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2017/066355

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X Y	WO 2013/074916 A1 (UNIV TEXAS [US]) 23 May 2013 (2013-05-23) paragraph [0015]; claims 1-37 -----	1,6,7, 17-23,25 1-28
X Y	WO 2014/191128 A1 (CELLECTIS [FR]) 4 December 2014 (2014-12-04) page 10, line 9 - line 23; claims 1-51 -----	1,6,8, 17-25 1-28

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No PCT/EP2017/066355

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