MULTI-CHAIN CHIMERIC ANTIGEN RECEPTOR AND USES THEREOF

Field of the invention

The present invention relates to chimeric antigen receptors (CAR). CARs are able to redirect immune cell specificity and reactivity toward a selected target exploiting the ligand-binding domain properties. In particular, the present invention relates to multi-chain Chimeric Antigen Receptor in which extracellular ligand binding and signaling domains are separate in different transmembrane polypeptides to improve their functions. The different transmembrane polypeptides composing the multi-chain CAR of the present invention, once assembled together can specifically bind to one or several ligand(s) in a target and induce activation of immune cells in which they are expressed and immune response. The present invention also relates to polynucleotides, vectors encoding such transmembrane polypeptides and isolated cells expressing said multi-chain CAR at their surface for immunotherapy. The present invention also relates to methods for engineering immune cells expressing multi-chain CAR at their surface. The invention opens the way to efficient adoptive immunotherapy strategies for treating cancer and viral infections.

Background of the invention

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

Novel specificities in T cells have been successfully generated through the genetic transfer of transgenic T cell receptors or chimeric antigen receptors (CARs) (Jena, Dotti et al. 2010). CARs are synthetic receptors consisting of a targeting moiety that is associated with one or more signaling domains in a single fusion molecule. In general, the binding moiety of a CAR consists of an antigen-binding domain of a single-chain antibody (scFv), comprising the light

and 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), and 4-1BB (CD137) have been added alone (second generation) or in combination (third generation) to enhance survival and increase proliferation of CAR modified T cells. CARs have successfully allowed T cells to be redirected against antigens expressed at the surface of tumor cells from various malignancies including lymphomas and solid tumors (Jena, Dotti et al. 2010).

Present CAR architectures are built on a design in which all relevant domains are contained within a single polypeptide (US 7,741,465). This design necessitates serial appending of signaling domains, thus necessitating moving some domains from their natural juxtamembrane positions, distal from the plasma membrane. Nevertheless, architectures in which ligands and signaling domains are separate in their normal juxtamembrane positions (i.e. adjacent to the cell membrane on the internal side of it) would be more desirable and deemed to allow improved function of costimulatory domains. The high affinity receptor for IgE (FcεRI), could afford such architecture. Indeed, FcεRI, which is present on mast cells and basophils, is a tetrameric complex consisting of a ligand binding alpha subunit, a beta subunit and a homodimer of two signal-transducing gamma subunits (Metzger, Alcaraz et al. 1986). FcεRI alpha domain consists of an extracellular domain containing two Ig-like domains that bind IgE, a transmembrane domain and a short cytoplasmic tail. Beta subunit contains four transmembrane segments separating amino and carboxy terminal cytoplasmic tails. The gamma chain consists essentially of a transmembrane region and cytoplasmic tail containing one immunoreceptor tyrosine-based activation motif (ITAM) (Cambier 1995).

The current protocol for treatment of patients using adoptive immunotherapy is based on autologous cell transfer. In this approach, T lymphocytes are recovered from patients, genetically modified or selected *ex vivo*, cultivated *in vitro* in order to amplify the number of cells if necessary and finally infused into the patient. In addition to lymphocyte infusion, the host may be manipulated in other ways that support the engraftment of the T cells or their

participation in an immune response, for example pre-conditioning (with radiation or chemotherapy) and administration of lymphocyte growth factors (such as IL-2). Each patient receives an individually fabricated treatment, using the patient's own lymphocytes (i.e. an autologous therapy).

Autologous therapies face substantial technical and logistic hurdles to practical application, their generation requires expensive dedicated facilities and expert personnel, they must be generated in a short time following a patient's diagnosis, and in many cases, pretreatment of the patient has resulted in degraded immune function, such that the patient's lymphocytes may be poorly functional and present in very low numbers. Because of these hurdles, each patient's autologous cell preparation is effectively a new product, resulting in substantial variations in efficacy and safety. Ideally, one would like to use a standardized therapy in which allogeneic therapeutic cells could be pre-manufactured, characterized in detail, and available for immediate administration to patients. By allogeneic it is meant that the cells are obtained from individuals belonging to the same species but are genetically dissimilar. However, the use of allogeneic cells presently has many drawbacks. In immune-competent hosts allogeneic cells are rapidly rejected, a process termed host versus graft rejection (HvG), and this substantially limits the efficacy of the transferred cells. In immune-incompetent hosts, allogeneic cells are able to engraft, but their endogenous TCR specificities recognize the host tissue as foreign, resulting in graft versus host disease (GvHD), which can lead to serious tissue damage and death. In order to effectively use allogeneic cells, both of these problems must be overcome.

In immunocompetent hosts, allogeneic cells are rapidly rejected by the host immune system. It has been demonstrated that, allogeneic leukocytes present in non-irradiated blood products will persist for no more than 5 to 6 days. (Boni, Muranski et al. 2008). Thus, to prevent rejection of allogeneic cells, the host's immune system must be effectively suppressed. Glucocorticoidsteroids are widely used therapeutically for immunosuppression (Coutinho and Chapman 2011). This class of steroid hormones binds to the glucocorticoid receptor (GR) present in the cytosol of T cells resulting in the translocation into the nucleus and the binding of specific DNA motifs that regulate the expression of a number of genes involved in the immunologic process. Treatment of T cells with glucocorticoid steroids results in reduced levels of cytokine production leading to T cell anergy and interfering in T

cell activation. Alemtuzumab, also known as CAMPATH1-H, is a humanized monoclonal antibody targeting CD52, a 12 amino acid glycosylphosphatidyl-inositol- (GPI) linked glycoprotein (Waldmann and Hale 2005). CD52 is expressed at high levels on T and B lymphocytes and lower levels on monocytes while being absent on granulocytes and bone marrow precursors. Treatment with Alemtuzumab, a humanized monoclonal antibody directed against CD52, has been shown to induce a rapid depletion of circulating lymphocytes and monocytes. It is frequently used in the treatment of T cell lymphomas and in certain cases as part of a conditioning regimen for transplantation. However, in the case of adoptive immunotherapy the use of immunosuppressive drugs will also have a detrimental effect on the introduced therapeutic T cells. Therefore, to effectively use an adoptive immunotherapy approach in these conditions, the introduced cells would need to be resistant to the immunosuppressive treatment.

On the other hand, T cell receptors (TCR) are cell surface receptors that participate in the activation of T cells in response to the presentation of antigen. The TCR is generally made from two chains, alpha and beta, which assemble to form a heterodimer and associates with the CD3-transducing subunits to form the T-cell receptor complex present on the cell surface. Each alpha and beta chain of the TCR consists of an immunoglobulin-like N-terminal variable (V) and constant (C) region, a hydrophobic transmembrane domain, and a short cytoplasmic region. As for immunoglobulin molecules, the variable region of the alpha and beta chains are generated by V(D)J recombination, creating a large diversity of antigen specificities within the population of T cells. However, in contrast to immunoglobulins that recognize intact antigen, T cells are activated by processed peptide fragments in association with an MHC molecule, introducing an extra dimension to antigen recognition by T cells, known as MHC restriction. Recognition of MHC disparities between the donor and recipient through the T cell receptor leads to T cell proliferation and the potential development of GVHD. It has been shown that normal surface expression of the TCR depends on the coordinated synthesis and assembly of all seven components of the complex (Ashwell and Klusner 1990). The inactivation of TCRalpha or TCRbeta can result in the elimination of the TCR from the surface of T cells preventing recognition of alloantigen and thus GVHD. However, TCR disruption results in the elimination of the CD3 signaling component and alters the means of further T cell expansion.

T-cell mediated immunity includes multiple sequential steps regulated by a balance between co-stimulatory and inhibitory signals that fine-tune the immunity response. The inhibitory signals referred to as immune checkpoints are crucial for the maintenance of self-tolerance and also to limit immune-mediated collateral tissue damage. The expression of immune checkpoints protein can be deregulated by tumours. The ability of tumours to co-opt these inhibitory pathways represents an important mechanism in immune resistance and limits the success of immunotherapy. One of promising approaches to activating therapeutic T-cell immune response is the blockade of these immune checkpoints (Pardoll 2012). Immune checkpoints represent significant barriers to activation of functional cellular immunity in cancer, and antagonistic antibodies specific for inhibitory ligands on T cells including CTLA4 and programmed death-1 (PD-1) are examples of targeted agents being evaluated in the clinics.

Cytotoxic–T-lymphocyte-associated antigen 4 (CTLA-4; also known as CD152) downregulates the amplitude of T cell activation and treatment with antagonist CTLA4 antibodies (ipilimumab) has shown a survival benefit in patients with melanoma (Robert and Mateus 2011). Programmed cell death protein 1 (PD1 or PDCD1 also known as CD279) represent another very promising target for immunotherapy (Pardoll and Drake 2012; Pardoll 2012). In contrast to CTLA-4, PD1 limits T cell effector functions in peripheral tissue at the time of an inflammatory response to infection and to limit autoimmunity. The first clinical trial with PD1 antibody shows some cases of tumour regression (Brahmer, Drake et al. 2010). Multiple additional immune checkpoint protein represent promising targets for therapeutic blockade based on recently studies.

In normal T-cells, T cell receptors emanate from the pre-T cell receptors (pTCR) which are expressed by immature thymocytes and are crucial for T cell development from the double negative (CD4- CD8-) to the double-positive (CD4+ CD8+) stages. Pre-T cells that succeed in productive rearrangements of the TCRbeta locus express a functional TCRbeta chain which pairs with an invariant preTalpha chain and CD3 signaling components to form the pre-TCR complex. The expression of the preTCR at the cell surface is necessary for triggering beta-selection, a process that induces the expansion of developing T cells, enforces allelic exclusion of the TCRbeta locus and results in the induction of rearrangements at the TCRalpha locus (von Boehmer 2005). After productive TCRalpha rearrangements and

substitution of pTalpha by TCRalpha to form a mature TCR, thymocytes undergo a second step of selection, referred to as positive or TCRalpha/beta selection upon binding of self peptide MHC complexes expressed on thymic epithelial cells. Thus, mature T cells recognize and respond to the antigen/MHC complex through their TCR. The most immediate consequence of TCR activation is the initiation of signaling pathways via the associated CD3 subunits that result in multiple events including clonal expansion of T cells, upregulation of activation markers on the cell surface and induction of cytotoxicity or cytokine secretion.

Because of the nature of selection of TCRbeta chains through pairing with preTalpha during thymic development, in T cells in which TCRalpha has been inactivated, the heterologous introduction of the pTalpha transgene can result in the formation of a preTCR. This pTCR can serve as a means of T cell activation or stimulation in a manner that is non-MHC dependent, thus for example allowing continued expansion of alpha/beta T-cells following TCRalpha inactivation. Importantly, the pTCR complex displays a similar biochemical composition as the TCR in terms of associated CD3 subunits (Carrasco, Ramiro et al. 2001). In addition, in contrast to the TCR, pre-TCR signaling may occur in part by a ligand independent event. The crystal structure of the pTCR extracellular domain has provided a structural basis for the possible ligand-independence of pTCR signaling. The pTCR has been shown to form a head to tail dimer where two pTalpha-TCRbeta heterodimers associate (Pang, Berry et al. 2010).

In the context of developing therapeutic grade engineered immune cells that can target malignant or infected cells, the inventors have sought for improved CAR architectures, which would be closer to natural ones and likely to behave accordingly using any extracellular mono or multi-specific ligand binding domains

As a result, they have designed a new generation of CARs involving separate polypeptide sub-units according to the present invention, referred to as "multi-chain CARs".

Summary of the invention

Chimeric Antigen Receptors (CAR) in the prior art present as single fusion molecules that necessitate serial appending of signaling domains. However, removing signaling domains from their natural juxtamembrane position interfere with their function. Thus, to overcome this drawback, the inventors have succeeded in designing so-called multi-chain CARs that

allow juxtamembrane position of all relevant signaling domains. In the multi-chain CARs the signaling domains are placed on different polypeptide chains, in such that they seat in juxtamembrane positions. For example, multi-chain CAR can be derived from FcεRI, by replacing the high affinity IgE binding domain of FcεRI alpha chain by an extracellular ligand-binding domain such as scFv, , whereas the N and/or C-termini tails of FcεRI beta and/or gamma chain are used to place signal transducing domain in normal juxtamembrane positions. The extracellular ligand binding domain has the role of redirecting T-cell specificity towards cell targets, while the signal transducing domains, left in juxtamembrane positions, activate the immune cell response.

The fact that the signaling domains in juxtamembrane position are present on polypeptide(s) distinct from that carrying the extracellular ligand binding domain, provides a more flexible architecture for CARs. Accordingly, additional signaling domains or co-stimulatory domains can be added or removed from the CAR depending on the intensity of interaction sought between the CAR's ligand binding domain and its receptor. This flexibility in the architecture of the CAR also permits to introduce additional extracellular ligand binding domain(s). This can be done by fusion of a second ligand binding domain to the polypeptide carrying the first ligand binding domain, or by introducing additional extracellular ligand binding domains. If this second ligand binding domain has a different specificity, this forms a bi-specific multi-chain CAR, and if there are additional ones, multi-specific multi-chain CAR. These are particularly advantageous in view of targeting a given cell or virus carrying two or more different receptors present at their surface. This increases the specificity of such multi-chain CAR towards said cell or virus. On another hand, the number of signaling domains may permit the modulation of the signal respectively to the different binding domains. In addition, each recombinant polypeptide forming the CAR can be expressed independently at different expression levels to further modulate activity of the CAR.

The present invention is drawn to this new CAR architecture, to the recombinant polynucleotides encoding thereof, as well as to the methods to engineer immune cells for specific cell recognition, whatever the purpose and nature of the immune cells.

The invention additionally provides methods for making such immune cells more suitable for immunotherapy purposes.

For instance, the immune cells can be further engineered to be non-alloreactive and/or resistant to immunosuppressive agents, in particular, by inactivating TCR alpha or beta genes in primary cells and/or by coupling the inactivation of genes encoding targets for different immunosuppressive agents, in particular CD52 and GR (Gluococorticoid Receptors) and further selecting the cells resistant to said immunosuppressive agents

In addition to the above conception of genetically modified immune cells, the present invention also relates to embodiments where immune cells are engineered to allow proliferation when TCRalpha is inactivated, for instance by transiently expressing preTalpha in the cell thereby restoring a functional CD3 complex in the absence of a functional alpha/beta TCR.

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

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

Brief description of the figures and Tables

In addition to the preceding features, the invention further comprises other features which will emerge from the description which follows, as well as to the appended drawings. A more complete appreciation of the invention and many of the attendant advantages thereof will be readily obtained as the same becomes better understood by reference to the following Figures in conjunction with the detailed description below.

Figure 1: Schematic representation of the normal relationship between T-cells and antigen presenting cell.

Figure 2: Schematic representation of the genetically modified therapeutic T-cells according to the invention and the patient's tumor cells.

Figure 3: Schematic representation of multi-chain CAR.

Figure 4: Schematic of different versions of multi-chain CARs. A. Schematic of the FcεRI receptor. B-C Different versions of multi-chain CARs (csm1 to csm10) comprising a scFv and a CD8 stalk region fused to the transmembrane domain of FcεRI alpha chain. At least one 41BB, CD28 and/or CD3 zeta domains can be fused to a FcεRI alpha, beta and/or gamma chain.

Figure 5: Schematic representation of one example of the method of engineering human allogenic cells for immunotherapy

Figure 6: Concentration in cells per milliliter of live CD52-positive or CD52-negative cells after treatment with anti-CD52 antibody (CAMPATH1-H) with complement or controls.

Figure 7: Comparison of the forward side scatter (FSC) distribution, an indicator of cell size, between TCR-positive and TCR-negative cells, or between CD52-positive and CD52-negative cells, and non activated cells as control.

Figure 8: Flow cytometry analysis of CD107a expression (marker of degranulation) on targeted CD52 and TCRalpha inactivated T cells. CD107 expression is analyzed on CD52+TCRαβ+ cells (first column), CD52-TCRαβ- cells (second column), CD52-TCRαβ+ cells (third column) and CD52+TCRαβ- cells (fourth column) before (A) and after incubation with Daudi cells (B); C) represents flow cytometry analysis of T cells further transfected with a CAR and incubated with Daudi cells; D) represents flow cytometry analysis of T cells transfected with a CAR but not incubated with Daudi cells and E) represents flow cytometry analysis of T cells transfected with a CAR and treated to PMA/ionomycin (positive control).

Figure 9: Deep sequencing analysis of CD52 and TRAC TALE-nucleases potential off-site targets. Figure 9 discloses "left half target" sequences as SEQ ID NOS: 149-165 and "right half target" sequences as SEQ ID NOS 166-182, all respectively, in order of appearance.

Figure 10: Analysis of PDCD1 and CTLA-4 genomic locus by T7-endonuclease assay. Arrows point to digested PCR products.

Figure 11: Schematic representation of some examples of preTalpha constructs.

Figure 12: Flow cytometry analysis of transduction efficiency (% BFP+ cells) and activity of the FL, Δ18, Δ48 pTalpha constructs (% CD3 surface expression) in TCR alpha inactivated Jurkat cells.

Figure 13: Schematic representation of a lentiviral construct coding for pTalpha protein (preTCRα).

Figure 14: A. Representation of the experimental protocol. **B.**Flow cytometry analysis of TCR alpha/beta, CD3 expression and BFP expression on TCRalpha inactivated T cells (KO) transduced with either BFP-2A-pTalphaΔ48 (KO/Δ48) or control BFP lentiviral vector (KO/BFP) before and after purification. **C.** Flow cytometry analysis of TCR alpha/beta and CD3 expression on purified TCR alpha inactivated cells transduced (BFPpos) or not (BFPneg) with BFP-2A-pTalphaΔ48 lentiviral vector. NEP represents non electroporated cells with TRAC TALE-nucleases.

Figure 15: A-B. Flow cytometry analysis of early activation marker CD69 (A), late activation marker CD25 (B) expression 24 and 48 hours after re-activation with anti-CD3/CD28 beads respectively on non electroporated cells (NEP) and TCRalpha inactivated cells (KO) transduced with BFP-2A-pTα-Δ48 lentiviral vector (pTα-Δ48), BFP-2A-pTα-Δ48.41BB lentiviral vector (pTα-Δ48.BB) or control BFP vector (BFP). pTα-Δ48 histograms correspond to the signal detected in TCR inactivated cells expressing pTα-Δ48 (BFP+ cells) while the KO histograms correspond to TCRalpha inactivated cells which do not express pTα-Δ48 (BFP- cells) pTα-Δ48.BB histograms correspond to the signal detected in TCR inactivated cells expressing pTα-Δ48.41BB (BFP+ cells) while the KO histograms correspond to TCRalpha inactivated cells which do not express pTα-Δ48.41BB (BFP- cells). NEP (non electroporated) histograms correspond to signal detected in non engineered cells. **C.** Flow cytometry analysis of the size of cells 72 hours after re-activation with anti-CD3/CD28 beads on non electroporated cells (NEP) and TCRalpha inactivated cells (KO) transduced with BFP-2A-pTα-Δ48 lentiviral vector (pTα-Δ48), BFP-2A-pTα-Δ48.41BB lentiviral vector (pTα-Δ48.BB) or control BFP vector (BFP). The values indicated in the upper part of each graph correspond to the geometrical mean of the fluorescence of each population.

Figure 16: Cell growth analysis of TCR alpha inactivated cells (KO) transduced with pTalpha-Δ48 (pTaΔ48) or control BFP vector (BFP) maintained in IL2 or in IL2 with anti- CD3/CD28 beads at different time points (x-axis). The BFP+ cells number is estimated at different time points for each condition and the fold induction of these cells (y-axis) was estimated with respect to the value obtained at day 2 post re-activation. The results are obtained from two

independent donors. For the second donor, cell growth was also determined for cells transduced with pTalpha-Δ48.41BB (pTa-Δ48.BB) and full-length pTalpha- (pTa-FL).

Figure 17: Flow cytometry analysis of GFP positive cells on PBMCs electroporated with the five different Cytopulse programs. A. NEP, EP#1 (GFP) and EP#2 (GFP); B. EP#3 (pUC), EP#4 (GFP) and EP#5 (GFP). The upper line corresponds to transfection of $6x10^6$ cells per cuvette, while the lower line corresponds to transfection of $3x10^6$ cells per cuvette.

Figure 18: Flow cytometry analysis of purified T cell mortality using viability dye (eFluor-450) and of GFP positive cells among the viable population after electroporation with GFP mRNA, GFP DNA and control pUC DNA. A. NT and NEP; B. EP (no DNA) and GFP DNA; C. pUC DNA and GFP mRNA. NEP corresponds to cells that were maintained in electroporation buffer but were not electroporated and NT corresponds to non electroporated cells maintained in culture medium.

Figure 19: Flow cytometry analysis of TCR alpha/beta and CD3 expression on human primary T cells following TRAC TALE-nuclease mRNA electroporation (top). Deep sequencing analysis of genomic DNA extracted from human primary T cells following TRAC TALE-nuclease mRNA electroporation (bottom) (SEQ ID NOS 183-192, respectively, in order of appearance).

Figure 20: A. Flow cytometry analysis of CAR expression (anti F(ab')2) after electroporation of T cells with or without mRNA encoding a single chain CAR. **B.** Flow cytometry analysis of CD107a expression (marker of degranulation) on electroporated T cells cocultured with daudi cells.

Figure 21: A. Representation of mRNA encoding a multi-chain CAR. **B.** Flow cytometry analysis of CAR expression (anti F(ab')2) on viable T cells electroporated with or without a polycistronic mRNA encoding a multi-chain CAR. **C.** Flow cytometry analysis of CD107a expression (marker of degranulation) on electroporated T cells cocultured with daudi cells.

Figure 22: Multi-chain CARs expression in human T cells after electroporation of polycistronic mRNAs.

Figure 23: The expression of the multi-subunit CARs is conditioned by the expression of the three chains: α, β and γ.

Figure 24: The human T cells transiently expressing the multi-chain CARs degranulate following coculture with target cells.

Figure 25: The human T cells transiently expressing the multi-chain CARs secrete cytokines following coculture with target cells.

Figure 26: The human T cells transiently expressing the multi-chain CARs lyse target cells.

Table 1: Description of the GR TALE-nucleases and sequences of the TALE-nucleases target sites in the human GR gene.

Table 2: Cleavage activity of the GR TALE-nucleases in yeast. Values are comprised between 0 and 1. Maximal value is 1.

Table 3: Percentage of targeted mutagenesis at endogenous TALE-nuclease target sites in 293 cells.

Table 4: Percentage of targeted mutagenesis at endogenous TALE-nuclease target sites in primary T lymphocytes.

Table 5: Description of the CD52, TRAC and TRBC TALE-nucleases and sequences of the TALE-nucleases target sites in the human corresponding genes.

Table 6: Additional target sequences for TRAC and CD52 TALE-nucleases.

Table 7: Percentage of indels for TALE-nuclease targeting CD52_T02, TRAC_T01, TRBC_T01 and TRBC_T02 targets.

Table 8: Percentages of CD52- negative, TCR-negative and CD52/TCR-double negative T lymphocytes after transfection of corresponding TALE-nuclease-expressing polynucleotides.

Table 9: Percentages of TCR-negative T lymphocytes after transfection of TRBC TALE-nuclease-expressing polynucleotides.

Table 10: Description of the CTLA4 and PDCD1 TALE-nucleases and sequences of the TALE-nucleases target sites in the human corresponding genes.

Table 11: Description of a subset of pTalpha constructs.

Table 12: Activity of the different pTalpha constructs in Jurkat TCR alpha inactivated cell. Activity was measured by flow cytometry analysis of CD3 expression on jurkat TCR alpha inactivated cell transfected with the different preTalpha constructs.

Table 13: Different cytopulse programs used to determine the minimal voltage required for electroporation in PBMC derived T-cells.

Table 14: Cytopulse program used to electroporate purified T-cells.

Table 15: Description of the FcR chains compositions of the multi-chain CARs versions.

Detailed description of the invention

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

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

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

specifically, Vols.154 and 155 (Wu et al. eds.) and Vol. 185, "Gene Expression Technology" (D. Goeddel, ed.); Gene Transfer Vectors For Mammalian Cells (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); Immunochemical Methods In Cell And Molecular Biology (Mayer and Walker, eds., Academic Press, London, 1987); Handbook Of Experimental Immunology, Volumes I-IV (D. M. Weir and C. C. Blackwell, eds., 1986); and Manipulating the Mouse Embryo, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

Multi-chain Chimeric Antigen Receptor (CAR)

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

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

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

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

The term "extracellular ligand-binding domain" as used herein is defined as an oligo- or polypeptide that is capable of binding a ligand. Preferably, the domain will be capable of interacting with a cell surface molecule. For example, the extracellular ligand-binding domain may be chosen to recognize a ligand that acts as a cell surface marker on target cells associated with a particular disease state. Thus examples of cell surface markers that may act as ligands include those associated with viral, bacterial and parasitic infections, autoimmune disease and cancer cells. In particular, the extracellular ligand-binding domain can comprise an antigen binding domain derived from an antibody against an antigen of the target. As non limiting examples, the antigen of the target can be a tumor-associated surface antigen, such as ErbB2 (HER2/neu), carcinoembryonic antigen (CEA), epithelial cell adhesion molecule (EpCAM), epidermal growth factor receptor (EGFR), EGFR variant III (EGFRvIII), CD19, CD20, CD30, CD40, disialoganglioside GD2, ductal-epithelial mucine, gp36, TAG-72,

glycosphingolipids, glioma-associated antigen, β-human chorionic gonadotropin, alphafetoprotein (AFP), lectin-reactive AFP, thyroglobulin, RAGE-1, MN-CA IX, human telomerase reverse transcriptase, RU1, RU2 (AS), intestinal carboxyl esterase, mut hsp70-2, M-CSF, prostase, prostase specific antigen (PSA), PAP, NY-ESO-1, LAGA-1a, p53, prostein, PSMA, surviving and telomerase, prostate-carcinoma tumor antigen-1 (PCTA-1), MAGE, ELF2M, neutrophil elastase, ephrin B2, CD22, insulin growth factor (IGF1)-I, IGF-II, IGFI receptor, mesothelin, a major histocompatibility complex (MHC) molecule presenting a tumor-specific peptide epitope, 5T4, ROR1, Nkp30, NKG2D, tumor stromal antigens, the extra domain A (EDA) and extra domain B (EDB) of fibronectin and the A1 domain of tenascin-C (TnC A1) and fibroblast associated protein (fap); a lineage-specific or tissue specific antigen such as CD3, CD4, CD8, CD24, CD25, CD33, CD34, CD133, CD138, CTLA-4, B7-1 (CD80), B7-2 (CD86), endoglin, a major histocompatibility complex (MHC) molecule, BCMA (CD269, TNFRSF 17), or a virus-specific surface antigen such as an HIV-specific antigen (such as HIV gp120); an EBV-specific antigen, a CMV-specific antigen, a HPV-specific antigen, a Lasse Virus-specific antigen, an Influenza Virus-specific antigen as well as any derivate or variant of these surface markers.

The extracellular ligand-binding domain can also comprise a peptide binding an antigen of the target, a peptide or a protein binding an antibody that binds an antigen of the target, a peptide or a protein ligand such as a growth factor, a cytokine or a hormone as non limiting examples binding a receptor on the target, or a domain derived from a receptor such as a growth factor receptor, a cytokine receptor or a hormone receptor as non limiting examples, binding a peptide or a protein ligand on the target. Preferably the target is a cell or a virus.

In a preferred embodiment, said extracellular ligand-binding domain is a single chain antibody fragment (scFv) comprising the light (V_L) and the heavy (V_H) variable fragment of a target antigen specific monoclonal antibody joined by a flexible linker. In a preferred embodiment, said scFv is an anti-CD19 scFV, preferably scFV-4G7 (Peipp et al., J Immunol Methods. 2004 Feb 15;285(2):265-80) (VH: SEQ ID NO: 193 and VL: SEQ ID NO: 194, scFV: SEQ ID NO: 195).

Other binding domain than scFv can also be used for predefined targeting of lymphocytes, such as camelid single-domain antibody fragments or receptor ligands like a vascular endothelial growth factor polypeptide, an integrin-binding peptide, heregulin or an IL-13

mutein, antibody binding domains, antibody hypervariable loops or CDRs as non limiting examples.

In a preferred embodiment said transmembrane polypeptide further comprises a stalk region between said extracellular ligand-binding domain and said transmembrane domain. The term "stalk region" used herein generally means any oligo- or polypeptide that functions to link the transmembrane domain to the extracellular ligand-binding domain. In particular, stalk region are used to provide more flexibility and accessibility for the extracellular ligand-binding domain. A stalk region may comprise up to 300 amino acids, preferably 10 to 100 amino acids and most preferably 25 to 50 amino acids. Stalk region may be derived from all or part of naturally occurring molecules, such as from all or part of the extracellular region of CD8, CD4 or CD28, or from all or part of an antibody constant region. Alternatively the stalk region may be a synthetic sequence that corresponds to a naturally occurring stalk sequence, or may be an entirely synthetic stalk sequence. In a preferred embodiment said stalk region is a part of human CD8 alpha chain (e.g. NP_001139345.1) (SEQ ID NO: 196).

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

Downregulation or mutation of target antigens is commonly observed in cancer cells, creating antigen-loss escape variants. Thus, to offset tumor escape and render immune cell more specific to target, the multi-chain CAR can comprise several extracellular ligand-binding domains, to simultaneously bind different elements in target thereby augmenting immune cell activation and function. In one embodiment, the extracellular ligand-binding domains can be placed in tandem on the same transmembrane polypeptide, and optionally can be separated by a linker. In another embodiment, said different extracellular ligand-binding domains can be placed on different transmembrane polypeptides composing the multi-chain CAR. In another embodiment, the present invention relates to a population of multi-chain CARs comprising each one different extracellular ligand binding domains. In a particular, the present invention relates to a method of engineering immune cells comprising providing an immune cell and expressing at the surface of said cell a population of multi-chain CAR each

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

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

The signal transducing domain or intracellular signaling domain of the multi-chain CAR of the invention is responsible for intracellular signaling following the binding of extracellular ligand binding domain to the target resulting in the activation of the immune cell and immune response. In other words, the signal transducing domain is responsible for the activation of at least one of the normal effector functions of the immune cell in which the multi-chain CAR is expressed. For example, the effector function of a T cell can be a cytolytic activity or helper activity including the secretion of cytokines. Thus, the term "signal tansducing domain" refers to the portion of a protein which transduces the effector signal function signal and directs the cell to perform a specialized function.

Preferred examples of signal transducing domain for use in multi-chain CAR can be the cytoplasmic sequences of the Fc receptor or T cell receptor and co-receptors that act in concert to initiate signal transduction following antigen receptor engagement, as well as any derivate or variant of these sequences and any synthetic sequence that as the same

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

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

A "co-stimulatory molecule" refers to the cognate binding partner on a T-cell that specifically binds with a co-stimulatory ligand, thereby mediating a co-stimulatory response by the cell,

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

In another particular embodiment, said signal transducing domain is a TNFR-associated Factor 2 (TRAF2) binding motifs, intracytoplasmic tail of costimulatory TNFR member family. Cytoplasmic tail of costimulatory TNFR family member contains TRAF2 binding motifs consisting of the major conserved motif (P/S/A)X(Q/E)E) or the minor motif (PXQXXD), wherein X is any amino acid. TRAF proteins are recruited to the intracellular tails of many TNFRs in response to receptor trimerization.

In a preferred embodiment, the signal transduction domain of the multi-chain CAR of the present invention comprises a part of co-stimulatory signal molecule selected from the group consisting of 4-1BB (GenBank: AAA53133.) and CD28 (NP_006130.1). In particular the signal transduction domain of the multi-chain CAR of the present invention comprises amino acid sequence selected from SEQ ID NO: 200 and SEQ ID NO: 201.

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

particular comprises a part of the FcεRI α (SEQ ID NO: 202), β (SEQ ID NO: 203) and/or γ chains (SEQ ID NO: 204) or variant thereof.

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

In a particular embodiment, the multi-chain CAR comprises a transmembrane polypeptide derived from a FcεRI chain. In more particular embodiment FcεRI chain is a FcεRI α chain, in which the extracellular domain is replaced by an extracellular ligand-binding domain, preferably by a scFV, more preferably scFv-4G7 (SEQ ID NO: 195).

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

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

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

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

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

In a preferred embodiment, the multi-chain CAR of the present invention comprise a part of a polypeptide with amino acid sequence selected from the group consisting of SEQ ID NO: 202 to SEQ ID NO: 204.

The term "a part of" used herein refers to any subset of the molecule, that is a shorter peptide. Alternatively, amino acid sequence functional variants of the polypeptide can be prepared by mutations in the DNA which encodes the polypeptide. Such variants or functional variants include, for example, deletions from, or insertions or substitutions of, residues within the amino acid sequence. Any combination of deletion, insertion, and substitution may also be made to arrive at the final construct, provided that the final construct possesses the desired activity, especially to exhibit a specific anti-target cellular immune activity. The functionality of the multi-chain CAR of the invention within a host cell is detectable in an assay suitable for demonstrating the signaling potential of said multi-chain CAR upon binding of a particular target. Such assays are available to the skilled person in the art. For example, this assay allows the detection of a signaling pathway, triggered upon binding of the target, such as an assay involving measurement of the increase of calcium ion release, intracellular tyrosine phosphorylation, inositol phosphate turnover, or interleukin (IL) 2, interferon γ, GM-CSF, IL-3, IL-4 production thus effected.

As non limiting example, different versions of multi-chain CAR are illustrated in Figure 4. In a more preferred embodiment, the multi-chain CAR of the present invention comprises a polypeptide with amino acid sequence selected from the group consisting of SEQ ID NO: 206 to SEQ ID NO: 213. In a preferred embodiment the multi-chain CAR comprise a polypeptide with amino acid sequence that has at least 70%, preferably at least 80%, more preferably at least 90 %, 95 % 97 % or 99 % sequence identity with amino acid sequence selected from the group consisting of SEQ ID NO: 206 to SEQ ID NO: 213.

Polynucleotides, vectors:

The present invention also relates to polynucleotides, vectors encoding the above described multi-chain CAR according to the invention. The present invention provides polynucleotides, including DNA and RNA molecules that encode the transmembrane polypeptides disclosed herein that can be included in the multi-chain CAR. In particular, the invention relates to a polynucleotide comprising a nucleic acid sequence encoding at least one transmembrane polypeptide composing the multi-chain CAR as described above. More particularly the invention relates to a polynucleotide comprising two or more nucleic acid sequences encoding transmembrane polypeptides composing the multi-chain CAR as described above. In a preferred embodiment, the present invention relates to a polynucleotide selected from the group consisting of: SEQ ID NO: 214 to SEQ ID NO: 223. In a preferred embodiment, the polynucleotide has at least 70%, preferably at least 80%, more preferably at least 90 %, 95 % 97 % or 99 % sequence identity with nucleic acid sequence selected from the group consisting of SEQ ID NO: 214 to SEQ ID NO: 223.

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

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

peptides have been used to express into the cell the different polypeptides of the multi-chain CAR. In a more preferred embodiment, the present invention relates to polynucleotides selected from the group consisiting of: SEQ ID NO: 224 to SEQ ID NO: 232.

To direct, transmembrane polypeptide such as FcεR into the secretory pathway of a host cell, a secretory signal sequence (also known as a leader sequence, prepro sequence or pre sequence) is provided in polynucleotide sequence or vector sequence. The secretory signal sequence may be that of FcεR, or may be derived from another secreted protein (e.g., t-PA) or synthesized *de novo*. The secretory signal sequence is operably linked to the transmembrane nucleic acid sequence, i.e., the two sequences are joined in the correct reading frame and positioned to direct the newly synthesized polypeptide into the secretory pathway of the host cell. Secretory signal sequences are commonly positioned 5' to the nucleic acid sequence encoding the polypeptide of interest, although certain secretory signal sequences may be positioned elsewhere in the nucleic acid sequence of interest (see, e.g., Welch et al., U.S. Patent No. 5,037,743; Holland et al., U.S. Patent No. 5,143,830). In a preferred embodiment the signal peptide comprises the residues 1 to 25 of the FcεRI alpha chain (NP_001992.1) and has the amino acid sequence SEQ ID NO: 205.

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

In a preferred embodiment, the polynucleotide according to the present invention comprises the nucleic acid sequence selected from the group consisting of: SEQ ID NO: 214 to SEQ ID NO: 223. The present invention relates to polynucleotides comprising a nucleic acid sequence that has at least 70%, preferably at least 80%, more preferably at least 90 %, 95 % 97 % or 99 % sequence identity with nucleic acid sequence selected from the group consisting of SEQ ID NO: 214 to SEQ ID NO: 222.

Methods of engineering an immune cell:

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

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

In another embodiment, said method further comprises a step of genetically modifying said cell by inactivating at least one gene expressing one component of the TCR, a target for an immunosuppressive agent, HLA gene and/or an immune checkpoint gene such as PDCD1 or CTLA-4. In a preferred embodiment, said gene is selected from the group consisting of TCRalpha, TCRbeta, CD52, GR, PD1 and CTLA-4. In a preferred embodiment said method further comprises introducing into said T cells a rare-cutting endonuclease able to selectively inactivate by DNA cleavage said genes. In a more preferred embodiment said rare-cutting endonuclease is TALE-nuclease. Preferred TALE-nucleases according to the invention are those recognizing and cleaving the target sequence selected from the group consisting of: SEQ ID NO: 1 to 6 (GR), SEQ ID NO: 37, 57 to 60 (TCRalpha), SEQ ID NO: 38 or 39 (TCRbeta), and SEQ ID NO: 40, SEQ ID NO: 61 to SEQ ID NO: 65 (CD52), SEQ ID NO: 74 to SEQ ID NO: 78 (PD1 and CTLA-4).

By inactivating a gene it is intended that the gene of interest is not expressed in a functional protein form. In particular embodiment, the genetic modification of the method relies on the expression, in provided cells to engineer, of one rare-cutting endonuclease such that said

rare-cutting endonuclease specifically catalyzes cleavage in one targeted gene thereby inactivating said targeted gene. The nucleic acid strand breaks caused by the rare-cutting endonuclease are commonly repaired through the distinct mechanisms of homologous recombination or non-homologous end joining (NHEJ). However, NHEJ is an imperfect repair process that often results in changes to the DNA sequence at the site of the cleavage. Mechanisms involve rejoining of what remains of the two DNA ends through direct re-ligation (Critchlow and Jackson 1998) or via the so-called microhomology-mediated end joining (Ma, Kim et al. 2003). Repair via non-homologous end joining (NHEJ) often results in small insertions or deletions and can be used for the creation of specific gene knockouts. Said modification may be a substitution, deletion, or addition of at least one nucleotide. Cells in which a cleavage-induced mutagenesis event, i.e a mutagenesis event consecutive to an NHEJ event, has occurred can be identified and/or selected by well-known method in the art.

In another embodiment, additional catalytic domain can be further introduced into the cell with said rare-cutting endonuclease to increase mutagenesis in order to enhance their capacity to inactivate targeted genes described in the present disclosure. In particular, said additional catalytic domain is a DNA end processing enzyme. Non limiting examples of DNA end-processing enzymes include 5-3' exonucleases, 3-5' exonucleases, 5-3' alkaline exonucleases, 5' flap endonucleases, helicases, hosphatase, hydrolases and template-independent DNA polymerases. Non limiting examples of such catalytic domain comprise of a protein domain or catalytically active derivate of the protein domain selected from the group consisting of hExoI (EXO1_HUMAN), Yeast ExoI (EXO1_YEAST), E.coli ExoI, Human TREX2, Mouse TREX1, Human TREX1, Bovine TREX1, Rat TREX1, TdT (terminal deoxynucleotidyl transferase) Human DNA2, Yeast DNA2 (DNA2_YEAST). In a preferred embodiment, said additional catalytic domain has a 3'-5'-exonuclease activity, and in a more preferred embodiment, said additional catalytic domain is TREX, more preferably TREX2 catalytic domain (WO2012/058458). In another preferred embodiment, said catalytic domain is encoded by a single chain TREX polypeptide. Said additional catalytic domain may be fused to a nuclease fusion protein or chimeric protein according to the invention optionally by a peptide linker.

Endonucleolytic breaks are known to stimulate the rate of homologous recombination. Thus, in another embodiment, the genetic modification step of the method further comprises a step of introduction into cells an exogeneous nucleic acid comprising at least a sequence homologous to a portion of the target nucleic acid sequence, such that homologous recombination occurs between the target nucleic acid sequence and the exogeneous nucleic acid. In particular embodiments, said exogenous nucleic acid comprises first and second portions which are homologous to region 5' and 3' of the target nucleic acid sequence, respectively. Said exogenous nucleic acid in these embodiments also comprises a third portion positioned between the first and the second portion which comprises no homology with the regions 5' and 3' of the target nucleic acid sequence. Following cleavage of the target nucleic acid sequence, a homologous recombination event is stimulated between the target nucleic acid sequence and the exogenous nucleic acid. Preferably, homologous sequences of at least 50 bp, preferably more than 100 bp and more preferably more than 200 bp are used within said donor matrix. Therefore, the exogenous nucleic acid is preferably from 200 bp to 6000 bp, more preferably from 1000 bp to 2000 bp. Indeed, shared nucleic acid homologies are located in regions flanking upstream and downstream the site of the break and the nucleic acid sequence to be introduced should be located between the two arms.

In particular, said exogenous nucleic acid successively comprises a first region of homology to sequences upstream of said cleavage, a sequence to inactivate one targeted gene selected from the group consisting of TCR alpha, TCR beta, CD52, GR, immune checkpoint genes and a second region of homology to sequences downstream of the cleavage. Said polynucleotide introduction step can be simultaneous, before or after the introduction or expression of said rare-cutting endonuclease. Depending on the location of the target nucleic acid sequence wherein break event has occurred, such exogenous nucleic acid can be used to knock-out a gene, e.g. when exogenous nucleic acid is located within the open reading frame of said gene, or to introduce new sequences or genes of interest. Sequence insertions by using such exogenous nucleic acid can be used to modify a targeted existing gene, by correction or replacement of said gene (allele swap as a non-limiting example), or to up- or down-regulate the expression of the targeted gene (promoter swap as non-limiting example), said targeted gene correction or replacement. In preferred embodiment,

inactivation of genes from the group consisting of TCR alpha, TCR beta, CD52, GR, immune checkpoint genes can be done at a precise genomic location targeted by a specific TALE-nuclease, wherein said specific TALE-nuclease catalyzes a cleavage and wherein said exogenous nucleic acid successively comprising at least a region of homology and a sequence to inactivate one targeted gene selected from the group consisting of TCR alpha, TCR beta, CD52, GR, immune checkpoint genes which is integrated by homologous recombination. In another embodiment, several genes can be, successively or at the same time, inactivated by using several TALE-nucleases respectively and specifically targeting one defined gene and several specific polynucleotides for specific gene inactivation.

By additional genomic modification step, can be intended also the inactivation of another gene selected from the group consisting of TCR alpha, TCR beta, CD52, GR, immune checkpoint genes. As mentioned above, said additional genomic modification step can be an inactivation step comprising:

(a) introducing into said cells at least one rare-cutting endonuclease such that said rare-cutting endonuclease specifically catalyzes cleavage in one targeted sequence of the genome of said cell.

(b) Optionally introducing into said cells a exogenous nucleic acid successively comprising a first region of homology to sequences upstream of said cleavage, a sequence to be inserted in the genome of said cell and a second region of homology to sequences downstream of said cleavage,

wherein said introduced exogenous nucleic acid inactivates a gene and integrates at least one exogenous polynucleotide sequence encoding at least one recombinant protein of interest. In another embodiment, said exogenous polynucleotide sequence is integrated within a gene selected from the group consisting of TCR alpha, TCR beta, CD52, GR, immune checkpoint genes.

- *Immunosuppressive resistant T cells:*

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

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

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

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

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

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

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

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

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

In a more preferred embodiment, said method comprises:

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

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

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

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

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

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

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

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

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

In another embodiment, said rare-cutting endonuclease can be a meganuclease, a Zinc finger nuclease or a TALE-nuclease. In a preferred embodiment, said rare-cutting endonuclease is a TALE-nuclease. Preferred TALE-nucleases according to the invention are those recognizing and cleaving the target sequence selected from the group consisting of:

- SEQ ID NO: 1 to 6 (GR), and

- SEQ ID NO: 40, 61 to 65 (CD52)

Said TALE-nucleases preferably comprise a polypeptide sequence selected from SEQ ID NO: 7 to SEQ ID NO: 18 and SEQ ID NO: 47 to SEQ ID NO: 48, in order to cleave the respective target sequences SEQ ID NO: 1 to 6 and SEQ ID NO: 40.

- *Highly active T cells for immunotherapy*

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

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

(b) expanding said cells.

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

protein expressed on certain CD4 and CD8 T cells; when engaged by its ligands (B7-1 and B7-2) on antigen presenting cells, T-cell activation and effector function are inhibited. Thus the present invention relates to a method of engineering T-cells, especially for immunotherapy, comprising genetically modifying T-cells by inactivating at least one protein involved in the immune check-point, in particular PD1 and/or CTLA-4.

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

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

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

(c) expanding said cells.

In a more preferred embodiment, said method comprises:

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

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

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

(d) expanding said cells.

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

2011; Mussolino, Morbitzer et al. 2011; Sander, Cade et al. 2011; Tesson, Usal et al. 2011; Weber, Gruetzner et al. 2011; Zhang, Cong et al. 2011; Deng, Yan et al. 2012; Li, Piatek et al. 2012; Mahfouz, Li et al. 2012; Mak, Bradley et al. 2012).

In the present invention new TALE-nucleases have been designed for precisely targeting relevant genes for adoptive immunotherapy strategies. Preferred TALE-nucleases according to the invention are those recognizing and cleaving the target sequence selected from the group consisting of: SEQ ID NO: 77 and SEQ ID NO: 78 (PD1), SEQ ID NO: 74 to SEQ ID NO: 76 (CTLA-4). The present invention also relates to TALE-nuclease polypeptides which comprise an amino acid sequence selected from the group consisting of SEQ ID NO: 79 to SEQ ID NO: 88.

The present invention also relates to polypeptides comprising an amino acid sequence that has at least 70%, preferably at least 80%, more preferably at least 90 %, 95 % 97 % or 99 % sequence identity with amino acid sequence selected from the group consisting of SEQ ID NO: 79 to SEQ ID NO: 88. Are also comprised in the scope of the present invention, polynucleotides, vectors encoding the above described rare-cutting endonucleases according to the invention. This method can be associated with any one of the different methods described in the present disclosure.

- *Non alloreactive T cells:*

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

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

(b) Expanding said cells.

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

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

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

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

(b) Expanding said cells.

In a more preferred embodiment, said method comprises:

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

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

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

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

(e) Expanding said cells.

In another embodiment, said rare-cutting endonuclease can be a meganuclease, a Zinc finger nuclease or a TALE-nuclease. In a preferred embodiment, said rare-cutting endonuclease is a TALE-nuclease. Preferred TALE-nucleases according to the invention are those recognizing and cleaving the target sequence selected from the group consisting of:

- SEQ ID NO: 37, 57 to 60 (TCRalpha),

- SEQ ID NO: 38 or 39 (TCRbeta),

Said TALE-nucleases preferably comprise a polypeptide sequence selected from SEQ ID NO: 41 to SEQ ID NO: 48, in order to cleave the respective target sequences SEQ ID NO: 37 to 39.

- *PreTalpha*

In another aspect, one another step of genetically modifying cell can be a method of expanding TCR alpha deficient T-cell comprising introducing into said T-cell pTalpha (also

named preTCRα) or a functional variant thereof and expanding said cells, optionally through stimulation of the CD3 complex. In a preferred embodiment, the method comprises:

a) Transforming said cells with nucleic acid encoding at least a fragment of pTalpha to support CD3 surface expression

b) Expressing said pTalpha into said cells

c) Expanding said cells, optionally through stimulation of the CD3 complex.

The invention also relates to a method of preparing T-cells for immunotherapy comprising steps of the method for expansion for T-cell.

In particular embodiment, the pTalpha polynucleotide sequence can be introduced randomly or else through homologous recombination, in particular the insertion could be associated with the inactivation of the TCRalpha gene.

According to the invention, different functional variants of pTalpha are used. A "functional variant" of the peptide refers to a molecule substantially similar to either the entire peptide or a fragment thereof. A "fragment" of the pTalpha or functional variant thereof of the present Invention, refers to any subset of the molecule, that is, a shorter peptide. Preferred pTalpha or functional variants can be full length pTalpha or a C-terminal truncated pTalpha version. C-terminal truncated pTalpha lacks in C-terminal end one or more residues. As non limiting examples, C-terminal truncated pTalpha version lacks 18 , 48, 62, 78, 92, 110 or 114 residues from the C-terminus of the protein (SEQ ID NO: 107 to SEQ ID NO: 114). Moreover, amino acid sequence variants of the peptide can be prepared by mutations in the DNA which encodes the peptide. Such functional variants include, for example, deletions from, or insertions or substitutions of, residues within the amino acid sequence. Any combination of deletion, insertion, and substitution may also be made to arrive at the final construct, provided that the final construct possesses the desired activity, in particular the restoration of a functional CD3 complex. In preferred embodiment, at least one mutation is introduced in the different pTalpha versions as described above to affect dimerization. As non limiting example, mutated residue can be at least W46R, D22A, K24A, R102A or R117A of the human pTalpha protein or aligned positions using CLUSTALW method on pTalpha family or homologue member. Preferably pTalpha or variant thereof as described above comprise the mutated residue W46R (SEQ ID NO:123) or the mutated residues D22A, K24A, R102A and

R117A (SEQ ID NO: 124). In particular embodiment, said pTalpha or variants are also fused to a signal-transducing domain such as CD28, OX40, ICOS, CD27, CD137 (4-1BB) and CD8 as non limiting examples (SEQ ID NO: 115 to SEQ ID NO: 120). The extracellular domain of pTalpha or variants as described above can be fused to a fragment of the TCRalpha protein, particularly the transmembrane and intracellular domain of TCRalpha (SEQ ID NO: 122). pTalpha variants can also be fused to the intracellular domain of TCRalpha (SEQ ID NO:121).

In another embodiment, said pTalpha versions are fused to an extracellular ligand-binding domain and more preferably pTalpha or functional variant thereof is fused to a single chain antibody fragment (scFV) comprising the light (V_L) and the heavy (V_H) variable fragment of a target antigen specific monoclonal antibody joined by a flexible linker. As a non limiting example, amino acid sequence of pTalpha or functional variant thereof is selected from the group consisting of SEQ ID NO: 107 to SEQ ID NO: 124.

Because some variability may arise from the genomic data from which these polypeptides derive, and also to take into account the possibility to substitute some of the amino acids present in these polypeptides without significant loss of activity (functional variants), the invention encompasses polypeptides variants of the above polypeptides that share at least 70%, preferably at least 80 %, more preferably at least 90 % and even more preferably at least 95 % identity with the sequences provided in this patent application.

The present invention is thus drawn to polypeptides comprising a polypeptide sequence that has at least 70%, preferably at least 80%, more preferably at least 90 %, 95 % 97 % or 99 % sequence identity with amino acid sequence selected from the group consisting of SEQ ID NO:107 to SEQ ID NO: 124.

By TCR alpha deficient T cell is intended an isolated T cell that lacks expression of a functional TCR alpha chain. This may be accomplished by different means, as non limiting examples, by engineering a T cell such that it does not express any functional TCR alpha on its cell surface or by engineering a T cell such that it produces very little functional TCR alpha chain on its surface or by engineering a T cell to express mutated or truncated form of TCR alpha chain.

TCR alpha deficient cells can no longer be expanded through CD3 complex. Thus, to overcome this problem and to allow proliferation of TCR alpha deficient cells, pTalpha or

functional variant thereof is introduced into said cells, thus restoring a functional CD3 complex. In a preferred embodiment, the method further comprises introducing into said T cells rare-cutting endonucleases able to selectively inactivate by DNA cleavage one gene encoding one component of the T-cell receptor (TCR). In particular embodiment, said rare-cutting endonuclease is a TALE-nucleases. As non limiting examples, TALE-nuclease is directed against one of the gene target sequences of TCRalpha selected from the group consisting of SEQ ID NO: 37 and SEQ ID NO: 57 to 60. Preferably, TALE-nucleases are selected from the group consisting of SEQ ID NO: 41 and SEQ ID NO: 42.

Are also encompassed in the present invention polypeptides encoding pTalpha, particularly functional variants described above. In a preferred embodiment the invention relates to a pTalpha or functional variant thereof fused to a signal transducing domain such as CD28, OX40, ICOS, CD137 and CD8. More particularly, the invention relates to pTalpha functional variant comprising amino acid sequence selected form the group consisting of SEQ ID NO: 107 to SEQ ID NO: 124. Are also encompassed in the present invention polynucleotides, vectors encoding pTalpha or functional variants thereof described above.

In the scope of the present invention are also encompassed isolated cells or cell lines susceptible to be obtained by said method. In particular said isolated cells or cell lines are obtained by introducing into said cells a pTalpha or a functional variant thereof to support CD3 surface expression. In a preferred embodiment, said isolated cell or cell line are further genetically modified by inactivating TCRalpha gene. This gene is preferably inactivating by at least one rare-cutting endonuclease. In a preferred embodiment said rare-cutting endonuclease is TALE-nuclease.

In the scope of the present invention are also encompassed isolated cells or cell lines susceptible to be obtained by said method to engineer cells, in particular T cells, in which at least one gene selected from the group consisting of TCR alpha, TCR beta, CD52, GR, immune checkpoint genes has been inactivated.

- *Bispecific antibodies*

According to a further embodiment, engineered T cells obtained by the different methods as previously described can be further exposed with bispecific antibodies. Said T-cells could be

exposed to bispecific antibodies *ex vivo* prior to administration to a patient or *in vivo* following administration to a patient. Said bispecific antibodies comprise two variable regions with distinct antigen properties that allow bringing the engineered cells into proximity to a target antigen. As a non limiting example, said bispecific antibody is directed against a tumor marker and lymphocyte antigen such as CD3 and has the potential to redirect and activate any circulating T cells against tumors.

Delivery methods

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

As non-limiting example, said multi-chain CAR, pTalpha or functional variant thereof, rare cutting endonucleases, TALE-nucleases or CAR optionally with DNA-end processing enzyme or exogenous nucleic acid can be introduced as transgenes encoded by one or as different plasmidic vectors. Different transgenes can be included in one vector which comprises a nucleic acid sequence encoding ribosomal skip sequence such as a sequence encoding a 2A peptide. 2A peptides, which were identified in the Aphthovirus subgroup of picornaviruses, causes a ribosomal "skip" from one codon to the next without the formation of a peptide bond between the two amino acids encoded by the codons (see Donnelly et al., J. of General Virology 82: 1013-1025 (2001); Donnelly et al., J. of Gen. Virology 78: 13-21 (1997); Doronina et al., Mol. And. Cell. Biology 28(13): 4227-4239 (2008); Atkins et al., RNA 13: 803-810 (2007)). By "codon" is meant three nucleotides on an mRNA (or on the sense strand of a DNA molecule) that are translated by a ribosome into one amino acid residue. Thus, two polypeptides can be synthesized from a single, contiguous open reading frame within an mRNA when the polypeptides are separated by a 2A oligopeptide sequence that is in frame. Such ribosomal skip mechanisms are well known in the art and are known to be used by several vectors for the expression of several proteins encoded by a single messenger RNA. As non-limiting example, in the present invention, 2A peptides have been used to express into the cell the rare-cutting endonuclease and a DNA end-processing enzyme or the different polypeptides of the multi-chain CAR.

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

Polypeptides may be synthesized *in situ* in the cell as a result of the introduction of polynucleotides encoding said polypeptides into the cell. Alternatively, said polypeptides could be produced outside the cell and then introduced thereto. Methods for introducing a polynucleotide construct into animal cells are known in the art and including as non limiting examples stable transformation methods wherein the polynucleotide construct is integrated into the genome of the cell, transient transformation methods wherein the polynucleotide construct is not integrated into the genome of the cell and virus mediated methods. Said polynucleotides may be introduced into a cell by for example, recombinant viral vectors (e.g. retroviruses, adenoviruses), liposome and the like. For example, transient transformation methods include for example microinjection, electroporation or particle bombardment. Said polynucleotides may be included in vectors, more particularly plasmids or virus, in view of being expressed in cells.

- *Electroporation*

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

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

to a method of transforming T cell comprising contacting said T cell with RNA and applying to T cell an agile pulse sequence consisting of:

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

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

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

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

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

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

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

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

In particular embodiments, as non limiting examples, said RNA encodes a rare-cutting endonuclase, one monomer of the rare-cutting endonuclease such as Half-TALE-nuclease, a Chimeric Antigen Receptor, at least one component of the multi-chain chimeric antigen

receptor, a pTalpha or functional variant thereof, an exogenous nucleic acid, one additional catalytic domain.

Modified T-cells

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

In the scope of the present invention is also encompassed an isolated immune cell, preferably a T-cell obtained according to any one of the methods previously described. Said immune cell refers to a cell of hematopoietic origin functionally involved in the initiation and/or execution of innate and/or adaptative immune response. Said immune cell according to the present invention can be derived from a stem cell. The stem cells can be adult stem cells, embryonic stem cells, more particularly non-human stem cells, cord blood stem cells, progenitor cells, bone marrow stem cells, induced pluripotent stem cells, totipotent stem cells or hematopoietic stem cells. Representative human cells are CD34+ cells. Said isolated cell can also be a dendritic cell, killer dendritic cell, a mast cell, a NK-cell, a B-cell or a T-cell selected from the group consisting of inflammatory T-lymphocytes, cytotoxic T-lymphocytes, regulatory T-lymphocytes or helper T-lymphocytes. In another embodiment, said cell can be derived from the group consisting of CD4+ T-lymphocytes and CD8+ T-lymphocytes. Prior to expansion and genetic modification of the cells of the invention, a source of cells can be obtained from a subject through a variety of non-limiting methods. Cells can be obtained from a number of non-limiting sources, including peripheral blood mononuclear cells, bone marrow, lymph node tissue, cord blood, thymus tissue, tissue from a site of infection, ascites, pleural effusion, spleen tissue, and tumors. In certain embodiments of the present

invention, any number of T cell lines available and known to those skilled in the art, may be used. In another embodiment, said cell can be derived from a healthy donor, from a patient diagnosed with cancer or from a patient diagnosed with an infection. In another embodiment, said cell is part of a mixed population of cells which present different phenotypic characteristics. In the scope of the present invention is also encompassed a cell line obtained from a transformed T- cell according to the method previously described. Modified cells resistant to an immunosuppressive treatment and susceptible to be obtained by the previous method are encompassed in the scope of the present invention.

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

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

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

(a) Recovering cells from said individual;

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

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

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

Activation and expansion of T cells

Whether prior to or after genetic modification of the T cells, the T cells can be activated and expanded generally using methods as described, for example, in U.S. Patents 6,352,694; 6,534,055; 6,905,680; 6,692,964; 5,858,358; 6,887,466; 6,905,681; 7,144,575; 7,067,318; 7,172,869; 7,232,566; 7,175,843; 5,883,223; 6,905,874; 6,797,514; 6,867,041; and U.S. Patent Application Publication No. 20060121005. T cells can be expanded *in vitro* or *in vivo.*

Generally, the T cells of the invention are expanded by contact with an agent that stimulates a CD3 TCR complex and a co-stimulatory molecule on the surface of the T cells to create an activation signal for the T-cell.

For example, chemicals such as calcium ionophore A23187, phorbol 12-myristate 13-acetate (PMA), or mitogenic lectins like phytohemagglutinin (PHA) can be used to create an activation signal for the T-cell.

As non limiting examples, T cell populations may be stimulated *in vitro* such as by contact with an anti-CD3 antibody, or antigen-binding fragment thereof, or an anti-CD2 antibody immobilized on a surface, or by contact with a protein kinase C activator (e.g., bryostatin) in

conjunction with a calcium ionophore. For co-stimulation of an accessory molecule on the surface of the T cells, a ligand that binds the accessory molecule is used. For example, a population of T cells can be contacted with an anti-CD3 antibody and an anti-CD28 antibody, under conditions appropriate for stimulating proliferation of the T cells. To stimulate proliferation of either CD4+ T cells or CD8+ T cells, an anti-CD3 antibody and an anti-CD28 antibody. For example, the agents providing each signal may be in solution or coupled to a surface. As those of ordinary skill in the art can readily appreciate, the ratio of particles to cells may depend on particle size relative to the target cell. In further embodiments of the present invention, the cells, such as T cells, are combined with agent-coated beads, the beads and the cells are subsequently separated, and then the cells are cultured. In an alternative embodiment, prior to culture, the agent-coated beads and cells are not separated but are cultured together. Conditions appropriate for T cell culture include an appropriate media (e.g., Minimal Essential Media or RPMI Media 1640 or, X-vivo 5, (Lonza)) that may contain factors necessary for proliferation and viability, including serum (e.g., fetal bovine or human serum), interleukin-2 (IL-2), insulin, IFN-g , 1L-4, 1L-7, GM-CSF, -10, - 2, 1L-15, TGFp, and TNF- or any other additives for the growth of cells known to the skilled artisan. Other additives for the growth of cells include, but are not limited to, surfactant, plasmanate, and reducing agents such as N-acetyl-cysteine and 2-mercaptoethanoi. Media can include RPMI 1640, A1M-V, DMEM, MEM, a-MEM, F-12, X-Vivo 1, and X-Vivo 20, Optimizer, with added amino acids, sodium pyruvate, and vitamins, either serum-free or supplemented with an appropriate amount of serum (or plasma) or a defined set of hormones, and/or an amount of cytokine(s) sufficient for the growth and expansion of T cells. Antibiotics, e.g., penicillin and streptomycin, are included only in experimental cultures, not in cultures of cells that are to be infused into a subject. The target cells are maintained under conditions necessary to support growth, for example, an appropriate temperature (e.g., 37° C) and atmosphere (e.g., air plus 5% C02). T cells that have been exposed to varied stimulation times may exhibit different characteristics

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

Therapeutic applications

In another embodiment, isolated cell obtained by the different methods or cell line derived from said isolated cell as previously described can be used as a medicament. In another embodiment, said medicament can be used for treating cancer or infections in a patient in need thereof. In another embodiment, said isolated cell according to the invention or cell line derived from said isolated cell can be used in the manufacture of a medicament for treatment of a cancer,viral infection or autoimmune disease in a patient in need thereof.

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

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

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

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

Said treatment can be ameliorating, curative or prophylactic. It may be either part of an autologous immunotherapy or part of an allogenic immunotherapy treatment. By autologous, it is meant that cells, cell line or population of cells used for treating patients are originating from said patient or from a Human Leucocyte Antigen (HLA) compatible donor. By allogeneic is meant that the cells or population of cells used for treating patients are not originating from said patient but from a donor.

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

Cells that can be used with the disclosed methods are described in the previous section. Said treatment can be used to treat patients diagnosed with cancer, viral infection, autoimmune disorders or Graft versus Host Disease (GvHD). Cancers that may be treated include tumors that are not vascularized, or not yet substantially vascularized, as well as vascularized

tumors. The cancers may comprise nonsolid tumors (such as hematological tumors, for example, leukemias and lymphomas) or may comprise solid tumors. Types of cancers to be treated with the multi-chain CARs of the invention include, but are not limited to, carcinoma, blastoma, and sarcoma, and certain leukemia or lymphoid malignancies, benign and malignant tumors, and malignancies e.g., sarcomas, carcinomas, and melanomas. Adult tumors/cancers and pediatric tumors/cancers are also included.

It can be a treatment in combination with one or more therapies against cancer selected from the group of antibodies therapy, chemotherapy, cytokines therapy, dendritic cell therapy, gene therapy, hormone therapy, laser light therapy and radiation therapy.

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

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

The administration of the cells or population of cells can consist of the administration of 10^4-10^9 cells per kg body weight, preferably 10^5 to 10^6 cells/kg body weight including all integer values of cell numbers within those ranges. The cells or population of cells can be administrated in one or more doses. In another embodiment, said effective amount of cells are administrated as a single dose. In another embodiment, said effective amount of cells are administrated as more than one dose over a period time. Timing of administration is within the judgment of managing physician and depends on the clinical condition of the patient. The cells or population of cells may be obtained from any source, such as a blood

bank or a donor. While individual needs vary, determination of optimal ranges of effective amounts of a given cell type for a particular disease or conditions within the skill of the art. An effective amount means an amount which provides a therapeutic or prophylactic benefit. The dosage administrated will be dependent upon the age, health and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment and the nature of the effect desired.

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

In certain embodiments of the present invention, cells are administered to a patient in conjunction with (e.g., before, simultaneously or following) any number of relevant treatment modalities, including but not limited to treatment with agents such as antiviral therapy, cidofovir and interleukin-2, Cytarabine (also known as ARA-C) or nataliziimab treatment for MS patients or efaliztimab treatment for psoriasis patients or other treatments for PML patients. In further embodiments, the T cells of the invention may be used in combination with chemotherapy, radiation, immunosuppressive agents, such as cyclosporin, azathioprine, methotrexate, mycophenolate, and FK506, antibodies, or other immunoablative agents such as CAM PATH, anti-CD3 antibodies or other antibody therapies, cytoxin, fludaribine, cyclosporin, FK506, rapamycin, mycoplienolic acid, steroids, FR901228, cytokines, and irradiation. These drugs inhibit either the calcium dependent phosphatase calcineurin (cyclosporine and FK506) or inhibit the p70S6 kinase that is important for growth factor induced signaling (rapamycin) (Liu et al., Cell 66:807-815, 1 1; Henderson et al., Immun. 73:316-321, 1991; Bierer et al., Citrr. Opin. mm n. 5:763-773, 93). In a further embodiment, the cell compositions of the present invention are administered to a patient in conjunction with (e.g., before, simultaneously or following) bone marrow transplantation, T cell ablative therapy using either chemotherapy agents such as, fludarabine, external-beam radiation therapy (XRT), cyclophosphamide, or antibodies such as OKT3 or CAMPATH, In another embodiment, the cell compositions of the present invention are administered following B-cell ablative therapy such as agents that react with CD20, e.g., Rituxan. For example, in one embodiment, subjects may undergo standard treatment with high dose chemotherapy followed by peripheral blood stem cell transplantation. In certain

embodiments, following the transplant, subjects receive an infusion of the expanded immune cells of the present invention. In an additional embodiment, expanded cells are administered before or following surgeiy. Said modified cells obtained by any one of the methods described here can be used in a particular aspect of the invention for treating patients in need thereof against Host versus Graft (HvG) rejection and Graft versus Host Disease (GvHD); therefore in the scope of the present invention is a method of treating patients in need thereof against Host versus Graft (HvG) rejection and Graft versus Host Disease (GvHD) comprising treating said patient by administering to said patient an effective amount of modified cells comprising inactivated TCR alpha and/or TCR beta genes.

Example of method to engineer human allogeneic cells for immunotherapy

For a better understanding of the invention, one example of method to engineer human allogenic cells for immunotherapy is illustrated in Figure 5. The method comprising a combination of one or several of the following steps:

1. Providing T-cells from a cell culture or from a blood sample from one individual patient or from blood bank and activating said T cells using anti-CD3/C28 activator beads. The beads provide both the primary and co-stimulatory signals that are required for activation and expansion of T cells.

2. a) Transducing said cells with pTalpha or functional variant thereof transgene to support CD3 surface expression and allow cell expansion through stimulation of CD3 complex. TCR disruption is expected to the elimination of the TCR complex and removes alloreactivity (GvHD) but may alter allogenic cells expansion due to the loss of CD3 signaling component. Transduced cells are expected to express pTalpha chain or functional variant thereof. This pTalpha chain pairs with TCRbeta chain and CD3 signaling components to form the preTCR complex and, thus restore a functional CD3 complex and support activation or stimulation of inactivated TCRalpha cells. Transduction of T-cells with pTalpha lentiviral vector can be realized before or after TCRalpha inactivation.

   b) Transducing said cells with multi-chain CARs allow redirecting T cells against antigens expressed at the surface of target cells from various malignancies including lymphomas and solid tumors. To improve the function of co-stimulatory domain, the inventors

have designed a multi-chain CAR derived from FcεRI as previously described. Transduction can be realized before or after the inactivation of TCRalpha and the other genes, such as CD52 genes.

3. Engineering non alloreactive and immunosuppressive resistant T cells:

a) It is possible to Inactivate TCR alpha in said cells to eliminate the TCR from the surface of the cell and prevent recognition of host tissue as foreign by TCR of allogenic and thus to avoid GvHD.

b) It is also possible to inactive one gene encoding target for immunosuppressive agent to render said cells resistant to immunosuppressive treatment to prevent graft rejection without affecting transplanted T cells. In this example, target of immunosuppressive agents is CD52 and immunosuppressive agent is a humanized monoclonal anti-CD52 antibody.

It has been shown by the inventors that the use of TALE-nuclease by allowing higher rates of DSB events within T-cells was particularly advantageous to achieve the above double inactivation in T-cells. Preferably, TCRalpha and CD52 genes are inactivated by electoporating T cells with mRNA coding for TALE-nuclease targeting said genes. It has been found by the inventors that using mRNA resulted into high transformation rate was less harmful to T-cells and so, was critical in the process of engineering T-cells. Then, inactivated T cells are sorted using magnetic beads. For example, T cells expressing CD52 are removed by fixation on a solid surface, and inactivated cells are not exposed of the stress of being passed through a column. This gentle method increases the concentration of properly engineered T-cells.

4. Expansion *in vitro* of engineered T-cells prior to administration to a patient or *in vivo* following administration to a patient through stimulation of CD3 complex. Before administration step, patients can be subjected to an immunosuppressive treatment such as CAMPATH1-H, a humanized monoclonal antibody anti-CD52.

5. Optionally exposed said cells with bispecific antibodies *ex vivo* prior to administration to a patient or *in vivo* following administration to a patient to bring the engineered cells into proximity to a target antigen.

Other definitions

- Unless otherwise specified, "a," "an," "the," and "at least one" are used interchangeably and mean one or more than one.- Amino acid residues in a polypeptide sequence are designated herein according to the one-letter code, in which, for example, Q means Gln or Glutamine residue, R means Arg or Arginine residue and D means Asp or Aspartic acid residue.

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

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

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

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

- by "polynucleotide successively comprising a first region of homology to sequences upstream of said double-stranded break, a sequence to be inserted in the genome of said cell and a second region of homology to sequences downstream of said double-stranded break" it is intended to mean a DNA construct or a matrix comprising a first and second portion that are homologous to regions 5' and 3' of a DNA target *in situ*. The DNA construct also comprises a third portion positioned between the first and second portion which comprise some homology with the corresponding DNA sequence *in situ* or alternatively comprise no homology with the regions 5' and 3' of the DNA target *in situ*. Following cleavage of the DNA target, a homologous recombination event is stimulated between the genome containing the targeted gene comprised in the locus of interest and this matrix, wherein the genomic sequence containing the DNA target is replaced by the third portion of the matrix and a variable part of the first and second portions of said matrix.

- 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 TALE-nuclease targets, targeted genomic sequences generally consist of two 17-bp long sequences (called half targets) separated by a 15-bp spacer. Each half-target is recognized by repeats of TALE-nucleases listed in tables 1, 5, 6 and 10 as non-limiting examples, encoded in plasmids, under the control of EF1-alpha promoter or T7 promoter. The nucleic acid target sequence is defined by the 5' to 3' sequence of one strand of said target, as indicated in tables 1, 5, 6 and 10.

- By chimeric antigen receptor (CAR) is intended molecules that combine a binding domain against a component present on the target cell, for example an antibody-based specificity for a desired antigen (e.g., tumor antigen) with a T cell receptor-activating intracellular domain to generate a chimeric protein that exhibits a specific anti-target cellular immune

activity. Generally, CAR consists of an extracellular single chain antibody (scFvFc) fused to the intracellular signaling domain of the T cell antigen receptor complex zeta chain (scFvFc:ζ) and have the ability, when expressed in T cells, to redirect antigen recognition based on the monoclonal antibody's specificity. One example of CAR used in the present invention is a CAR directing against CD19 antigen and can comprise as non limiting example the amino acid sequence : SEQ ID NO: 73

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

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

Viral vectors include retrovirus, adenovirus, parvovirus (e. g. adenoassociated viruses), coronavirus, negative strand RNA viruses such as orthomyxovirus (e. g., influenza virus), rhabdovirus (e. g., rabies and vesicular stomatitis virus), paramyxovirus (e. g. measles and Sendai), positive strand RNA viruses such as picornavirus and alphavirus, and double-stranded DNA viruses including adenovirus, herpesvirus (e. g., Herpes Simplex virus types 1 and 2, Epstein-Barr virus, cytomegalovirus), and poxvirus (e. g., vaccinia, fowlpox and

canarypox). Other viruses include Norwalk virus, togavirus, flavivirus, reoviruses, papovavirus, hepadnavirus, and hepatitis virus, for example. Examples of retroviruses include: avian leukosis-sarcoma, mammalian C-type, B-type viruses, D type viruses, HTLV-BLV group, lentivirus, spumavirus (Coffin, J. M., Retroviridae: The viruses and their replication, In Fundamental Virology, Third Edition, B. N. Fields, et al., Eds., Lippincott-Raven Publishers, Philadelphia, 1996).

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

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

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

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

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

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

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

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

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

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

- As used herein, the term "locus" is the specific physical location of a DNA sequence (e.g. of a gene) on a chromosome. The term "locus" can refer to the specific physical location of a rare-cutting endonuclease target sequence on a chromosome. Such a locus can comprise a target sequence that is recognized and/or cleaved by a rare-cutting endonuclease according

to the invention. It is understood that the 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 "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 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 12 base pairs (bp) in length, more preferably of 14-55 bp. Rare-cutting endonucleases significantly increase HR by inducing DNA double-strand breaks (DSBs) at a defined locus (Rouet, Smih et al. 1994; Choulika, Perrin et al. 1995; Pingoud and Silva 2007). Rare-cutting endonucleases can for example be a homing endonuclease (Paques and Duchateau 2007), a chimeric Zinc-Finger nuclease (ZFN) resulting from the fusion of engineered zinc-finger domains with the catalytic domain of a restriction enzyme such as FokI (Porteus and Carroll 2005) or a chemical endonuclease (Eisenschmidt, Lanio et al. 2005; Arimondo, Thomas et al. 2006). In chemical endonucleases, a chemical or peptidic cleaver is conjugated either to a polymer of nucleic acids or to another DNA recognizing a specific target sequence, thereby targeting the cleavage activity to a specific sequence. Chemical endonucleases also encompass synthetic nucleases like conjugates of orthophenanthroline, a DNA cleaving molecule, and triplex-forming oligonucleotides (TFOs), known to bind specific DNA sequences (Kalish and Glazer 2005). Such chemical endonucleases are comprised in the term "endonuclease" according to the present invention.

Rare-cutting endonucleases can also be for example TALE-nucleases, a new class of chimeric nucleases using a FokI catalytic domain and a DNA binding domain derived from Transcription Activator Like Effector (TALE), a family of proteins used in the infection process by plant pathogens of the Xanthomonas genus (Boch, Scholze et al. 2009; Moscou and Bogdanove 2009; Christian, Cermak et al. 2010; Li, Huang et al.). The functional layout of a

FokI-based TALE-nuclease (TALE-nuclease) is essentially that of a ZFN, with the Zinc-finger DNA binding domain being replaced by the TALE domain. As such, DNA cleavage by a TALE-nuclease requires two DNA recognition regions flanking an unspecific central region. Rare-cutting endonucleases encompassed in the present invention can also be derived from TALE-nucleases.

Rare-cutting endonuclease can be a homing endonuclease, also known under the name of meganuclease. Such homing endonucleases are well-known to the art (Stoddard 2005). Homing endonucleases recognize a DNA target sequence and generate a single- or double-strand break. Homing endonucleases are highly specific, recognizing DNA target sites ranging from 12 to 45 base pairs (bp) in length, usually ranging from 14 to 40 bp in length. The homing endonuclease according to the invention may for example correspond to a LAGLIDADG endonuclease ("LAGLIDADF" disclosed as SEQ ID NO: 127), to a HNH endonuclease, or to a GIY-YIG endonuclease. Preferred homing endonuclease according to the present invention can be an I-*CreI* variant.

- By a "TALE-nuclease" (TALEN) is intended a fusion protein consisting of a nucleic acid-binding domain typically derived from a Transcription Activator Like Effector (TALE) and one nuclease catalytic domain to cleave a nucleic acid target sequence. The catalytic domain is preferably a nuclease domain and more preferably a domain having endonuclease activity, like for instance I-TevI, ColE7, NucA and Fok-I. In a particular embodiment, the TALE domain can be fused to a meganuclease like for instance I-CreI and I-OnuI or functional variant thereof. In a more preferred embodiment, said nuclease is a monomeric TALE-Nuclease. A monomeric TALE-Nuclease is a TALE-Nuclease that does not require dimerization for specific recognition and cleavage, such as the fusions of engineered TAL repeats with the catalytic domain of I-TevI described in WO2012138927. Transcription Activator like Effector (TALE) are proteins from the bacterial species *Xanthomonas* comprise a plurality of repeated sequences, each repeat comprising di-residues in position 12 and 13 (RVD) that are specific to each nucleotide base of the nucleic acid targeted sequence. Binding domains with similar modular base-per-base nucleic acid binding properties (MBBBD) can also be derived from new modular proteins recently discovered by the applicant in a different bacterial species. The new modular proteins have the advantage of displaying more sequence variability than TAL repeats. Preferably, RVDs associated with recognition of the different nucleotides are

HD for recognizing C, NG for recognizing T, NI for recognizing A, NN for recognizing G or A, NS for recognizing A, C, G or T, HG for recognizing T, IG for recognizing T, NK for recognizing G, HA for recognizing C, ND for recognizing C, HI for recognizing C, HN for recognizing G, NA for recognizing G, SN for recognizing G or A and YG for recognizing T, TL for recognizing A, VT for recognizing A or G and SW for recognizing A. In another embodiment, critical amino acids 12 and 13 can be mutated towards other amino acid residues in order to modulate their specificity towards nucleotides A, T, C and G and in particular to enhance this specificity. TALE-nuclease have been already described and used to stimulate gene targeting and gene modifications (Boch, Scholze et al. 2009; Moscou and Bogdanove 2009; Christian, Cermak et al. 2010; Li, Huang et al.). Engineered TAL-nucleases are commercially available under the trade name TALEN™ (Cellectis, 8 rue de la Croix Jarry, 75013 Paris, France).

- The term "cleavage" refers to the breakage of the covalent backbone of a 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 DNA/RNA hybrid cleavage can result in the production of either blunt ends or staggered ends.

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

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

to specific polypeptides described herein and preferably exhibiting substantially the same functions, as well as polynucleotide encoding such polypeptides, are contemplated.

- "similarity" describes the relationship between the amino acid sequences of two or more polypeptides. BLASTP may also be used to identify an amino acid sequence having at least 70%, 75%, 80%, 85%, 87.5%, 90%, 92.5%, 95%, 97.5%, 98%, 99% sequence similarity to a reference amino acid sequence using a similarity matrix such as BLOSUM45, BLOSUM62 or BLOSUM80. Unless otherwise indicated a similarity score will be based on use of BLOSUM62. When BLASTP is used, the percent similarity is based on the BLASTP positives score and the percent sequence identity is based on the BLASTP identities score. BLASTP "Identities" shows the number and fraction of total residues in the high scoring sequence pairs which are identical; and BLASTP "Positives" shows the number and fraction of residues for which the alignment scores have positive values and which are similar to each other. Amino acid sequences having these degrees of identity or similarity or any intermediate degree of identity of similarity to the amino acid sequences disclosed herein are contemplated and encompassed by this disclosure. The polynucleotide sequences of similar polypeptides are deduced using the genetic code and may be obtained by conventional means. For example, a functional variant of pTalpha can have 70%, 75%, 80%, 85%, 87.5%, 90%, 92.5%, 95%, 97.5%, 98%, 99% sequence similarity to the amino acid sequence of SEQ ID NO : 107. A polynucleotide encoding such a functional variant would be produced by reverse translating its amino acid sequence using the genetic code.

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

such as but not limited to, CD27, CD28, 4-IBB, OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LTGHT, NKG2C, B7-H3, a ligand that specifically binds with CD83.

A "co-stimulatory molecule" refers to the cognate binding partner on a Tcell that specifically binds with a co-stimulatory ligand, thereby mediating a co-stimulatory response by the cell, such as, but not limited to proliferation. Co-stimulatory molecules include, but are not limited to an MHC class I molecule, BTLA and Toll ligand receptor.

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

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

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

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

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.

The above description is presented to enable a person skilled in the art to make and use the invention, and is provided in the context of a particular application and its requirements. Various modifications to the preferred embodiments will be readily apparent to those skilled in the art, and the generic principles defined herein may be applied to other embodiments and applications without departing from the spirit and scope of the invention. Thus, this invention is not intended to be limited to the embodiments shown, but is to be accorded the widest scope consistent with the principles and features disclosed herein.

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 be limiting unless otherwise specified.

Examples

Example 1: TALE-nucleases cleaving the human GR gene

6 heterodimeric TALE-nucleases targeting exons of the human GR gene were designed and produced. Table 1 below indicates the target sequences cleaved by each TALE-nuclease. GR TALE-nuclease was composed of two independent entities (called half TALE-nucleases) each containing a repeat sequence engineered to bind and cleave GR target sequences consisting of two 17-bp long sequences (called half targets) separated by a 15-bp spacer.

Target name	Target sequence	Repeat sequence	Half TALE-nuclease sequence
GRex2	TATTCACTGATGGACTC caaagaatcattaac TCCTGGTAGAGAAGAAA (SEQ ID NO: 1)	Repeat GRex2-LPT9-L1 (SEQ ID NO: 7)	GRex2-L TALEN (SEQ ID NO: 19)
		Repeat -GRex2-LPT9-R1 (SEQ ID NO: 8)	GRex2-R TALEN (SEQ ID NO: 20)

GRex3T2	TGCCTGGTGTGCTCTGA tgaagcttcaggatg TCATTATGGAGTCTTAA (SEQ ID NO: 2)	Repeat -GRex3T2-L1 (SEQ ID NO: 9)	GRex3T2-L TALEN (SEQ ID NO: 21)
		Repeat -GRex3T2-R1 (SEQ ID NO: 10)	GRex3T2-R TALEN (SEQ ID NO: 22)
GRex3T4	TGCTCTGATGAAGCTTC aggatgtcattatgg AGTCTTAACTTGTGGAA (SEQ ID NO: 3)	Repeat -GRex3T4-L1 (SEQ ID NO: 11)	GRex3T4-L TALEN (SEQ ID NO: 23)
		Repeat -GRex3T4-R1 (SEQ ID NO: 12)	GRex3T4-R TALEN (SEQ ID NO: 24)
GRex5T1	TGGTGTCACTGTTGGAG gttattgaacctgaa GTGTTATATGCAGGATA (SEQ ID NO: 4)	Repeat -GRex5T1-LPT8-L1 (SEQ ID NO: 13)	GRex5T1-L TALEN (SEQ ID NO: 25)
		Repeat -GRex5T1-LPT8-R1 (SEQ ID NO: 14)	GRex5T1-R TALEN (SEQ ID NO: 26)
GRex5T2	TATGATAGCTCTGTTCC agactcaacttggag GATCATGACTACGCTCA (SEQ ID NO: 5)	Repeat -GRex5T2-L1 (SEQ ID NO: 15)	GRex5T2-L TALEN (SEQ ID NO: 27)
		Repeat GRex5T2-R1 (SEQ ID NO: 16)	GRex5T2-R TALEN (SEQ ID NO: 28)
GRex5T3	TTATATGCAGGATATGA tagctctgttccaga CTCAACTTGGAGGATCA (SEQ ID NO: 6)	Repeat -GRex5T3-L1 (SEQ ID NO: 17)	GRex5T3-L TALEN (SEQ ID NO: 29)
		Repeat -GRex5T3-R1 (SEQ ID NO: 18)	GRex5T3-R TALEN (SEQ ID NO: 30)

Table 1: Description of the GR TALE-nucleases and sequences of the TALE-nucleases target sites in the human GR gene.

The amino acid sequences of the N-terminal, C-terminal domains and repeat are based on the AvrBs3 TALE (ref: GenBank: X16130.1). The C-terminal and the N-terminal domains are separated by two BsmBI restriction sites. The repeat arrays (SEQ ID NO: 7 to 18), targeting the desired sequences (SEQ ID NO: 1 to 6) were synthesized using a solid support method composed of consecutive restriction/ligation/washing steps (International PCT application WO2013/017950). In brief, the first block (coding for a di-repeat) was immobilized on a solid support through biotin/streptavidin interaction, the second block (tri-repeat) was then ligated to the first and after SfaNI digestion a third bloc (tri-repeat) was coupled. The process was repeated using tri- or di-repeat blocks upon obtaining the desired repeat array. The product was then cloned in a classical pAPG10 cloning plasmid for amplification in *E. coli* and sequenced. The repeat array sequences thus obtained were subcloned in a yeast expression TALE vector using type IIS restriction enzymes BsmBI for the receiving plasmid and BbvI and SfaNI for the inserted repeat sequence. DNA coding for the half TALE-nuclease, containing a TALE derived DNA binding domain fused to the catalytic domain of the FokI restriction

enzyme, was amplified in *E. coli*, recovered by standard miniprep techniques and sequenced to assess the integrity of the insert.

Activity of GR TALE-nucleases in yeast:

Nuclease activity of the six GR-TALE-nucleases were tested at 37°C and 30°C in our yeast SSA assay previously described (International PCT Applications WO 2004/067736 and in (Epinat, Arnould et al. 2003; Chames, Epinat et al. 2005; Arnould, Chames et al. 2006; Smith, Grizot et al. 2006) on targets containing the two TALE target sequences facing each other on the DNA strand separated by a spacer of 15 bps resulting in SEQ ID NO: 1 to 6. All the yeast target reporter plasmids containing the TALE-nuclease DNA target sequences were constructed as previously described (International PCT Applications WO 2004/067736 and in (Epinat, Arnould et al. 2003; Chames, Epinat et al. 2005; Arnould, Chames et al. 2006; Smith, Grizot et al. 2006). TALE-nuclease cleavage activity levels, in yeast, of individual clones on the targets are presented in table 2.

Target	Half TALE-nuclease transfected	yeast gal37°C	yeast gal30°C
GRex2	Grex2-L TALEN	1	1
	Grex2-R TALEN		
GRex3T2	GRex3T2-L TALEN	0,92	0,87
	GRex3T2-R TALEN		
GRex3T4	GRex3T4-L TALEN	0,94	0,87
	GRex3T4-R TALEN		
GRex5T1	GRex5T1-L TALEN	0,48	0,36
	GRex5T1-R TALEN		
GRex5T2	GRex5T2-L TALEN	0,97	0,91
	GRex5T2-R TALEN		
GRex5T3	GRex5T3-L TALEN	1	0,98
	GRex5T3-R TALEN		

Table 2: Cleavage activity of the GR TALE-nucleases in yeast.

Values are comprised between 0 and 1. Maximal value is 1.

Activity of GR TALE-nucleases in HEK293 cells:

Each TALE-nuclease construct was subcloned using restriction enzyme digestion in a mammalian expression vector under the control of a pEF1alpha long promoter.

One million HEK293cells were seeded one day prior to transfection. Cells were co-transfected with 2.5 µg of each of two plasmids encoding left and right half of GRex2, GRex3T2, GRex3T4, GRex5T1, GRex5T2 or GRex5T3 TALE-nuclease recognizing the two half targets genomic sequences of interest in the GR gene under the control of EF1alpha promoter using 25µL of lipofectamine (Invitrogen) according to the manufacturer's instructions. As a control, cells were co-transfected with 2.5 µg of each of the two plasmids encoding the left and the right half of TALE-nucleases targeting the T-cell receptor alpha constant chain region (TRAC_T01) target site ((TRAC_T01-L and -R TALE-nuclease (SEQ ID NO: 41 and SEQ ID NO: 42, TRAC_T01 target site (SEQ ID NO: 37)) under the control of EF1alpha promoter. The double strand break generated by TALE-nucleases in GR coding sequence induces non homologous end joining (NHEJ), which is an error-prone mechanism. Activity of TALE-nucleases is measured by the frequency of insertions or deletions at the genomic locus targeted.

2 or 7 days post transfection cells were harvested and locus specific PCRs were performed on genomic DNA extracted using the following composite primers: 5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG-3' (forward adaptator sequence, SEQ ID NO: 126)-10N (TAG)- locus specific forward sequence for GR exon 2: 5'-GGTTCATTTAACAAGCTGCC-3' (SEQ ID NO: 127; composite primer disclosed as SEQ ID NO: 31), for GR exon 3: 5'-GCATTCTGACTATGAAGTGA-3' (SEQ ID NO: 128; composite primer disclosed as SEQ ID NO: 32) and for GR exon 5: 5'-TCAGCAGGCCACTACAGGAGTCTCACAAG-3' (SEQ ID NO: 129; composite primer disclosed as SEQ ID NO: 33) and the reverse composite primer 5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG-3' (reverse adaptor sequence, SEQ ID No: 130))-locus specific reverse sequence for GR exon 2 : 5'-AGCCAGTGAGGGTGAAGACG-3' (SEQ ID NO: 131; composite primer disclosed as SEQ ID NO: 34), for GR exon 3 : 5'-GGGCTTTGCATATAATGGAA-3' (SEQ ID NO: 132; composite primer disclosed as SEQ ID NO: 35) and for GR exon 5 : 5'-CTGACTCTCCCCTTCATAGTCCCCAGAAC-3' (SEQ ID NO: 133; composite primer disclosed as SEQ ID NO: 36).

PCR products were sequenced by a 454 sequencing system (454 Life Sciences). Approximately 10,000 sequences were obtained per PCR product and then analyzed for the presence of site-specific insertion or deletion events. Table 3 indicates the percentage of the sequences showing insertions or deletions at the TALE-nuclease target site among the total number of sequences in the sample. In table 3 are listed for GRex2, GRex3T2 and GRex3T4 the results of a representative experiment.

In all cases tested, the % of mutagenesis was similar at day 7 compared to the one of the sample at day 2 post transfection. The nature of the mutagenic events was also analyzed, revealing a majority of deletions in all cases compared to insertions.

Target	% Indels at 2 days with GR TALE-nuclease transfection	% Indels at 7 days with GR TALE-nuclease transfection	% Indels at 2 days with TRAC_T01 TALE-nuclease control transfection
GRex2	20.3	24.9	0.5
GRex3T2	9.3	9.8	0
GRex3T4	19	18.3	0.0
GRex5T1	11.2	NA	0.7
GRex5T2	3.4	NA	0
GRex5T3	8.3	NA	0

Table 3: Percentage of targeted mutagenesis at endogenous TALE-nuclease Target sites in HEK293 cells.

Activity of GR TALE-nucleases in primary T lymphocytes:

Each TALE-nuclease construct was subcloned using restriction enzyme digestion in an expression vector under the control of a T7 promoter.

mRNA encoding TALE-nucleases cleaving GR genomic sequences were synthesized from each plasmid carrying the coding sequences downstream from the T7 promoter. T lymphocytes isolated from peripheral blood were activated for 5 days using anti-CD3/CD28 activator beads (Life technologies) and 5 million cells were transfected by electroporation with 10 µg of each of 2 mRNAs encoding both half TALE-nucleases using a CytoLVT-P instrument (BTX-Harvard apparatus). T cells transfected with 10µg of each of the 2 mRNAs encoding both half TALE-nucleases targeting the CD52 gene (CD52_T02-L and -R TALEN (SEQ ID NO: 55 and 56), target sequence CD52_T02 SEQ ID NO: 40) are used as a control.

3 and 7 days after transfection, genomic DNA was isolated from transfected cells and locus specific PCRs were performed using the primers described previously. PCR products were sequenced by a 454 sequencing system (454 Life Sciences). Approximately 10,000 sequences were obtained per PCR product and then analyzed for the presence of site-specific insertion or deletion events; results are in Table 4.

Target	% Indels at day 3 with GR TALE-nuclease transfection	% Indels at day 7 with GR TALE-nuclease transfection	% Indels at day 3 with CD52 TALE-nuclease control transfection
GRex2	26.2	30.7	0.7
GRex3T2	1.09	0.86	0.02
GRex3T4	6.3	6.93	0
GRex5T1	0.04	0.035	0.05
GRex5T2	1.3	1.0	0.22
GRex5T3	17.4	NA	0.41

Table 4: Percentage of targeted mutagenesis at endogenous TALE-nuclease target sites in primary T lymphocytes.

Example 2: TALE-nucleases cleaving the human CD52 gene, the human T-cell receptor alpha constant chain (TRAC) and the human T-cell receptor beta constant chains 1 and 2 (TRBC)

As described in example 1, heterodimeric TALE-nucleases targeting respectively CD52, TRAC and TRBC genes were designed and produced. The targeted genomic sequences consist of two 17-bp long sequences (called half targets) separated by an 11 or 15-bp spacer. Each half-target is recognized by repeats of half TALE-nucleases listed in table 5. The human genome contains two functional T-cell receptor beta chains (TRBC1 and TRBC2). During the development of alpha/beta T lymphocytes, one of these two constant chains is selected in each cell to be spliced to the variable region of TCR-beta and form a functional full length beta chain. The 2 TRBC targets were chosen in sequences conserved between TRBC1 and TRBC2 so that the corresponding TALE-nuclease would cleave both TRBC1 and TRBC2 at the same time.

Target	Target sequence	Repeat sequence	Half TALE-nuclease
TRAC_T01	TTGTCCCACAGATATCC Agaaccctgaccctg	Repeat TRAC_T01-L (SEQ ID NO: 41)	TRAC_T01-L TALEN (SEQ ID NO: 49)

	CCGTGTACCAGCTGAGA (SEQ ID NO: 37)	Repeat TRAC_T01-R (SEQ ID NO: 42)	TRAC_T01-R TALEN (SEQ ID NO: 50)
TRBC_T01	TGTGTTTGAGCCATCAG aagcagagatctccc ACACCCAAAAGGCCACA (SEQ ID NO: 38)	Repeat TRBC_T01-L (SEQ ID NO: 43)	TRBC_T01-L TALEN (SEQ ID NO: 51)
		Repeat TRBC_T01-R (SEQ ID NO: 44)	TRBC_T01-R TALEN (SEQ ID NO: 52)
TRBC_T02	TTCCCACCCGAGGTCGC tgtgtttgagccatca GAAGCAGAGATCTCCCA (SEQ ID NO: 39)	Repeat TRBC_T02-L (SEQ ID NO: 45)	TRBC_T02-L TALEN (SEQ ID NO: 53)
		Repeat TRBC_T02-R (SEQ ID NO: 46)	TRBC_T02-R TALEN (SEQ ID NO: 54)
CD52_T02	TTCCTCCTACTCACCAT cagcctcctggttat GGTACAGGTAAGAGCAA (SEQ ID NO: 40)	Repeat CD52_T02-L (SEQ ID NO: 47)	CD52_T02-L TALEN (SEQ ID NO: 55)
		Repeat CD52_T02-R (SEQ ID NO: 48)	CD52_T02-R TALEN (SEQ ID NO: 56)

Table 5: Description of the CD52, TRAC and TRBC TALE-nucleases and sequences of the TALE-nucleases target sites in the human corresponding genes.

Other target sequences in TRAC and CD52 genes have been designed, which are displayed in Table 6.

Target	Target sequence
TRAC_T02	TTTAGAAAGTTCCTGTG atgtcaagctggtcg AGAAAAGCTTTGAAACA (SEQ ID NO: 57)
TRAC_T03	TCCAGTGACAAGTCTGT ctgcctattcaccga TTTTGATTCTCAAACAA (SEQ ID NO: 58)
TRAC_T04	TATATCACAGACAAAAC tgtgctagacatgag GTCTATGGACTTCAAGA (SEQ ID NO: 59)
TRAC_T05	TGAGGTCTATGGACTTC aagagcaacagtgct GTGGCCTGGAGCAACAA (SEQ ID NO: 60)
CD52_T01	TTCCTCTTCCTCCTAC caccatcagcctcct TTACCTGTACCATAAC (SEQ ID NO: 61)
CD52_T04	TTCCTCCTACTCACCA cagcctcctgg TCTTACCTGTACCATA (SEQ ID NO: 62)
CD52_T05	TCCTACTCACCATCAG ctcctggttat TTGCTCTTACCTGTAC (SEQ ID NO: 63)

CD52_T06	TTATCCCACTTCTCCT ctacagatacaaact TTTTGTCCTGAGAGTC (SEQ ID NO: 64)
CD52_T07	TGGACTCTCAGGACAA acgacaccagccaaa TGCTGAGGGGCTGCTG (SEQ ID NO: 65)

Table 6: Additional target sequences for TRAC and CD52 TALE-nucleases.

Activity of CD52-TALE-nuclease, TRAC-TALE-nuclease and TRBC-TALE-nuclease in HEK293 cells

Each TALE-nuclease construct was subcloned using restriction enzyme digestion in a mammalian expression vector under the control of pEF1alpha long promoter. One million HEK293 cells were seeded one day prior to transfection. Cells were co-transfected with 2.5 µg of each of the two plasmids encoding the TALE-nucleases recognizing the two half targets in the genomic sequence of interest in the CD52 gene, T-cell receptor alpha constant chain region (TRAC) or T-cell receptor beta constant chain region (TRBC) under the control of the EF1-alpha promoter or 5 µg of a control pUC vector (pCLS0003) using 25 µl of lipofectamine (Invitrogen) according to the manufacturer's instructions. The double stranded cleavage generated by TALE-nucleases in CD52 or TRAC coding sequences is repaired in live cells by non homologous end joining (NHEJ), which is an error-prone mechanism. Activity of TALE-nucleases in live cells is measured by the frequency of insertions or deletions at the genomic locus targeted. 48 hours after transfection, genomic DNA was isolated from transfected cells and locus specific PCRs were performed using the following composite primers: 5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG (forward adaptor sequence, SEQ ID NO: 126)- 10N (TAG)- locus specific forward sequence for CD52: 5'-CAGATCTGCAGAAAGGAAGC-3' (SEQ ID NO: 134; composite primer disclosed as SEQ ID NO: 66), for TRAC: 5'-ATCACTGGCATCTGGACTCCA-3' (SEQ ID NO: 135; composite primer disclosed as SEQ ID NO: 67), for TRBC1: 5'-AGAGCCCCTACCAGAACCAGAC-3' (SEQ ID NO: 136; composite primer disclosed as SEQ ID NO: 68), or for TRBC2: 5'- GGACCTAGTAACATAATTGTGC-3' (SEQ ID NO: 137; composite primer disclosed as SEQ ID NO: 69), and the reverse composite primer 5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG (reverse adaptor sequence, SEQ ID NO: 130)-endogenous locus specific reverse sequence for CD52: 5'-CCTGTTGGAGTCCATCTGCTG-3' (SEQ ID NO: 138; composite primer disclosed as SEQ ID NO: 70), for TRAC: 5'-

CCTCATGTCTAGCACAGTTT-3' (SEQ ID NO: 139; composite primer disclosed as SEQ ID NO: 71), for TRBC1 and TRBC2: 5'- ACCAGCTCAGCTCCACGTGGT-3' (SEQ ID NO: 140; composite primer disclosed as SEQ ID NO: 72). PCR products were sequenced by a 454 sequencing system (454 Life Sciences). Approximately 10,000 sequences were obtained per PCR product and then analyzed for the presence of site-specific insertion or deletion events; results are in Table 7.

Target	% Indels with TALE-nuclease transfection	% Indels with pUC control transfection
CD52_T02	28.0	0.9
TRAC_T01	41.9	0.3
TRBC_T01 in constant chain 1	3.81	0
TRBC_T01 in constant chain 2	2.59	0
TRBC_T02 in constant chain 1	14.7	0
TRBC_T02 in constant chain 1	5.99	0

Table 7: Percentages of indels for TALE-nuclease targeting CD52_T02, TRAC_T01, TRBC_T01 and TRBC_T02 targets.

Activity of CD52-TALE-nuclease, TRBC-TALE-nuclease and TRAC-TALE-nuclease in primary T lymphocytes

Each TALE-nuclease construct was subcloned using restriction enzyme digestion in a mammalian expression vector under the control of the T7 promoter.

mRNA encoding TALE-nuclease cleaving CD52 TRAC and TRBC genomic sequence were synthesized from plasmid carrying the coding sequences downstream from the T7 promoter. T lymphocytes isolated from peripheral blood were activated for 5 days using anti-CD3/CD28 activator beads (Life technologies) and 5 million cells were then transfected by electroporation with 10 µg of each of 2 mRNAs encoding both half TALE-nuclease (or non coding RNA as controls) using a CytoLVT-P instrument. As a consequence of the insertions and deletions induced by NHEJ, the coding sequence for CD52 and/or TRAC will be out of frame in a fraction of the cells resulting in non-functional genes. 5 days after electroporation, cells were labeled with fluorochrome-conjugated anti-CD52 or anti-TCR antibody by flow cytometry for the presence of CD52 or TCR at their cell surface. Since all T

lymphocytes expanded from peripheral blood normally express CD52 and TCR, the proportion of CD52-negative or TCR-negative cells is a direct measure of TALE-nuclease activity. In table 8 are listed the results of a representative experiment. The table 9 shows the results of a representative experiment testing the efficiency of TRBC TALE-nucleases.

ARN transfected	% CD52-negative cells	% TCR-negative cells	% CD52/TCR double negative cells
non coding RNA	1,21	1,531	0,111
TALEN CD52_T02	49,2	1,6	0,78
TALEN TRAC_T01	2,16	44,8	0,97
TALEN CD52_T02 + TALEN TRAC_T01	29,3	39,6	15,5

Table 8: Percentages of CD52- negative, TCR-negative and CD52/TCR-double negative T lymphocytes after transfection of corresponding TALE-nuclease-expressing polynucleotides.

ARN transfected	% TCR-negative cells
no RNA	1,22
TALEN TRBC_T01	6,52
TALEN TRBC_T02	23,5

Table 9: Percentages of TCR-negative T lymphocytes after transfection of TRBC TALE-nuclease-expressing polynucleotides.

Functional analysis of T cells with targeted CD52 gene

The goal of CD52 gene inactivation is to render T lymphocytes resistant to anti-CD52 antibody mediated immunosuppression. As described in the previous paragraph, T lymphocytes were transfected with mRNA encoding TALE-nuclease cleaving CD52. 7 days after transfection, cells were treated with 50µg/ml anti-CD52 monoclonal antibody (or rat IgG as control) with or without 30% rabbit complement (Cedarlane). After 2 hours of incubation at 37°C, the cells were labeled with a fluorochrome-conjugated anti-CD52

antibody together with a fluorescent viability dye (eBioscience) and analyzed by flow cytometry to measure the frequency of CD52-positive and CD52-negative cells among live cells. Figure 6 shows the result of a representative experiment, demonstrating that CD52-negative cells are completely resistant to complement-mediated anti-CD52 antibody toxicity.

Functional analysis of T cells with targeted TRAC gene

The goal of TRAC gene inactivation is to render T lymphocytes unresponsive to T-cell receptor stimulation. As described in the previous paragraph, T lymphocytes were transfected with mRNA encoding TALE-nuclease cleaving TRAC or CD52. 16 days after transfection, cells were treated with up to 5µg/ml of phytohemagglutinin (PHA, Sigma-Aldrich), a T-cell mitogen acting through the T cell receptor. Cells with a functional T-cell receptor should increase in size following PHA treatment. After three days of incubation, cells were labeled with a fluorochrome-conjugated anti-CD52 or anti-TCR antibody and analyzed by flow cytometry to compare the cell size distribution between TCR-positive and TCR-negative cells, or between CD52-positive and CD52-negative cells. Figure 7 shows that TCR-positive cells significantly increase in size after PHA treatment whereas TCR-negative cells have the same size as untreated cells indicating that TRAC inactivation rendered them unresponsive to TCR-signaling. By contrast, CD52-positive and CD52-negative increase in size to same extent.

Functional analysis of T cells with targeted CD52 and TRAC genes

To verify that genome engineering did not affect the ability of T cells to present anti-tumor activity when provided with a chimeric antigen receptor (CAR), we transfected T cells that had been targeted with CD52-TALE-nuclease and TRAC-TALE-nuclease with 10µg of RNA encoding an anti-CD19 CAR (SEQ ID NO: 73). 24 hours later, T cells were incubated for 4 hours with CD19 expressing Daudi cells. The cell surface upregulation of CD107a, a marker of cytotoxic granule release by T lymphocytes (called degranulation) was measured by flow cytometry analysis (Betts, Brenchley et al. 2003). The results are included in Figure 8 and show that CD52-negative/TCRαβ-negative cells and CD52-positive/TCRαβ-positive have the same ability to degranulate in response to PMA /ionomycin (positive control) or CD19+ Daudi cells. CD107 upregulation is dependent on the presence of a CD19+. These data

suggest that genome engineering has no negative impact on the ability of T cells to mount a controlled anti-tumor response.

Genomic safety of CD52-TALE-nuclease and TRAC-TALE-nuclease in primary T lymphocytes

As our constructs include nuclease subunits, an important question is whether multiple TALE-nuclease transfection can lead to genotoxicity and off-target cleavage at 'close match' target sequences or by mispairing of half-TALE-nucleases. To estimate the impact of TRAC-TALE-nuclease and CD52-TALE-nuclease on the integrity of the cellular genomes, we listed sequences in the human genome that presented the potential for off-site cleavage. To generate this list, we identified all the sequences in the genome with up to 4 substitutions compared to the original half targets and then identified the pairs of potential half targets in a head to head orientation with a spacer of 9 to 30 bp from each other. This analysis included sites potentially targeted by homodimers of one half-TALE-nuclease molecule or heterodimers formed by one CD52 half TALE-nuclease and one TRAC half-TALE-nuclease. We scored the potential off-site targets based on the specificity data taking into account the cost of individual substitutions and the position of the substitutions (where mismatches are better tolerated for bases at the 3' end of the half target). We obtained 173 unique sequences with a score reflecting an estimation of the likelihood of cleavage. We selected the 15 top scores and analyzed by deep sequencing the frequency of mutations found at these loci in T cells simultaneously transfected with CD52 and TRAC TALE-nuclease and purified by magnetic separation as CD52-negative, TCRαβ-negative. Results are in Figure 9. The highest frequency of insertion/deletion is $7x10^{-4}$. These results make the putative offsite target at least 600 times less likely to be mutated than the intended targets. The TALE-nuclease reagents used in this study therefore appear extremely specific.

Example 3: TALE-nucleases cleaving the human CTLA4 gene and the human PDCD1 gene.

As described in example 1, heterodimeric TALE-nucleases targeting respectively PDCD1 and CTLA4 genes were designed and produced. The targeted genomic sequences consist of two 17-bp long sequences (called half targets) separated by an 11 or 15-bp spacer. Each half-target is recognized by repeats of half TALE-nucleases listed in table 10.

Target	Target sequence	Repeat sequence	Half TALE-nuclease
CTLA4_T01	TGGCCCTGCACTCTCCT gttttttcttctctt CATCCCTGTCTTCTGCA (SEQ ID NO: 74)	Repeat CTLA4_T01-L (SEQ ID NO: 79)	CTLA4_T01-L TALEN (SEQ ID NO: 89)
		Repeat CTLA4_T01-R (SEQ ID NO: 80)	CTLA4_T01-R TALEN (SEQ ID NO: 90)
CTLA4_T03	TTTTCCATGCTAGCAAT gcacgtggcccagcc TGCTGTGGTACTGGCCA (SEQ ID NO : 75)	Repeat CTLA4_T03-L (SEQ ID NO: 81)	CTLA4_T03-L TALEN (SEQ ID NO: 91)
		Repeat CTLA4_T03-R (SEQ ID NO: 82)	CTLA4_T03-R TALEN (SEQ ID NO: 92)
CTLA4_T04	TCCATGCTAGCAATGCA cgtggcccagcctgc TGTGGTACTGGCCAGCA (SEQ ID NO: 76)	Repeat CTLA4_T04-L (SEQ ID NO: 84)	CTLA4_T04-L TALEN (SEQ ID NO: 93)
		Repeat CTLA4_T04-R (SEQ ID NO: 85)	CTLA4_T04-R TALEN (SEQ ID NO: 94)
PDCD1_T01	TTCTCCCCAGCCCTGCT cgtggtgaccgaagg GGACAACGCCACCTTCA (SEQ ID NO : 77)	Repeat PDCD1_T01-L (SEQ ID NO: 86)	PDCD1_T01-L TALEN (SEQ ID NO: 95)
		Repeat PDCD1_T01-R (SEQ ID NO: 87)	PDCD1_T01-R TALEN (SEQ ID NO: 96)
PDCD1_T03	TACCTCTGTGGGGCCAT ctccctggcccccaa GGCGCAGATCAAAGAGA (SEQ ID NO : 78)	Repeat PDCD1_T03-L (SEQ ID NO: 88)	PDCD1_T03-L TALEN (SEQ ID NO: 97)
		Repeat PDCD1_T03-R (SEQ ID NO: 89)	PDCD1_T03-R TALEN (SEQ ID NO: 98

Table 10: Description of the CTLA4 and PDCD1 TALE-nucleases and sequences of the TALE-nucleases target sites in the human corresponding genes.

Activity of CTLA4-TALE-nuclease and PDCD1-TALE-nuclease in HEK293 cells

Each TALE-nuclease construct was subcloned using restriction enzyme digestion in a mammalian expression vector under the control of the pEF1alpha long promoter. One million HEK293 cells were seeded one day prior to transfection. Cells were co-transfected with 2.5 µg of each of two plasmids encoding the TALE-nucleases recognizing the two half targets in the genomic sequence of interest in the PDCD1 and CTLA-4 gene under the control of the EF1-alpha promoter or 5 µg of a control pUC vector (pCLS0003) using 25 µl of lipofectamine (Invitrogen) according to the manufacturer's instructions. The double stranded cleavage generated by TALE-nucleases in PDCD1 or CTLA-4 coding sequences is repaired in live cells by non homologous end joining (NHEJ), which is an error-prone mechanism. Activity of TALE-nucleases in live cells is measured by the frequency of insertions or deletions at the genomic locus targeted. 48 hours after transfection, genomic

DNA was isolated from transfected cells and locus specific PCRs were performed using the following composite primers: 5'-CCATCTCATCCCTGCGTGTCTCCGACTCAG (forward adaptor sequence, SEQ ID NO: 126)- 10N (TAG)- locus specific forward sequence for CTLA4_T01: 5'-CTCTACTTCCTGAAGACCTG-3' (SEQ ID NO: 141; composite primer disclosed as SEQ ID NO: 99) , for CTLA4_T03/T04: 5'-ACAGTTGAGAGATGGAGGGG-3' (SEQ ID NO: 142; composite primer disclosed as SEQ ID NO: 100), for PDCD1_T01: 5'-CCACAGAGGTAGGTGCCGC-3' (SEQ ID NO: 143; composite primer disclosed as SEQ ID NO: 101) or for PDCD1_T03: 5'-GACAGAGATGCCGGTCACCA-3' (SEQ ID NO: 144; composite primer disclosed as SEQ ID NO: 102) and the reverse composite primer 5'-CCTATCCCCTGTGTGCCTTGGCAGTCTCAG (reverse adaptor sequence, SEQ ID NO: 130)- endogenous locus specific reverse sequence for CTLA4_T01: 5'-TGGAATACAGAGCCAGCCAA-3' (SEQ ID NO: 145; composite primer disclosed as SEQ ID NO: 103), for CTLA4_T03/T04: 5'-GGTGCCCGTGCAGATGGAAT-3' (SEQ ID NO: 146; composite primer disclosed as SEQ ID NO: 104), for PDCD1_T01: 5'-GGCTCTGCAGTGGAGGCCAG-3' (SEQ ID NO: 147; composite primer disclosed as SEQ ID NO: 105) or for PDCD1_T03: 5'-GGACAACGCCACCTTCACCT-3' (SEQ ID NO: 148; composite primer disclosed as SEQ ID NO: 106).

PCR products were analyzed by T7-endonuclease assay: briefly, after denaturation and reannealing of the PCR product, T7 endonuclease will specifically digest mismatched DNA composed of wild type and mutated strands. The digestion product is then resolved by polyacrylamide gel electrophoresis. The presence of a digested product is indicative of mutated sequences induced by TALE-nuclease activity. Results are displayed in Figure 10 where arrows point to the digested PCR products. They demonstrate that PDCD1_T1, PDCD1_T3, CTLA4_T1, CTLA4_T3 and CTLA4_T4 TALE-nucleases all exhibit mutagenic nuclease activity at their target sites.

Example 4: pTalpha permits CD3 surface expression in inactivated TCR alpha T lymphocytes:

Description of the different preTalpha versions:

The human pTalpha gene encodes a transmembrane glycoprotein comprising an extracellular Ig-like domain, a hydrophobic transmembrane domain and a large C-terminal

intracytoplasmic tail. Different versions derived from human pTalpha glycoprotein have been designed and are described in Table 11 and represented in figure 11.

PTalpha versions	Description	SEQ ID
pTalpha-FL	Full-length of human pTalpha glycoprotein	107
pTalpha-Δ18	Truncated Human pTalpha glycoprotein lacking 18 residues from the C-terminus.	108
pTalpha-Δ48	Truncated Human pTalpha glycoprotein lacking 48 residues from the C-terminus.	109
pTalpha-Δ62	Truncated Human pTalpha glycoprotein lacking 62 residues from the C-terminus.	110
pTalpha-Δ78	Truncated Human pTalpha glycoprotein lacking 78 residues from the C-terminus.	111
pTalpha-Δ92	Truncated Human pTalpha glycoprotein lacking 92 residues from the C-terminus.	112
pTalpha-Δ110	Truncated Human pTalpha glycoprotein lacking 110 residues from the C-terminus.	113
pTalpha-Δ114	Truncated Human pTalpha glycoprotein lacking 114 residues from the C-terminus.	114
pTalpha-FL-CD28	Full-length of human pTalpha glycoprotein fused in C-terminus with CD28 activation domain.	115
pTalpha-FL-CD8	Full-length of human pTalpha glycoprotein fused in C-terminus with CD8 activation domain.	116
pTalpha-FL-4-1BB	Full-length of human pTalpha glycoprotein fused in C-terminus with 4-1BB activation domain..	117
pTalpha-Δ48-CD28	pTalpha-Δ48 glycoprotein fused in C-terminus with CD28 activation domain.	118
pTalpha -Δ48-CD8	pTalpha-Δ48 glycoprotein fused in C-terminus with CD8 activation domain.	119
pTalpha -Δ48-41BB	pTalpha-Δ48 glycoprotein fused in C-terminus with 4-1BB activation domain.	120
pTalpha-Δ114/TCRα.IC	pTalpha-Δ114 glycoprotein fused in C-terminus with the intracellular domain of TCRalpha	121
pTalpha-EC/TCRα.TM.IC	pTalpha extracellular domain fused in C-terminus with the transmembrane and intracellular domain of TCRalpha.	122
pTalpha-Δ48-1xMUT	pTalpha-Δ48 glycoprotein with mutated residue W46R.	123

preTalpha-Δ48-4xMUT	pTalpha-Δ48 glycoprotein with mutated residues D22A, K24A, R102A, R117A	124

Table 11: Description of a subset of pTalpha constructs

The different preTalpha constructs tested include:

1) **pTalpha deletion mutants:** Different deletions were generated in the intracellular cytoplasmic tail of the human pTalpha protein (which comprises 114 amino acids) (SEQ ID NO: 107). The constructs tested include the full length version of the protein (FL) and mutants in which 18, 48, 62, 78, 92, 110 and 114 amino acids were deleted from the C-terminus of the protein (SEQ ID NO: 108 to SEQ ID NO: 114).
2) **pTalpha mutants containing intracellular activation domains:** The FL and Δ48 variants where fused to the CD8, CD28 or 41BB intracellular activation domains at their C-terminus (SEQ ID NO: 115 to SEQ ID NO: 120).
3) **pTalpha/TCRα chimeric mutants**: In one of the constructs, the TCRα intracellular domain (IC) was fused to a tail-less version (Δ114) of pTalpha (SEQ ID NO: 121). A second construct was also generated in which the pTalpha extracellular domain was fused to the transmembrane (TM) and the IC domains from TCRα (SEQ ID NO: 122).
4) **pTalpha dimerization mutants:** Some mutations have been described in the literature as being capable to alter the oligomerisation/dimerisation ability of the preTCR complex. These mutants are proposed to allow preTCR expression at the cell surface, without inducing the constitutive signaling (supposed to be induced upon preTCR oligomerization). The mutations have been introduced in the pTalphaΔ48 variant and are:
    - 1xMUT: W46R (SEQ ID NO: 123)
    - 4x MUT: D22A, K24A, R102A, R117A (SEQ ID NO: 124)

Activity of different preTalpha constructs in TRAC inactivated Jurkat cells:

In order to screen different pTalpha variants for their ability to restore CD3 surface expression in TCRalpha inactivated cells, a cell line was generated in which the TCRalpha gene was disrupted using TALEN targeting TRAC. Jurkat cells (a T-cell leukemia cell line) were transfected with plasmids coding for the TALEN cleaving TRAC using CytoPulse electroporation, and the KO cells ($TCR_{\alpha/\beta}^{NEG}$; $CD3^{NEG}$) where then purified by negative selection using CD3 magnetic beads. The KO population (JKT_KOx3 cells) was amplified and

used for screening of the different pTalpha variants. Screening was performed by transfection of one million of JKT_KOx3 cells with 15 µg of plasmid coding the different pTalpha variants under control of the EF1α promoter, followed by analysis by flow cytometry of CD3 cell surface expression 48h after transfection. Figure 12 is a representative example of the transfection efficiencies (% of BFP+ cells) and activity of the FL, Δ18 and Δ48 pTalpha constructs in JKT_KOx3 cells, based on the % of CD3+ cells, determined by flow cytometry. The results from the different constructs are grouped in Table 12.

Mutant	ID	% $CD3_{LOW}$	SD
0	NEG	4,69	1,53
1	preTCRa-FL	31,18	4,15
2	preTCRα-Δ18	20,13	4,56
3	preTCRα-Δ48	44,86	3,90
4	preTCRα-Δ62	32,42	2,95
5	preTCRα-Δ78	24,75	3,87
6	preTCRα-Δ92	20,63	3,70
7	preTCRα-Δ110	18,18	3,49
8	preTCRα-Δ114	4,29	2,74
9	preTCRα-FL-CD8	18,16	5,30
10	preTCRα-FL-CD28	5,67	2,77
11	preTCRα-FL-41BB	27,27	3,66
12	preTCRα-Δ48–CD8	11,56	6,01
13	preTCRα-Δ48–CD28	12,22	4,72
14	preTCRα-Δ48–41BB	35,93	4,55
15	preTCRα-Δ114/TCRα.IC	3,94	1,95
16	preTCRα-EC/TCRα.TM.IC	17,80	4,47
17	preTCRα-Δ48-1xMUT	26,88	4,37
18	preTCRα-Δ48-4xMUT	7,59	1,06

Table 12: Activity of the different pTalpha constructs in Jurkat TCR alpha inactivated cells. Activity was measured by flow cytometry analysis of CD3 expression in jurkat TCR alpha inactivated cells transfected with the different preTalpha constructs.

Activity of pTalpha-FL and pTalpha-Δ48 in TCR alpha inactivated primary T lymphocytes:

In order to test the ability of pTalpha-FL and pTalpha-Δ48 versions to induce CD3 surface expression in TCR alpha inactivated T lymphocytes, pTalpha-FL and pTalpha-Δ48 coding sequences were cloned into a self-inactivating pLV-SFFV-BFP-2A-PCTRA lentiviral vector that

codes for Blue Fluorescent protein (BFP) under the SFFV promoter followed by the self-cleaving T2A peptide (figure 13).

T lymphocytes isolated from peripheral blood were activated for 72 hours using anti-CD3/CD28 activator beads (Life technologies) and 4.5 million cells were transfected by electroporation with 10 µg mRNA encoding the TALE-nuclease targeting TCR alpha constant chain region (TRAC) using a CytoLVT-S instrument (BTX-Harvard Harbour). Two days after electroporation, T cells were transduced with either the LV-SFFV-BFP-2A- pTalpha-Δ48 or LV-SFFV-BFP-2A-control lentiviral vectors. CD3 negative and CD3low T cells were then purified using anti-CD3 magnetic beads (Miltenyi Biotech). This experimental protocol is represented in Figure 14A.

Figure 14B represents flow cytometry analysis of TCRalpha/beta, CD3 cell surface expression, and BFP expression on TCRalpha inactivated T cells (KO) transduced with either BFP-2A-pTalphaΔ48 (KO/Δ48) or control BFP lentiviral vector (KO/BFP) before and after purification with CD3 beads. TCRalpha inactivated cells transduced with the BFP-T2A-pTalpha-Δ48 vector (BFP+ cells) show higher levels of CD3 compared to non transduced cells (BFP- cells). No differences are observed among cells transduced with the control BFP vector. These results indicate that pTalpha mediates restoration of CD3 expression at the cell surface of TCRalpha inactivated cells. In contrast, TCRalpha/beta staining remains, as expected, unchanged in cells transduced or not with the pTalpha-Δ48 expressing vector.

<u>pTalpha-mediated CD3 expression supports activation of TCR-deficient T-cells:</u>

To determine the capacity of pTalpha to transduce cell activation signals, expression of early and later activation markers was analyzed on TCR alpha inactivated T cells transduced with pTalpha-Δ48 and pTalpha-Δ48.41BB. TCR alpha inactivated T cells transduced with pTalpha-Δ48 and pTalpha-Δ48.41BB were generated from primary human T-cells as described in previous section and in Figure 14A.

To detect signaling via CD3, cells were re-activated using anti-CD3/CD28-coated beads 3 days after purification of TCR alpha inactivated T cells with CD3 beads (figure 14A). Cells were stained with fluorochrome-conjugated anti-CD69 (early activation marker) and anti-CD25 (late activation marker), 24 and 48 hours after re-activation respectively and analyzed by flow cytometry (Figure 15A-B). As represented in figure 15A-B, TCR alpha inactivated cells

expressing pTalpha-Δ48 (KO/pTα-Δ48) or pTalpha-Δ48.41BB (KO/pTα-Δ48.BB) show upregulation of the activation markers, to levels similar to those observed in TCRalpha/beta expressing cells (NEP: non electroporated cells).

Another indicator of T cell activation is an increase in cell size which is sometimes referred to as "blasting". The capacity of the preTCR complexes to induce "blasting" was measured by flow cytometry analysis of the cell size 72 hours after re-activation using anti-CD3/CD28-beads (Figure 15C). Stimulation with anti-CD3/CD28 beads induced comparable increases in cell size in cells expressing TCRalpha/beta complexes vs. cells expressing pTalpha-Δ48 or pTalpha-Δ48.41BB. Taken together, these results suggest that preTCR complexes are competent to transduce signals that efficiently couple to the mechanisms mediating activation marker upregulation.

pTalpha mediated CD3 expression supports expansion of TCR-deficient primary T-cells using stimulatory anti-CD3/CD28 antibodies

To evaluate the capacity of preTCR complexes to support long term cell proliferation, proliferation of cells generated as previously described was measured. Ten days after the initial activation, cells were maintained in IL2 (non-Re-act) or in IL2 with anti-CD3/CD28 beads (Re-act). For each condition, cells were counted and analyzed by flow cytometry at the different time points to estimate the number of BFP+ cells. The growth of TCRalpha inactivated cells (KO) transduced with BFP or BFP-T2A-preTCRα-Δ48 vectors was compared, and the fold induction of these cells was estimated with respect to the value obtained at day 2 post re-activation. Figure 16 shows the results obtained with two independent donors. In both cases, TCRalpha inactivated cells expressing pTalpha-Δ48 displayed greater expansion than TCR alpha inactivated cells expressing only the BFP control vector. For the second donor, TCRalpha inactivated cells expressing pTalpha-Δ48.41BB or full-length pTalpha were also included, displaying also greater expansion than TCRalpha inactivated cells expressing only the BFP control vector.

Example 5: optimization of mRNA transfection in T cells using Cytopulse Technology.

Determination of the optimized cytopulse program

A first set of experiments were performed on non activated PBMCs in order to determine a voltage range in which cells could be transfected. Five different programs were tested as described in Table 13.

	Group 1				Group 2				Group 3			
Cyto-pulse program	Pul-ses	V	duration (ms)	Interval (ms)	Pul-ses	V	duration (ms)	Interval (ms)	Pul-ses	V	duration (ms)	Interval (ms)
1	1	600	0.1	0.2	1	600	0.1	100	4	130	0.2	2
2	1	900	0.1	0.2	1	900	0.1	100	4	130	0.2	2
3	1	1200	0.1	0.2	1	1200	0.1	100	4	130	0.2	2
4	1	1200	0.1	10	1	900	0.1	100	4	130	0.2	2
5	1	900	0.1	20	1	600	0.1	100	4	130	0.2	2

Table 13 : Different cytopulse programs used to determine the minimal voltage required for electroporation in PBMC derived T-cells.

3 or 6 million of cells were electroporated in 0.4 cm gap cuvette (30 or $15x10^6$ cells/ml) with 20 µg of plasmids encoding GFP and control plasmids pUC using the different Cytopulse programs. 24 hours post electroporation, GFP expression was analyzed in electroporated cells by flow cytometry to determine the efficiency of transfection. The data shown in Figure 17 indicates the minimal voltage required for plasmid electroporation in PBMC derived T cells. These results demonstrate that the cytopulse program 3 and 4 allow an efficient transformation of T cells (EP#3 and #4).

Electroporation of mRNA of purified Tcells activated

After determining the best cytopulse program that allows an efficient DNA electroporation of T cells, we tested whether this method was applicable to the mRNA electroporation.

$5x10^6$ purified T cells preactivated 6 days with PHA/IL2 were resupended in cytoporation buffer T (BTX-Harvard apparatus) and electroporated in 0.4 cm cuvettes with 10µg of mRNA encoding GFP or 20µg of plasmids encoding GFP or pUC using the preferred cytopulse program as determined in the previous section (table 14).

	Group 1				Group 2				Group 3			
Cyto-pulse program	Pulse	V	duration (ms)	Interval (ms)	Pulse	V	duration (ms)	Interval (ms)	Pulse	V	duration (ms)	Interval (ms)
3	1	1200	0.1	0.2	1	1200	0.1	100	4	130	0.2	2

Table 14: Cytopulse program used to electroporate purified T-cells.

48h after transfection cells were stained with viability dye (eFluor-450) and the cellular viability and % of viable GFP+ cells was determined by flow cytometry analysis (Figure 18).

The data shown in Figure 18 indicates that the electroporation of RNA with the optimal condition determined here is no toxic and allows transfection of more than 95% of the viable cells.

In synthesis, the whole dataset shows that T-cells can be efficiently transfected either with DNA or RNA. In particular, RNA transfection has no impact on cellular viability and allows uniform expression levels of the transfected gene of interest in the cellular population.

Efficient transfection can be achieved early after cellular activation, independently of the activation method used (PHA/IL-2 or CD3/CD28-coated-beads). The inventors have succeeded in transfecting cells from 72h after activation with efficiencies of >95%. In addition, efficient transfection of T cells after thawing and activation can also be obtained using the same electroporation protocol.

mRNA electroporation in primary human T cells for TALE-nuclease functional expression

After demonstrating that mRNA electroporation allow efficient expression of GFP in primary human T cells, we tested whether this method was applicable to the expression of other proteins of interest. Transcription activator-like effector nucleases (TALE-nuclease) are site-specific nucleases generated by the fusion of a TAL DNA binding domain to a DNA cleavage domain. They are powerful genome editing tools as they induce double-strand breaks at practically any desired DNA sequence. These double-strand breaks activate Non-homologous end-joining (NHEJ), an error-prone DNA repair mechanism, potentially leading to inactivation of any desired gene of interest. Alternatively, if an adequate repair template is introduced into the cells at the same time, TALE-nuclease-induced DNA breaks can be repaired by homologous recombination, therefore offering the possibility of modifying at will the gene sequence.

We have used mRNA electroporation to express a TALE-nuclease designed to specifically cleave a sequence in the human gene coding for the alpha chain of the T cell antigen receptor (TRAC). Mutations induced in this sequence are expected to result in gene inactivation and loss of TCRαβ complex from the cell surface. TRAC TALE-nuclease RNA or non coding RNA as control are transfected into activated primary human T lymphocytes

using Cytopulse technology. The electroporation sequence consisted in 2 pulses of 1200 V followed by four pulses of 130 V as described in Table 14.

By flow cytometry analysis of TCR surface expression 7 days post electroporation (Figure 19, top panel), we observed that 44% of T cells lost the expression of TCRαβ. We analyzed the genomic DNA of the transfected cells by PCR amplification of the TRAC locus followed by 454 high throughput sequencing. 33% of alleles sequenced (727 out of 2153) contained insertion or deletion at the site of TALE-nuclease cleavage. Figure 19 (bottom panel) shows examples of the mutated alleles.

These data indicate that electroporation of mRNA using cytopulse technology results in functional expression of TRAC TALE-nuclease.

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

$5X10^6$ T cells preactivated several days (3-5) with anti-CD3/CD28 coated beads and IL2 were resuspended in cytoporation buffer T, and electroporated in 0.4cm cuvettes without mRNA or with 10µg of mRNA encoding a single chain CAR (SEQ ID NO: 73) using the program described in Table 14.

24 hours post electroporation, cells were stained with a fixable viability dye eFluor-780 and a PE-conjugated goat anti mouse IgG F(ab')2 fragment specific to assess the cell surface expression of the CAR on the live cells. The data is shown in the figure 20. It indicates that the vast majority of the live T cells electroporated with the monocitronic mRNA described previously express the CAR at their surface.

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

The data shown in figure 20 indicates that the majority of the cells electroporated with the monocistronic mRNA described previously degranulate in the presence of target cells expressing CD19. These results clearly demonstrate that the CAR expressed at the surface of electroporated T cells is active.

Electroporation of T cells with a polycistronic mRNA encoding for an anti-CD19 multisubunit chimeric antigen receptor (CAR):

$5X10^6$ T cells preactivated several days (3-5) with anti CD3/CD28 coated beads and IL2 were electroporated in cytoporation buffer T, and electroporated in 0.4cm cuvettes without mRNA or with 45µg of mRNA encoding a multi-chain CAR (SEQ ID NO: 226), Figure 21A and figure 4B (csm4)) using the program described in Table 14.

24 hours post electroporation, cells were stained with a fixable viability dye eFluor-780 and a PE-conjugated goat anti mouse IgG F(ab')2 fragment specific to assess the cell surface expression of the CAR on the live cells. The data shown in Figure 21 indicates that the vast majority of the live T cells electroporated with the polycistronic mRNA described previously express the CAR at their surface.

24 hours post electroporation, T cells were cocultured with Daudi ($CD19^+$) for 6 hours and analyzed by flow cytometry to detect the expression of the degranulation marker CD107a at their surface. The data shown in Figure 21 indicates that the majority of the cells electroporated with the polycistronic mRNA described previously degranulate in the presence of target cells expressing CD19. These results clearly demonstrate that the CAR expressed at the surface of electroporated T cells is active.

Example 6: Multi-chain CARs

A. Design of multi-chain CARs

Nine multi-chain CARs targeting the CD19 antigen were designed based on the high affinity receptor for IgE (FcεRI). The FcεRI expressed on mast cells and basophiles triggers allergic reactions. It is a tetrameric complex composed of a single α subunit (SEQ ID NO: 202), a single β subunit (SEQ ID NO: 203) and two disulfide-linked γ subunits (SEQ ID NO: 204). The α subunit contains the IgE-binding domain. The β and γ subunits contain ITAMs (SEQ ID NO: 198 and SEQ ID NO: 199) that mediate signal transduction Figure 4A. The multi-chain CARs were designed as described in Figures 4B, C and Table 15. In every multi-chain CAR, the extracellular domain of the FcRα chain was deleted and replaced by the 4G7scFv and the CD8α hinge (SEQ ID NO: 206). In the multi-chain CARs csm2, csm4, csm5, csm8 and csm10, the ITAM of the FcRβ chain and/or the FcRγ chain was deleted. In the multi-chain CARs csm2, csm4, csm5, csm6, csm8, csm9 and csm10, the 3 ITAMs of CD3ζ (SEQ ID NO: 197) were added to the FcRβ chain or the FcRγ chain. In the multi-chain CARs csm2, csm4, csm5, csm6, csm7, csm8, csm9 and csm10, the 4-1BB intracellular domain (SEQ ID NO: 200) was added to the FcRα chain, the FcRβ chain or the FcRγ chain. In the multi-chain CAR csm10, the CD28 intracellular domain was added to the FcRα chain (SEQ ID NO: 201).

Multi-chain CAR versions	alpha chain	beta chain	gamma chain
Csm1	FcRα-4G7scFV-CD8 (SEQ ID NO: 206)	FcRβ (SEQ ID NO: 203)	FcRγ (SEQ ID NO: 204)
Csm2	FcRα-4G7scFv-CD8 (SEQ ID NO: 206)	FcRβ (SEQ ID NO: 203)	FcRγ-ΔITAM-41BB-CD3ζ (SEQ ID NO: 212)
Csm4	FcRα-4G7scFv-CD8 (SEQ ID NO: 206)	FcRβ-ΔITAM-41BB (SEQ ID NO: 208)	FcRγ-ΔITAM-CD3ζ (SEQ ID NO: 213)
Csm5	FcRα-4G7scFv-CD8 (SEQ ID NO: 206)	FcRβ-ΔITAM-41BB-CD3ζ (SEQ ID NO: 209)	FcRγ (SEQ ID NO: 204)
Csm6	FcRα-4G7scFv-CD8 (SEQ ID NO: 206)	FcRβ-41BB (SEQ ID NO: 210)	FcRγ-CD3ζ (SEQ ID NO: 214)
Csm7	FcRα-4G7scFv-CD8-4-1BB (SEQ ID NO: 207)	FcRβ (SEQ ID NO: 203)	FcRγ (SEQ ID NO: 204)
Csm8	FcRα-4G7scFv-CD8-4-1BB	FcRβ-ΔITAM-CD3ζ (SEQ ID NO: 211)	FcRγ (SEQ ID NO: 204)

	(SEQ ID NO: 207)		
Csm9	FcRα-4G7scFv-CD8-4-1BB (SEQ ID NO: 207)	FcRβ (SEQ ID NO: 203)	FcRγ-CD3ζ (SEQ ID NO: 214)
Csm10	FcRα-4G7scFv-CD8-CD28 (SEQ ID NO: 206)	FcRβ-ΔITAM-41BB (SEQ ID NO: 208)	FcRγ-ΔITAM-CD3ζ (SEQ ID NO: 213)

Table 15: Description of the chains compositions of the multi-chain CARs versions.

B. Transiently expressed multi-chain CARs can trigger T cells activation

Multi-chain CARs can be expressed in human T cells after electroporation of polycistronic mRNA.

T cells were electroporated with capped and polyadenylated polycistronic mRNA (Figure 21A), that were produced using the mMESSAGE mMACHINE kit and linearized plasmids as template. The plasmids used as template contained the T7 RNA polymerase promoter followed by a polycistronic DNA sequence encoding csm1, csm2, csm4, csm5, csm6, csm7, csm8, csm9 or csm10 (SEQ ID NO: 224 to 232).

The electroporation of the polycistronic mRNAs into the human T cells was done using the CytoLVT-S device (Cellectis), according to the following protocol: $5X10^6$ T cells preactivated several days (3-5) with anti CD3/CD28 coated beads and IL2 were resuspended in cytoporation buffer T, and electroporated in 0.4cm cuvettes with 45µg of mRNA using the PBMC3 program Table 14.

24 hours post electroporation, human T cells engineered using polycistronic mRNAs encoding the multi-chain CARs were labeled with a fixable viability dye eFluor-780 and a PE-conjugated goat anti mouse IgG F(ab')2 fragment specific, and analysed by flow cytometry. The data shown in Figure 22 indicate that the live T cells engineered using polycistronic mRNAs express the multi-chain CARs csm1, csm2, csm4, csm5, csm6, csm7, csm8, csm9 and csm10.

As described in the literature for the FcεRI, the expression of the multi-subunit CARs is conditioned by the expression of the 3 chains. The α chain alone is less expressed than the α + γ chains complex, and the α + γ chains complex is less expressed than the α + β + γ chains complex Figure 23.

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

24 hours post electroporation, human T cells engineered using polycistronic mRNAs encoding the multi-chain CARs were co-cultured with target (Daudi) or control (K562) cells for 6 hours. The CD8+ T cells were then analyzed by flow cytometry to detect the expression of the degranulation marker CD107a at their surface. The data shown in Figure 24 indicate that the human CD8+ T cells expressing the multi-chain CARs csm1, csm2, csm4, csm5, csm6, csm7, csm8, csm9 and csm10 degranulate in coculture with CD19 expressing target cells but not in coculture with control cells.

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

24 hours post electroporation, human T cells engineered using polycistronic mRNAs encoding the multi-chain CARs were co-cultured with target (Daudi) or control (K562) cells for 24 hours. The supernatants were then harvested and analysed using the TH1/TH2 cytokine cytometric bead array kit to quantify the cytokines produced by the T cells. The data shown in Figure 25A-C indicate that the human T cells expressing the multi-chain CARs csm1, csm2, csm4, csm5, csm6, csm7, csm8, csm9 and csm10 produce IFNγ, IL8 and IL5 in coculture with CD19 expressing target cells but not in coculture with control cells.

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

24 hours post electroporation, human T cells engineered using polycistronic mRNAs encoding the multi-chain CARs were co-cultured with target (Daudi) or control (K562) cells for 4 hours. The target cells were then analysed by flow cytometry to analyse their viability. The data shown in Figure 26 indicate that the cells expressing the multi-chain CARs csm1, csm2, csm4, csm5, csm6, csm7, csm8, csm9 and csm10 lyse the CD19 expressing target cells but not the control cells.

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CLAIMS

1) A multi-chain Chimeric Antigen Receptor (CAR) comprising at least:

   - one transmembrane polypeptide comprising at least one extracellular ligand-binding domain, wherein the at least one extracellular ligand-binding domain interacts with a cell surface molecule; and
   - one transmembrane polypeptide comprising at least one signal-transducing domain;

   wherein the signal transducing domain(s) of the multi-chain Chimeric Antigen Receptor is present on a polypeptide distinct from that carrying the extracellular ligand-binding domain(s).

2) The multi-chain Chimeric Antigen Receptor of claim 1 wherein at least one transmembrane polypeptide comprises a part of Fc receptor.

3) The multi-chain Chimeric Antigen Receptor of claim 2 wherein said part of Fc receptor is selected from the group consisting of: (a) FcεRI alpha chain, (b) FcεRI beta chain and (c) FcεRI gamma chain.

4) The multi-chain Chimeric Antigen Receptor of claim 3 wherein said part of Fc receptor is a part of FcεRI alpha chain.

5) The multi-chain Chimeric Antigen Receptor according to claim 3 or 4 comprising a part of FcεRI alpha chain and a part of FcεRI beta chain such that said FcεRI chains dimerize together to form a dimeric Chimeric Antigen Receptor.

6) The multi-chain Chimeric Antigen Receptor according to claim 3 or 4 comprising a part of FcεRI alpha chain and a part of FcεRI gamma chain such that said FcεRI chains trimerize together to form a trimeric Chimeric Antigen Receptor.

7) The multi-chain Chimeric Antigen Receptor according to claim 3 or 4 comprising a part of FcεRI alpha chain, a part of FcεRI beta chain and a part of FcεRI gamma chain such that said FcεRI chains tetramerize together to form a tetrameric Chimeric Antigen Receptor.

8) The multi-chain Chimeric Antigen Receptor according to any one of claims 1 to 7, wherein the extracellular ligand-binding domain recognizes a ligand that acts as a cell surface marker on target cells associated with a particular disease state.

9) The multi-chain Chimeric Antigen Receptor according to any one of claims 1 to 8 wherein said extracellular ligand-binding domain is a single chain antibody fragment (scFv).

10) The multi-chain Chimeric Antigen Receptor according to any one of claims 1 to 9 wherein said transmembrane polypeptide further comprises a stalk region.

11) The multi-chain Chimeric Antigen Receptor according to any one of claims 1 to 10 wherein said signal transducing domain is selected from the group consisting of: TCR zeta chain, FCεRβ chain, FcεRIγ chain, immunoreceptor tyrosine-based activation motif (ITAM).

12) The multi-chain Chimeric Antigen Receptor according to any one of claims 1 to 11 wherein said CAR comprises a co-stimulatory domain.

13) The multi-chain Chimeric Antigen Receptor according to any one of claims 1 to 11 further comprising a co-stimulatory molecule selected from the group consisting of: CD28, OX40, ICOS, CD137 and CD8.

14) The multi-chain Chimeric Antigen Receptor according to any one of claims 1 to 13 comprising polypeptides selected from the group consisting of SEQ ID NO: 202 to SEQ ID NO: 204 and SEQ ID NO: 206 to SEQ ID NO: 213.

15) A polynucleotide comprising two or more nucleic acid sequences encoding the transmembrane polypeptides composing the multi-chain CAR according to any one of claims 1 to 14.

16) A vector comprising a polynucleotide of claim 15.

17) A method of engineering an immune cell comprising:

(a) Providing an immune cell;

(b) Expressing at the surface of said cells at least one multi-chain Chimeric Antigen Receptor according to any one of the claims 1 to 14.

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

(a) Providing an immune cell;

(b) Introducing into said cell at least one polynucleotide encoding polypeptides composing at least one multi-chain Chimeric Antigen Receptor according to any one of claims 1 to 14;

(c) Expressing said polynucleotides into said cell.

19) The method of engineering an immune cell of claim 17 comprising:

(a) Providing an immune cell;

(b) Expressing at the surface of said cell a population of multi-chain Chimeric Antigen Receptors according to any one of the claims 1 to 14 each one comprising different extracellular ligand-binding domains.

20) The method of engineering an immune cell of claim 18 comprising:

(a) Providing an immune cell;

(b) Introducing into said cell at least one polynucleotide encoding polypeptides composing a population of multi-chain Chimeric Antigen Receptors according to any one of claims 1 to 14 each one comprising different extracellular ligand binding domains.

(c) Expressing said polynucleotides into said cell.

21) The method of engineering an immune cell of any one of claims 17 to 20 further comprising the step of d) inactivating in said immune cell at least one gene selected from the group consisting of TCR alpha, TCR beta, CD52, GR and immune checkpoints.

22) An isolated immune cell obtainable from the method according to any one of claims 17 to 21.

23) An isolated immune cell comprising at least one multi-chain Chimeric Antigen Receptor according to any one of claims 1 to 14.

24) An isolated cell according to claim 22 or 23 derived from inflammatory T-lymphocytes, cytotoxic T-lymphocytes, regulatory T-lymphocytes or helper T-lymphocytes.

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