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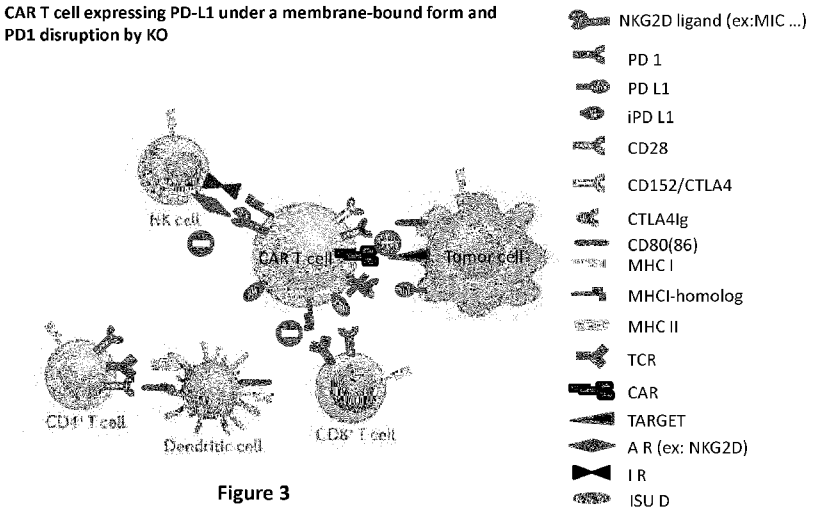

Figure 3

(57) Abstract: The present invention relates to methods for developing engineered immune cells such as T-cells for immunotherapy that have a higher potential of persistence and/or engraftment in host organism. IN particular, this method involves an inactivation of at least one gene involved in self/non self recognition, combined with a step of contact with at least one non-endogenous immunosuppressive polypeptide. The invention allows the possibility for a standard and affordable adoptive immunotherapy, whereby the risk of GvH is reduced.

METHODS FOR ENGINEERING ALLOGENEIC T CELL TO INCREASE THEIR PERSISTENCE AND/OR ENGRAFTMENT INTO PATIENTS

Field of the invention

The present invention relates to methods for developing engineered non-alloreactive T-cells for immunotherapy and more specifically to methods for increasing the persistence and/or the engraftment of allogeneic immune cells. This method involves at least a step of inactivation of a gene implicated in the self/non-selfrecognition by the use of preferably specific rare-cutting endonuclease, followed by a step of contact of said engineered immune cells with at least one non-endogenous immunosuppressive polypeptide (such as PD-L1 ligand and/or CTLA-4 Ig). This invention also relates to engineered immune cells and functional derivatives thereof, Chimeric Antigen Receptor (CAR), multichain CAR and their use thereof to enhance the efficiency of immunotherapy. The invention opens the way to a safer strategy by reducing the risk of graft versus host disease GvHD and allows an affordable adoptive immunotherapy.

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).

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.

Adaptive immune response is a complex biological system where numerous cellular components interact. Professional Antigen Presenting Cells (APC) are able to process foreign bodies and expose them to helper T cells in the context of MHC Class II molecules. Activated helper T cells will in turn stimulate B cells response and cytotoxic T (CTL) cells response. CTL recognize foreign peptides presented by MHC Class I molecules but in the case of alloreactivity, recognize and kill cells bearing foreign MHC Class I. MHC Class I molecules are composed of 2 entities: the highly polymorphic, transmembrane heavy chain and a small invariant polypeptide, the β2-microglobuline (β2-m) encoded by B2M gene. The expression of the MHC Class I heavy chain at the cell surface requires its association with the β2-m. Hence, abrogation of β2-m expression in CAR T cells will impair MHC Class I expression and make them invisible to host CTL. However, MHC Class I deficient CAR T cells are susceptibe to lysis by host NK cells, which target cells lacking MHC Class I molecules [Ljunggren HG et al.(1990), Immunl Today. 11:237-244].

NK cells exert cytotoxic functions towards the cells they interact with based on the balance between activating and inhibitory signals they received through different monomorphic or polymorphic receptors. One central activating receptor on human NK cells is NKG2D and its ligands include proteins such as MICA, MICB, ULBP1, ULBP2, ULBP3 [Raulet DH, (2003), Nature Reviews Immunology 3 (10): 781–79]. On the other hand, the inhibitory signal is mediated through the interaction between NK receptors like LIR-1/ILT2 and MHC Class I molecules [Ljunggren HG et al.(1990), Immunl Today. 11:237-244]. Some viruses such as cytomegaloviruses have aquired mechanisms to avoid NK cell mediate immune surveillance. HCMV genome encodes proteins that are able to prevent MHC Class I surface expression (i.e. US2, US3, US6 and US11) while expressing a MHC class I homolog protein (UL18) that acts as a decoy to block NK-mediated cell lysis [Kim, Y et al. (2008), PLOS Pathogens. 4: e1000123, and Wilkinson G. et al. (2010). J Clin Virol. 41(3):206-212]. Moreover, HCMV interferes with the NKG2D pathway by secreting a protein able to bind NKG2D ligands and prevent their surface expression [Welte SA et al. (2003), Eur J Immunol 33 (1): 194–203]. In tumor cells, some mechanisms have evolved to evade NKG2D response by secreting NKG2D ligands such as ULBP2, MICB or MICA (Waldhauer I, Steinle A (2003). Proteolytic release of soluble UL16-binding protein 2 from tumor cells. Cancer Res 2006; 66(5): 2520-2526; Salih HR et al. (2006), Hum Immunol. 2006 Mar;67(3):188-95; Salih HR et al. (2003) Blood. 2003 Aug 15;102(4):1389-96; Salih HR et al. (2002) J Immunol.;169(8):4098-102].

Many strategies are used by viruses to escape host immune system Tumor cells expressing a retroviral envelope escape immune rejection in vivo [Mangeney M et al.(1998). Proc. Natl. Acad. Sci. 95: 14920; Quintana F. et al. (2005). J. Clin. Invest. 115:2149 ; Bloch I. et al. (2007), FASEB J. 21:393]. It has been shown that retroviruses like Moloney murine leukemia virus as well as lentiviruses (HIV 1

and HIV 2) exert immunosuppressive activity through their envelope protein gp41 [Morozov V. et al. (2012), Retrovorology. 9:67; Denner J. et al. (2013), PLOS ONE. 8:e55199; Schlecht-Louf G et al (2014). J. Virology. 88:992). Although the primary function of this viral protein is to promote fusion between viral and cell membrane, different domains of gp41 can inhibit T cell activation and proliferation. The first one, termed ISU (for ImmunoSuppressive Unit) is located in the C-terminal part of the N-helical repeat of gp41 (Mangeney M et al.(1998). Proc. Natl. Acad. Sci. 95: 14920; Morozov V. et al. (2012), Retrovorology. 9:67; Denner J. et al. (2013), PLOS ONE. 8:e55199; Schlecht-Louf G et al (2014). J. Virology. 88:992). Its mode of action is not well established but it seems to interfere with calcium influx and PKC (protein Kinase C) function. The second one, termed FP (fusion peptide) is located in the N terminal part of the protein and interacts directly with TCRalpha chain, preventing TCR complex assembly [Cohen T et al (2010), PLOS Pathogens. 6:e1001085; Faingold O et al, (2012), J. Biol. Chem. 287:33503]. Both ISU and FP have been shown to be inmmunosuppressive as whole protein transmembrane protein, truncated transmembrane protein or synthetic peptides.

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.

WO2013/173223A application describes a method for immunotherapy wherein the PD-1-PD-L1 pathway is disrupted by the administration of antibodies against PD-1 and/or PD-L1. This inhibitory immunoregulator is used as biomarker to enable patient selection and guide on-treatment management.

Pegram et al. (2012) have shown that tumor-targeted T cells modified to secrete the interferon IL-12 can eradicate systemic tumors in murine model, without the need of prior conditioning such as irradiation, lymphodepleting chemotherapy and/or additional cytokine support.

Rong et al. (2014) have demonstrated that the expression of both CTLA-4 Ig and PD-L1 are required in human embryonic stem cells (hESCs) to confer immune protection as neither was sufficient on their own. This approach has been used to support allograft of human Embryonic Stem Cells into mice.

In all 3 above prior art (WO2013/173223A, Pegram et al., and Rong Z et al), self-recognition systems, such as TCR, were maintained functional, which limited the persistence of the engrafted cells into the host. However, to be able to use allogeneic CAR T cells as treatment in cancer immunotherapy or other indications, one must mitigate the risk of graft vs. host disease (GvHD) as well as the risk of rejection of therapeutic cells by the patient. Allogenic cells can survive in patients having received lymphodepletion regimen but their therapeutic activity is limited by the duration of the lymphodepletion.

To extend their survival and enhance their therapeutic activity, the inventors describe here a method to prevent the rejection of therapeutic allogeneic T cells, while the patient's immune system may be still active. This method consists in creating a local immune protection by engineering therapeutic cells to ectopically express and/or secrete immunosuppressive polypeptides at or through the cell membrane. They found that a various panel of such polypeptides in particular antagonists of immune checkpoints, or derived from viral envelope or NKG2D ligand could enhance persistence and/or an engraftment of allogeneic immune cells into the host. For a better efficacy, this local immunosuppressive effect is completed by the inactivation of gene involved in the self/non-self recognition, making these engineered immune cell for engraftment, available as an “off the shelf” product.

Summary of the invention

The present invention discloses methods to engineer immune cell, such as T cells, to make them suitable for immunotherapy purposes by increasing their persistence and/or easing their

engraftment in host organism, reducing thereby the risk of graft versus host disease (GvHD). More particularly, the invention relates to a method, wherein at least one endogenous gene encoding a polypeptide involved in the self and non-self antigen recognition is inactivated in one immune cells, followed by contacting said engineered immune cells with at least one non-endogenous immunosuppressive polypeptide.

In one aspect, the inactivation is performed on TCR and/or beta2M gene, preferably by using a specific rare-cutting endonuclease, such as a TALE-nuclease.

In a further aspect, in order to prevent depletion of adoptively transferred allogeneic immune cells by host-versus-graft (HvG)- i.e. host immune cells attacking those allogeneic transferred immune cells- the contacting step is realized by the expression of inactive PD1 and/or CTLA-4 ligand by the immune cell itself. Other alternatives according to the invention provide with the expression of viral MHC homolog, NKG2D ligand, and/or viral *env* immune suppressive domain (ISU) or the viral FP protein.

Also, still within the scope of the present invention, the incubation of engineered immune cells with at least one non-endogenous immunosuppressive polypeptide may be used instead of the expression of immunosuppressive polypeptides. CD80/CD86 antibodies are preferred as immune suppressive polypeptide to be used in said incubation.

The modified immune cells relevant for immunotherapy may further comprise exogenous recombinant polynucleotides encoding Chimeric Antigen Receptors (CAR) for specific cell recognition. The resulting isolated cells or cell lines comprising any of the proteins, polypeptides or vectors described in this specification are dedicated for use as therapeutic products, ideally as “off the shelf” products with reduced graft-versus-host disease (GvHD) risk and extended life span.

Methods for treating or preventing cancer or infections in a patient by administrating such engineered immune cells area also described.

Brief description of the figures and Tables

In addition to the above, the invention further comprises features which will emerge from the description that 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.

Legends of the figures

Figure 1: Schematic representation of the potential interactions between an allogeneic CAR T cell with diverse host immune cells (CD8+ and CD4+ T cell, APC such as dendritic cell and NK cell), the CAR T cell having no additional genetic modification. Sign (+) represents activation and sign (-) inhibition. CAR T cell is activated by encountering targeted tumor cell which displays an antigen cell surface recognized by the scFvs of the CAR. Interaction between allogeneic CAR T cell and host NK cell is inhibited by the recognition of the MHC I by the inhibitor of the NK cell. Activation of the host cytotoxic T cell (CD8+ T cell) takes place by the binding by TCR of the MHC I components of the CAR T cell. Also, the action of host NK cell on allogeneic T CAR cell is inhibited via the MHCI recognition.

Figure 2: Schematic representation of the potential interactions between an allogeneic CAR T cell with diverse host immune cells (CD8+ and CD4+ T cell, APC such as dendritic cell and NK cell); the CAR T cell expressing secreted CTLA-4 Igs. Sign (+) represents activation and sign (-) inhibition. CAR T cell is activated by encountering targeted tumor cell which displays an antigen cell surface recognized by the scFvs of the CAR. The interaction between NK cell and CAR T cell remains unchanged. The secreted CLTA-4 Igs bind to the CD80/CD86 antigen on the surface of APC cell and tumor cell, therefore inactivating the interaction between APC cell (such a dendritic cell here) and the CAR T cell. Thus, the secretion of CLTA-4 Igs creates a local immune protection.

Figure 3: Schematic representation of the potential interactions between an allogeneic CAR T cell with diverse host immune cells (CD8+ and CD4+ T cell, APC such as dendritic cell and NK cell); the CAR T cell expressing membrane-bound PD-L1 and whose PD-1 gene is inactivated by KO. Sign (+) represents activation and sign (-) inhibition. The potential interaction between CAR T cell with the tumor cell and the NK cell remain unchanged. The expression of PD-L1 by the allogeneic CAR T cell makes it insensitive to the host CD8+ T cell due to the binding PD-L1 to the PD-1 receptor of the latter. Thus, the PD-L1 triggers T cells inhibitory pathway in the patient's T cells, and this effect is more pronounced when the PD-1 gene of the allogeneic is inactivated.

Figure 4: Schematic representation of the potential interactions between an allogeneic CAR T cell with diverse host immune cells (CD8+ and CD4+ T cell, APC such as dendritic cell and NK cell); the CAR T cell expressing secreted PD-L1 and whose PD-1 gene is inactivated by KO. Sign (+) represents activation and sign (-) inhibition. The potential interaction between CAR T cell with the tumor cell and with the NK cell remain unchanged. The secreted PD-L1 by the allogeneic CAR T cell can bind to host CD8+ and CD4+ T cell by their PD-1 receptors, inhibiting the PD-L1/PD-1 pathway. Thus, the PD-L1

triggers T cells inhibitory pathway in the patient's T cells, and this effect is more pronounced when the PD-1 gene of the allogeneic is inactivated.

Figure 5: Schematic representation of the potential interactions between an allogeneic CAR T cell with diverse host immune cells (CD8+ and CD4+ T cell, APC such as dendritic cell and NK cell), the CAR T cell expressing a viral *env* immunosuppressive domain (ISU). Sign (+) represents activation and sign (-) inhibition. The potential interaction between CAR T cell with the tumor cell and with the NK cell remain unchanged. The expression of viral ISU appears to inhibit the recognition of the allogeneic CAR T cell by the host T cells and APCs cells maybe by the reduced production of IL-10 interleukin, and thus creating an immunosuppressive effect.

Figure 6: Schematic representation of the potential interactions between an allogeneic CAR T cell with diverse host immune cells (CD8+ and CD4+ T cell, APC such as dendritic cell and NK cell), the CAR T cell having its B2M gene inactivated by KO. Sign (+) represents activation and sign (-) inhibition. The potential interaction between CAR T cell with the tumor cell remains unchanged. The inactivation of B2M gene which is one component of the MCHI, renders the latter non-functional in regards to the interactions with host cytotoxic T cell (CD8+) and with NK cell. Then, NK cell can exert its activation on allogeneic CAR T cell via activator pathway such NKG2D/NKG2D ligand.

Figure 7: Schematic representation of the potential interactions between an allogeneic CAR T cell with diverse host immune cells (CD8+ and CD4+ T cell, APC such as dendritic cell and NK cell), the CAR T cell having its B2M gene inactivated by KO and expressing viral MHCI homolog. Sign (+) represents activation and sign (-) inhibition. The potential interaction between CAR T cell with the tumor cell remains unchanged. As for the preceding figure (only B2M KO), the interaction between CAR T cell and host CD8+ T cell is alleviated. In this case, the expression of viral MHCI homolog renders the interaction with NK cell inoperative via MHCI/inhibitor receptor. The double genetic modification of allogeneic CAR T cells by KO of B2M combined with the expression of viral MHCI homolog strengthens their immunosuppressive protection.

Figure 8: Schematic representation of the potential interactions between an allogeneic CAR T cell with diverse host immune cells (CD8+ and CD4+ T cell, APC such as dendritic cell and NK cell), the CAR T cell having its B2M gene inactivated by KO and expressing a soluble NKG2D ligand. Sign (+) represents activation and sign (-) inhibition. The potential interaction between CAR T cell with the tumor cell remains unchanged. As for the preceding figure (only B2M KO), the interaction between CAR T cell and host CD8+ T cell is alleviated. The expression of soluble NKG2D ligand is another way to inactivate the interaction with NK cell. In this case, the soluble NKG2D ligand can bind to NKG2D

receptor on NK cell but exerts no action, in contrast to the NKG2D ligand of CAR T cell with which it exerts an inhibitory competition. The double genetic modification of allogeneic CAR T cells by KO of B2M combined with the expression of soluble NKG2D ligand strengthens their immunosuppressive protection.

Figure 9: General scheme of allogeneic CAR T cell adoptive immunotherapy illustrating the potential host versus graft reaction (HvG). Here is represented an example with anti-CD19 chimeric antigen receptor (CAR) which is aimed to treat patients suffering from acute lymphoblastic leukemia (ALL). After the steps of purification of allogeneic T cells from a healthy donor, their activation and expansion, the CAR transduction and finally their infusion into an ALL patient, there is a high risk of host immune attack (HvG) against these allogeneic CAR T cells.

Figure 10: Scheme describing the re-expression of PD-L1 to prevent the HvG reaction taking place in the host patient after allogeneic CAR T cells adoptive transfer. **A**. Scheme of HvG reaction prevention via re-expression of PD-L1 at the cell surface of primary T cells. **B**. Scheme of HvG reaction in the absence of PD-L1 expression.

Figure 11: Scheme describing the HvG reaction including the host antigen presenting cells (APC) and host T cells along with the names of the receptors involved in their activation in the presence of Allogeneic T cells. A. Scheme of HvG reaction. B. Scheme of HvG prevention in excretion of CTLA4-Ig by allogeneic T cells.

Figure 12: A, B &C Characterization of the expression of PD-L1 at the surface of T cells or CAR tool T cells by flow cytometry.

Figure 13: Specific cell lysis activity of engineered CAR T cell expressing PD-L1 toward relevant and non-relevant tumor cells (Daudi and K562 cells respectively). **A** and **B** indicate different blood donors.

Figure 14: ELISA detection of CTLA4a Ig and CTLA4b Ig excretion by CAR T cells in the culture media. **A**. Standard curve used to quantify the amount of CTLA4 Ig in the culture media. **B**. Detection of CTLA4 a and b Ig in the culture media supernatant of engineered CAR T cells transfected with 10 or 20 µg of mRNA encoding either CTLA4a Ig or CTLA4b Ig.

Figure 15: Specific Cell lysis activity of Engineered CAR T cell expressing CTLA4a Ig toward relevant and non-relevant tumor cells (Daudi and K562 cells respectively).

Figure 16: FACS analysis of β2-m expression in T cells. Untransfected (top) and transfected T cells (middle and bottom) are analysed by FACS for viability (left) and β2-m expression (right).

Figure 17: Detection of CTLA4Ig in the culture media supernatant of engineered CAR T cells transduced with a control LV (PD-L1), CTLA4Ig LV alone or co transduced with PD-L1 and CTLA4Ig LV at a MOI of 5. Supernatants from 14 day old culture are tested by ELISA at 1/1000e dilution (hatched bar) or 1/5000e dilution (dark bar). The grey bar represents the mean titer from both dilution.

Figure 18: Mixed Lymphocytes reaction for quantification of CFSE negative T cells from donor 1 (grey bars), quantification of CD3+ T cells (black bars) and T cells viability (hatched bars) in response to the indicated stimulations. D1 and D2 correspond to donor 1 and donor 2 respectively. From left to right: (a) PBMCs from donor 1 without any treatment have been cultured alone; (b) PBMCs from donor 1, which have been submitted to a treatment with increasing concentration of PHA (PhytoHemAgglutinin -10µg/ml, a T cell mitogen) are cultured alone; (c) PBMCs from donor 1 are co-cultured with untransduced T cells from donor 2; (d) PBMCs from donor 1 are co-cultured with PD-L1 transduced T cells from donor 2; (e) PBMCs from donor 1 are co-cultured with CTLA4Ig transduced T cells from donor 2; (f) PBMCs from donor 1 are co-cultured with PD-L1 and CTLA4Ig co- transduced T cells from donor 2.

Figure 19: Cytotoxicity assay in which indicated differently engineered CAR T cells (as shown under the graph) are incubated at a E:T ratio of 10:1 with specific target cells (MOLM-13; expressing CD123 antigen) and control negative cells (Daudi) for 4 hours. Target cell death is measured by flow cytometry and is normalized for non-specific killing (Daudi).

Figure 20. Cytotoxicity assay in which Indicated differently engineered CAR T cells (as shown under the graph) are incubated at E:T ratio of 10:1 (hatched bar), 5:1 (dark bars) and 1:1 (grey bars) with specific target cells (MOLM-13) and control negative cells (Daudi) for 4 hours. Target cell death is measured by flow cytometry and is normalized for non-specific killing (Daudi).

Figure 21. Outline scheme for in vivo experiment. NOG mice are first injected with MOLM-13 (luc/GFP) tumor cell line 7 days before engineered CAR T cells injection. Tumor progression is monitored via bioluminescence (Biolum.) analysis and overall survival.

Figure 22. Engineered T cells are monitored for the CAR CD123 and PD-L1 cell surface expression by flow cytometry.

Figure 23. Bioluminescence imaging from D-1 to D14 showing the tumors in the NOG mice. The dark spots in the photos represent the tumors. The different groups of T cells injected into the mice are presented as follows. In Figure 23A : untransfected T cells (no CAR T cells) or T cells transfected by anti-CD123 CAR (CAR T CD123). In Figure 23B : T cells transfected by anti-CD123 CAR and transduced with CTLA4Ig (CAR T CD123/CTLA4Ig); T cells transfected by anti-CD123 CAR and transduced with PD-L1 (CAR T CD123/PDL1); T cells transfected by anti-CD123 CAR and transduced with CTLA4Ig and with PD-L1(CAR T CD123/PDL1/CTLA4Ig).

Figure 24: Schematic representation of the different single chain chimeric antigen receptor CAR Architecture (V1 to V6) with the components: VH and VL chains specific to antigen, hinge, transmembrane domain (TM), co-stimulatory domain (4-1BB) and signaling transduction domain (CD3zeta), optionally with linker(s).

Table 1: Description of the β2m TALE-nucleases sequences

Table 2: Polynucleotide sequence of 2 pairs of TALENs are presented and for 2 different PDC1 gene targets

Table 3: Polynucleotide sequences of plasmidic constructs expressing CLTA-4a, CTLA-4b and PD-L1.

Table 4: Polypeptide sequences of ISU domain variants from diverse virus.

Table 5: Polypeptide sequences of a viral MHC homolog (UL18) and a panel of NKG2D ligands.

Table 6: Aminoacid sequences of FP polypeptide from natural and artificial origins.

Table 7: Description of the TRAC and TRBC TALE-nucleases and sequences of the TALE-nucleases target sites in the human corresponding genes.

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).

In a general aspect, the present invention relates to methods for new adoptive immunotherapy strategies in treating cancer and infections.

The present invention relates to the following main embodiments:

1) Method to increase the persistence and/or the engraftment of allogeneic immune cells, comprising:

a) providing allogeneic cells;

b) modifying said cells by inactivating at least one endogenous gene encoding a polypeptide involved in the self and non-self antigen recognition;

and;

c) contacting said immune cells with at least one non-endogenous immunosuppressive polypeptide.

2) The method of embodiment 1, wherein said immune cells are hematopoietic cells.

3) The method of embodiment 1 or embodiment 2, wherein said immune cells are primary cells.

4) The method according to any one of embodiment 1 to 3, wherein said expression or contact in step c) does not specifically inhibit T regulatory cells.

5) The method of any one of embodiment 1 to 4, wherein said expression or contact in step c) does specifically inhibit CD8+ T cells.

6) The method according to embodiment 1 to embodiment 5, wherein the step c) is performed by the expression in said immune cells at least one non-endogenous polynucleotide directing the secretion of at least one non-endogenous immunosuppressive polypeptide.

7) The method according to embodiment 1 to embodiment 6, wherein the step c) contains additionally an inactivation of the expression of a gene encoding PD-1.

8) The method according to embodiment 7, wherein inactivation of PD-1 gene is performed by using a polynucleotide encoding TALE-nucleases of SEQ ID N°11-12 and 13-14.

9) The method according to any one of embodiment 1 to 8, wherein said polypeptide in step b) is chosen amongst TCR, MHC class of class I component, b-2 microglobulin (B2M), TAP1 and large multifunctional protease 2.

10) The method according to any one of embodiment 1 to 9, wherein said polypeptide in step c) is chosen amongst PDL-1, CTLA-4, viral MHC homolog, NKG2D ligand, viral *env* immune suppressive domain (ISU) or the viral FP protein.

11) The method according to any one of embodiment 1 to 10, wherein step b) is performed by the inactivation of the B2M and step c) is performed by the expression of inactive PDL-1 ligand in said allogeneic immune cells.

12) The method according to any one of embodiment 1 to 11, wherein the additional modification in step c) is performed by the expression of CTLA-4 immunoglobulins in said allogeneic immune cells.

13) The method according to embodiment 12, wherein the nucleic acid molecule encoding CTLA-4 immunoglobulins to be expressed shares at least 80%, preferably 90% and more preferably 95% of identity with SEQ ID NO: 16-17.

14) The method according to any one of embodiment 1 to 13, wherein step b) is performed by the inactivation of the TCR and step c) is performed by the expression of inactive PDL-1 ligand by said allogeneic immune cells.

15) The method according to anyone of embodiment 1 to 10, wherein step c) is performed by expressing viral *env* immune suppressive domain (ISU) chosen from FeLV, MLV, HERV or the viral FP protein.

16) The method of anyone of embodiment 1 to 10, wherein step b) is performed by the inactivation of the B2M and step c) is performed by the expression of viral *env* immune suppressive domain (ISU) by said allogeneic immune cells.

17) The method of anyone of embodiment 1 to 10, wherein step b) is performed by the inactivation of the TCR and step c) is performed by the expression of viral *env* immune suppressive domain (ISU) by said allogeneic immune cells.

18) The method according to embodiment 16 or 17, wherein the nucleic acid molecule encoding viral *env* immune suppressive domain (ISU) to be expressed shares at least 80%, preferably 90% and more preferably 95% of identity with SEQ ID NO: 19-38.

19) The method of anyone of embodiment 1 to 10, wherein step b) is performed by the inactivation of the B2M and step c) is performed by the expression of viral FP protein by said allogeneic immune cells.

20) The method of anyone of embodiment 1 to 10, wherein step b) is performed by the inactivation of the TCR and step c) is performed by the expression of viral FP protein by said allogeneic immune cells.

21) The method according to embodiment 19 or 20, wherein the nucleic acid molecule encoding viral FP protein to be expressed shares at least 80%, preferably 90% and more preferably 95% of identity with SEQ ID NO: 48-50.

22) The method of anyone of embodiment 1 to 10, wherein step b) is performed by the inactivation of the B2M and step c) is performed by the expression of NKG2G ligand by said allogeneic immune cells.

23) The method of anyone of embodiment 1 to 10, wherein step b) is performed by the inactivation of the TCR and step c) is performed by the expression of NKG2G ligand by said allogeneic immune cells.

24) The method according to embodiment 22 or 23, wherein the nucleic acid molecule encoding NKG2G ligand to be expressed shares at least 80%, preferably 90% and more preferably 95% of identity with SEQ ID NO: 40-47.

25) The method according to anyone of embodiment 1 to 10, wherein the viral MHC homolog in step b) ii) is UL18.

26) The method of anyone of embodiment 1 to 10, wherein step b) is performed by the inactivation of the B2M and step c) is performed by the expression of viral MHC homolog UL18 protein by said allogeneic immune cells.

27) The method of anyone of embodiment 1 to 10, wherein step b) is performed by the inactivation of the TCR and step c) is performed by the expression of viral MHC homolog UL18 protein by said allogeneic immune cells.

28) The method according to embodiment 26 or 27, wherein the nucleic acid molecule encoding viral MHC homolog UL18 to be expressed shares at least 80%, preferably 90% and more preferably 95% of identity with SEQ ID NO: 39.

29) The method according to any one of embodiment 1 to 5 or embodiments 8-9, wherein the step c) is performed by the incubation of said immune in at least one non-endogenous immunosuppressive polypeptide.

30) The method according to embodiment 29, wherein said non-endogenous immunosuppressive polypeptide is anti-CD80 or anti-CD86 mAbs.

31) The method according to anyone of embodiment 1 or 30, wherein gene inactivation in step b) is performed by using a TAL-nuclease, meganuclease, zing-finger nuclease (ZFN), or RNA guided endonuclease.

32) The method according to anyone of embodiment 1 or 31, wherein gene inactivation in step b) is performed using a TAL-nuclease.

33) The method according to any one of embodiment 1 to 31, wherein gene inactivation in step b) is performed by using a RNA-guided endonucleases.

34) The method according to embodiment 33, wherein the RNA-guided endonuclease is Cas9.

35) The method according to any one of embodiment 1-10, 11-15, 17-18, 20-21, 23-25 or 27-34 wherein gene inactivation in step b) is performed by using a nucleic acid molecule that inhibits the expression of a gene encoding TCR.

36) The method according to embodiment 32, wherein inactivation of TCR gene is performed by using the TALE-nucleases of SEQ ID N°52-53, 55-56, 62-63 and 65-66.

37) The method according to any one of embodiments 1-12, 13-16, 18-19, 21-22, 24-26 or 28-34 wherein inactivation gene in step b) is performed by using a nucleic acid molecule that inhibits the expression of a gene encoding B2M.

38) The method according to embodiment 32, wherein inactivation of B2M gene is performed by using the TALE-nucleases of SEQ ID N°2-3, 5-6 and 8-9.

39) The method according to any one of embodiments 1 to 38, further comprising the step of:

d) introducing into said T-cell an exogenous nucleic acid molecule comprising a nucleotide sequence coding for a Chimeric Antigen Receptor (CAR) directed against at least one antigen expressed at the surface of a malignant or infected cell.

40) The method according to embodiment 39 wherein said Chimeric Antigen Receptor comprises scFv (VH and VL chains) having as antigenic target sequence of over 80% identity, preferably over 90%, and more preferably over 95% with SEQ ID NO 67 (CD19 antigen), SEQ ID NO 68 (CD38 antigen), SEQ ID NO 69 (CD123 antigen), SEQ ID NO 70 (CS1 antigen), SEQ ID NO 71 (BCMA antigen), SEQ ID NO 72 (FLT-3 antigen), SEQ ID NO 73 (CD33 antigen), SEQ ID NO 74 (CD70 antigen), SEQ ID NO 75 (EGFR-3v antigen) and SEQ ID NO 76 (WT1 antigen).

41) The method according to any one of embodiments 1 to 40, further comprising the step of:

d) expanding the resulting engineered T-cell.

42) An engineered, preferably isolated, T-cell, obtainable by using the method according to anyone of embodiment 1 to 41.

43) The engineered T-cell according to embodiment 42 for use as a medicament.

44) The engineered T-cell according to embodiment 42 or embodiment 43 for use in the treatment of a cancer or viral infection.

45) The engineered T-cell according to any one of embodiments 42 to 44 for use in the treatment of lymphoma.

46) The engineered T-cell according to any one of embodiments 42 to 45, wherein said T-cell originates from a patient to be treated.

47) The engineered T-cell according to any one of embodiments 42 to 45, wherein said T-cell originates from a donor.

48) A composition comprising at least one engineered T-cell according to any one of embodiments 42 to 47.

The present invention relates more particularly to the following embodiments:

1) Method to increase the persistence and/or the engraftment of allogeneic immune cells in presence of host immune cells, comprising:

a) providing allogeneic cells;

b) modifying said cells by inactivating at least one endogenous gene encoding a polypeptide involved in the response against self and non-self antigen recognition;

and;

c) contacting said host immune cells with at least one non-endogenous immunosuppressive polypeptide which has the effect to prevent them from interacting with allogeneic immune cells.

2) The method according to embodiment 1, wherein said polypeptide in step b) is chosen amongst TCR, MHC class of class I component, b-2 microglobulin (B2M), TAP1 and large multifunctional protease 2.

3) Method according to embodiment 1 or 2, wherein said immunosuppressive polypeptide in step c) is present under a membrane-bound form and/or under a secreted form.

4) Method of anyone of embodiment 1 to 3, wherein the step c) is performed by the expression in said immune cells at least one non-endogenous polynucleotide encoding for one non-endogenous immunosuppressive polypeptide bound to the membrane surface of said immune cells.

5) Method of anyone of embodiment 1 to 4, wherein said one non-endogenous immunosuppressive polypeptide bound to the membrane surface of said immune cells is a PD-L1 ligand.

6) The method according to embodiment 5, wherein the nucleic acid molecule encoding PD-L1 ligand under a membrane-bound form to be expressed shares at least 80%, preferably 90% and more preferably 95% of identity with SEQ ID NO:18.

7) Method of any one of embodiment 1 to 3, wherein said immunosuppressive polypeptide is present under a secreted form.

8) The method according to any one of embodiment 1-3 or embodiment 7, wherein the step c) is performed by the expression in said immune cells at least one non-endogenous polynucleotide encoding for one non-endogenous immunosuppressive polypeptide under a secreted form in said immune cells.

9) The method according to embodiment 8, wherein the nucleic acid molecule encoding CTLA-4 immunoglobulins to be expressed shares at least 80%, preferably 90% and more preferably 95% of identity with SEQ ID NO: 16-17.

10) The method according to any one of embodiment 1-8, wherein step c) is performed by contacting said host immune cells with both non-endogenous immunosuppressive polypeptide PD-L1 ligand and CTLA-4 immunoglobulins.

11) The method according to embodiment 10, wherein step c) is performed by the step c) is performed by the expression in said immune cells of both non-endogenous immunosuppressive polypeptide PD-L1 ligand and CTLA-4 immunoglobulins.

12) The method according to embodiment 11, wherein said secretion of at least one non-endogenous immunosuppressive polypeptide is PD-L1 ligand under a secreted form.

13) The method according to anyone of embodiment 9 to 12, wherein the nucleic acid molecules encoding PD-L1 ligand under a membrane-bound form and CTLA-4 immunoglobulins to be expressed in said allogeneic immune cells shares at least 80%, preferably 90% and more preferably 95% of identity with respectively SEQ ID NO:18 and SEQ ID NO: 16-17.

14) The method according to anyone of embodiment 1 to 13, wherein said immune cells are primary cells.

15) The method according to any one of embodiment 1 to 3, wherein said expression or contact in step c) does not specifically inhibit T regulatory cells.

16) The method according to any one of embodiment 1 to 15, wherein said expression or contact in step c) does specifically inhibit host CD8+ T cells.

17) The method according to anyone of embodiment 1 to 16, wherein the step c) contains additionally an inactivation of the expression of a PD-1 gene.

18) The method according to any one of embodiment 1 to 3, wherein the step c) is performed by the expression in said immune cells at least one non-endogenous polynucleotide encoding for PD-L1 ligand under a membrane-bound form, and a further modification of said allogeneic cells is performed by an inactivation of the expression of PD-1 gene.

19) The method according to any one of embodiment 1 to 18, wherein the step c) is performed by the expression in said immune cells at least one non-endogenous polynucleotide directing the secretion of CTLA4 Ig, and a further modification of said allogeneic cells is performed by an inactivation of the expression of PD-1 gene.

20) The method according to any one of embodiment 1 to 19, wherein step c) is performed by the step c) is performed by the expression in said immune cells of both non-endogenous immunosuppressive polypeptide PD-L1 ligand and CTLA-4 immunoglobulins, and a further modification of said allogeneic immune cells is performed by an inactivation of the expression of PD-1 gene.

21) The method according to embodiment 20, wherein inactivation of PD-1 gene is performed by using a polynucleotide encoding TALE-nucleases of SEQ ID N°11-12 and 13-14.

22) The method according to any one of embodiment 1 to 16, wherein said polypeptide in step c) is chosen amongst PD-L1, CTLA-4, viral MHC homolog, NKG2D ligand, viral *env* immune suppressive domain (ISU) or the viral FP protein.

23) The method according to any one of embodiment 1 to 4, wherein step b) is performed by the inactivation of the B2M and step c) is performed by the expression of PD-L1 ligand in said allogeneic immune cells.

24) The method according to any one of embodiment 1 to 4, wherein step b) is performed by the inactivation of the TCR and step c) is performed by the expression of PD-L1 ligand by said allogeneic immune cells.

25) The method according to anyone of embodiment 1 to 4, wherein step c) is performed by expressing viral *env* immune suppressive domain (ISU) chosen from FeLV, MLV, HERV or the viral FP protein.

26) The method according to anyone of embodiment 1 to 4 or embodiment 25, wherein step b) is performed by the inactivation of the B2M and step c) is performed by the expression of viral *env* immune suppressive domain (ISU) by said allogeneic immune cells.

27) The method according to anyone of embodiment 1 to 4, wherein step b) is performed by the inactivation of the TCR and step c) is performed by the expression of viral *env* immune suppressive domain (ISU) by said allogeneic immune cells.

28) The method according to embodiment 26 or 27, wherein the nucleic acid molecule encoding viral *env* immune suppressive domain (ISU) to be expressed shares at least 80%, preferably 90% and more preferably 95% of identity with SEQ ID NO: 19-38.

29) The method according to anyone of embodiment 1 to 4, wherein step b) is performed by the inactivation of the B2M and step c) is performed by the expression of viral FP protein by said allogeneic immune cells.

30) The method according to anyone of embodiment 1 to 4 or embodiment 29, wherein step b) is performed by the inactivation of the TCR and step c) is performed by the expression of viral FP protein by said allogeneic immune cells.

31) The method according to embodiment 29 or 30, wherein the nucleic acid molecule encoding viral FP protein to be expressed shares at least 80%, preferably 90% and more preferably 95% of identity with SEQ ID NO: 48-50.

32) The method according to anyone of embodiment 1 to 4, wherein step b) is performed by the inactivation of the B2M and step c) is performed by the expression of NKG2G ligand by said allogeneic immune cells.

33) The method according to anyone of embodiment 1 to 4 or embodiment 32, wherein step b) is performed by the inactivation of the TCR and step c) is performed by the expression of NKG2G ligand by said allogeneic immune cells.

34) The method according to embodiment 32 or 33, wherein the nucleic acid molecule encoding NKG2G ligand to be expressed shares at least 80%, preferably 90% and more preferably 95% of identity with SEQ ID NO: 40-47.

35) The method according to anyone of embodiment 1 to 4, wherein the viral MHC homolog in step b) ii) is UL18.

36) The method according to anyone of embodiment 1 to 4 or embodiment 35, wherein step b) is performed by the inactivation of the B2M and step c) is performed by the expression of viral MHC homolog UL18 protein by said allogeneic immune cells.

37) The method according to anyone of embodiment 1 to 4 or embodiment 36, wherein step b) is performed by the inactivation of the TCR and step c) is performed by the expression of viral MHC homolog UL18 protein by said allogeneic immune cells.

38) The method according to embodiment 36 or 37, wherein the nucleic acid molecule encoding viral MHC homolog UL18 to be expressed shares at least 80%, preferably 90% and more preferably 95% of identity with SEQ ID NO: 39.

39) The method according to any one of embodiment 1 to 38, wherein the step c) is performed by the incubation of said immune in at least one non-endogenous immunosuppressive polypeptide.

40) The method according to embodiment 39, wherein said non-endogenous immunosuppressive polypeptide is anti-CD80 or anti-CD86 mAbs.

41) The method according to anyone of embodiment 1 to 40, wherein gene inactivation in step b) is performed by using a TAL-nuclease, meganuclease, zing-finger nuclease (ZFN), or RNA guided endonuclease.

42) The method according to embodiment 41, wherein gene inactivation in step b) is performed using a TAL-nuclease.

43) The method according to embodiment 41, wherein gene inactivation in step b) is performed by using a RNA-guided endonucleases.

44) The method according to embodiment 43 wherein the RNA-guided endonuclease is Cas9.

45) The method according to any one of embodiments 1-18, 20-22, 24-25, 27-28, 30-32 or 34-41 wherein gene inactivation in step b) is performed by using a nucleic acid molecule that inhibits the expression of a gene encoding TCR.

46) The method according to embodiment 45, wherein inactivation of TCR gene is performed by using the TALE-nucleases of SEQ ID N°52-53, 55-56, 62-63 and 65-66.

47) The method according to any one of embodiments 1-17, 20-23, 25-26, 28-29, 31-33 or 35-41 wherein inactivation gene in step b) is performed by using a nucleic acid molecule that inhibits the expression of a gene encoding B2M.

48) The method according to embodiment 47, wherein inactivation of B2M gene is performed by using the TALE-nucleases of SEQ ID N°2-3, 5-6 and 8-9.

49) The method according to any one of embodiments 1 to 48, further comprising the step of:

d) introducing into said T-cell an exogenous nucleic acid molecule comprising a nucleotide sequence coding for a Chimeric Antigen Receptor (CAR) directed against at least one antigen expressed at the surface of a malignant or infected cell.

50) The method according to embodiment 49 wherein said Chimeric Antigen Receptor comprises scFv (VH and VL chains) having as antigenic target sequence of over 80% identity, preferably over 90%, and more preferably over 95% with SEQ ID NO 67 (CD19 antigen), SEQ ID NO 68 (CD38 antigen), SEQ ID NO 69 (CD123 antigen), SEQ ID NO 70 (CS1 antigen), SEQ ID NO 71 (BCMA antigen), SEQ ID NO 72 (FLT-3 antigen), SEQ ID NO 73 (CD33 antigen), SEQ ID NO 74 (CD70 antigen), SEQ ID NO 75 (EGFR-3v antigen) and SEQ ID NO 76 (WT1 antigen)

51) The method according to anyone of embodiment 1 to 50, wherein step c) is performed by the step c) is performed by the expression in said allogeneic immune cells of non-endogenous immunosuppressive polypeptide PD-L1 ligand and / or CTLA-4 immunoglobulins, said allogeneic immune cells being further modified by the expression of an anti-CD123 Chimeric Antigen Receptor.

52) The method according to embodiment 51, wherein a further modification of said allogeneic immune cells is performed by an inactivation of the expression of the PD-1 gene.

53) The method according to any one of embodiments 1 to 52, further comprising the step of:

d) expanding the resulting engineered T-cell.

54) An engineered, preferably isolated, T-cell, obtainable by using the method according to anyone of embodiment 1 to 53.

55) The engineered T-cell according to embodiment 54 for use as a medicament.

56) The engineered T-cell according to embodiment 54 or embodiment 55 for use in the treatment of a cancer or viral infection.

57) The engineered T-cell according to any one of embodiments 54 to 56 for use in the treatment of lymphoma or leukemia.

58) The engineered T-cell according to any one of embodiments 54 to 57, wherein said T-cell originates from a patient to be treated.

59) The engineered T-cell according to any one of embodiments 54 to 57, wherein said T-cell originates from a donor.

60) A composition comprising at least one engineered T-cell according to any one of embodiments 54 to 59.

More details about the above aspects of the invention are provided in the description below.

Non alloreactive and highly persistent T cells for immunotherapy

According to a first aspect of the present invention, the inventors have shown that some genes, when they are expressed in allogeneic immune cells, could allow an increase of their persistence in the host organism for a better efficacy.

The present invention relates to a method to increase the persistence and/or the engraftment of allogeneic immune cells, preferably in presence of host immune cells, comprising:

i) providing allogeneic cells;

ii) modifying said cells by inactivating at least one endogenous gene encoding a polypeptide involved in the response against self and non-self antigen recognition;

and;

iii) contacting said host immune cells with at least one non-endogenous immunosuppressive polypeptide.

Said non-endogenous immunosuppressive polypeptide is expected to have the effect of preventing host immune cells from interacting with allogeneic immune cells.

“Persistence” refers to the ability of cells to resist rejection and remain and/or increase in number over time (e.g., days, weeks, months, years) in vivo. In general, the engineered immune cells

of the present invention can be found in patient's blood at least 10 days, preferably at least 20 days, more preferably at least 25 days and even more preferably at least 30 days after infusion into said patient.

"Engraftment" refers to the process of cellular contact and incorporation into an existing site of interest *in vivo*.

By "increased persistence and/or engraftment", is meant that the number of allogeneic immune cells, engineered to render them persistent, remains higher during the course of the treatment, compared to the case where non-engineered ones (i.e non persistent) are administered to the patient. Such improved persistence and/or engraftment in allogeneic immune cells (e.g.. T cells) to be injected to a patient are part of the immunological tolerance (or "tolerisation") which describes a state of unresponsiveness of the host immune system with respect to said immune cells, whereas said immune cells retain the capacity to elicit an immune response.

Inactivation of gene involved in the self and non-self antigen recognition

By *"self and non-self antigen recognition"*, it is intended the screening performed by the cellular immune system whereby peptides are presented by host cells on Major Histocompatibility Complex (MHC) molecules to assess if cells are infected by foreign organisms. This screening involves other transmembrane structures such as for instance TCR, or TAP1/TAP2 or protease 2.

By inactivating a gene it is intended that the gene of interest is not expressing 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.

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.

According to a preferred embodiment, the gene inactivation is preferably performed by using a TAL-nuclease, meganuclease, zing-finger nuclease (ZFN), or RNA/DNA guided endonuclease, such as Cas9, Cpf1 or Argonaute.

According to a more preferred embodiment, the inactivation of said gene involved in the self and non-self antigen recognition is performed by using TALE-nucleases. This can be accomplished 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 previously cited. 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. 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).

According to another preferred embodiment, the inactivation of said gene involved in the self and non-self antigen recognition is performed by RNA-guided endonuclease such as Cas9 or DNA-guided endonuclease, such as Argonaute based techniques as described in WO2014189628.

The present invention relates to a method to increase the persistence and/or the engraftment of allogeneic cells which comprises a step of inactivation of at least one gene involved in the self/non-self recognition. By "*gene involved in self/non-self recognition*" is meant a gene encoding a polypeptide that is structurally part of an external receptor or ligand, which is deemed necessary for the detection and destruction of allogeneic cells by the immune system. Such genes preferably code for at least one component of TCR, MHC, in particular class I MHC, beta-2 microglobulin (B2M), TAP1 or large multifunctional protease 2.

In a preferred embodiment, the gene to be inactivated is TCR or B2M, more preferably TCR.

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: 2-3, 5-6 and 8-9 for inactivation of β2m and SEQ ID N°52-53, 55-56, 62-63 and 65-66. (TCR).

TALE-nucleases cleaving human β2m

mRNA encoding the TALE-nucleases targeting exons of the human β2m gene were ordered from Cellectis Bioresearch (8, rue de la Croix Jarry, 75013 PARIS). Table 1 below indicates the target sequences cleaved by each of the two independent entities (called half TALE-nucleases) each containing a repeat sequence engineered to bind and cleave between target sequences consisting of two 17-bp long sequences (called half targets) separated by a 15-bp spacer.

Target name	SEQ ID NO :	Half TALE-nuclease sequence
T01 Beta2M target	**1**	TCTCGCTCCGTGGCCTTAGCTGTGCTCGCGCTACTCTCTCTTTCTGGCCTGGAGGCTA
T01 TALEN Beta2M	**2**	ATGGGCGATCCTAAAAAGAAACGTAAGGTCATCGATTACCCATACGATGTTCCAGATTACGCTATCGATATCGCCGATCTACGCACGCTCGGCTACAGCCAGCAGCAACAGGAGAAGATCAAACCGAAGGTTCGTTCGACAGTGGCGCAGCACCACGAGGCACTGGT

LEFT		CGGCCACGGGTTTACACACGCGCACATCGTTGCGTTAAGCCAACACCCGGCAGCGT TAGGGACCGTCGCTGTCAAGTATCAGGACATGATCGCAGCGTTGCCAGAGGCGAC ACACGAAGCGATCGTTGGCGTCGGCAAACAGTGGTCCGGCGCACGCGCTCTGGAG GCCTTGCTCACGGTGGCGGGAGAGTTGAGAGGTCCACCGTTACAGTTGGACACAG GCCAACTTCTCAAGATTGCAAAACGTGGCGGCGTGACCGCAGTGGAGGCAGTGCA TGCATGGCGCAATGCACTGACGGGTGCCCCGCTCAACTTGACCCCCCAGCAGGTGG TGGCCATCGCCAGCAATAATGGTGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCT GTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCCCAGCAGGTGGTGGCCATC GCCAGCAATAATGGTGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCG GTGCTGTGCCAGGCCCACGGCTTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCC ACGATGGCGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGT GCCAGGCCCACGGCTTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCCACGATGG CGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCC CACGGCTTGACCCCCCAGCAGGTGGTGGCCATCGCCAGCAATGGCGGTGGCAAGC AGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTT GACCCCCCAGCAGGTGGTGGCCATCGCCAGCAATGGCGGTGGCAAGCAGGCGCTG GAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCGG AGCAGGTGGTGGCCATCGCCAGCAATATTGGTGGCAAGCAGGCGCTGGAGACGG TGCAGGCGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCCCAGCAGGT GGTGGCCATCGCCAGCAATAATGGTGGCAAGCAGGCGCTGGAGACGGTCCAGCG GCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCGGAGCAGGTGGTGGCC ATCGCCAGCCACGATGGCGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGC CGGTGCTGTGCCAGGCCCACGGCTTGACCCCCCAGCAGGTGGTGGCCATCGCCAG CAATGGCGGTGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCT GTGCCAGGCCCACGGCTTGACCCCCCAGCAGGTGGTGGCCATCGCCAGCAATAAT GGTGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAG GCCCACGGCTTGACCCCCCAGCAGGTGGTGGCCATCGCCAGCAATGGCGGTGGCA AGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGG CTTGACCCCCCAGCAGGTGGTGGCCATCGCCAGCAATAATGGTGGCAAGCAGGCG CTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCC CGGAGCAGGTGGTGGCCATCGCCAGCCACGATGGCGGCAAGCAGGCGCTGGAGA CGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCCCAGCAG GTGGTGGCCATCGCCAGCAATGGCGGTGGCAAGCAGGCGCTGGAGACGGTCCAG CGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCTCAGCAGGTGGTGG CCATCGCCAGCAATGGCGGCGGCAGGCCGGCGCTGGAGAGCATTGTTGCCCAGTT ATCTCGCCCTGATCCGGCGTTGGCCGCGTTGACCAACGACCACCTCGTCGCCTTGG CCTGCCTCGGCGGGCGTCCTGCGCTGGATGCAGTGAAAAAGGGATTGGGGGATCC TATCAGCCGTTCCCAGCTGGTGAAGTCCGAGCTGGAGGAGAAGAAATCCGAGTTG AGGCACAAGCTGAAGTACGTGCCCCACGAGTACATCGAGCTGATCGAGATCGCCC GGAACAGCACCCAGGACCGTATCCTGGAGATGAAGGTGATGGAGTTCTTCATGAA GGTGTACGGCTACAGGGGCAAGCACCTGGGCGGCTCCAGGAAGCCCGACGGCGC CATCTACACCGTGGGCTCCCCCATCGACTACGGCGTGATCGTGGACACCAAGGCCT ACTCCGGCGGCTACAACCTGCCCATCGGCCAGGCCGACGAAATGCAGAGGTACGT GGAGGAGAACCAGACCAGGAACAAGCACATCAACCCCAACGAGTGGTGGAAGGT GTACCCCTCCAGCGTGACCGAGTTCAAGTTCCTGTTCGTGTCCGGCCACTTCAAGG GCAACTACAAGGCCCAGCTGACCAGGCTGAACCACATCACCAACTGCAACGGCGCC GTGCTGTCCGTGGAGGAGCTCCTGATCGGCGGCGAGATGATCAAGGCCGGCACCC TGACCCTGGAGGAGGTGAGGAGGAAGTTCAACAACGGCGAGATCAACTTCGCGG

		CCGACTGATAA
T01 TALEN Beta2M RIGHT	3	ATGGGCGATCCTAAAAAGAAACGTAAGGTCATCGATAAGGAGACCGCCGCTGCCA AGTTCGAGAGACAGCACATGGACAGCATCGATATCGCCGATCTACGCACGCTCGG CTACAGCCAGCAGCAACAGGAGAAGATCAAACCGAAGGTTCGTTCGACAGTGGCG CAGCACCACGAGGCACTGGTCGGCCACGGGTTTACACACGCGCACATCGTTGCGTT AAGCCAACACCCGGCAGCGTTAGGGACCGTCGCTGTCAAGTATCAGGACATGATC GCAGCGTTGCCAGAGGCGACACACGAAGCGATCGTTGGCGTCGGCAAACAGTGGT CCGGCGCACGCGCTCTGGAGGCCTTGCTCACGGTGGCGGGAGAGTTGAGAGGTCC ACCGTTACAGTTGGACACAGGCCAACTTCTCAAGATTGCAAAACGTGGCGGCGTGA CCGCAGTGGAGGCAGTGCATGCATGGCGCAATGCACTGACGGGTGCCCCGCTCAA CTTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCAATATTGGTGGCAAGCAGGCG CTGGAGACGGTGCAGGCGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCC CCCAGCAGGTGGTGGCCATCGCCAGCAATAATGGTGGCAAGCAGGCGCTGGAGAC GGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCGGAGCAG GTGGTGGCCATCGCCAGCCACGATGGCGGCAAGCAGGCGCTGGAGACGGTCCAG CGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCGGAGCAGGTGGTGG CCATCGCCAGCCACGATGGCGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTT GCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCCCAGCAGGTGGTGGCCATCGCC AGCAATGGCGGTGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTG CTGTGCCAGGCCCACGGCTTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCCACG ATGGCGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCA GGCCCACGGCTTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCCACGATGGCGGC AAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACG GCTTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCAATATTGGTGGCAAGCAGGC GCTGGAGACGGTGCAGGCGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACC CCCCAGCAGGTGGTGGCCATCGCCAGCAATAATGGTGGCAAGCAGGCGCTGGAGA CGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCCCAGCAG GTGGTGGCCATCGCCAGCAATAATGGTGGCAAGCAGGCGCTGGAGACGGTCCAGC GGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCGGAGCAGGTGGTGGC CATCGCCAGCCACGATGGCGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTG CCGGTGCTGTGCCAGGCCCACGGCTTGACCCCGGAGCAGGTGGTGGCCATCGCCA GCCACGATGGCGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCT GTGCCAGGCCCACGGCTTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCAATATT GGTGGCAAGCAGGCGCTGGAGACGGTGCAGGCGCTGTTGCCGGTGCTGTGCCAG GCCCACGGCTTGACCCCCCAGCAGGTGGTGGCCATCGCCAGCAATAATGGTGGCA AGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGG CTTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCAATATTGGTGGCAAGCAGGCG CTGGAGACGGTGCAGGCGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCC CTCAGCAGGTGGTGGCCATCGCCAGCAATGGCGGCGGCAGGCCGGCGCTGGAGA GCATTGTTGCCCAGTTATCTCGCCCTGATCCGGCGTTGGCCGCGTTGACCAACGAC CACCTCGTCGCCTTGGCCTGCCTCGGCGGGCGTCCTGCGCTGGATGCAGTGAAAAA GGGATTGGGGGATCCTATCAGCCGTTCCCAGCTGGTGAAGTCCGAGCTGGAGGAG AAGAAATCCGAGTTGAGGCACAAGCTGAAGTACGTGCCCCACGAGTACATCGAGC TGATCGAGATCGCCCGGAACAGCACCCAGGACCGTATCCTGGAGATGAAGGTGAT GGAGTTCTTCATGAAGGTGTACGGCTACAGGGGCAAGCACCTGGGCGGCTCCAGG AAGCCCGACGGCGCCATCTACACCGTGGGCTCCCCCATCGACTACGGCGTGATCGT GGACACCAAGGCCTACTCCGGCGGCTACAACCTGCCCATCGGCCAGGCCGACGAA

		ATGCAGAGGTACGTGGAGGAGAACCAGACCAGGAACAAGCACATCAACCCCAACG AGTGGTGGAAGGTGTACCCCTCCAGCGTGACCGAGTTCAAGTTCCTGTTCGTGTCC GGCCACTTCAAGGGCAACTACAAGGCCCAGCTGACCAGGCTGAACCACATCACCAA CTGCAACGGCGCCGTGCTGTCCGTGGAGGAGCTCCTGATCGGCGGCGAGATGATC AAGGCCGGCACCCTGACCCTGGAGGAGGTGAGGAGGAAGTTCAACAACGGCGAG ATCAACTTCGCGGCCGACTGATAA
T02 Beta2M target	**4**	TCCAAAGATTCAGGTTTACTCACGTCATCCAGCAGAGAATGGAAAGTCAA
T02 TALEN Beta2M LEFT	**5**	ATGGGCGATCCTAAAAAGAAACGTAAGGTCATCGATTACCCATACGATGTTCCAGA TTACGCTATCGATATCGCCGATCTACGCACGCTCGGCTACAGCCAGCAGCAACAGG AGAAGATCAAACCGAAGGTTCGTTCGACAGTGGCGCAGCACCACGAGGCACTGGT CGGCCACGGGTTTACACACGCGCACATCGTTGCGTTAAGCCAACACCCGGCAGCGT TAGGGACCGTCGCTGTCAAGTATCAGGACATGATCGCAGCGTTGCCAGAGGCGAC ACACGAAGCGATCGTTGGCGTCGGCAAACAGTGGTCCGGCGCACGCGCTCTGGAG GCCTTGCTCACGGTGGCGGGAGAGTTGAGAGGTCCACCGTTACAGTTGGACACAG GCCAACTTCTCAAGATTGCAAAACGTGGCGGCGTGACCGCAGTGGAGGCAGTGCA TGCATGGCGCAATGCACTGACGGGTGCCCCGCTCAACTTGACCCCGGAGCAGGTG GTGGCCATCGCCAGCCACGATGGCGGCAAGCAGGCGCTGGAGACGGTCCAGCGG CTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCGGAGCAGGTGGTGGCCA TCGCCAGCCACGATGGCGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCC GGTGCTGTGCCAGGCCCACGGCTTGACCCCGGAGCAGGTGGTGGCCATCGCCAGC AATATTGGTGGCAAGCAGGCGCTGGAGACGGTGCAGGCGCTGTTGCCGGTGCTGT GCCAGGCCCACGGCTTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCAATATTGG TGGCAAGCAGGCGCTGGAGACGGTGCAGGCGCTGTTGCCGGTGCTGTGCCAGGCC CACGGCTTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCAATATTGGTGGCAAGC AGGCGCTGGAGACGGTGCAGGCGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTT GACCCCCCAGCAGGTGGTGGCCATCGCCAGCAATAATGGTGGCAAGCAGGCGCTG GAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCGG AGCAGGTGGTGGCCATCGCCAGCAATATTGGTGGCAAGCAGGCGCTGGAGACGG TGCAGGCGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCCCAGCAGGT GGTGGCCATCGCCAGCAATGGCGGTGGCAAGCAGGCGCTGGAGACGGTCCAGCG GCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCCCAGCAGGTGGTGGCC ATCGCCAGCAATGGCGGTGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGC CGGTGCTGTGCCAGGCCCACGGCTTGACCCCGGAGCAGGTGGTGGCCATCGCCAG CCACGATGGCGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTG TGCCAGGCCCACGGCTTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCAATATTG GTGGCAAGCAGGCGCTGGAGACGGTGCAGGCGCTGTTGCCGGTGCTGTGCCAGG CCCACGGCTTGACCCCCCAGCAGGTGGTGGCCATCGCCAGCAATAATGGTGGCAA GCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGC TTGACCCCCCAGCAGGTGGTGGCCATCGCCAGCAATAATGGTGGCAAGCAGGCGC TGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCC CCAGCAGGTGGTGGCCATCGCCAGCAATGGCGGTGGCAAGCAGGCGCTGGAGAC GGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCCCAGCAG GTGGTGGCCATCGCCAGCAATGGCGGTGGCAAGCAGGCGCTGGAGACGGTCCAG CGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCTCAGCAGGTGGTGG CCATCGCCAGCAATGGCGGCGGCAGGCCGGCGCTGGAGAGCATTGTTGCCCAGTT

		ATCTCGCCCTGATCCGGCGTTGGCCGCGTTGACCAACGACCACCTCGTCGCCTTGG CCTGCCTCGGCGGGCGTCCTGCGCTGGATGCAGTGAAAAAGGGATTGGGGGATCC TATCAGCCGTTCCCAGCTGGTGAAGTCCGAGCTGGAGGAGAAGAAATCCGAGTTG AGGCACAAGCTGAAGTACGTGCCCCACGAGTACATCGAGCTGATCGAGATCGCCC GGAACAGCACCCAGGACCGTATCCTGGAGATGAAGGTGATGGAGTTCTTCATGAA GGTGTACGGCTACAGGGGCAAGCACCTGGGCGGCTCCAGGAAGCCCGACGGCGC CATCTACACCGTGGGCTCCCCCATCGACTACGGCGTGATCGTGGACACCAAGGCCT ACTCCGGCGGCTACAACCTGCCCATCGGCCAGGCCGACGAAATGCAGAGGTACGT GGAGGAGAACCAGACCAGGAACAAGCACATCAACCCCAACGAGTGGTGGAAGGT GTACCCCTCCAGCGTGACCGAGTTCAAGTTCCTGTTCGTGTCCGGCCACTTCAAGG GCAACTACAAGGCCCAGCTGACCAGGCTGAACCACATCACCAACTGCAACGGCGCC GTGCTGTCCGTGGAGGAGCTCCTGATCGGCGGCGAGATGATCAAGGCCGGCACCC TGACCCTGGAGGAGGTGAGGAGGAAGTTCAACAACGGCGAGATCAACTTCGCGG CCGACTGATAA
T02 TALEN Beta2M RIGHT	6	ATGGGCGATCCTAAAAAGAAACGTAAGGTCATCGATAAGGAGACCGCCGCTGCCA AGTTCGAGAGACAGCACATGGACAGCATCGATATCGCCGATCTACGCACGCTCGG CTACAGCCAGCAGCAACAGGAGAAGATCAAACCGAAGGTTCGTTCGACAGTGGCG CAGCACCACGAGGCACTGGTCGGCCACGGGTTTACACACGCGCACATCGTTGCGTT AAGCCAACACCCGGCAGCGTTAGGGACCGTCGCTGTCAAGTATCAGGACATGATC GCAGCGTTGCCAGAGGCGACACACGAAGCGATCGTTGGCGTCGGCAAACAGTGGT CCGGCGCACGCGCTCTGGAGGCCTTGCTCACGGTGGCGGGAGAGTTGAGAGGTCC ACCGTTACAGTTGGACACAGGCCAACTTCTCAAGATTGCAAAACGTGGCGGCGTGA CCGCAGTGGAGGCAGTGCATGCATGGCGCAATGCACTGACGGGTGCCCCGCTCAA CTTGACCCCCCAGCAGGTGGTGGCCATCGCCAGCAATAATGGTGGCAAGCAGGCG CTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCC CGGAGCAGGTGGTGGCCATCGCCAGCAATATTGGTGGCAAGCAGGCGCTGGAGA CGGTGCAGGCGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCGGAGCA GGTGGTGGCCATCGCCAGCCACGATGGCGGCAAGCAGGCGCTGGAGACGGTCCA GCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCCCAGCAGGTGGTG GCCATCGCCAGCAATGGCGGTGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTG TTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCCCAGCAGGTGGTGGCCATCGC CAGCAATGGCGGTGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGT GCTGTGCCAGGCCCACGGCTTGACCCCCCAGCAGGTGGTGGCCATCGCCAGCAAT GGCGGTGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGC CAGGCCCACGGCTTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCCACGATGGCG GCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCA CGGCTTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCCACGATGGCGGCAAGCAG GCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGA CCCCGGAGCAGGTGGTGGCCATCGCCAGCAATATTGGTGGCAAGCAGGCGCTGGA GACGGTGCAGGCGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCCCAG CAGGTGGTGGCCATCGCCAGCAATGGCGGTGGCAAGCAGGCGCTGGAGACGGTC CAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCCCAGCAGGTGG TGGCCATCGCCAGCAATGGCGGTGGCAAGCAGGCGCTGGAGACGGTCCAGCGGC TGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCGGAGCAGGTGGTGGCCAT CGCCAGCCACGATGGCGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCC GGTGCTGTGCCAGGCCCACGGCTTGACCCCCCAGCAGGTGGTGGCCATCGCCAGC AATGGCGGTGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGT GCCAGGCCCACGGCTTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCCACGATGG

		CGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCC CACGGCTTGACCCCCCAGCAGGTGGTGGCCATCGCCAGCAATGGCGGTGGCAAGC AGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTT GACCCCTCAGCAGGTGGTGGCCATCGCCAGCAATGGCGGCGGCAGGCCGGCGCTG GAGAGCATTGTTGCCCAGTTATCTCGCCCTGATCCGGCGTTGGCCGCGTTGACCAA CGACCACCTCGTCGCCTTGGCCTGCCTCGGCGGGCGTCCTGCGCTGGATGCAGTGA AAAAGGGATTGGGGGATCCTATCAGCCGTTCCCAGCTGGTGAAGTCCGAGCTGGA GGAGAAGAAATCCGAGTTGAGGCACAAGCTGAAGTACGTGCCCCACGAGTACATC GAGCTGATCGAGATCGCCCGGAACAGCACCCAGGACCGTATCCTGGAGATGAAGG TGATGGAGTTCTTCATGAAGGTGTACGGCTACAGGGGCAAGCACCTGGGCGGCTC CAGGAAGCCCGACGGCGCCATCTACACCGTGGGCTCCCCCATCGACTACGGCGTG ATCGTGGACACCAAGGCCTACTCCGGCGGCTACAACCTGCCCATCGGCCAGGCCGA CGAAATGCAGAGGTACGTGGAGGAGAACCAGACCAGGAACAAGCACATCAACCCC AACGAGTGGTGGAAGGTGTACCCCTCCAGCGTGACCGAGTTCAAGTTCCTGTTCGT GTCCGGCCACTTCAAGGGCAACTACAAGGCCCAGCTGACCAGGCTGAACCACATCA CCAACTGCAACGGCGCCGTGCTGTCCGTGGAGGAGCTCCTGATCGGCGGCGAGAT GATCAAGGCCGGCACCCTGACCCTGGAGGAGGTGAGGAGGAAGTTCAACAACGG CGAGATCAACTTCGCGGCCGACTGATAA
T03 Beta2M target	**7**	TTAGCTGTGCTCGCGCTACTCTCTCTTTCTGGCCTGGAGGCTATCCA
T03 TALEN Beta2M LEFT	8	ATGGGCGATCCTAAAAAGAAACGTAAGGTCATCGATTACCCATACGATGTTCCAGA TTACGCTATCGATATCGCCGATCTACGCACGCTCGGCTACAGCCAGCAGCAACAGG AGAAGATCAAACCGAAGGTTCGTTCGACAGTGGCGCAGCACCACGAGGCACTGGT CGGCCACGGGTTTACACACGCGCACATCGTTGCGTTAAGCCAACACCCGGCAGCGT TAGGGACCGTCGCTGTCAAGTATCAGGACATGATCGCAGCGTTGCCAGAGGCGAC ACACGAAGCGATCGTTGGCGTCGGCAAACAGTGGTCCGGCGCACGCGCTCTGGAG GCCTTGCTCACGGTGGCGGGAGAGTTGAGAGGTCCACCGTTACAGTTGGACACAG GCCAACTTCTCAAGATTGCAAAACGTGGCGGCGTGACCGCAGTGGAGGCAGTGCA TGCATGGCGCAATGCACTGACGGGTGCCCCGCTCAACTTGACCCCGGAGCAGGTG GTGGCCATCGCCAGCAATATTGGTGGCAAGCAGGCGCTGGAGACGGTGCAGGCG CTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCCCAGCAGGTGGTGGCCAT CGCCAGCAATAATGGTGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCG GTGCTGTGCCAGGCCCACGGCTTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCC ACGATGGCGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGT GCCAGGCCCACGGCTTGACCCCCCAGCAGGTGGTGGCCATCGCCAGCAATGGCGG TGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCC CACGGCTTGACCCCCCAGCAGGTGGTGGCCATCGCCAGCAATAATGGTGGCAAGC AGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTT GACCCCCCAGCAGGTGGTGGCCATCGCCAGCAATGGCGGTGGCAAGCAGGCGCTG GAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCCC AGCAGGTGGTGGCCATCGCCAGCAATAATGGTGGCAAGCAGGCGCTGGAGACGG TCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCGGAGCAGGT GGTGGCCATCGCCAGCCACGATGGCGGCAAGCAGGCGCTGGAGACGGTCCAGCG GCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCCCAGCAGGTGGTGGCC ATCGCCAGCAATGGCGGTGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGC CGGTGCTGTGCCAGGCCCACGGCTTGACCCCGGAGCAGGTGGTGGCCATCGCCAG

		CCACGATGGCGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTG TGCCAGGCCCACGGCTTGACCCCCCAGCAGGTGGTGGCCATCGCCAGCAATAATG GTGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGC CCACGGCTTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCCACGATGGCGGCAAG CAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCT TGACCCCCCAGCAGGTGGTGGCCATCGCCAGCAATAATGGTGGCAAGCAGGCGCT GGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCG GAGCAGGTGGTGGCCATCGCCAGCCACGATGGCGGCAAGCAGGCGCTGGAGACG GTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCCCAGCAGGT GGTGGCCATCGCCAGCAATGGCGGTGGCAAGCAGGCGCTGGAGACGGTCCAGCG GCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCTCAGCAGGTGGTGGCC ATCGCCAGCAATGGCGGCGGCAGGCCGGCGCTGGAGAGCATTGTTGCCCAGTTAT CTCGCCCTGATCCGGCGTTGGCCGCGTTGACCAACGACCACCTCGTCGCCTTGGCC TGCCTCGGCGGGCGTCCTGCGCTGGATGCAGTGAAAAAGGGATTGGGGGATCCTA TCAGCCGTTCCCAGCTGGTGAAGTCCGAGCTGGAGGAGAAGAAATCCGAGTTGAG GCACAAGCTGAAGTACGTGCCCCACGAGTACATCGAGCTGATCGAGATCGCCCGG AACAGCACCCAGGACCGTATCCTGGAGATGAAGGTGATGGAGTTCTTCATGAAGG TGTACGGCTACAGGGGCAAGCACCTGGGCGGCTCCAGGAAGCCCGACGGCGCCAT CTACACCGTGGGCTCCCCCATCGACTACGGCGTGATCGTGGACACCAAGGCCTACT CCGGCGGCTACAACCTGCCCATCGGCCAGGCCGACGAAATGCAGAGGTACGTGGA GGAGAACCAGACCAGGAACAAGCACATCAACCCCAACGAGTGGTGGAAGGTGTAC CCCTCCAGCGTGACCGAGTTCAAGTTCCTGTTCGTGTCCGGCCACTTCAAGGGCAA CTACAAGGCCCAGCTGACCAGGCTGAACCACATCACCAACTGCAACGGCGCCGTGC TGTCCGTGGAGGAGCTCCTGATCGGCGGCGAGATGATCAAGGCCGGCACCCTGAC CCTGGAGGAGGTGAGGAGGAAGTTCAACAACGGCGAGATCAACTTCGCGGCCGA CTGATAA
T03 TALEN Beta2M RIGHT	9	ATGGGCGATCCTAAAAAGAAACGTAAGGTCATCGATAAGGAGACCGCCGCTGCCA AGTTCGAGAGACAGCACATGGACAGCATCGATATCGCCGATCTACGCACGCTCGG CTACAGCCAGCAGCAACAGGAGAAGATCAAACCGAAGGTTCGTTCGACAGTGGCG CAGCACCACGAGGCACTGGTCGGCCACGGGTTTACACACGCGCACATCGTTGCGTT AAGCCAACACCCGGCAGCGTTAGGGACCGTCGCTGTCAAGTATCAGGACATGATC GCAGCGTTGCCAGAGGCGACACACGAAGCGATCGTTGGCGTCGGCAAACAGTGGT CCGGCGCACGCGCTCTGGAGGCCTTGCTCACGGTGGCGGGAGAGTTGAGAGGTCC ACCGTTACAGTTGGACACAGGCCAACTTCTCAAGATTGCAAAACGTGGCGGCGTGA CCGCAGTGGAGGCAGTGCATGCATGGCGCAATGCACTGACGGGTGCCCCGCTCAA CTTGACCCCCCAGCAGGTGGTGGCCATCGCCAGCAATAATGGTGGCAAGCAGGCG CTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCC CCCAGCAGGTGGTGGCCATCGCCAGCAATAATGGTGGCAAGCAGGCGCTGGAGAC GGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCGGAGCAG GTGGTGGCCATCGCCAGCAATATTGGTGGCAAGCAGGCGCTGGAGACGGTGCAG GCGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCCCAGCAGGTGGTGG CCATCGCCAGCAATGGCGGTGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTT GCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCGGAGCAGGTGGTGGCCATCGCC AGCAATATTGGTGGCAAGCAGGCGCTGGAGACGGTGCAGGCGCTGTTGCCGGTGC TGTGCCAGGCCCACGGCTTGACCCCCCAGCAGGTGGTGGCCATCGCCAGCAATAAT GGTGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAG GCCCACGGCTTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCCACGATGGCGGCA AGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGG

		CTTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCCACGATGGCGGCAAGCAGGCG CTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCC CCCAGCAGGTGGTGGCCATCGCCAGCAATGGCGGTGGCAAGCAGGCGCTGGAGA CGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCGGAGCA GGTGGTGGCCATCGCCAGCCACGATGGCGGCAAGCAGGCGCTGGAGACGGTCCA GCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCGGAGCAGGTGGTG GCCATCGCCAGCCACGATGGCGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGT TGCCGGTGCTGTGCCAGGCCCACGGCTTGACCCCGGAGCAGGTGGTGGCCATCGC CAGCAATATTGGTGGCAAGCAGGCGCTGGAGACGGTGCAGGCGCTGTTGCCGGTG CTGTGCCAGGCCCACGGCTTGACCCCCCAGCAGGTGGTGGCCATCGCCAGCAATAA TGGTGGCAAGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAG GCCCACGGCTTGACCCCCCAGCAGGTGGTGGCCATCGCCAGCAATAATGGTGGCA AGCAGGCGCTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGG CTTGACCCCGGAGCAGGTGGTGGCCATCGCCAGCCACGATGGCGGCAAGCAGGCG CTGGAGACGGTCCAGCGGCTGTTGCCGGTGCTGTGCCAGGCCCACGGCTTGACCC CTCAGCAGGTGGTGGCCATCGCCAGCAATGGCGGCGGCAGGCCGGCGCTGGAGA GCATTGTTGCCCAGTTATCTCGCCCTGATCCGGCGTTGGCCGCGTTGACCAACGAC CACCTCGTCGCCTTGGCCTGCCTCGGCGGGCGTCCTGCGCTGGATGCAGTGAAAAA GGGATTGGGGGATCCTATCAGCCGTTCCCAGCTGGTGAAGTCCGAGCTGGAGGAG AAGAAATCCGAGTTGAGGCACAAGCTGAAGTACGTGCCCCACGAGTACATCGAGC TGATCGAGATCGCCCGGAACAGCACCCAGGACCGTATCCTGGAGATGAAGGTGAT GGAGTTCTTCATGAAGGTGTACGGCTACAGGGGCAAGCACCTGGGCGGCTCCAGG AAGCCCGACGGCGCCATCTACACCGTGGGCTCCCCCATCGACTACGGCGTGATCGT GGACACCAAGGCCTACTCCGGCGGCTACAACCTGCCCATCGGCCAGGCCGACGAA ATGCAGAGGTACGTGGAGGAGAACCAGACCAGGAACAAGCACATCAACCCCAACG AGTGGTGGAAGGTGTACCCCTCCAGCGTGACCGAGTTCAAGTTCCTGTTCGTGTCC GGCCACTTCAAGGGCAACTACAAGGCCCAGCTGACCAGGCTGAACCACATCACCAA CTGCAACGGCGCCGTGCTGTCCGTGGAGGAGCTCCTGATCGGCGGCGAGATGATC AAGGCCGGCACCCTGACCCTGGAGGAGGTGAGGAGGAAGTTCAACAACGGCGAG ATCAACTTCGCGGCCGACTGATAA

Table 1: Description of the β2m TALE-nucleases sequences

TALE-nucleases cleaving human PD-1 gene

In addition to the inactivation of said gene involved in the self and non-self antigen recognition, further genetic engineering may be sought such as the inactivation of one or several genes encoding immune checkpoints as described in WO2014/184744,

In a preferred embodiment, to the inactivation of at least one gene involved in self/non self recognition, an additional inactivation is performed on a gene encoding PD-1. PD-1 corresponds to the human Programmed Death 1 (also known as PDCD1 or CD279, RefSeq accession number: NM_005018 for the human gene). This PD-1 inhibition, preferably by TALEN-mediated disruption, has

the objective to render allogeneic immune cells resistant to their self or reciprocal inhibition by PD-L1 (also known as CD274 or B7 homolog 1 (B7-H1), and has RefSeq N°NM_001267706 for human gene).

According to a preferred embodiment, said inactivation of PD-1 gene is performed by using a polynucleotide encoding TALE-nucleases as presented in the following Table 2.

Target	Target sequence	Half TALE-nuclease
PDCD1_T01	TTCTCCCCAGCCCTGCT cgtggtgaccgaagg GGACAACGCCACCTTCA (SEQ ID NO : 10)	PDCD1_T01-L TALEN (SEQ ID NO: 11)
		PDCD1_T01-R TALEN (SEQ ID NO: 12)
PDCD1_T03	TACCTCTGTGGGGCCAT ctccctggcccccaa GGCGCAGATCAAAGAGA (SEQ ID NO : 13)	PDCD1_T03-L TALEN (SEQ ID NO: 14)
		PDCD1_T03-R TALEN (SEQ ID NO: 15

Table 2: Polynucleotide sequences of 2 pairs of TALENs are presented for 2 different PDC1 (or PD-1) gene targets

According to one embodiment of the present invention, said step c) of the method is performed by the expression in said immune cells of at least one non-endogenous polynucleotide encoding for PD-L1 ligand bound to the membrane, and a further modification of said allogeneic cells is performed by an inactivation of the expression of PD-1 gene.

According to another embodiment of the present invention, said step c) is performed by the expression in said immune cells at least one non-endogenous polynucleotide corresponding to secreted CTLA4 immunoglobulins, and a further modification of said allogeneic cells is performed by an inactivation of the expression of a gene encoding PD-1.

According to a preferred embodiment of the present invention, said step c) is performed by the step c) is performed by the expression in said immune cells of both non-endogenous

immunosuppressive polypeptide PD-L1 ligand and CTLA-4 immunoglobulins, and a further modification of said allogeneic immune cells is performed by an inactivation of the expression of a gene encoding PD-1.

Expression of non-endogenous immunosuppressive polypeptide

According to a preferred embodiment, said step c) of the method of the invention is performed by the expression in said immune cells of at least one non-endogenous polynucleotide encoding for one non-endogenous immunosuppressive polypeptide bound to the membrane of said immune cells.

According to one embodiment, said non endogenous immunosuppressive polypeptide is present under a membrane-bound form and/or under a secreted form.

By *"non-endogenous polypeptide"* is meant a polypeptide not normally expressed by a donor's immune cell, preferably a polypeptide expressed by an exogenous polynucleotide that has been imported into the immune's cell genome. For instance, IL12 is not considered hereby as being a non-endogenous polypeptide because it is expressed from a preexisting gene from the donor's immune cell.

By *"not naturally expressed"* is meant that the polynucleotide sequence encoding said polypeptide is either not originally present in the genome of the immune cell (e.g.: CTLA4 Ig), or said polynucleotide sequence is present in the genome but the polypeptide is expressed in the native immune cell (i.e. non-engineered) at a much lower level – generally at least 50%, preferably at least 75%, more preferably at least 100% and even more preferably 200% lower than the expression level observed into the engineered immune cell in the same experimental or treatment conditions.

By "immunosuppressive" is meant that the expression of said non-endogenous polypeptide has the effect of alleviating the immune response of the patient host against the donor's immune cells.

According to a preferred aspect of the invention, said non endogenous immunosuppressive polypeptide is selected amongst PD-L1, CTLA-4-Ig, viral MHC homolog, NKG2D ligand, viral *env* immune suppressive domain (ISU) or the viral FP protein.

According to one embodiment, the method comprises as step c) an expression in immune cells at least one non-endogenous polynucleotide corresponding to a non-endogenous secreted immunosuppressive polypeptide.

According to a more preferred embodiment, said one non-endogenous immunosuppressive polypeptide bound to the membrane of said immune cells is a PD-L1 ligand under a membrane-bound form.

Expression of CTLA-4-Ig

According to one embodiment, the non-endogenous immunosuppressive polypeptide to be expressed in said allogeneic immune cells is a ligand of CTLA-4 protein, preferably a CTLA4 immunoglobulin. Cytotoxic T-Lymphocyte Antigen 4 (CTLA-4) is also known as CD152, GenBank accession number AF414120.1).

According to a preferred embodiment, the polypeptide corresponding to CTLA-4 immunoglobulin to be expressed in said allogeneic immune cells comprises SEQ ID NO: 16 (CTLA-4a) or SEQ ID NO:17 (CTLA4b), or shares at least 80%, preferably 90% and more preferably 95% identity with SEQ ID NO: 16 or SEQ ID NO:17.

The interaction between the allogeneic T cell and host immune cells is schematically represented in Figure 2 (expression of CTLA4-Ig) in regard to the situation in Figure 1 (no expression).

According to one preferred embodiment, the nucleic acid molecule encoding CTLA-4a Ig and CTLA-4b Ig to be expressed shares respectively at least 80%, preferably 90% and more preferably 95% of identity with SEQ ID NO: 16 and SEQ ID NO: 17 as presented in the following Table 3.

Name of construct	Expression	SEQ ID NO :	Polypeptide sequence
pCLS27068	CTLA4a expression plasmid	**16**	MGGVLLTQRTLLSLVLALLFPSMASMAMHVAQPAVVLASSRGIASFVCEYAS PGKATEVRVTVLRQADSQVTEVCAATYMMGNELTFLDDSICTGTSSGNQVN LTIQGLRAMDTGLYICKVELMYPPPYYLGIGNGTQIYVIDPEPCPDSDQEPKSS DKTHTSPPSPAPELLGGSSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVK FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVS NKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAV EWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHE ALHNHYTQKSLSLSPGKGS
pCLS27066	CTLA4b expression plasmid	**17**	MGGVLLTQRTLLSLVLALLFPSMASMAMHVAQPAVVLASSRGIASFVCEYAS PGKYTEVRVTVLRQADSQVTEVCAATYMMGNELTFLDDSICTGTSSGNQVN LTIQGLRAMDTGLYICKVELMYPPPYYEGIGNGTQIYVIDPEPCPDSDQEPKSS DKTHTSPPSPAPELLGGSSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEVK FNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVS NKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDIAV EWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHE ALHNHYTQKSLSLSPGKGS
pCLS27069	PD-L1 expression plasmid	**18**	MGRIFAVFIFMTYWHLLNAFTVTVPKDLYVVEYGSNMTIECKFPVEKQLDLA ALIVYWEMEDKNIIQFVHGEEDLKVQHSSYRQRARLLKDQLSLGNAALQITDV KLQDAGVYRCMISYGGADYKRITVKVNAPYNKINQRILVVDPVTSEHELTCQA EGYPKAEVIWTSSDHQVLSGKTTTTNSKREEKLFNVTSTLRINTTTNEIFYCTFR RLDPEENHTAELVIPELPLAHPPNERTHLVILGAILLCLGVALTFIFRLRKGRMM DVKKCGIQDTNSKKQSDTHLEETGS

Table 3: polynucleotide sequences of plasmidic constructs expressing CLTA-4a, CTLA-4b and PD-L1.

According to one embodiment, the engineered immune cells are incubated with a non-endogenous immunosuppressive polypeptide which is anti-CD80 or anti-CD86 mAbs.

Expression of PD-L1

PD-L1 (other names: CD274, Programmed cell death 1 ligand; ref. UniProt for the human polypeptide sequence Q9NZQ7) encodes a type I transmembrane protein of 290 amino acids consisting of a Ig V-like domain, a Ig C-like domain, a hydrophobic transmembrane domain and a cytoplasmic tail of 30 amino acids.

According to a preferred embodiment of the invention, the non-endogenous immunosuppressive polypeptide to be expressed in said allogeneic immune cells is a ligand of PD-L1, more especially under a membrane-bound form.

Such membrane-bound form of PD-L1 ligand is meant in the present invention under a native form (wild-type) or under a truncated form such as, for instance, by removing the intracellular domain, or with one or more mutation(s) (Wang S et al, 2003, J Exp Med. 2003; 197(9): 1083–1091). PD1 is not considered as being a membrane-bound form of PD-L1 ligand according to the present invention.

According to a more preferred embodiment, the nucleic acid molecule encoding PD-L1 ligand under a membrane-bound form to be expressed is of SEQ ID NO:18, or shares at least 80%, preferably 90% and more preferably 95% of identity with SEQ ID NO:18 (corresponding to the wild-type form of PDL1-ligand).

According to another embodiment, said at least one non-endogenous immunosuppressive polypeptide is PD-L1 ligand under a secreted form. Such recombinant secreted PD-L1 (or soluble PD-L1) may be generated by fusing the extracellular domain of PD-L1 to the Fc portion of immunoglobuline (Haile ST et al, 2014, Cancer Immunol Res. 2(7): 610–615 ; Song MY et al, 2015, Gut. 64(2):260-71). This recombinant PD-L1 neutralizes PD-1 and abrogates PD-1-mediated T-cell inhibition.

The interaction between the allogeneic T cell and host immune cells is schematically represented in Figure 3 (expression of membrane-bound PD-L1) in regard to the situation to Figure 1 (no expression). Figure 3 represents also the situation when the PD-1 gene is disrupted by KO.

According to an alternative to the precedent embodiment, the non-endogenous immunosuppressive polypeptide to be expressed in said allogeneic immune cells is a PD-L1 ligand under a secreted form. The interaction between the allogeneic T cell and host immune cells is schematically represented in Figure 4 (expression of secreted PD-L1 ligand) in regard to the situation to Figure 1 (no expression). Figure 4 represents also the situation when the PD-1 gene is disrupted by KO.

According to one preferred embodiment, the nucleic acid molecule encodes formembrane-bound PD-L1 to be expressed which is of SEQ ID NO: 18, or shares at least 80%, preferably 90% and more preferably 95% of identity with SEQ ID NO: 18.

Co-expression of PD-L1 ligand with CTLA4 Ig

The present invention relates also to a method to increase the persistence and/or the engraftment of allogeneic immune cells in presence of host immune cells, wherein step c) is performed by contacting said host immune cells with both non-endogenous immunosuppressive polypeptide PD-L1 ligand and CTLA-4 immunoglobulins.

According to a preferred embodiment, step c) of the method is performed by the step c) is performed by the expression in said immune cells of both non-endogenous immunosuppressive polypeptide PD-L1 ligand and CTLA-4 immunoglobulins.

According to a preferred embodiment, the nucleic acid molecules encode for PD-L1 ligand under a membrane-bound form and for CTLA-4 immunoglobulins to be expressed during step c) of the method in said allogeneic immune cells, said PD-L1 ligand and CTLA-4 Ig having respectively SEQ ID NO:18 and SEQ ID NO: 16-17, or sharing at least 80%, preferably 90% and more preferably 95% of identity with respectively SEQ ID NO:18 and SEQ ID NO: 16-17.

Expression of ISU domain

According to another embodiment, the non-endogenous immunosuppressive polypeptide to be expressed in said allogeneic immune cells is a viral *env* immusuppressive domain (ISU), which is derived for instance from HIV-1, HIV-2, SIV, MoMuLV, HTLV-I, -II, MPMV, SRV-1, Syncitin 1 or 2, HERV-K or FELV .

The interaction between the allogeneic T cell and host immune cells is schematically represented in Figure 5 (expression of viral ISU domain) in regard to the situation to Figure 1 (no expression).

The following Table 4 shows variants of ISU domain from diverse virus which can be expressed within the present invention.

SEQ ID #	Position														virus
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	Origin
SEQ ID N°19-24	L	Q	A	R	I/V	L	A	V	E	R	Y	L	K/R/Q	D	HIV-1
SEQ ID N°25-30	L	Q	A	R	V	T	A	I	E	K	Y	L	K/A/Q	D/H	HIV-2
SEQ ID N° 31	L	Q	A	R	L	L	A	V	E	R	Y	L	K	D	SIV
SEQ ID N° 32	L	Q	N	R	R	G	L	D	L	L	F	L	K	E	MoMuLV
SEQ ID N° 33	A	Q	N	R	R	G	L	D	L	L	F	W	E	Q	HTLV-I, -II
SEQ ID N° 34	L	Q	N	R	R	G	L	D	L	L	T	A	E	Q	MPMV, SRV-1
SEQ ID N° 35	L	Q	N	R	R	A	L	D	L	L	T	A	E	R	Syncitin 1
SEQ ID N° 36	L	Q	N	R	R	G	L	D	M	L	T	A	A	Q	Syncitin 2
SEQ ID N° 37	L	A	N	Q	I	N	D	L	R	Q	T	V	I	W	HERV-K
SEQ ID N° 38	L	Q	N	R	R	G	L	D	I	L	F	L	Q	E	FELV

Table 4: ISU domain variants from diverse viruses

Accordingly, in certain embodiments, the non-endogenous immunosuppressive polypeptide to be expressed in said allogeneic immune cells is an ISU domain of SEQ ID NO.19-38.

Expression of viral MHC homolog

According to another embodiment, the non-endogenous immunosuppressive polypeptide to be expressed in said allogeneic immune cells is a viral MHC homolog, such as for instance UL18.

In one embodiment, said non-endogenous immunosuppressive polypeptide is a MHC homolog comprising a chimeric beta2m -UL18 of SEQ ID NO.39, or sharing at least 80%, preferably 90% and more preferably 95% of identity with respectively SEQ ID NO:39.

The interaction between the allogeneic T cell and host immune cells is schematically represented in Figure 7 (expression of viral MHC homolog) in regard to the situation to Figure 6 (no expression). In both figures, the MHC class I is inactivated by disrupting (KO) the beta2M gene.

Expression of NKG2D ligand

Some viruses such as cytomegaloviruses have acquired mechanisms to avoid NK cell mediate immune surveillance and interfere with the NKG2D pathway by secreting a protein able to bind NKG2D ligands and prevent their surface expression (Welte, S.A.; Sinzger, C.; Lutz, S.Z.; Singh-Jasuja, H.; Sampaio, K.L.; Eknigk, U.; Rammensee, H.G.; Steinle, A. 2003 "Selective intracellular retention of virally induced NKG2D ligands by the human cytomegalovirus UL16 glycoprotein". Eur. J. Immunol., 33, 194–203). In tumors cells, some mechanisms have evolved to evade NKG2D response by secreting NKG2D ligands such as ULBP2, MICB or MICA (Salih HR, Antropius H, Gieseke F, Lutz SZ, Kanz L, et al. (2003) Functional expression and release of ligands for the activating immunoreceptor NKG2D in leukemia. Blood 102: 1389–1396)

According to another embodiment, the non-endogenous immunosuppressive polypeptide to be expressed in said allogeneic immune cells is an NKG2D ligand. The interaction between the allogeneic T cell and host immune cells is schematically represented in Figure 8 (expression of soluble NKG2D ligand) in regard to the situation to Figure 6 (no expression). In both figures, the MHC class I is inactivated by disrupting (KO) the beta2M gene.

The following Table 5 represents a viral MHC homolog (UL18) and a panel of NKG2D ligands and their polypeptide sequence to be expressed according to the present invention.

	SEQ ID NO:	Polypeptide sequence
Chimeric B2M-UL18	39	MALPVTALLLPLALLLHAARPSRSVALAVLALLSLSGLEAIQRTPKIQVYSRHPAENGKSNFLN CYVSGFHPSDIEVDLLKNGERIEKVEHSDLSFSKDWSFYLLYYTEFTPTEKDEYACRVNHVTLS QPKIVKWDRDMGGGGSGGGGSGGGGSGGGGSMTMWCLTLFVLWMLRVVGMHVLRY GYTGIFDDTSHMTLTVVGIFDGQHFFTYHVNSSDKASSRANGTISWMANVSAAYPTYLDGE RAKGDLIFNQTEQNLLELEIALGYRSQSVLTWTHECNTTENGSFVAGYEGFGWDGETLMELK DNLTLWTGPNYEISWLKQNKTYIDGKIKNISEGDTTIQRNYLKGNCTQWSVIYSGFQTPVTH PVVKGGVRNQNDNRAEAFCTSYGFFPGEINITFIHYGNKAPDDSEPQCNPLLPTFDGTFHQG CYVAIFCNQNYTCRVTHGNWTVEIPISVTSPDDSSSGEVPDHPTANKRYNTMTISSVLLALLL CALLFAFLHYFTTLKQYLRNLAFAWRYRKVRSS
SP-MICAed	40	MGGVLLTQRTLLSLVLALLFPSMASMEPHSLRYNLTVLSWDGSVQSGFLTEVHLDGQPFLRC DRQKCRAKPQGQWAEDVLGNKTWDRETRDLTGNGKDLRMTLAHIKDQKEGLHSLQEIRV CEIHEDNSTRSSQHFYYDGELFLSQNLETKEWTMPQSSRAQTLAMNVRNFLKEDAMKTKTH YHAMHADCLQELRRYLKSGVVLRRTVPPMVNVTRSEASEGNITVTCRASGFYPWNITLSWR QDGVSLSHDTQQWGDVLPDGNGTYQTWVATRICQGEEQRFTCYMEHSGNHSTHPVPSG KVLVLQSHW
SP-MICBed	41	MGGVLLTQRTLLSLVLALLFPSMASMAEPHSLRYNLMVLSQDESVQSGFLAEGHLDGQPFL RYDRQKRRAKPQGQWAEDVLGAKTWDTETEDLTENGQDLRRTLTHIKDQKGGLHSLQEIR VCEIHEDSSTRGSRHFYYDGELFLSQNLETQESTVPQSSRAQTLAMNVTNFWKEDAMKTKT HYRAMQADCLQKLQRYLKSGVAIRRTVPPMVNVTCSEVSEGNITVTCRASSFYPRNITLTWR QDGVSLSHNTQQWGDVLPDGNGTYQTWVATRIRQGEEQRFTCYMEHSGNHGTHPVPSG KVLVLQSQRTD
SP-ULBP1ed	42	MGGVLLTQRTLLSLVLALLFPSMASMGWVDTHCLCYDFIITPKSRPEPQWCEVQGLVDERP FLHYDCVNHKAKAFASLGKKVNVTKTWEEQTETLRDVVDFLKGQLLDIQVENLIPIEPLTLQA RMSCEHEAHGHGRGSWQFLFNGQKFLLFDSNNRKWTALHPGAKKMTEKWEKNRDVTMF FQKISLGDCKMWLEEFLMYWEQMLDPT
SP-ULBP2ed	43	MGGVLLTQRTLLSLVLALLFPSMASMGRADPHSLCYDITVIPKFRPGPRWCAVQGQVDEKT FLHYDCGNKTVTPVSPLGKKLNVTTAWKAQNPVLREVVDILTEQLRDIQLENYTPKEPLTLQA RMSCEQKAEGHSSGSWQFSFDGQIFLLFDSEKRMWTTVHPGARKMKEKWENDKVVAMS FHYFSMGDCIGWLEDFLMGMDSTLEPSAG
SP-ULBP3ed	44	MGGVLLTQRTLLSLVLALLFPSMASMDAHSLWYNFTIIHLPRHGQQWCEVQSQVDQKNFL SYDCGSDKVLSMGHLEEQLYATDAWGKQLEMLREVGQRLRLELADTELEDFTPSGPLTLQV RMSCECEADGYIRGSWQFSFDGRKFLLFDSNNRKWTVVHAGARRMKEKWEKDSGLTTFFK MVSMRDCKSWLRDFLMHRKKRLEPT
SP-N2DL4ed	45	MGGVLLTQRTLLSLVLALLFPSMASMHSLCFNFTIKSLSRPGQPWCEAQVFLNKNLFLQYNS DNNMVKPLGLLGKKVYATSTWGELTQTLGEVGRDLRMLLCDIKPQIKTSDPSTLQVEMFCQ REAERCTGASWQFATNGEKSLLFDAMNMTWTVINHEASKIKETWKKDRGLEKYFRKLSKG DCDHWLREFLGHWEAMPEPTVSPVNASDIHWSSSSLPD
SP-RET1Ged	46	MGGVLLTQRTLLSLVLALLFPSMASMGLADPHSLCYDITVIPKFRPGPRWCAVQGQVDEKTF LHYDCGSKTVTPVSPLGKKLNVTTAWKAQNPVLREVVDILTEQLLDIQLENYIPKEPLTLQAR MSCEQKAEGHGSGSWQLSFDGQIFLLFDSENRMWTTVHPGARKMKEKWENDKDMTMS FHYISMGDCTGWLEDFLMGMDSTLEPSAGAPPTMSSGTAQPR
SP-RAETILed	47	MGGVLLTQRTLLSLVLALLFPSMASMRRDDPHSLCYDITVIPKFRPGPRWCAVQGQVDEKT FLHYDCGNKTVTPVSPLGKKLNVTMAWKAQNPVLREVVDILTEQLLDIQLENYTPKEPLTLQ ARMSCEQKAEGHSSGSWQFSIDGQTFLLFDSEKRMWTTVHPGARKMKEKWENDKDVAM SFHYISMGDCIGWLEDFLMGMDSTLEPSAG

Table 5: Polypeptide sequence of a viral MHC homolog (UL18) and a panel of NKG2D ligands.

Accordingly, in certain embodiments, said non-endogenous immunosuppressive polypeptide to be expressed in engineered immune cells comprises or consists of a NKG2D ligand of SEQ ID NO.40-47, or sharing at least 80%, preferably 90% and more preferably 95% of identity with respectively SEQ ID NO:40-47.

Expression of FP polypeptide

According to another embodiment, the non-endogenous immunosuppressive polypeptide to be expressed in said allogeneic immune cells is a FP polypeptide such as gp41. The following Table 6 represents several FP polypeptide from natural and artificial origins.

	Position									
	1	2	3	4	5	6	7	8	9	Origin
SEQ ID N°48	G	A	L	F	L	G	F	L	G	HIV-1 gp41
SEQ ID N°49	A	G	F	G	L	L	L	G	F	Synthetic
SEQ ID N°50	A	G	L	F	L	G	F	L	G	Synthetic

Table 6: Aminoacid sequences of FP polypeptide from natural and artificial origins

Accordingly, in certain embodiments, said non-endogenous immunosuppressive polypeptide to be expressed in engineered immune cells is a FP polypeptide comprising or consisting of SEQ ID NO.48-50, or sharing at least 80%, preferably 90% and more preferably 95% of identity with respectively SEQ ID NO:48-50.

Non alloreactive and immunosuppressive resistant T cells

Combinations of gene inactivation with gene expression

The inventors present here a method for increasing the persistence and/or the engraftment to apply on allogeneic immune cells, wherein a series of genetic modifications may be performed. Amongst those, are encompassed diverse combinations of both at least one inactivation gene involved in the self/non self-recognition and at least one expression of non-endogenous immunosuppressive polypeptide.

According to a preferred embodiment, the genetic modifications are performed by the inactivation of the B2M and/or TCR gene combined with the expression in said allogeneic immune

cells of PD-L1 ligand and/or CTLA-4 immunoglobulins and/or viral *env* immune suppressive domain (ISU) and/or viral FP protein and/or NKG2G ligand viral MHC homolog such as for instance UL18.

Are also comprised in the scope of the present invention, polynucleotides, vectors encoding the above described rare-cutting endonucleases according to the invention.

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 allogeneic immune cells such as T cells, in which at least one endogenous gene encoding a polypeptide involved in the self and non-self antigen recognition is inactivated and at least one non-endogenous immunosuppressive polypeptide is allowed to contact said all allogeneic immune cells.

In a particular aspect, the present invention relates to a method of engineering immune cells such as T-cells, especially for immunotherapy.

In a particular embodiment, the method comprises:

i) providing allogeneic cells;

ii) modifying said cells by inactivating at least one endogenous gene encoding a polypeptide involved in the self and non-self antigen recognition;
and;

iii) contacting said immune cells with at least one non-endogenous immunosuppressive polypeptide.

In another particular aspect, the present invention relates to a method of engineering immune cells such as T-cells, especially for immunotherapy.

In a particular embodiment, the method comprises:

i) providing allogeneic cells;

ii) modifying said cells by inactivating at least one endogenous gene encoding a polypeptide involved in the self and non-self antigen recognition;
and;

iii) expressing in said immune cells at least one non-endogenous immunosuppressive polypeptide.

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-tunes the response.

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 another embodiment, the genetic modification step of the method relies on the inactivation of more than two genes. The genetic modification is preferably operated ex-vivo.

TALE-nucleases cleaving human TCR genes (TRAC and TRBC)

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.

Although human TCR genes may be disrupted in allogeneic immune cells as taught in WO WO2013176915, the present invention encompasses the situation where such inactivation is combined with any of the foregoing inactivation of self/non-self recognition genes and ectopic expression of at least one non-endogenous immunosuppressive polypeptide previously mentioned.

The following Table 7 presents nucleotide sequences for 5 TRAC and 2 TRBC targets and some of their corresponding left and right TALEN. Additional sequences can be found in the applications WO2014/184741 and WO2014/184744.

Target	Target sequence	Half TALE-nuclease
TRAC_T00	TGATCCTCTTGTCCCACAGATATCC Agaaccctgaccctg CCGTGTACCAGCTGAGAGA (SEQ ID NO 51)	TRAC_T00-L TALEN (SEQ ID NO: 52)
		TRAC_T00-R TALEN (SEQ ID NO: 53)
TRAC_T01	TTGTCCCACAGATATCC Agaaccctgaccctg CCGTGTACCAGCTGAGA (SEQ ID NO: 54)	TRAC_T01-L TALEN (SEQ ID NO: 55)
		TRAC_T01-R TALEN (SEQ ID NO: 56)
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)	
TRBC_T01	TGTGTTTGAGCCATCAG aagcagagatctccc ACACCCAAAAGGCCACA (SEQ ID NO: 61	TRBC_T01-L TALEN (SEQ ID NO: 62)
		TRBC_T01-R TALEN (SEQ ID NO: 63)
TRBC_T02	TTCCCACCCGAGGTCGC tgtgtttgagccatca GAAGCAGAGATCTCCCA (SEQ ID NO: 64)	TRBC_T02-L TALEN (SEQ ID NO: 65)
		TRBC_T02-R TALEN (SEQ ID NO: 66)

Table 7: Description of the TRAC and TRBC TALE-nucleases and sequences of the TALE-nucleases target sites in the human corresponding genes.

5

Single chain CAR

According to one aspect of the invention, the method comprises the step of introducing into said T-cell an exogenous nucleic acid molecule comprising a nucleotide sequence coding for a Chimeric Antigen Receptor (CAR) directed against at least one antigen expressed at the surface of a malignant or infected cell. They may be designed according to single-chain or multi-chain architectures.

In one embodiment, the Chimeric Antigen Receptor (CAR) is a single-chain CAR.

In a preferred embodiment, said extracellular ligand-binding domain is a scFv. 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.

As preferred examples of scFv according to the invention, VH and VL chains have as antigenic target sequence of over 80% identity, preferably over 90%, and more preferably over 95% with SEQ ID NO 67 (CD19 antigen), SEQ ID NO 68 (CD38 antigen), SEQ ID NO 69 (CD123 antigen), SEQ ID NO 70 (CS1 antigen), SEQ ID NO 71 (BCMA antigen), SEQ ID NO 72 (FLT-3 antigen), SEQ ID NO 73 (CD33 antigen), SEQ ID NO 74 (CD70 antigen), SEQ ID NO 75 (EGFR-3v antigen) and SEQ ID NO 76 (WT1 antigen). Other examples of surface antigens of tumoral cells to be targeted are CLL1, Hsp70, CD22, MUC16, PRAME, TSPAN10, ROR1, GD3, CT83 and mesothelin.

According to an embodiment, the present invention relates to a method as described above, wherein step c) is performed by the step c) is performed by the expression in said allogeneic immune cells of non-endogenous immunosuppressive polypeptide PD-L1 ligand and / or CTLA-4 immunoglobulins, said allogeneic immune cells being further modified by the expression of an anti-CD123 Chimeric Antigen Receptor.

According to a preferred embodiment, said anti-CD123 CAR/PD-L1 ligand/CTLA-4 Ig expressed allogeneic immune cells are further modified during step c) of the method to undergo an inactivation of the expression of the PD-1 gene.

Said polypeptide of a) further may comprise 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.

Said polypeptide may further comprise at least one signal-transducing domain. In a most preferred embodiment, said signal-transducing domain is selected from the group consisting of CD28, OX40, ICOS, CD137 and CD8.

Said C-terminal cytoplasmic tail of FcεRI alpha, beta and/or gamma chain fragment further comprises TNFR-associated Factor 2 (TRAF2) binding motifs. In a most preferred embodiment, said C-terminal cytoplasmic tail of FcεRI alpha, beta and/or gamma chain is replaced by 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.

Said intracytoplasmic domain of FcεRI alpha, beta and/or gamma chain is replaced by intracytoplasmic domain of TCR zeta chain (also named CD3 zeta). In another preferred embodiment, said intracytoplasmic domain of FcεRI alpha, beta and/or gamma chain comprises at least one additional immunoreceptor tyrosine-based activation motif (ITAM). 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 include those derived from TCRzeta, FCRgamma, FCRbeta, CD3gamma, CD3delta, CD3epsilon, CD5, CD22, CD79a, CD79b, and CD66d.

For instance, an example of single-chain CAR is depicted by the SEQ ID NO: 77.

In a preferred embodiment, said above CAR is single-chain CAR chosen in the group consisting of anti-CD123 single-chain CAR, anti-CS1 single-chain CAR, anti-CD38 single-chain CAR, anti-CLL1 single-chain CAR, anti-Hsp70 single-chain CAR, anti-EGFRvIII single-chain CAR, anti-BCMA single-chain CAR, anti-CD33 single-chain CAR, anti-FLT3 single-chain CAR, anti-CD70 single-chain CAR, anti-WT1 single-chain CAR, anti-MUC16 single-chain CAR, anti-PRAME single-chain CAR, anti-TSPAN10 single-chain CAR, anti-ROR1 single-chain CAR, anti-GD3 single-chain CAR, anti-CT83 single-chain CAR and mesothelin single-chain CAR;

- said CAR being expressed in an immune cell has one of the polypeptide structure selected from V1, V3 or V5, as illustrated in Figure 24;
- said structure comprising:
    - an extra cellular ligand binding-domain comprising VH and VL from a monoclonal antibody selected in the group consisting of anti-CD123 mAb, anti-CS1 mAb, anti-CD38 mAb, anti-CLL1 mAb, anti-Hsp70 mAb, anti-EGFRvIII mAb, anti-BCMA mAb, anti-CD33 mAb, anti-FLT3 mAb, anti-CD70 mAb, anti-WT1 mAb, anti-MUC16 mAb, anti-PRAME mAb, anti-TSPAN10 mAb, anti-ROR1 mAb, anti-GD3 mAb, anti-CT83 mAb and anti-mesothelin mAb respectively;
    - a hinge chosen in the group consisting of CD8alpha, FcERIIIgamma and IgG1;
    - a CD8α transmembrane domain;
    - a cytoplasmic domain including a CD3 zeta signaling domain and;
    - a 4-1BB co-stimulatory domain.

All the other components chosen in the architecture of the CAR including transmembrane domain (i.e CD8αTM), co-stimulatory domain (ie. 4-1BB), hinge (CD8alpha, FcERIIIgamma, IgG1), cytoplasmic signaling domain (ITAM CD3zeta) may be those already described in the above WO2015140268 and WO2015121454 applications.

As examples, VH and VL may be those described in the applications WO2015140268 for anti-CD123, WO2015121454 for anti-CS1 and anti-CD38.

Multi-chain Chimeric Antigen Receptor (CAR)

In another embodiment, the invention relates to a multi-chain chimeric antigen receptor (CAR) particularly adapted to the production and expansion of engineered immune cells such as T-cells of the present invention. The multi-chain CAR comprising at least two of the following components:

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

b) one polypeptide comprising a part of N- and C- terminal cytoplasmic tail and the transmembrane domain of FcεRI beta chain and/or

c) two polypeptides comprising each a part of intracytoplasmic tail and the transmembrane domain of FcεRI gamma chain, whereby different polypeptides multimerize together spontaneously to form dimeric, trimeric or tetrameric CAR.

CAR of the present invention can also be “multi-chain CARs” as previously mentioned, which means that the extracellular binding domain and the signaling domains are preferably located on different polypeptide chains, whereas co-stimulatory domains may be located on the same or a third polypeptide. Such multi-chain CARs can be derived from FcεRI (Ravetch et al, 1989), by replacing the high affinity IgE binding domain of FcεRI alpha chain by an extracellular ligand-binding domain such as scFv, whereas the N and/or C-termini tails of FcεRI beta and/or gamma chains are fused to signal transducing domains and co-stimulatory domains respectively. The extracellular ligand binding domain has the role of redirecting T-cell specificity towards cell targets, while the signal transducing domains activate or reduce the immune cell response. The fact that the different polypeptides derive from the alpha, beta and gamma polypeptides from FcεRI are transmembrane polypeptides sitting in juxtamembrane position provides a more flexible architecture to CARs, improving specificity towards the targeted molecule and reducing background activation of immune cells .Multi-chain architectures are more particularly disclosed in WO2014039523.

In another embodiment, said CAR which are expressed in the immune cell such as described earlier is chosen in the group consisting of anti-CD123 multi-chain CAR, anti-CS1 multi-chain CAR, anti-CD38 multi-chain CAR, anti-CLL1 multi-chain CAR or anti-Hsp70 multi-chain CAR.

In another preferred embodiment, said above CAR is multi-chain CAR chosen in the group consisting of anti-CD123 multi-chain CAR, anti-CS1 multi-chain CAR, anti-CD38 multi-chain CAR, anti-CLL1 multi-chain CAR, anti-Hsp70 multi-chain CAR, anti-EGFRvIII multi-chain CAR, anti-BCMA multi-chain CAR, anti-CD33 multi-chain CAR, anti-FLT3 multi-chain CAR, anti-CD70 multi-chain CAR, anti-WT1 multi-chain CAR, anti-MUC16 multi-chain CAR, anti-PRAME multi-chain CAR, anti-TSPAN10 multi-chain CAR, anti-ROR1 multi-chain CAR, anti-GD3 multi-chain CAR, anti-CT83 multi-chain CAR and mesothelin multi-chain CAR.

Such multi-chain CAR architectures are disclosed in WO2014/039523, especially in Figures 2 to 4, and from page 14 to 21, which are herein incorporated by reference.

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 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.

Are also comprised in the scope of the present invention, polynucleotides, vectors encoding the above described multi-chain CAR according to the invention.

In a particular embodiment, the invention relates to a method of preparing immune cells such as T-cells for immunotherapy comprising introducing into said T-cells the different polypeptides composing said multi-chain CAR and expanding said cells.

The present invention also relates isolated cells or cell lines susceptible to be obtained by said method to engineer cells. In particular said isolated cell comprises exogenous polynucleotide sequences encoding polypeptides composing said multi-chain CAR.

Bispecific antibodies

According to a further embodiment, engineered immune cells such as 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 pTalpha or functional variants thereof, rare cutting endonuclease, TALE-nuclease, CAR or multi-chain CAR optionally with DNA-end processing enzyme or exogenous nucleic acid into a cell.

As non-limiting example, rare cutting endonucleases, TALE-nucleases, gene encoding non-endogenous immunosuppressive polypeptide, CAR or multi-chain 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 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*

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.

The method of transforming T cell may comprise 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.

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, an exogenous nucleic acid, one additional catalytic domain.

Activation and expansion of immune cells

Whether prior to or after genetic modification of the immune cells such as T cells, the immune 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 a surface having attached thereto an agent that stimulates a CD3 TCR complex associated signal and a ligand that stimulates a co-stimulatory molecule on the surface of the T cells.

In particular, immune cells such as 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 immune cells can be contacted with an anti-CD3 antibody and an anti-CD28 antibody, under conditions appropriate for stimulating proliferation of the immune 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. Cell surface proteins may be ligated by allowing paramagnetic beads to which anti-CD3 and anti-CD28 are attached (3x28 beads) to contact the T cells. In one embodiment the cells (for example, 4 to 10 T cells) and beads (for example, DYNABEADS® M-450 CD3/CD28 T paramagnetic beads at a ratio of 1:1) are combined in a buffer, preferably PBS (without divalent cations such as, calcium and magnesium). Again, those of ordinary skill in the art can readily appreciate any cell concentration may be used. The mixture may be

cultured for several hours (about 3 hours) to about 14 days or any hourly integer value in between. In another embodiment, the mixture may be cultured for 21 days. 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). Immune cells such as 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.

Engineered immune cells and their interaction with host immune cells

In the scope of the present invention is also encompassed an isolated immune cell obtained according to any one of the methods previously described. Said immune cell according to the present invention can be derived from a hematopoietic 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.

According to an embodiment, said immune cells are hematopoietic cells, and more preferably primary cells.

According to a preferred embodiment, the engineered allogeneic immune cells, after contacting at least one non-endogenous immunosuppressive polypeptide, do not induce specifically the inhibition of T regulatory cells.

According to a more preferred embodiment, the engineered allogeneic immune cells, after contacting at least one non-endogenous immunosuppressive polypeptide, induce specifically an inhibition of CD8+ T cells.

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. Immune cells such as T 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. Any number of immune 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. 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 immune cell such as 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 endogenous gene encoding a polypeptide involved in the self and non-self antigen recognition, such as TCR, MHC class of class I component, b-2 microglobulin (B2M), TAP1 and large multifunctional protease 2. Furthermore, said engineered allogeneic immune cells are contacted with at least one non-endogenous immunosuppressive polypeptide, either by expressing at least one secreted non-endogenous immunosuppressive polypeptide or by incubating said immune cells with at least one non-endogenous immunosuppressive polypeptide.

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 or a viral infection 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 such as T-cell obtainable by any one of the methods previously described;

(b) Administrating said transformed immune cell such as T-cells to said patient,

On one embodiment, said immune cell such as T cells of the invention can undergo robust *in vivo* immune cell such as 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 immune cells such as 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 immune 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 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, mycophenolic 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.

Other definitions

- 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 "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 2,7 and 11 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 2 and 7.

- 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: 6

- 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 non limiting 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, 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" 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.

- "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.

-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

The inventors propose to explore three different strategies to prevent allogeneic CAR T cells depletion via HvG (Figure 9). As presented in Example 1, the first one consists of expressing PD-L1 at the surface of CAR T cell. The presence of such antigen is likely to inhibit host T cells via PD1/PD-L1 inhibition pathway and thus decrease their cytolytic activity toward CAR T cell (Figure 10A). Without such decoy system and after a certain length of time, host T cells are expected to attack and deplete allogeneic CAR T cells (Figure 10B). The second strategy consists of engineering CAR T cells to make them excrete CTLA4 Ig, a chimeric construction made out of CTLA4 protein fused the constant region of IgG. Release of CTLA4 Ig in the extracellular medium is likely to bind to CD86/CD80 exposed at the surface of antigen presenting cells (APC) and prevent them to activate host T cells via CD28/CD80 or CD28/CD86 interactions. The HvG reaction, involving host APC and host T cells interaction/activation, is displayed in Figure 11A and the prevention of CAR T cell rejection via excretion of CTLA4 Ig is displayed in Figure 11B. The third strategy, consisting of combining the two aforementioned strategies, could also been used to prevent HvG reaction and allow CAR T cells to proliferate in the setting of an allogeneic cell adoptive transfer.

In the following Examples 3 to 7, to prolong their survival and enhance their therapeutic activity, the inventors describe a method to prevent NK-cell mediated rejection of therapeutic allogeneic T cells by engineering the allogenic T cells through the inactivation of the B2M gene using specific TALEN, combined to either: i) the expression of a chimeric single chain molecule composed of UL18 and β2-m (B2M-UL18) or ii) the secretion of NKG2D ligands. The particularity resides in applying to primary T cells a mechanism occuring normally in tumor cells or virally infected cells. Thus, the mechanism of action is potentially different: in tumor cells, shedding NKG2D ligands leads to their decreased presence at the surface whereas in engineered cells, secreted the NKG2D ligand(s) would serve as a decoy for several other NKG2D ligands potentially still present at the T cell surface.

In the following Examples 8 to 11, are presented a method where allogenic CAR T cells are engineered in order to express immunosuppressive polypeptides from viral proteins (ISU or FP as membrane-bound or secreted peptides), allowing inhibition of patient T cells and therefore allowing efficient persistence of allogenic CAR T cells infused into patient.

GENERAL METHODS

Primary T-cell cultures

T cells were purified from Buffy coat samples provided by EFS (Etablissement Français du Sang, Paris, France) using Ficoll gradient density medium. The PBMC layer was recovered and T cells

were purified using a commercially available T-cell enrichment kit. Purified T cells were activated in X-Vivo™-15 medium (Lonza) supplemented with 20ng/mL Human IL-2, 5% Human, and Dynabeads Human T activator CD3/CD28 at a bead:cell ratio 1:1 (Life Technologies).

scCAR mRNA transfection

Transfections were done at Day 4 or Day 11 after T-cell purification and activation. 5 millions of cells were transfected with 15µg of mRNA encoding the different scCAR constructs. scCAR mRNAs were produced using T7 mRNA polymerase transfections done using Cytopulse technology, by applying two 0.1 mS pulses at 3000V/cm followed by four 0.2 mS pulses at 325V/cm in 0.4cm gap cuvettes in a final volume of 200µl of "Cytoporation buffer T" (BTX Harvard Apparatus). Cells were immediately diluted in X-Vivo™-15 media and incubated at 37°C with 5% CO_2. IL-2 was added 2h after electroporation at 20ng/mL.

Cytotoxicity assay

T-cells were incubated in 96-well plates (100,000 cells/well), together with 10,000 target cells (expressing CD123) and 10,000 control (CD123neg) cells in the same well. Target and control cells were labelled with fluorescent intracellular dyes (CFSE or Cell Trace Violet) before co-culturing them with CD123 CAR+ T-cells. The co-cultures were incubated for 4 hours at 37°C with 5% CO_2. After this incubation period, cells were labelled with a fixable viability dye and analyzed by flow cytometry. Viability of each cellular population (target cells or CD123 control cells) was determined and the % of specific cell lysis was calculated. Cytotoxicity assays were carried out 48h after mRNA transfection.

T-cell transduction

Transduction of T-cells with recombinant lentiviral vectors expression the scCAR was carried out three days after T-cell purification/activation. scCAR detection at the surface of T-cells was done using a recombinant protein consisting on the fusion of the extracellular domain of the human CD123 protein, together with a murine IgG1 Fc fragment. Binding of this protein to the scCAR molecule was detected with a fluorochrome-conjugated secondary antibody targeting the mouse Fc portion of the protein, and analyzed by flow cytometry.

Example 1. Transgenic expression of PD-L1 at the surface of primary T cells and CAR T cells

In these experiments, it is shown that - human activated T cells, transfected or transduced with PD-L1 encoding vectors (mRNA or lentivirus) express detectable levels of PD-L1 at the cell surface.

Expression of PD-L1

This example describes expression of PD-L1 at the surface of T cells or CAR T cells along with the impact of such expression on their cytolytic activity toward tumor cells.To express PD-L1 at the surface of primary T cells, primary T cells were first purified from buffy-coat samples, activated transduced by a lentiviral particle containing an anti-CD19 CAR tool (pCLS23856, SEQ ID NO 77) and transfected according to the procedure described in Galetto R et al. (2014) Molecular Therapy - Methods & Clinical Development 1, Article number: 14021 doi:10.1038/mtm.2014.2. Briefly regarding transduction, 2 days post activation by Dynabeads human T activator CD3/CD28, T cells were incubated with lentiviral particles containing anti-CD19 CAR tool at 5 MOI.

Transfection of mRNA

Regarding transfection, 5 days after their activation, 5 million of CAR T cells or T cells were transfected with 20 µg of mRNA encoding PD-L1 (pCLS27069, SEQ ID NO 18). Transfection was performed using Agilpulse technology, by applying two 0.1 mS pulses at 3,000 V/cm followed by four 0.2 mS pulses at 325 V/cm in 0.4 cm gap cuvettes and a final volume of 200 µl of Cytoporation buffer T (BTX Harvard Apparatus, Holliston, Massachusetts). Cells were then immediately diluted in X-Vivo-15 media supplemented by 20 ng/ml IL-2 (final concentration) and 5% human serum AB. Transfected T cells were eventually diluted at 1×10^6/ml and kept in culture at 37°C in the presence of 5% CO_2 and 20 ng/ml IL-2 (final concentration) and 5% human AB serum for further characterization. One day post transfection, CAR T cells were recovered to characterize the expression of PD-L1 at their cell surface and to determine the impact of such expression on their specific cytolytic activity toward relevant tumor cells targeted by their anti CD19 tool CAR.

Our results showed that PD-L1 is expressed in CAR T cell transfected with mRNA encoding PD-L1 (>90% of cells express PD-L1, Figure 12) whereas no expression could be detected in mock transfected T cells or CAR T cells. Similar results were obtained with untransduced T cells indicating that PD-L1 is successfully expressed on CAR T cells and T cells (Figure 12).

Transfection with lentivirus vector (LV)

A LV vector containing the PD-L1 cDNA was produced. Primary T cells were first purified from buffy-coat samples, activated, transduced either with a lentiviral particle containing PD-L1

(pCLS27062 of SEQ ID NO.18) at a MOI of 5. Three days post transduction, transduced T cells were recovered to characterize the expression of PD-L1 at their cell surface.

The results showed that PD-L1 is expressed in T cell transduced with LV vector encoding PD-L1 alone (>70% of cells express PD-L1, result not shown) whereas no expression could be detected in untransduced T cells.

Specific cell lysis

Regarding the specific cell lysis activity of CAR T cells toward relevant tumor cells (Daudi) determined using the flow-based assay described in Zhao, Y. et al. (2010) Cancer *Res* **70**, 9053-9061, our result showed that the re-expression of PD-L1 at the surface of CAR T cell does not markedly affect their activity (Figure 13). This result is reproducible with CAR T cells engineered out of different blood donor (Figure 13, see results obtained with Mock CAR T cells B and PD-L1 CAR T cells B).

Example 2. Transgenic expression and excretion of Abatacept and Belatacept (CTLA4 Ig) by primary T cells and CAR T cells

Transfection of mRNA and with lentivirus vector (LV)

Abatacept and belatacept (marketed as Orencia and Nulojix respectively) are fusion proteins composed of the Fc region of the immunoglobulin IgG1 fused to the extracellular domain of CTLA-4.

This example describes the expression and excretion of CTLA4a Ig and CTLA4b Ig (Abatacept, or Belatacept pCLS27068 SEQ ID NO 3 and pCLS27066, SEQ ID NO 4 respectively) and by LV containing the CTLA4Ig (pCLS27064 of SEQ ID NO.16) at a MOI of 5 by primary T cells in the culture media. Abatacept is described in Moreland L et al; (2006) Nature Reviews Drug Discovery 5, 185-186.Belatacept is described by Larsen CP et al. (2005) "*Rational development of LEA29Y (belatacept), a high-affinity variant of CTLA4-Ig with potent immunosuppressive properties*". Am J Transplant. 5(3):443-53.

CTLA4 a/b Ig expression

To express CTLA4 Ig by primary T cells, primary T cells were first purified from buffy-coat samples, activated transduced by a lentiviral particle containing an anti-CD19 CAR tool (pCLS23856, SEQ ID NO 77) and transfected according to the procedure described as in example 1. Regarding transfection, 5 days after their activation, 5 million of CAR T cells or T cells were transfected with 20 µg of mRNA encoding CTLA4a or b Ig (Abatacept, or Belatacept pCLS27068 SEQ ID NO:16 and pCLS27066, SEQ ID NO:17 respectively) and cultured according to the protocol described in example 1. One day post transfection, CAR T cells were recovered to characterize their ability to excrete CTLA4 Ig in the culture media via ELISA and to determine the impact of such expression/excretion on

their specific cytolytic activity toward relevant and non-relevant tumor cells targeted (Daudi and K562 respectively).

Our results showed that transfection of primary T cells by mRNA encoding CTLA4 a or b Ig resulted in the appearance of the corresponding fusion proteins in the culture media. The quantity of CTLA4 Ig in the culture media was approximatively proportional to the amount of mRNA transfected with a maximum of CTLA4a Ig and CTLA4b Ig of 2.1 and 3.1 pg/mL respectively in our experimental condition. As expected, the culture media of mock transfected T cell did not contain any detectable CTLA4 Ig protein. These results indicated that CTLA4a Ig and CTLA4b Ig were successfully expressed by primary T cells and excreted in the culture media.

Specific cytolytic activity

To study the impact of CTLA4 Ig on the activity of CAR T cells, their cytolytic activity toward relevant and non-relevant tumor cell lines was determined using a flow based assay described in Example 1. Our results showed that the Mock transfected CAR T cell and CTLA4 Ig CAR T cells displayed significant cytolytic activity toward Daudi cells. Altogether, these results indicated that primary T cells successfully expressed and excreted CTLA4 Ig while retaining their antitumor activity.

Example 3. Transgenic expression and excretion of CTLA4 Ig and CTLA4 Ig /PD-L1 ligand by primary T cells

In this experiment, in T cells co-transduced with LV vectors encoding PD-L1 and CTLA4Ig as described before. Figure 17 shows the level of CTLA4 Ig secreted in the supernatant by T cell transduced with LV vector encoding CTLA4Ig alone (mean= 250pg/µl) or in T cells co transduced with LV vectors encoding PD-L1 and CTLA4Ig (mean=270 pg/µl) whereas no expression could be detected in PD-L1-transduced T cells. The results showed that PD-L1 is expressed in T cell transduced with LV vector encoding PD-L1 alone (>70% of cells express PD-L1, Figure 17) or in T cells co transduced with LV vectors encoding PD-L1 and CTLA4Ig (59%) whereas no expression could be detected in untransduced T cells or CTLA4Ig-transduced T cells.

Example 4: Mixed reaction assay (MLR) to test allogeneic T cells response

Rationale and protocol of the experiment

In order to test whether overexpression of PD-L1 and/or CTLA4Ig by CAR T cells would have an impact on the host immune system, it was set up an in vitro assay in which naïve PBMCs from donor 1 are co-cultured with T cell from an HLA-mismatched donor 2. Briefly, PBMCs (donor 1) are labeled with CFSE and mixed with unlabeled, mitomycin-treated or irradiated engineered T cells

(donor 2) meaning that they cannot proliferate. After a period of 6 days, flow cytometry analysis is performed with the following gating strategy: FSC/SSC -> viable cells -> CD3+ (T cells from donor 1 PBMCs) -> CFSE. Decrease of CFSE staining is indicative of cell division and thus of allogeneic response of donor 1's T cells due to the presence of HLA mismatched donor 2's T cells.

A series of experiments has been set as follows from left to right in Figure 18:

- (a) PBMCs from donor 1 without any treatment have been cultured alone,
- (b) PBMCs from donor 1, which have been submitted to a treatment with increasing concentration of PHA (PhytoHemAgglutinin), a T cell mitogen) are cultured alone;
- (c) PBMCs from donor 1 are co-cultured with untransduced T cells from donor 2
- (d) PBMCs from donor 1 are co-cultured with PD-L1 transduced T cells from donor 2;
- (e) PBMCs from donor 1 are co-cultured with CTLA4Ig transduced T cells from donor 2;
- (f) PBMCs from donor 1 are co-cultured with PD-L1 and CTLA4Ig co- transduced T cells from donor 2.

Results

From figure 18, it appears that CD3+ T cells do not proliferate when not co-cultured (a) or in the presence au autologous T cells (c). As a control (b), PBMCs from donor 1, as expected CFSE positive population is decreasing as PHA concentration increases. CD3+ T cells do proliferate (disappearance of CFSE positive population) in the presence of allogeneic T cells (c). When allogeneic T cells are engineered to express PD-L1 (d), CTLA4Ig (e) or both (f), it is observed that responder T cells keep a bright CFSE staining, leading to the conclusion that the expression PD-L1 and/or CTLA4Ig by engineered T cells inhibit the responder proliferation. Thus, the results obtained in Figure 18 show that engineered T cells expressing PD-L1, CTLA4Ig or both are less prone to stimulate allogeneic T cells response in an in vitro mixed lymphocytes reaction (MLR) assay. Moreover, the results show a cumulative effect when both PD-L1, CTLA4Ig are expressed.

Example 5: Cytotoxicity assay testing anti-CD123 CAR T cells expressing PD-L1 and/or CTLA4Ig for their capacity to kill MOLM13 target cells

This experiment is aimed to test T cells expressing PD-L1 and/or CTLA4Ig for their capacity to kill specific target cells through the expression of a CAR molecule.

T cells that have transduced with PD-L1 LV, CTLA4IG or both, have been transfected with 20µg of mRNA encoding anti-CD123 CAR (SEQ ID NO.69) After a period of 2 days, the cytotoxic assay is performed using MOLM13 cell line as specific target cells.

Results from cytotoxicity assays, Figure 19 and Figure 20 show that engineered T cells expressing PD-L1, CTLA4Ig or both, and further engineered to express a CD123 CAR molecule, sustain their capacity to kill specific target . Furthermore, these data suggest that expression of PD-L1, CTLA4Ig or both, increase their intrinsic cytolytic activity.

Example 6: In vivo experiments

The aim of these experiments is to verify that modified T cells are still able to eradicate cognate tumor cells in vivo. Thus, an in vivo experiment has been conducted to investigate whether the expression of PD-L1, CTLA4Ig or both impact the CAR T cells anti-tumor activity. The protocol outline is shown in Figure 21.

Activated T cells were obtained for the following groups of treatment:

- Non-transduced T cells;
- T cells transduced with CD123 CAR lentivirus (LV);
- T cells transduced with CD123 CAR LV and PD-L1 LV;
- T cells transduced with CD123 CAR LV, CTLA4Ig LV;
- T cells transduced with CD123 CAR LV, PD-L1 LV and CTLA4Ig LV.

After 2 days of transduction, T cells were amplified in G-Rex for in vivo experiments. After 19 days cells were recovered and counted.

Figure 22 shows that engineered T cells expressing PD-L1, CTLA4Ig or both, and further engineered to express a CD123 CAR molecule sustain similar proliferative capacity as compared to CAR CD123 engineered T cells.

T cells thus obtained were injected in NOG mice for in vivo experiment.

Anti-tumor mouse model

Immunodeficient NOG mice were intravenously (iv) injected with (CD123 expressing_MOLM13-Luciferase cells as an AML xenograft mouse model. Mice were then iv injected (either 2 or 7 days after injection of the tumor cell line) with different doses of CD123 CAR+ T-cells to be tested, or with T-cells that were not transduced with the CD123 CAR lentiviral vector. Bioluminescent signals were determined at the day of T-cell injection (D0), at D7 and D14 after T-cell injection in order to follow tumoral progression on the different animals.

Bioluminescence analysis results from Figure 23A and Figure 23B indicate that all groups of mice injected with engineered CAR T cells eradicate efficiently the tumor as compared to control group, and a clear anti-tumor activity of engineered CAR T cells in vivo.

Example 7 Efficient B2M gene knock out using specific B2M TALEN.

Specific TALEN targeting a sequence (SEQ ID N°1) within the first coding exon of the B2M gene (GenBank accession number NC_000015) has been produced (Left DNA binding domain RVDs: NN-NN-HD-HD-NG-NG-NI-NN-HD-NG-NN-NG-NN-HD-NG-NG with SEQ ID NO: 2, and Right DNA binding domain RVDs: NI-NN-HD-HD-NG-HD-HD-NI-NN-NN-HD-HD-NI-NN-NI-NG with SEQ ID NO: 3).

To test the ability of this B2M specific TALEN to promote error-prone NHEJ events at the B2M locus, 2 or 10 µg of mRNA encoding TALEN were electroporated in Primary T cells using Pulse Agile technology according to the manufacturer protocol. Three days post transfection, cells were recovered and labeled with a specific β2-microglobulin antibody coupled to the PhycoErythrin fluorochrome. Cells are then analyzed by flow cytometry for viability and β2-m expression. The results are shown on Figure 16. On the top panel, nearly 100% of untransfected T cells express β2-m (top right panel). Transfection of T cells with the specific B2M TALEN reduces dramatically β2-m expression since 38% (middle right) and 80 % of T cells (bottom right panel) become beta2-m negative when transfected with 2 µg or 10 µg of TALEN mRNA respectively. These data indicates that B2M knock-out in T cells can be achieved with high efficacy.

Example 8: Production and expression of the single chain molecule B2M-UL18 in T cells.

HCMV UL18 encodes a type I transmembrane glycoprotein that shares a high level of AA sequence identity with MHC Class I molecules that associates with beta2-m and binds endogenous

peptides. Since our goal is to express this molecule in T cells where B2M gene has been invalidated, our strategy is to produce a chimeric molecule where beta2-m and UL18 is fused as a single chain polypeptide. SEQ ID N°39 shows the amino-acid sequence of the chimeric protein. Lentiviral particles containing the chimeric B2M-UL18 are transduced into T cells. Expression of transgene is monitored by FACS analysis using a beta2-m antibody. The results from this experiment aim to show that a B2M-UL18 chimeric protein is efficiently expressed in T cells.

Example 9: Production and expression of NKG2D ligands in T cells

NKG2D natural ligands are transmembrane or GPI-anchored proteins. In order to achieve secretion of these molecules by T cells, the extra-cellular domains of NKG2D ligands have been fused in their N-terminus to a secretory peptide form . Amino-acid sequences of secreted chimeric NKG2D ligands are listed below (SEQ ID NO:40 to SEQ ID NO:47). Lentiviral particles containing the chimeric NKG2D ligands are transduced into T cells. Expression of transgene in culture supernatant is monitored by Western Blot analysis using specific antibodies. The results from this experiment aim to show that chimeric NKG2D ligand proteins are efficiently expressed in T cells.

Example 10: beta2-M deficient CAR T cells are not recognized by allogenic T cells

PBMCs from healthy donor A is co-cultured with irradiated or mitomycin-treated engineered beta2-m deficient T cells from donor B. As a control, PBMCs from healthy donor A is co-cultured with irradiated or mitomycin-treated engineered beta2-m positive T cells from donor B. 7 days later, cells proliferation from donor A is measured by XTT colorimetric assay or by CFSE dilution (FACS analysis). Although cell proliferation is observed in control, no or limited cell proliferation is observed when engineered T cells do not express beta2-m. The results from this experiment aim to show that alloreactive T cells are not able to recognize and proliferate against beta2-m deficient T cells.

Example 11: Efficient inhibition of NK mediated engineered T cells lysis

NK cells are purified from healthy donor A PBMCs. As targets, engineered T cells from healthy donor B are produced and listed below. a) engineered T cells (negative control), b) beta2-m deficient engineered T cells (positive control), c) beta2-m deficient engineered T cells expressing B2M-UL18 (SEQ ID N° 39), d-k) beta2-m deficient engineered T cells expressing respectively SP-MICAed (SEQ ID

N° 40), SP-MICBed (SEQ ID No 41), SP-ULBP1ed (SEQ ID N° 42), SP-ULBP2ed (SEQ ID N° 43), SP-ULBP3ed (SEQ ID N°44), SP-N2DL4ed (SEQ ID N° 45), SP-RET1Ged (SEQ ID N°46), SP-RAETILed (SEQ ID N°47). Cytotoxicity mediated by NK cells was determined by a CFSE labeling assay. Target cells were labeled with CFSE, washed in PBS, mixed with NK cells at various E:T cell ratios and incubated for 4h at 37°C. Cells are then analysed by flow cytometry and percentages of CFSE positive engineered T cells are measured, indicating the survival of engineered T cells in the presence of NK cells. It is intended that although NK mediated cell lysis is observed in the positive control (beta2-m deficient engineered T cells), no or limited NK mediated cell lysis is observed when beta2-m deficient engineered T cells engineered T cells express B2M-UL18 (SEQ ID N° 39) or secreted NKG2D ligands (SP-MICAed (SEQ ID N° 40), SP-MICBed (SEQ ID N° 41), SP-ULBP1ed (SEQ ID N°42), SP-ULBP2ed (SEQ ID N° 43), SP-ULBP3ed (SEQ ID N° 44), SP-N2DL4ed (SEQ ID N° 45), SP-RET1Ged (SEQ ID N° 46), SP-RAETILed (SEQ ID N° 47)). The results from this experiment aim to show that allogenic NK cells cytotoxicity activity is impaired when chimeric molecules, express in engineered T cells, act as decoy either for inhibitory signal receptor (B2M-UL18) or for stimulatory signal receptor (NKG2D ligands).

Example 12: Expression of ISU in engineered T cells

Lentiviral particles bearing either the envelope protein from Moloney Murine Leukemia Virus (MMLV) (SEQ ID N°78), a transmembrane truncated form of the envelope protein from MMLV (SEQ ID N°79) or secreted 14-mer ISU peptides (6 variants from HIV-1 virus SEQ ID N°19 to 24; 6 variants from HIV-2 virus SEQ ID N°25 to 30; from SIV, MoMuLV, HTLV-1, MPMV, Syncitin 1, Syncitin 2, HERV-K and FELV virus with respectively SEQ ID N°32, 33, 34, 35, 36, 37, and 38) are transduced into T cells. Expression of membrane bound transgene is monitored by FACS analysis whereas expression of secreted ISU peptide is monitored in cell culture supernatant by western blot. The results from this experiment aim to show that both forms of ISU are efficiently expressed in T cells.

Example 13: Expression of FP peptides in engineered T cells

Lentiviral particles bearing secreted 9-mer FP polypeptides (1 from HIV-1 virus and 2 from artificial sequence with respective SEQ ID N°48 and 49-50) are transduced into T cells. Expression of secreted FP peptides is monitored in cell culture supernatant by western blot. The results from this experiment aim to show that secreted FP peptides are efficiently expressed in T cells.

Example 14: Efficient inhibition of T cells proliferation towards engineered T cells expressing ISU

PBMCs from healthy donor A is co-cultured with irradiated or mitomycin-treated engineered T cells from donor B, expressing ISU. As a control, PBMCs from healthy donor A is co-cultured with irradiated or mitomycin-treated engineered T cells from donor B that do not express ISU. 7 days later, cells proliferation from donor A is measured by XTT colorimetric assay or by CFSE dilution (FACS analysis). Although cell proliferation is observed in control, no or limited cell proliferation is observed when engineered T cells express membrane bound or secreted ISU. The results from this experiment aim to show that alloreactive T cells proliferation is inhibited when engineered T cells express ISU.

Example 15: Efficient inhibition of T cells proliferation towards engineered T cells expressing FP

PBMCs from healthy donor A is co-cultured with irradiated or mitomycin-treated engineered T cells from donor B, expressing FP. As a control, PBMCs from healthy donor A is co-cultured with irradiated or mitomycin-treated engineered T cells from donor B that do not express FP. 7 days later, cells proliferation from donor A is measured by XTT colorimetric assay or by CFSE dilution (FACS analysis). Although cell proliferation is observed in control, no or limited cell proliferation is observed when engineered T cells express secreted FP. The results from this experiment aim to show that alloreactive T cells proliferation is inhibited when engineered T cells express FP.

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CLAIMS

1) Method to increase the persistence and/or the engraftment of allogeneic immune cells in presence of host immune cells, comprising:

a) providing allogeneic cells;

b) modifying said cells by inactivating at least one endogenous gene encoding a polypeptide involved in the response against self and non-self antigen recognition;

and;

c) contacting said host immune cells with at least one non-endogenous immunosuppressive polypeptide which has the effect to prevent them from interacting with allogeneic immune cells.

2) The method according to claim 1, wherein said polypeptide in step b) is chosen amongst TCR, MHC class of class I component, b-2 microglobulin (B2M), TAP1 and large multifunctional protease 2.

3) Method according to claim 1 or claim 2, wherein said immunosuppressive polypeptide in step c) is present under a membrane-bound form and/or under a secreted form.

4) Method according to anyone of claim 1 to 3, wherein the step c) is performed by the expression in said immune cells at least one non-endogenous polynucleotide encoding for one non-endogenous immunosuppressive polypeptide bound to the membrane surface of said immune cells.

5) Method according to anyone of claim 1 to 4, wherein said one non-endogenous immunosuppressive polypeptide bound to the membrane surface of said immune cells is a PD-L1 ligand.

6) The method according to claim 5, wherein the nucleic acid molecule encoding PD-L1 ligand under a membrane-bound form to be expressed shares at least 80%, preferably 90% and more preferably 95% of identity with SEQ ID NO:18.

7) Method of any one of claim 1 to 3, wherein said immunosuppressive polypeptide is present under a secreted form.

8) The method according to any one of claim 1 to 3 or claim 7, wherein the step c) is performed by the expression in said immune cells at least one non-endogenous polynucleotide encoding for one non-endogenous immunosuppressive polypeptide under a secreted form in said immune cells.

9) The method according to claim 8, wherein the nucleic acid molecule encoding CTLA-4 immunoglobulins to be expressed shares at least 80%, preferably 90% and more preferably 95% of identity with SEQ ID NO: 16-17.

10) The method according to any one of claim 1 to 9, wherein step c) is performed by contacting said host immune cells with both non-endogenous immunosuppressive polypeptide PD-L1 ligand and CTLA-4 immunoglobulins.

11) The method according to claim 10 wherein step c) is performed by the step c) is performed by the expression in said immune cells of both non-endogenous immunosuppressive polypeptide PD-L1 ligand and CTLA-4 immunoglobulins.

12) The method according to anyone of claim 1 to 3, wherein said secretion of at least one non-endogenous immunosuppressive polypeptide is PD-L1 ligand under a secreted form.

13) The method according to anyone of claim 1 to 12, wherein the nucleic acid molecules encoding PD-L1 ligand under a membrane-bound form and CTLA-4 immunoglobulins to be expressed in said allogeneic immune cells shares at least 80%, preferably 90% and more preferably 95% of identity with respectively SEQ ID NO:18 and SEQ ID NO: 16-17.

14) The method according to claim 1 or claim 2, wherein said immune cells are primary cells.

15) The method according to anyone of claim 1 to 14, wherein the step c) contains additionally an inactivation of the expression of a PD-1 gene.

16) The method according to anyone of claim 1 to 14, wherein the step c) is performed by the expression in said immune cells at least one non-endogenous polynucleotide encoding for PD-L1 ligand under a membrane-bound form, and a further modification of said allogeneic cells is performed by an inactivation of the expression of PD-1 gene.

17) The method according to anyone of claim 1 to 14, wherein the step c) is performed by the expression in said immune cells at least one non-endogenous polynucleotide directing the secretion of CTLA4 Ig, and a further modification of said allogeneic cells is performed by an inactivation of the expression of PD-1 gene.

18) The method according to anyone of claim 1 to 14, wherein step c) is performed by the step c) is performed by the expression in said immune cells of both non-endogenous immunosuppressive polypeptide PD-L1 ligand and CTLA-4 immunoglobulins, and a further modification of said allogeneic immune cells is performed by an inactivation of the expression of PD-1 gene.

19) The method according to anyone of claim 15 to 18, wherein inactivation of PD-1 gene is performed by using a polynucleotide encoding TALE-nucleases of SEQ ID N°11-12 and 13-14.

20) The method according to anyone of claim 1 to 4, wherein said polypeptide in step c) is chosen amongst PD-L1, CTLA-4, viral MHC homolog, NKG2D ligand, viral *env* immune suppressive domain (ISU) or the viral FP protein.

21) The method according to anyone of claim 1 to 19, wherein step b) is performed by the inactivation of the B2M and step c) is performed by the expression of PD-L1 ligand in said allogeneic immune cells.

22) The method according to any one of claim 1 to 19, wherein step b) is performed by the inactivation of the TCR and step c) is performed by the expression of PD-L1 ligand by said allogeneic immune cells.

23) The method according to anyone of claim 1 to 19, wherein step b) is performed by the inactivation of the B2M and step c) is performed by the expression of NKG2G ligand by said allogeneic immune cells.

24) The method according to anyone of claim 1 to 19, wherein step b) is performed by the inactivation of the B2M and step c) is performed by the expression of viral MHC homolog UL18 protein by said allogeneic immune cells.

25) The method according to anyone of claim 1 to 19, wherein step b) is performed by the inactivation of the TCR and step c) is performed by the expression of viral MHC homolog UL18 protein by said allogeneic immune cells.

26) The method according to any one of claim 1 to 25, wherein the step c) is performed by the incubation of said immune in at least one non-endogenous immunosuppressive polypeptide.

27) The method according to claim 26, wherein said non-endogenous immunosuppressive polypeptide is anti-CD80 or anti-CD86 mAbs.

28) The method according to anyone of claim 1 to 27, wherein gene inactivation in step b) is performed by using a TAL-nuclease, meganuclease, zing-finger nuclease (ZFN), or RNA guided endonuclease.

29) The method to claim 28, wherein gene inactivation in step b) is performed using a TAL-nuclease.

30) The method according to anyone of claim 2, 22 or 25, wherein gene inactivation in step b) is performed by using a nucleic acid molecule that inhibits the expression of a gene encoding TCR.

31) The method according to claim 30, wherein inactivation of TCR gene is performed by using the TALE-nucleases of SEQ ID N°52-53, 55-56, 62-63 and 65-66.

32) The method according to anyone of claims 2 or 21-24, wherein inactivation gene in step b) is performed by using a nucleic acid molecule that inhibits the expression of a gene encoding B2M.

33) The method according to claim 32, wherein inactivation of B2M gene is performed by using the TALE-nucleases of SEQ ID N°2-3, 5-6 and 8-9.

34) The method according to any one of claims 1 to 33, further comprising the step of:

d) introducing into said T-cell an exogenous nucleic acid molecule comprising a nucleotide sequence coding for a Chimeric Antigen Receptor (CAR) directed against at least one antigen expressed at the surface of a malignant or infected cell.

35) The method according to claim 34 wherein said Chimeric Antigen Receptor comprises scFv (VH and VL chains) having as antigenic target sequence of over 80% identity, preferably over 90%, and more preferably over 95% with SEQ ID NO 67 (CD19 antigen), SEQ ID NO 68 (CD38 antigen), SEQ ID NO 69 (CD123 antigen), SEQ ID NO 70 (CS1 antigen), SEQ ID NO 71 (BCMA antigen), SEQ ID NO 72 (FLT-3 antigen), SEQ ID NO 73 (CD33 antigen), SEQ ID NO 74 (CD70 antigen), SEQ ID NO 75 (EGFR-3v antigen) and SEQ ID NO 76 (WT1 antigen)40) The method according to any one of claims 1 to 4470, further comprising the step of:

e) expanding the resulting engineered T-cell.

36) An engineered, preferably isolated, T-cell, obtainable by using the method according to anyone of claim 1 to 35.

37) The engineered T-cell according to claim 36 for use as a medicament.

38) The engineered T-cell according to claim 36 or claim 37 for use in the treatment of a cancer or viral infection.

39) The engineered T-cell according to anyone of claims 36 to 38, wherein said T-cell originates from a patient to be treated.

40) The engineered T-cell according to anyone of claims 36 to 39, wherein said T-cell originates from a donor.

41) A composition comprising at least one engineered T-cell according to any one of claims 36 to 40.

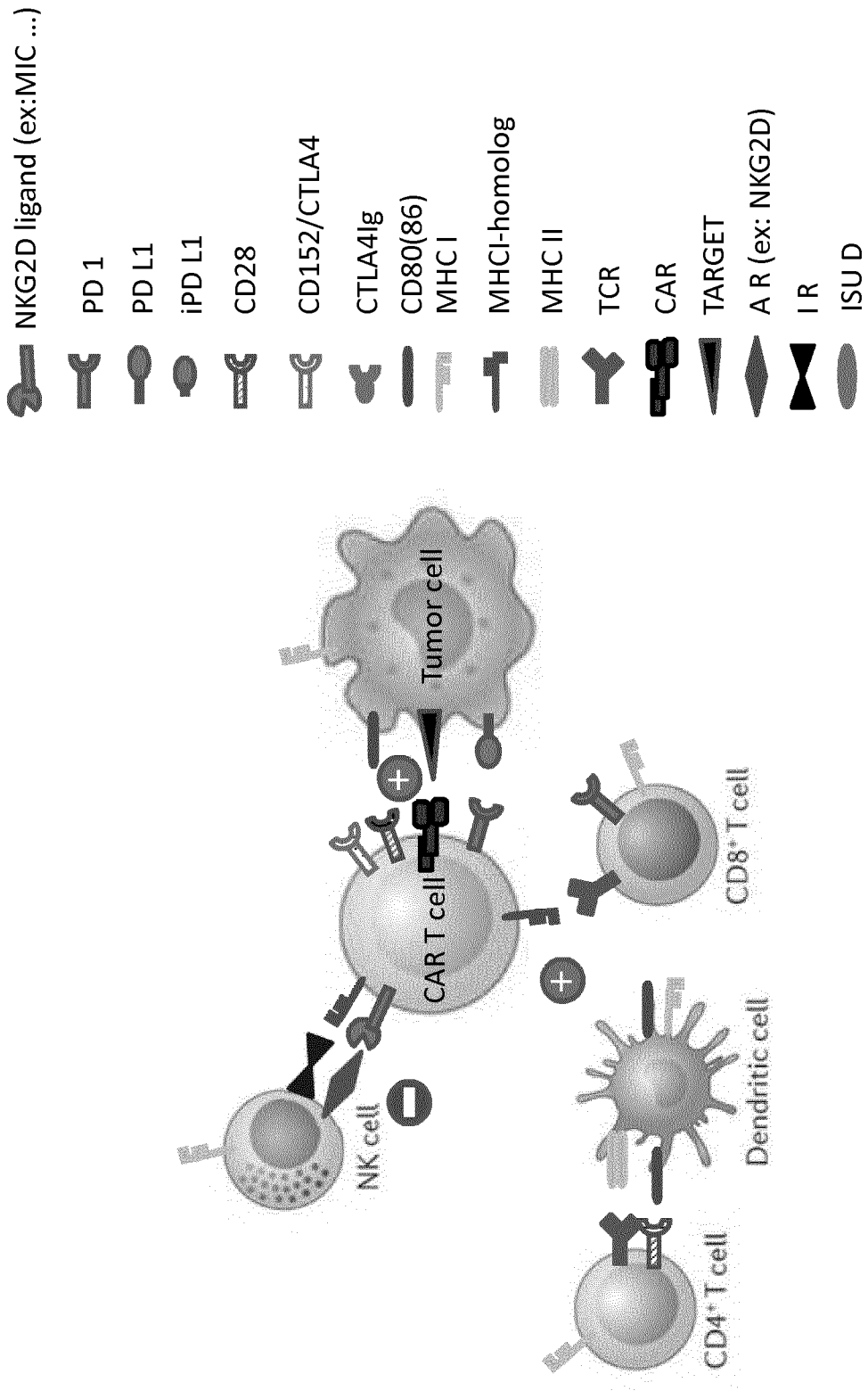
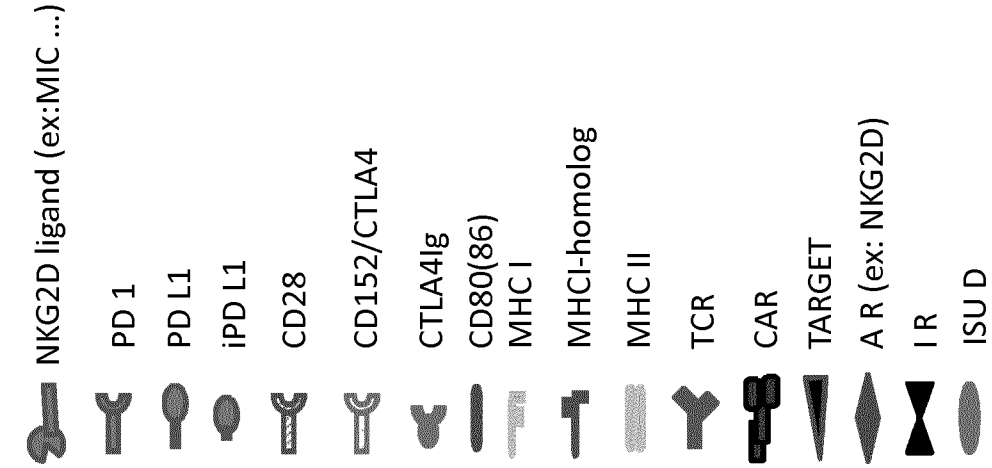

NKG2D ligand (ex:MIC ...)
PD 1
PD L1
iPD L1
CD28
CD152/CTLA4
CTLA4Ig
CD80(86)
MHC I
MHCI-homolog
MHC II
TCR
CAR
TARGET
A R (ex: NKG2D)
I R
ISU D
Tumor cell
CAR T cell
NK cell
CD8+ T cell
Dendritic cell
CD4+ T cell

Figure 1


NKG2D ligand (ex:MIC ...)
PD 1
PD L1
iPD L1
CD28
CD152/CTLA4
CTLA4Ig
CD80(86)
MHC I
MHCI-homolog
MHC II
TCR
CAR
TARGET
A R (ex: NKG2D)
I R
ISU D

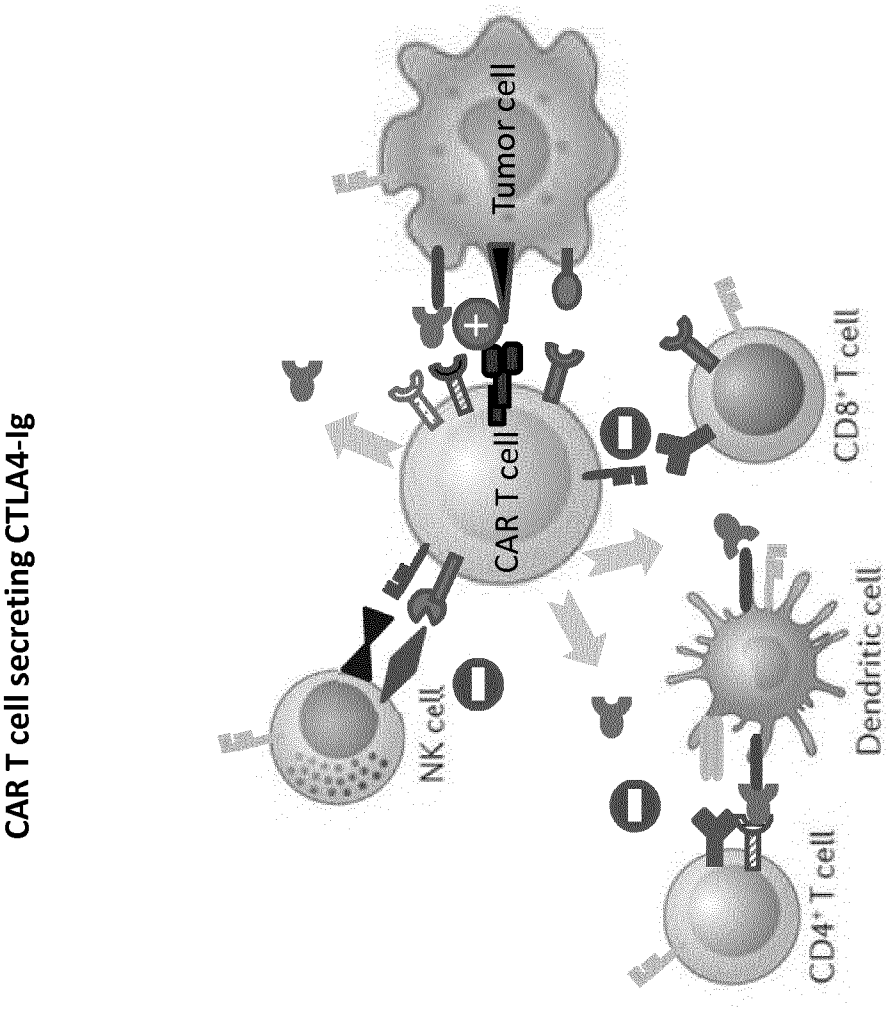

CAR T cell secreting CTLA4-Ig
Tumor cell
CAR T cell
NK cell
CD8+ T cell
Dendritic cell
CD4+ T cell

Figure 2

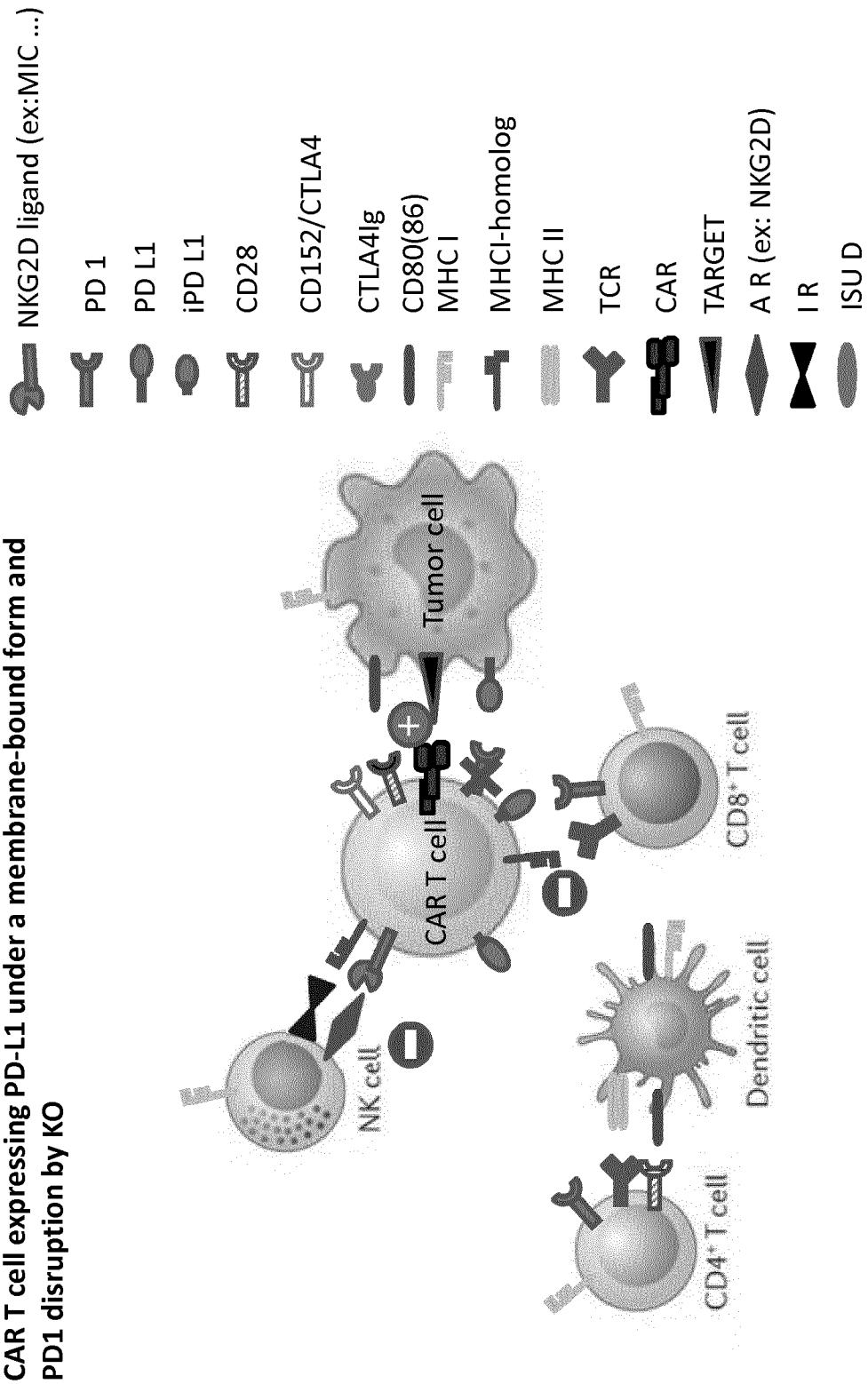

CAR T cell expressing PD-L1 under a membrane-bound form and PD1 disruption by KO
NKG2D ligand (ex:MIC ...)
PD 1
PD L1
iPD L1
CD28
CD152/CTLA4
CTLA4Ig
CD80(86)
MHC I
MHCI-homolog
MHC II
TCR
CAR
TARGET
A R (ex: NKG2D)
I R
ISU D
Tumor cell
CAR T cell
NK cell
CD8+ T cell
Dendritic cell
CD4+ T cell

Figure 3

CAR T cell expressing PD-L1 under a secreted form and disrupting PD1 by KO

Figure 4

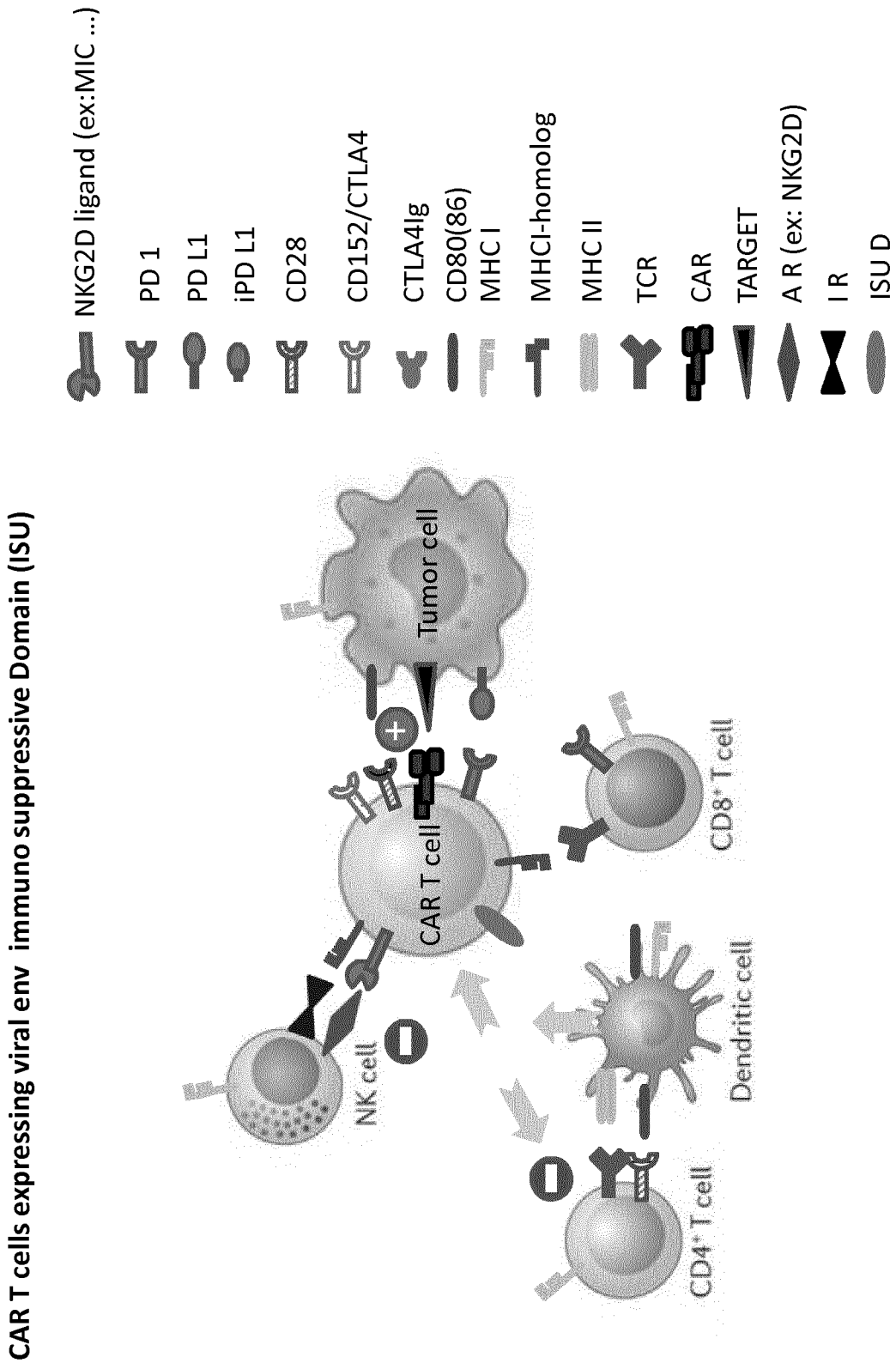

CAR T cells expressing viral env immuno suppressive Domain (ISU)
NKG2D ligand (ex:MIC ...)
PD 1
PD L1
iPD L1
CD28
CD152/CTLA4
CTLA4Ig
CD80(86)
MHC I
MHCI-homolog
MHC II
TCR
CAR
TARGET
A R (ex: NKG2D)
I R
ISU D
Tumor cell
CAR T cell
NK cell
CD8+ T cell
Dendritic cell
CD4+ T cell

Figure 5

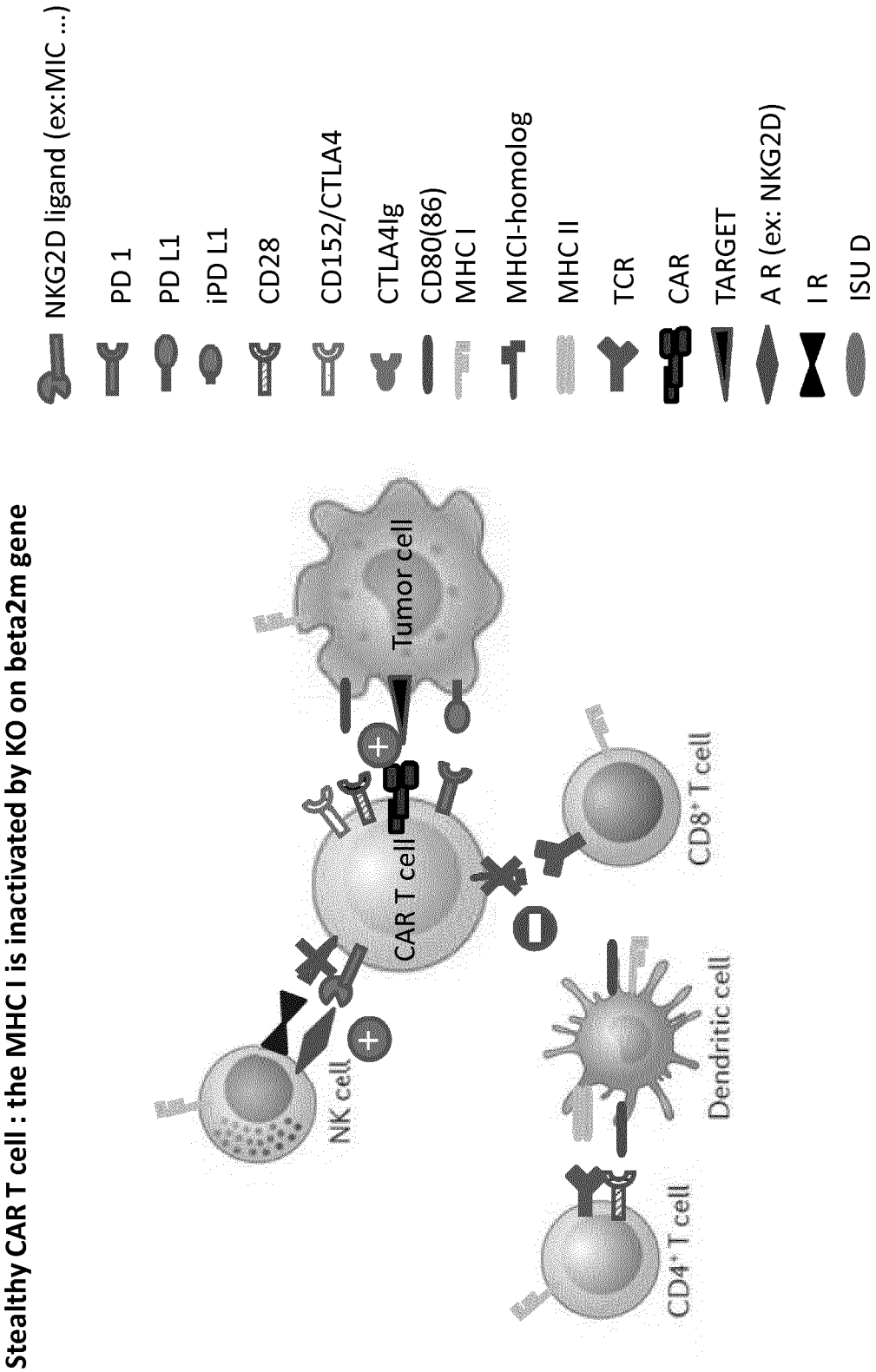

Stealthy CAR T cell : the MHC I is inactivated by KO on beta2m gene
NKG2D ligand (ex:MIC ...)
PD 1
PD L1
iPD L1
CD28
CD152/CTLA4
CTLA4Ig
CD80(86)
MHC I
MHCI-homolog
MHC II
TCR
CAR
TARGET
A R (ex: NKG2D)
I R
ISU D
Tumor cell
CAR T cell
NK cell
CD8+ T cell
Dendritic cell
CD4+ T cell

Figure 6

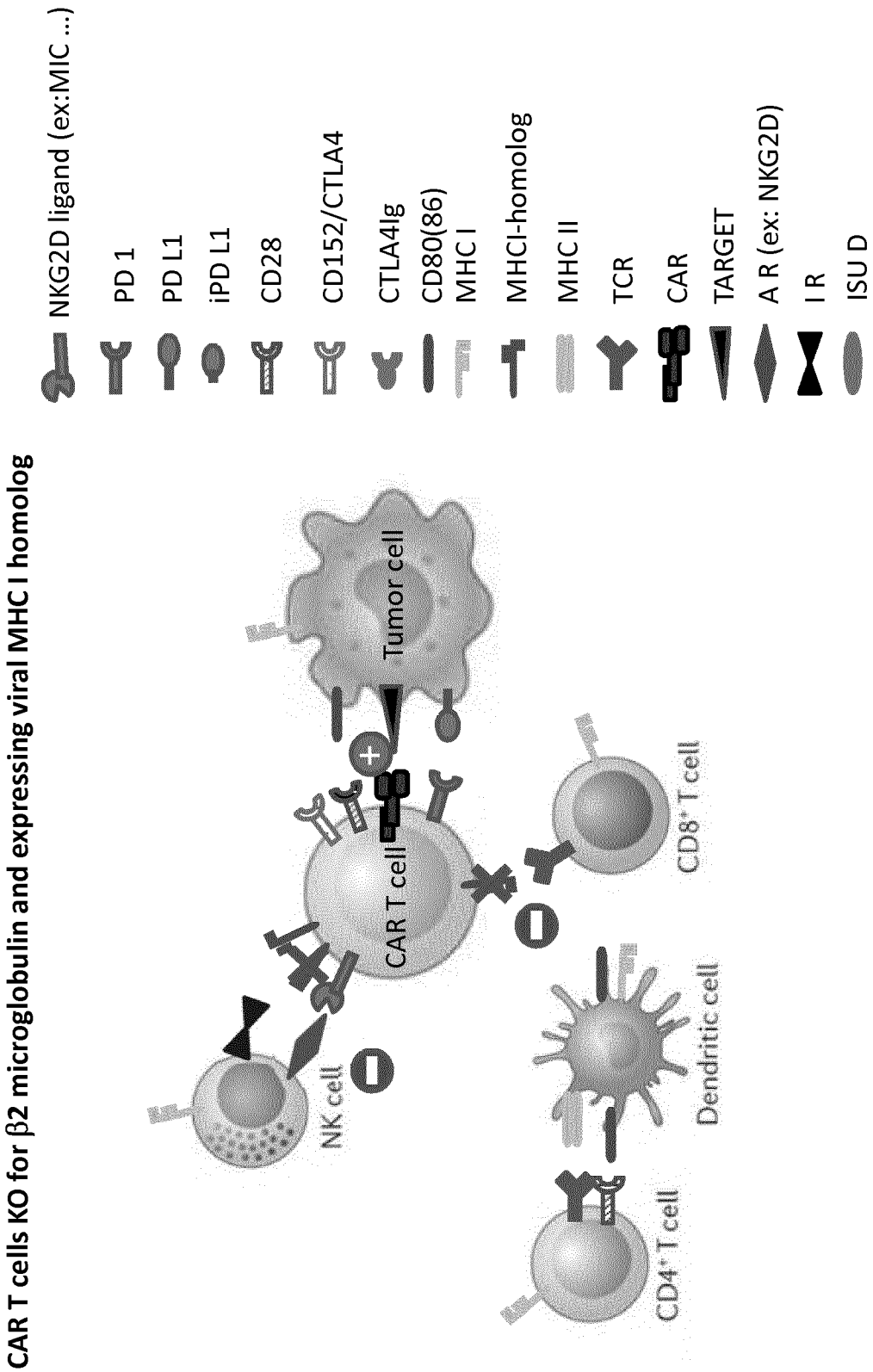

CAR T cells KO for β2 microglobulin and expressing viral MHC I homolog
NKG2D ligand (ex:MIC ...)
PD 1
PD L1
iPD L1
CD28
CD152/CTLA4
CTLA4Ig
CD80(86)
MHC I
MHCI-homolog
MHC II
TCR
CAR
TARGET
A R (ex: NKG2D)
I R
ISU D
Tumor cell
CAR T cell
NK cell
CD8+ T cell
Dendritic cell
CD4+ T cell

Figure 7

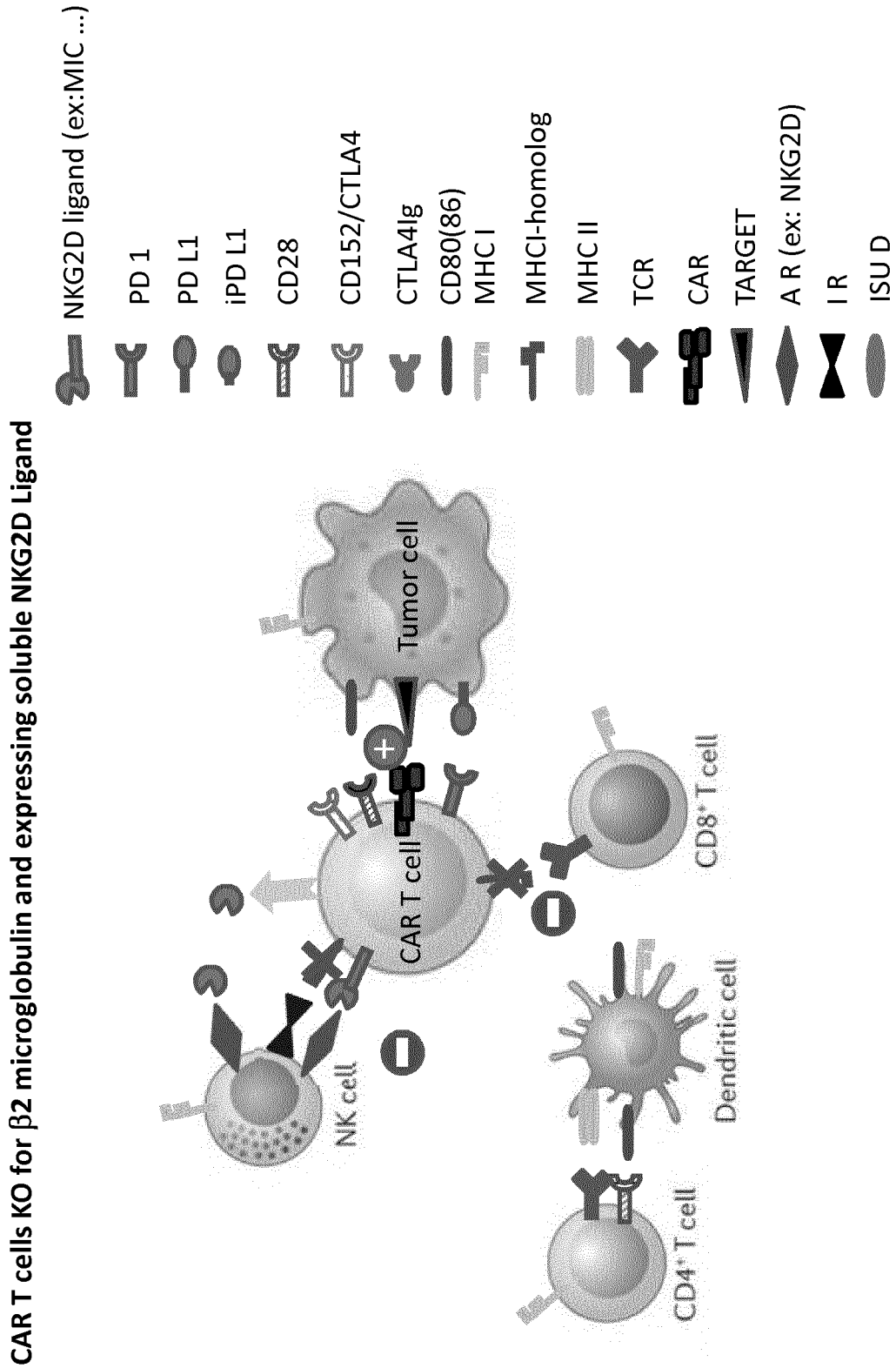

CAR T cells KO for β2 microglobulin and expressing soluble NKG2D Ligand
NKG2D ligand (ex:MIC ...)
PD 1
PD L1
iPD L1
CD28
CD152/CTLA4
CTLA4Ig
CD80(86)
MHC I
MHCI-homolog
MHC II
TCR
CAR
TARGET
A R (ex: NKG2D)
I R
ISU D
Tumor cell
CAR T cell
NK cell
CD8+ T cell
Dendritic cell
CD4+ T cell

Figure 8

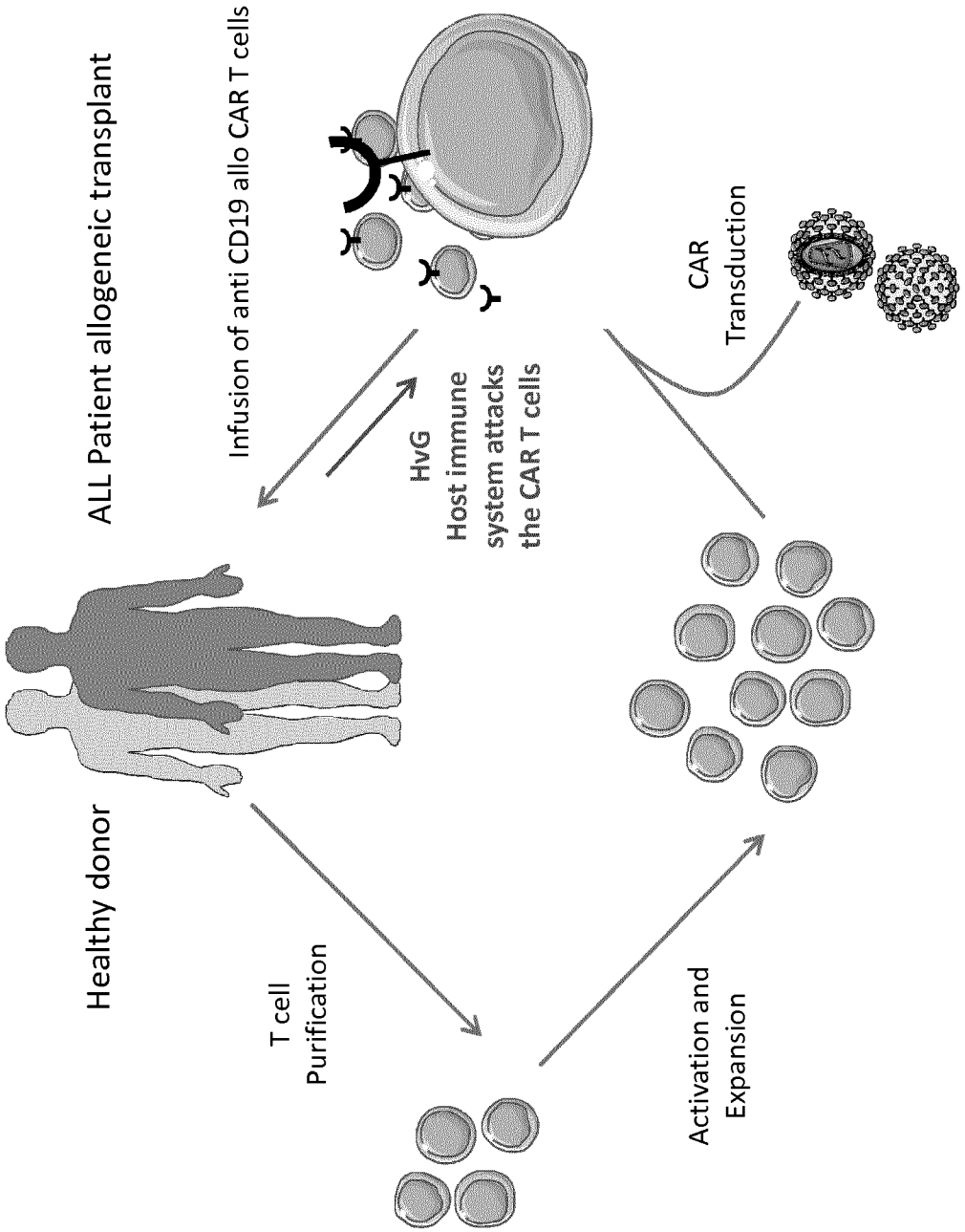

Healthy donor
T cell
Purification
Activation and
Expansion
CAR
Transduction
ALL Patient allogeneic transplant
Infusion of anti CD19 allo CAR T cells
HvG
Host immune
system attacks
the CAR T cells

Figure 9

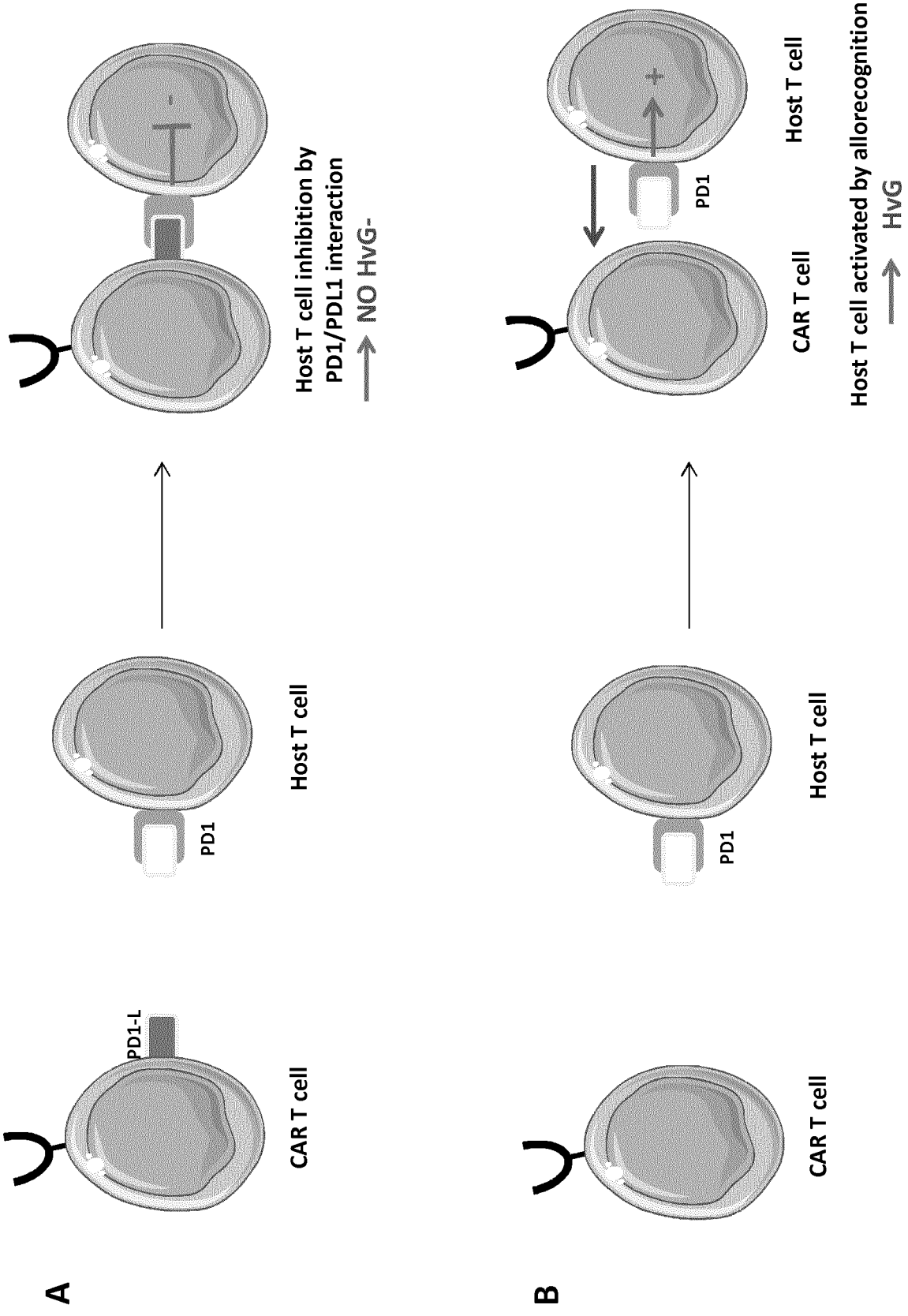

A
PD1-L
CAR T cell
PD1
Host T cell
Host T cell inhibition by
PD1/PDL1 interaction
NO HvG-
B
CAR T cell
PD1
Host T cell
PD1
CAR T cell
Host T cell
Host T cell activated by allorecognition
HvG

Figure 10

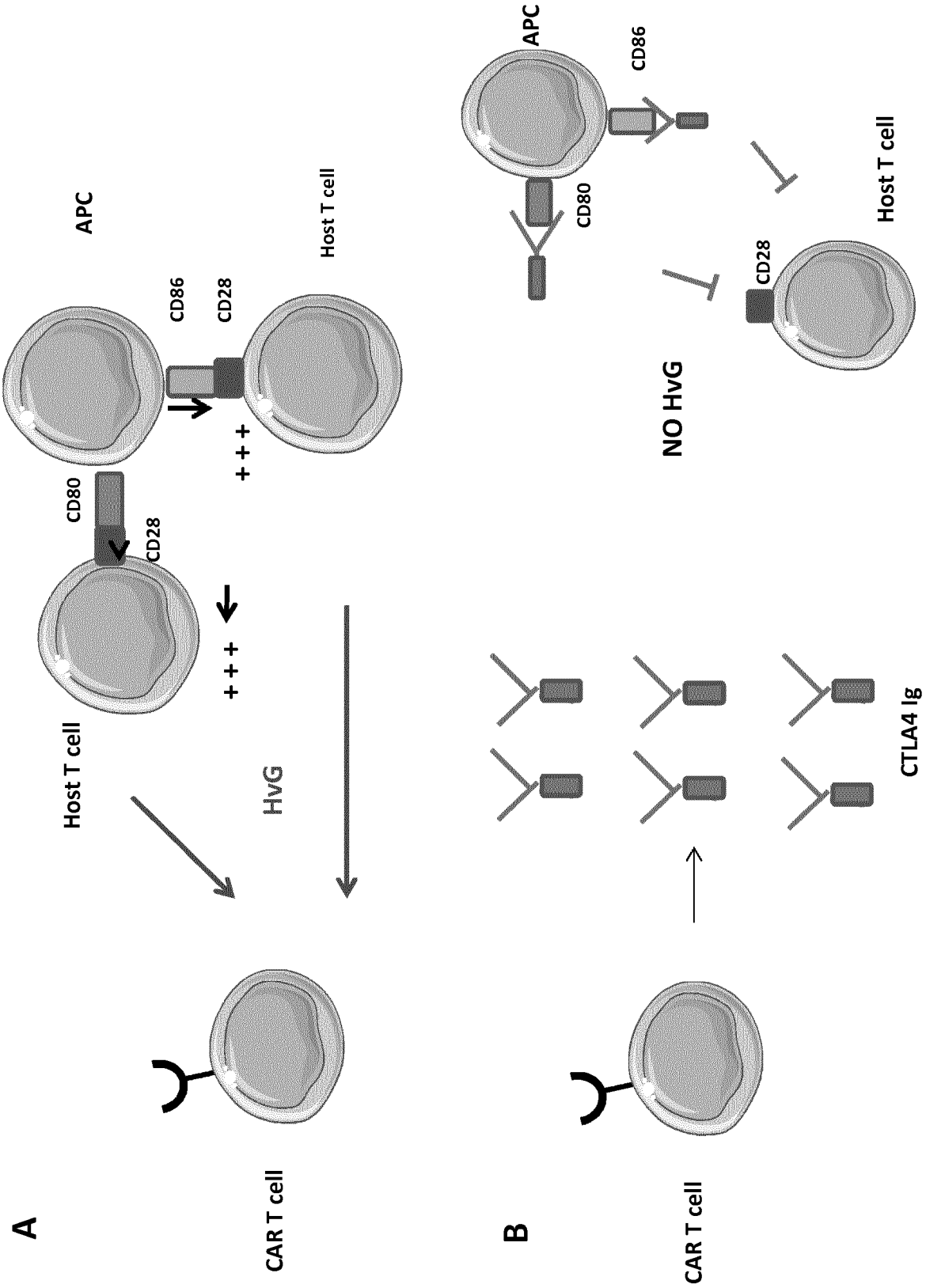

A
CAR T cell
Host T cell
CD80
CD28
+ + +
HvG
APC
CD86
CD28
Host T cell
+ + +
B
CAR T cell
CTLA4 Ig
NO HvG
APC
CD86
CD80
CD28
Host T cell

Figure 11

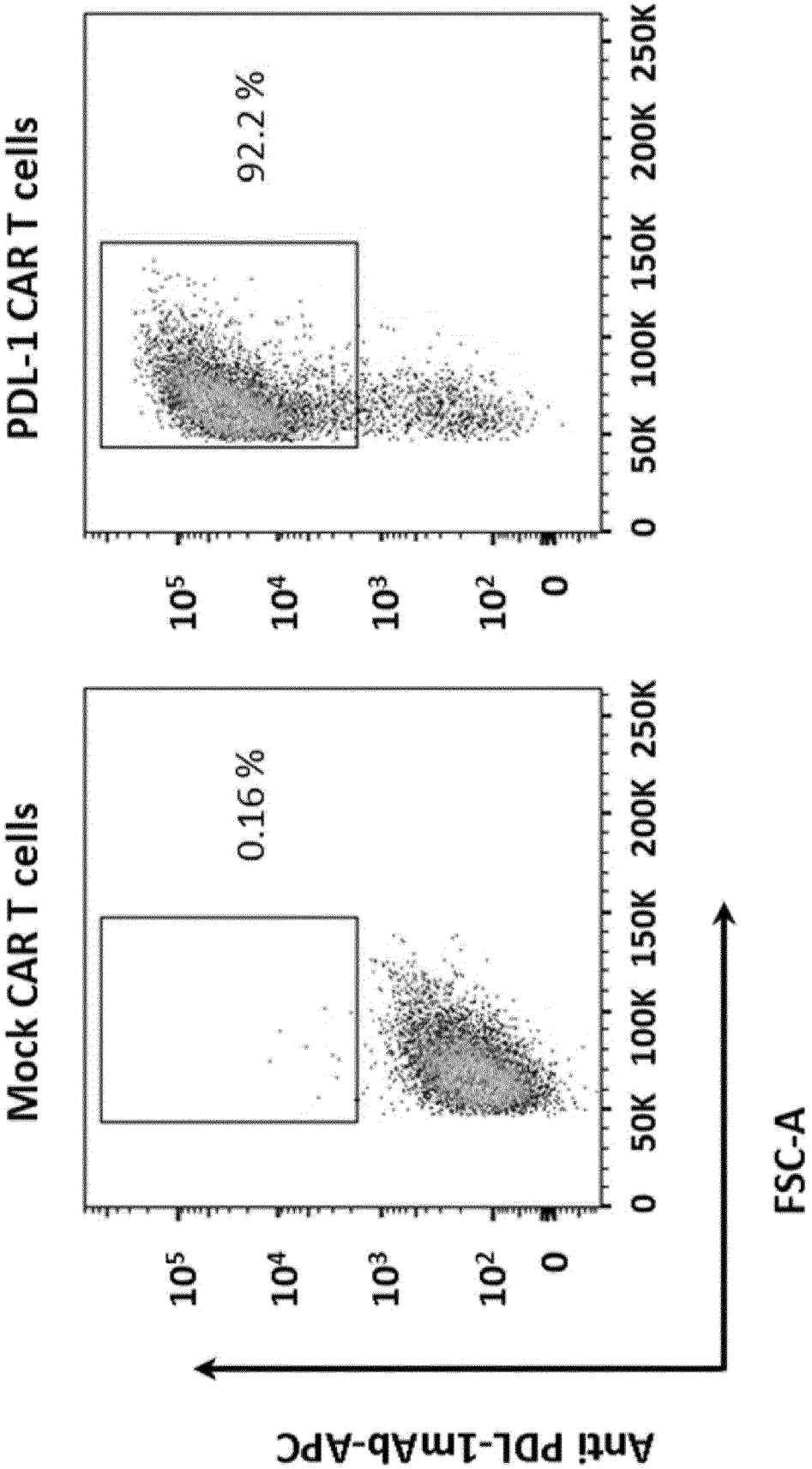

Mock CAR T cells
0.16 %
PDL-1 CAR T cells
92.2 %
Anti PDL-1mAb-APC
FSC-A
0
50K
100K
150K
200K
250K
10^2
10^3
10^4
10^5

Figure 12A

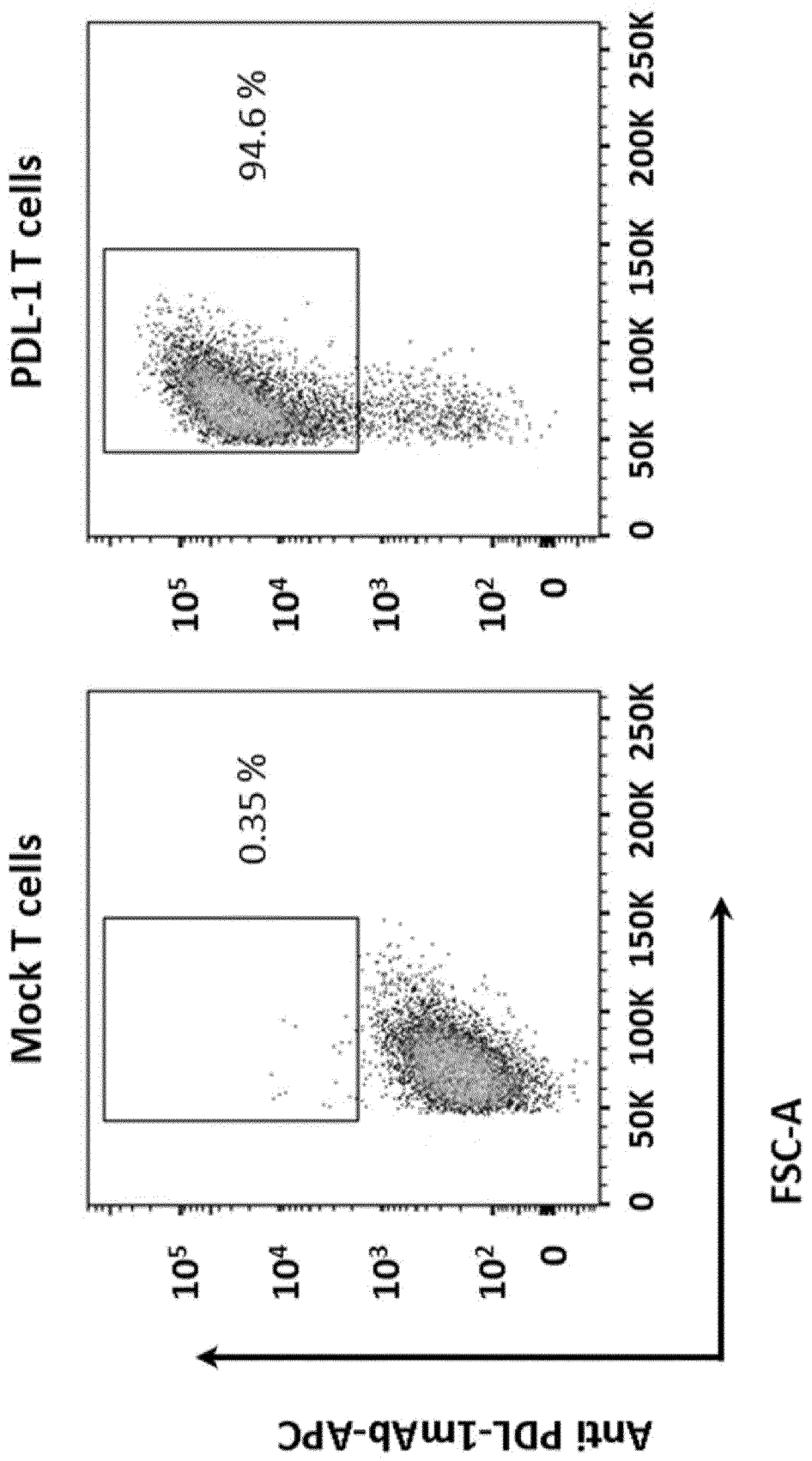

Mock T cells
PDL-1 T cells
0.35 %
94.6 %
Anti PDL-1mAb-APC
FSC-A
0
50K
100K
150K
200K
250K
10^2
10^3
10^4
10^5

Figure 12B

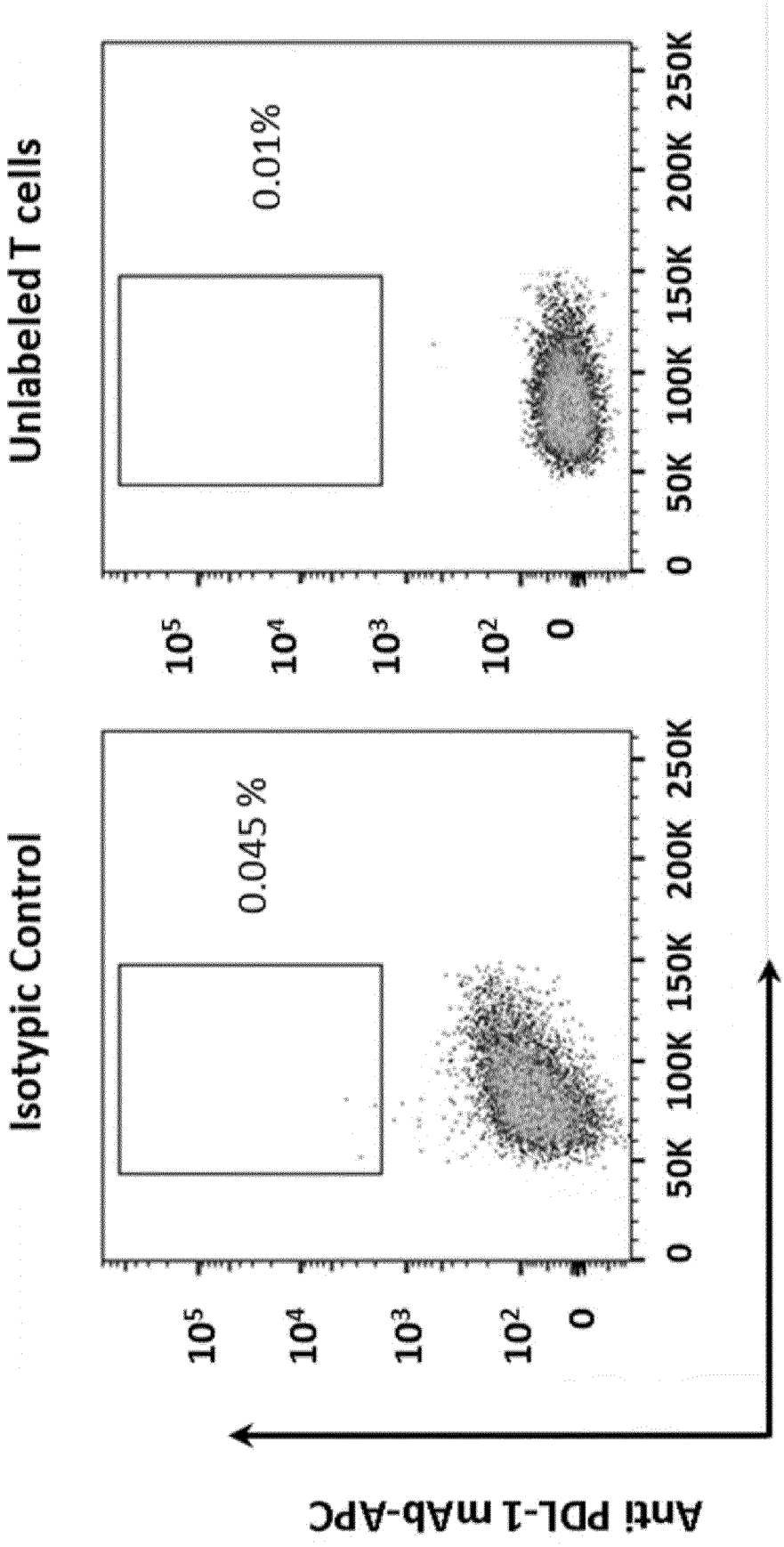

Isotypic Control
0.045 %
Unlabeled T cells
0.01%
Anti PDL-1 mAb-APC
FSC-A
0
50K
100K
150K
200K
250K
10^2
10^3
10^4
10^5

Figure 12C

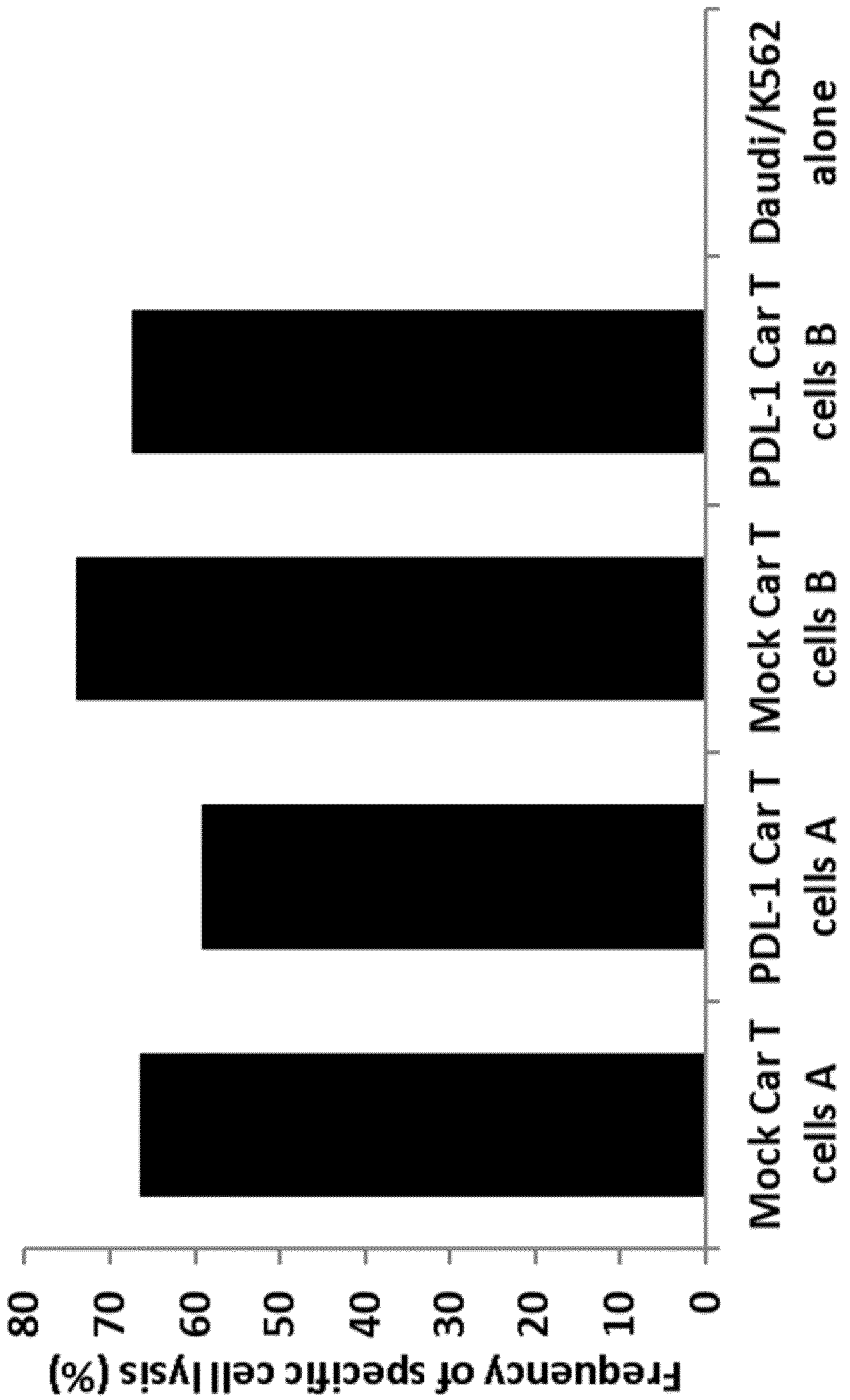

Frequency of specific cell lysis (%)
0
10
20
30
40
50
60
70
80
Mock Car T cells A
PDL-1 Car T cells A
Mock Car T cells B
PDL-1 Car T cells B
Daudi/K562 alone

Figure 13

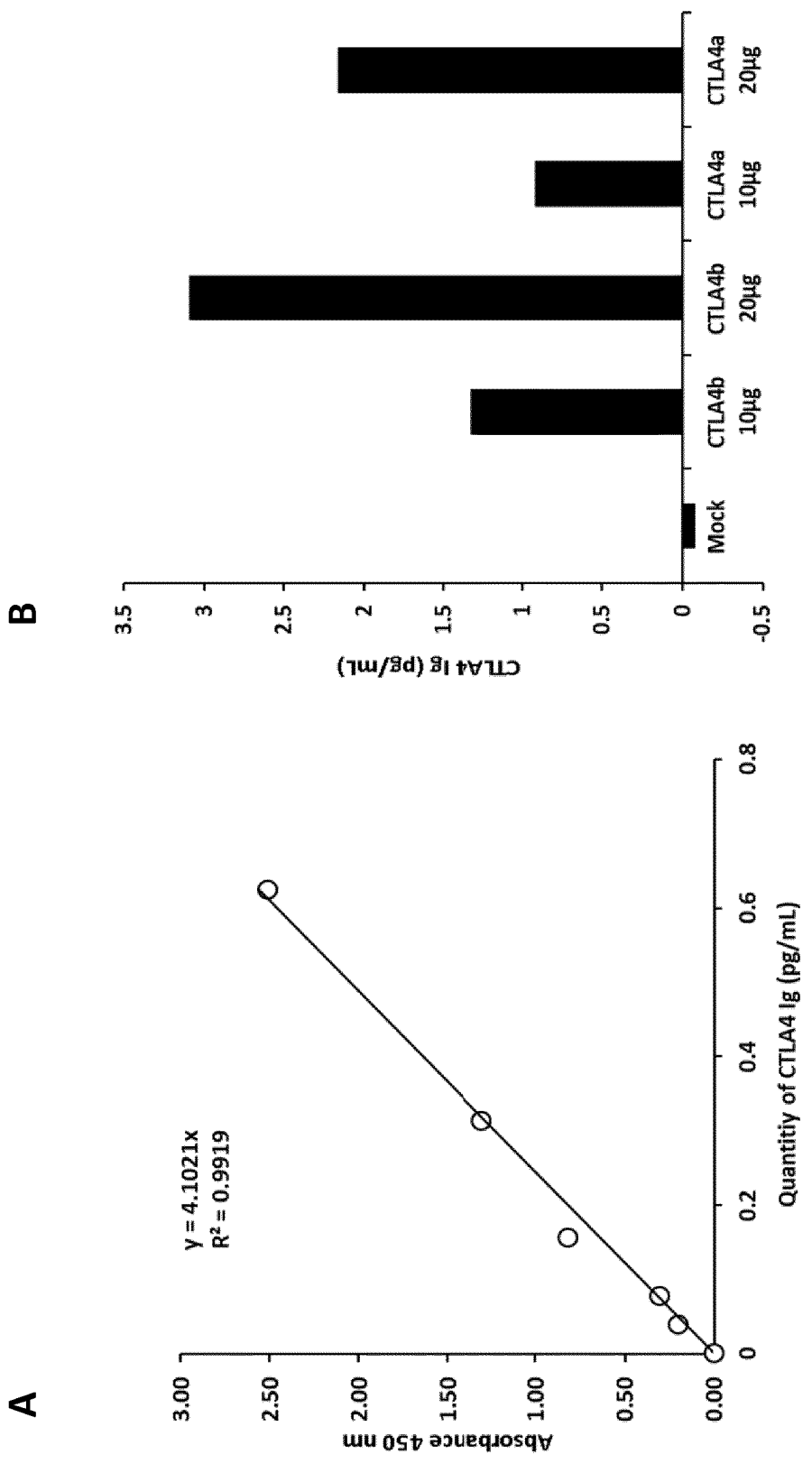

A
y = 4.1021x
R² = 0.9919
Absorbance 450 nm
Quantitiy of CTLA4 Ig (pg/mL)
3.00
2.50
2.00
1.50
1.00
0.50
0.00
0
0.2
0.4
0.6
0.8
B
CTLA4 Ig (pg/ml)
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
Mock
CTLA4b 10µg
CTLA4b 20µg
CTLA4a 10µg
CTLA4a 20µg

Figure 14

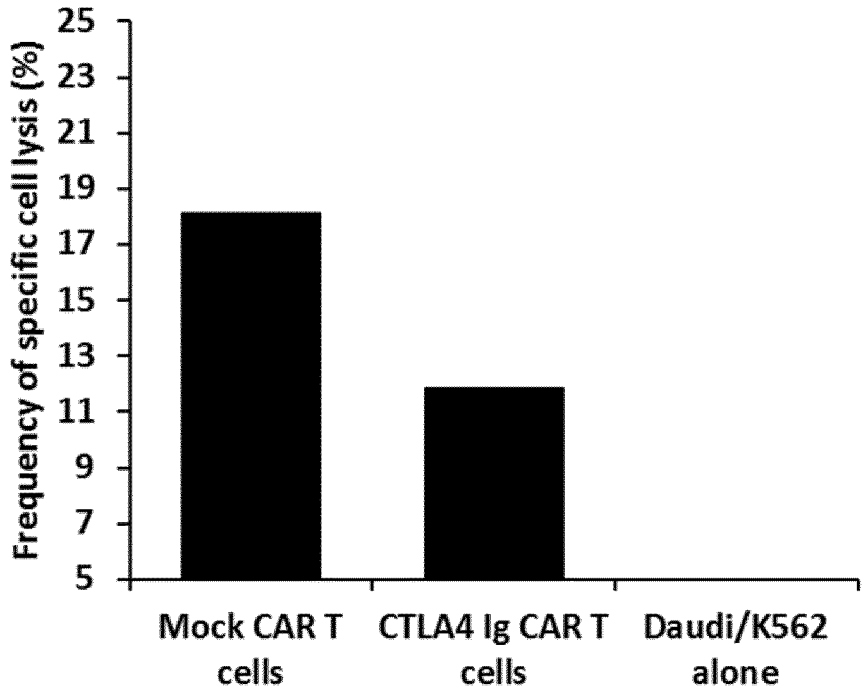

Frequency of specific cell lysis (%)
25
23
21
19
17
15
13
11
9
7
5
Mock CAR T cells
CTLA4 Ig CAR T cells
Daudi/K562 alone

Figure 15

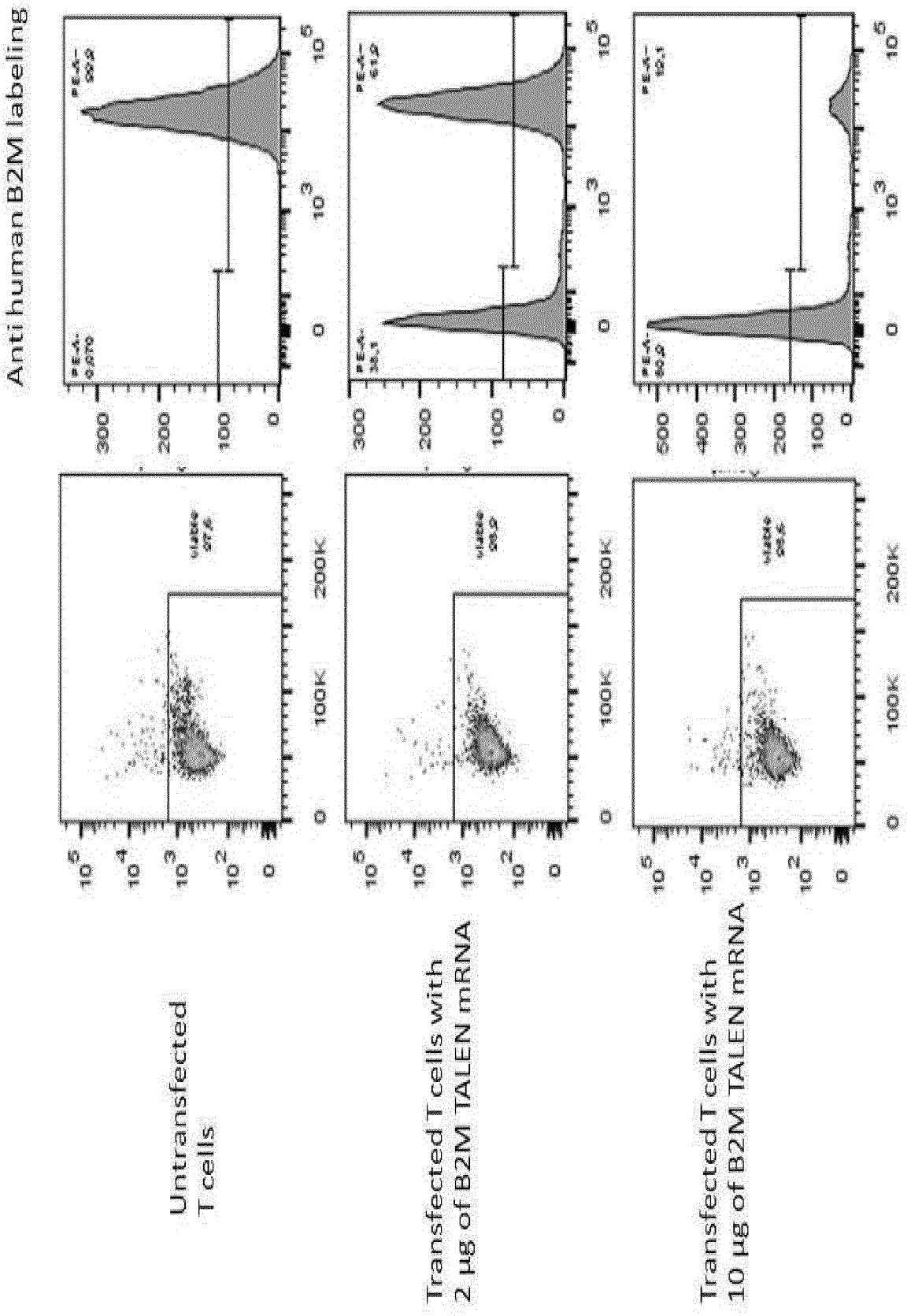

Anti human B2M labeling
Untransfected T cells
Transfected T cells with 2 µg of B2M TALEN mRNA
Transfected T cells with 10 µg of B2M TALEN mRNA

Figure 16

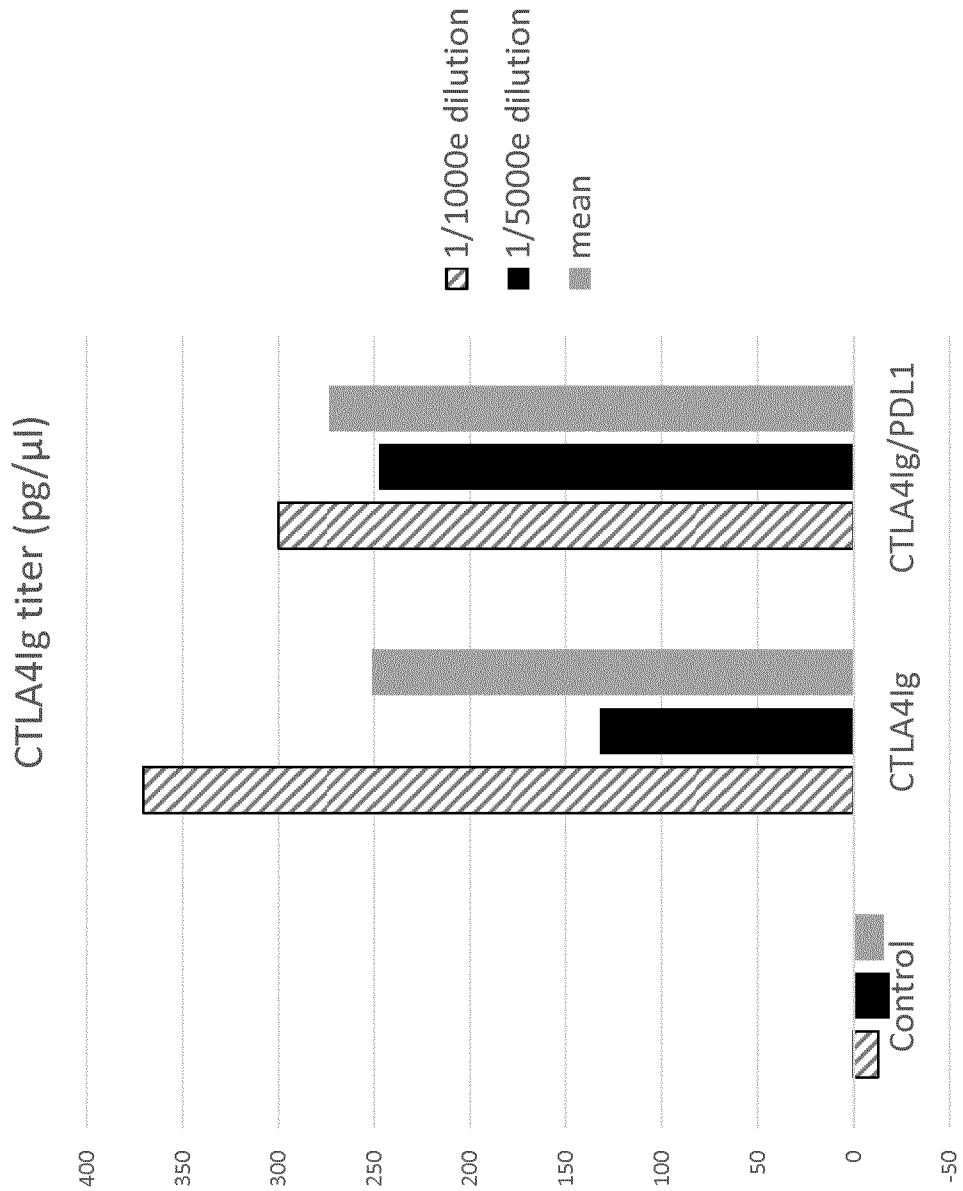

CTLA4Ig titer (pg/µl)
400
350
300
250
200
150
100
50
0
-50
Control
CTLA4Ig
CTLA4Ig/PDL1
1/1000e dilution
1/5000e dilution
mean

Figure 17

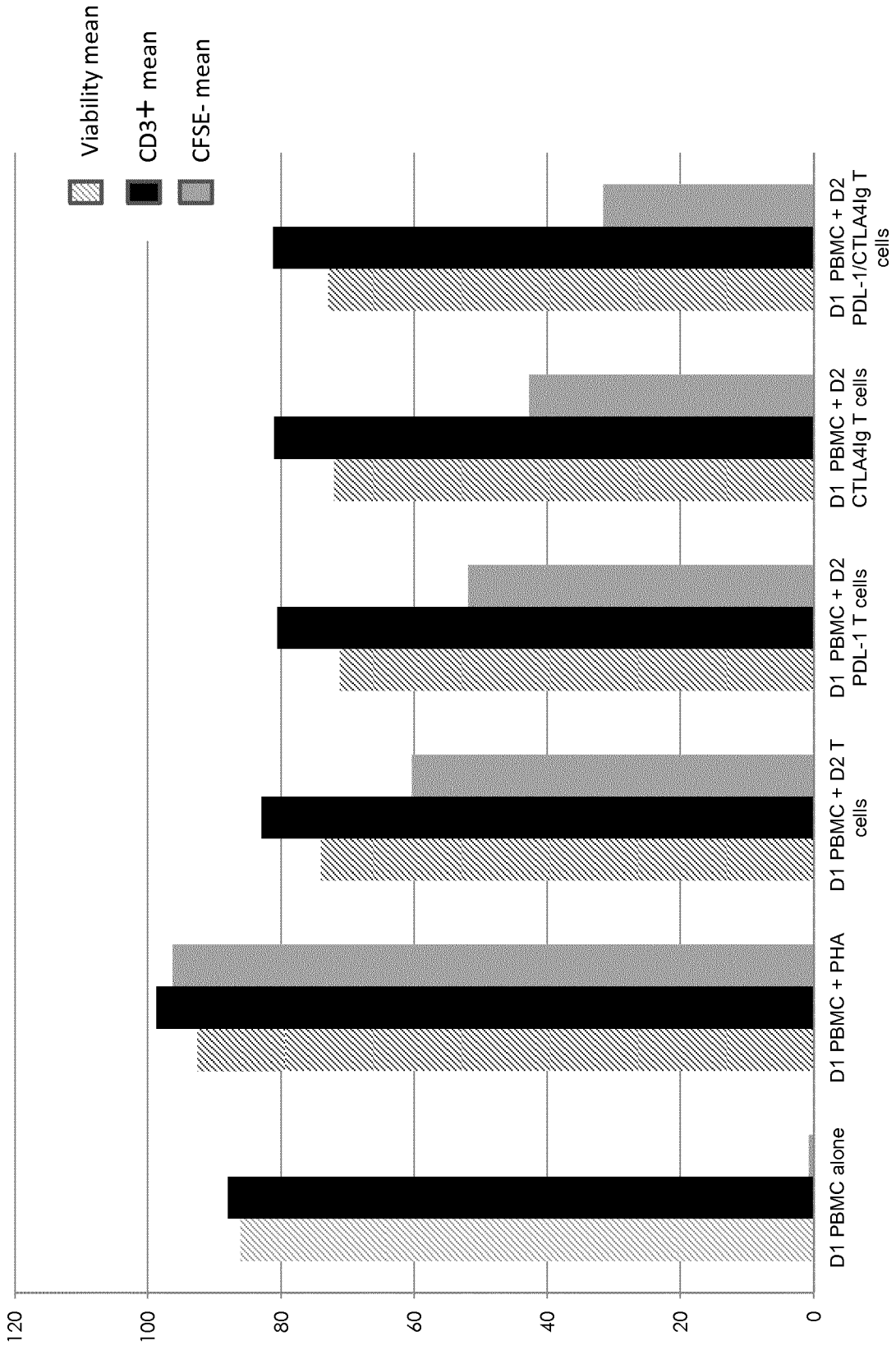

Viability mean
CD3+ mean
CFSE- mean
120
100
80
60
40
20
0
D1 PBMC alone
D1 PBMC + PHA
D1 PBMC + D2 T cells
D1 PBMC + D2 PDL-1 T cells
D1 PBMC + D2 CTLA4Ig T cells
D1 PBMC + D2 PDL-1/CTLA4Ig T cells

Figure 18

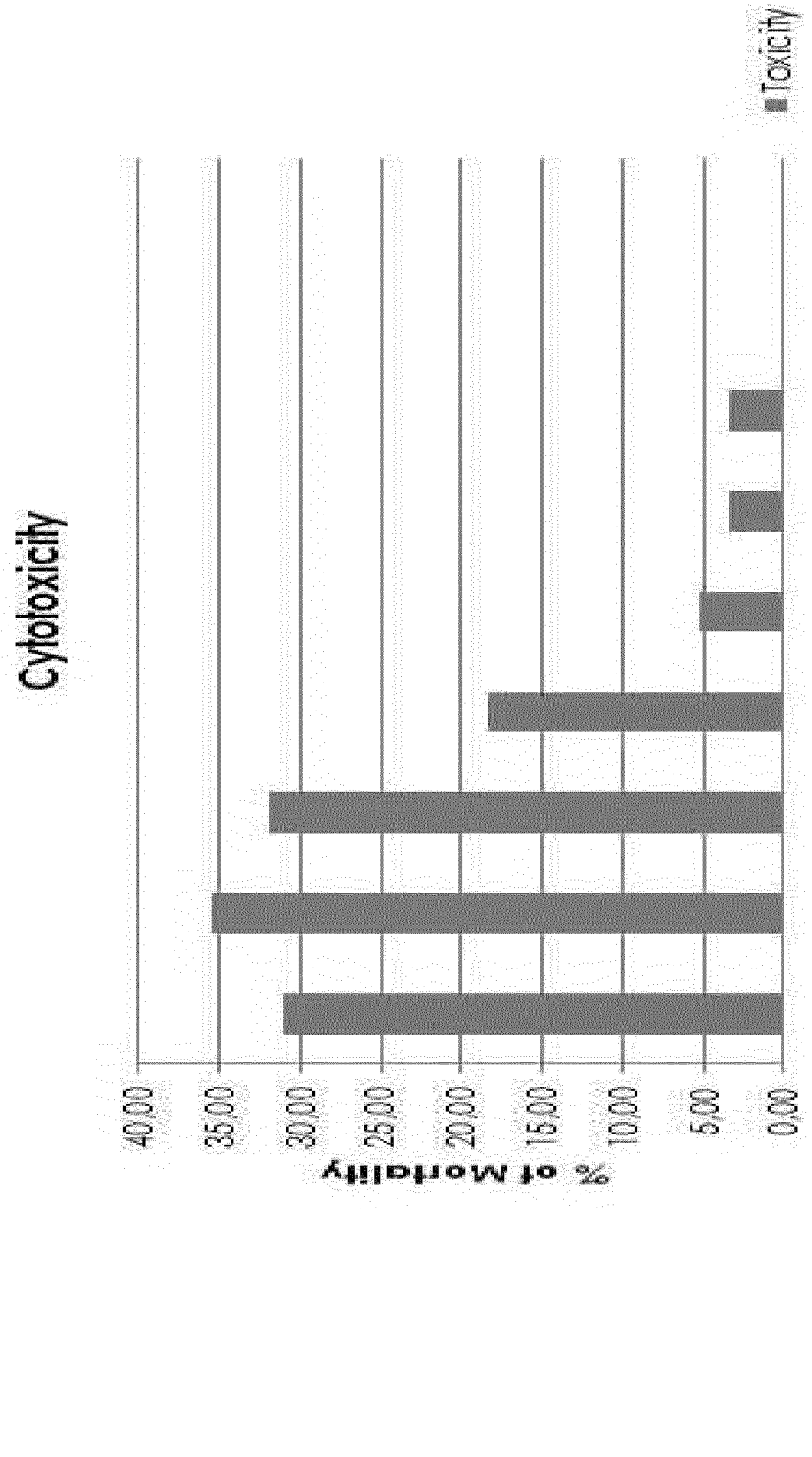

Cytoloxicity
% of Mortality
40,00
35,00
30,00
25,00
20,00
15,00
10,00
5,00
0,00
Toxicity
T cells transduced with: PDL1 CTLA4Ig PDL1/ CTLA4Ig - PDL1 CTLA4Ig PDL1/ CTLA4Ig -
T cells transfected with & CAR CD123 mRNA + + + + - - - -

Figure 19

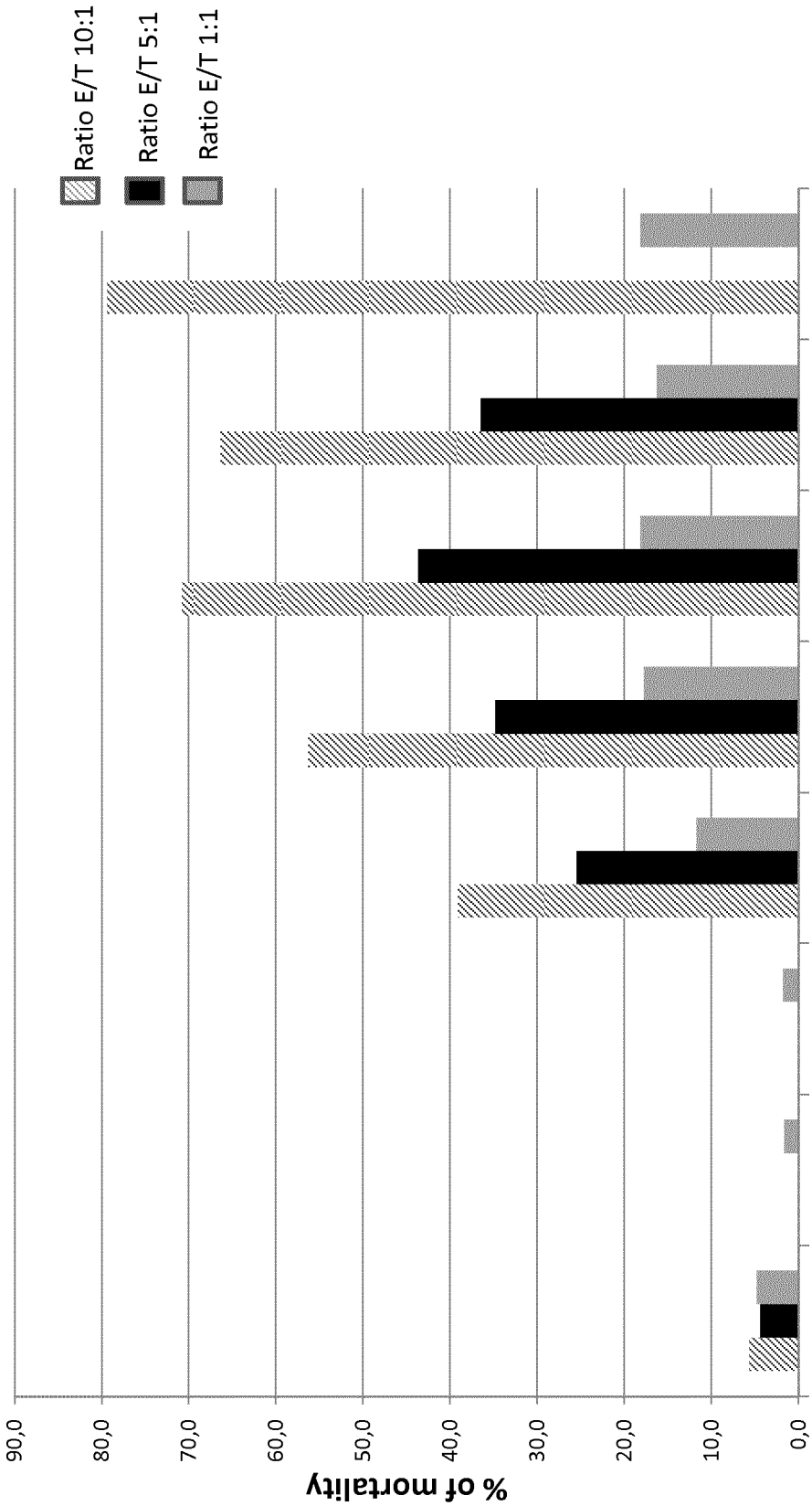

Ratio E/T 10:1
Ratio E/T 5:1
Ratio E/T 1:1
% of mortality
90,0
80,0
70,0
60,0
50,0
40,0
30,0
20,0
10,0
0,0
Target alone
LT WT
LT KO PD1
LT CAR CD123
LT CAR CD123/KO PD1
LT CAR CD123/CTLA4Ig/KO PD1
LT CAR CD123/PD-L1/KO PD1
LT CAR CD123/CTLA4Ig/PD-L1/KO PD1

Figure 20

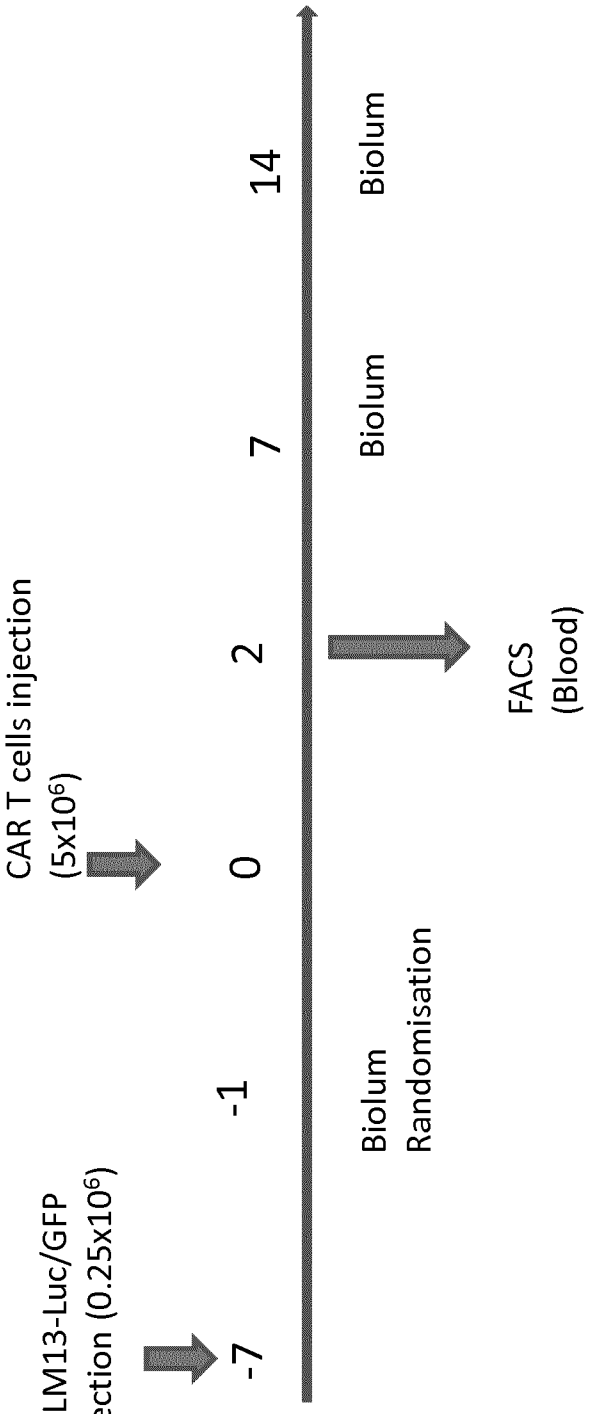

MOLM13-Luc/GFP
injection (0.25x10^6)
-7
-1
Biolum
Randomisation
CAR T cells injection
(5x10^6)
0
2
FACS
(Blood)
7
Biolum
14
Biolum

Figure 21

Group	% CAR+ cells	% PDL1+ cells	CTLA4Ig concentration (pg/µl)
CAR CD123	98.3	n.a.	n.a.
CAR CD123/PDL1	97.3	96.6	n.a.
CAR CD123/CTLA4Ig	97.7	n.a.	250
CAR CD123/PDL1/CTLA4Ig	97.1	91.9	275

Figure 22

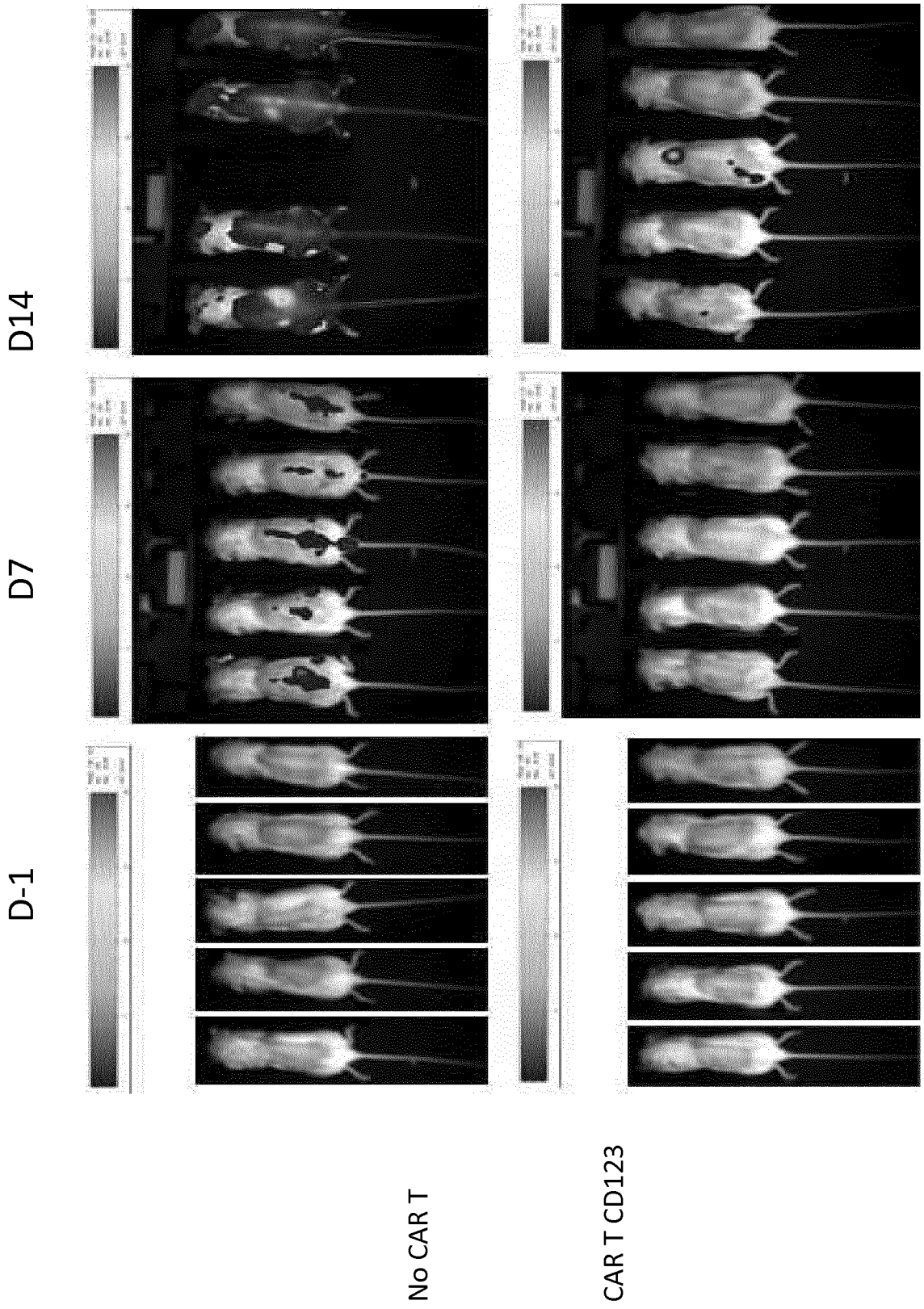


Figure 23A

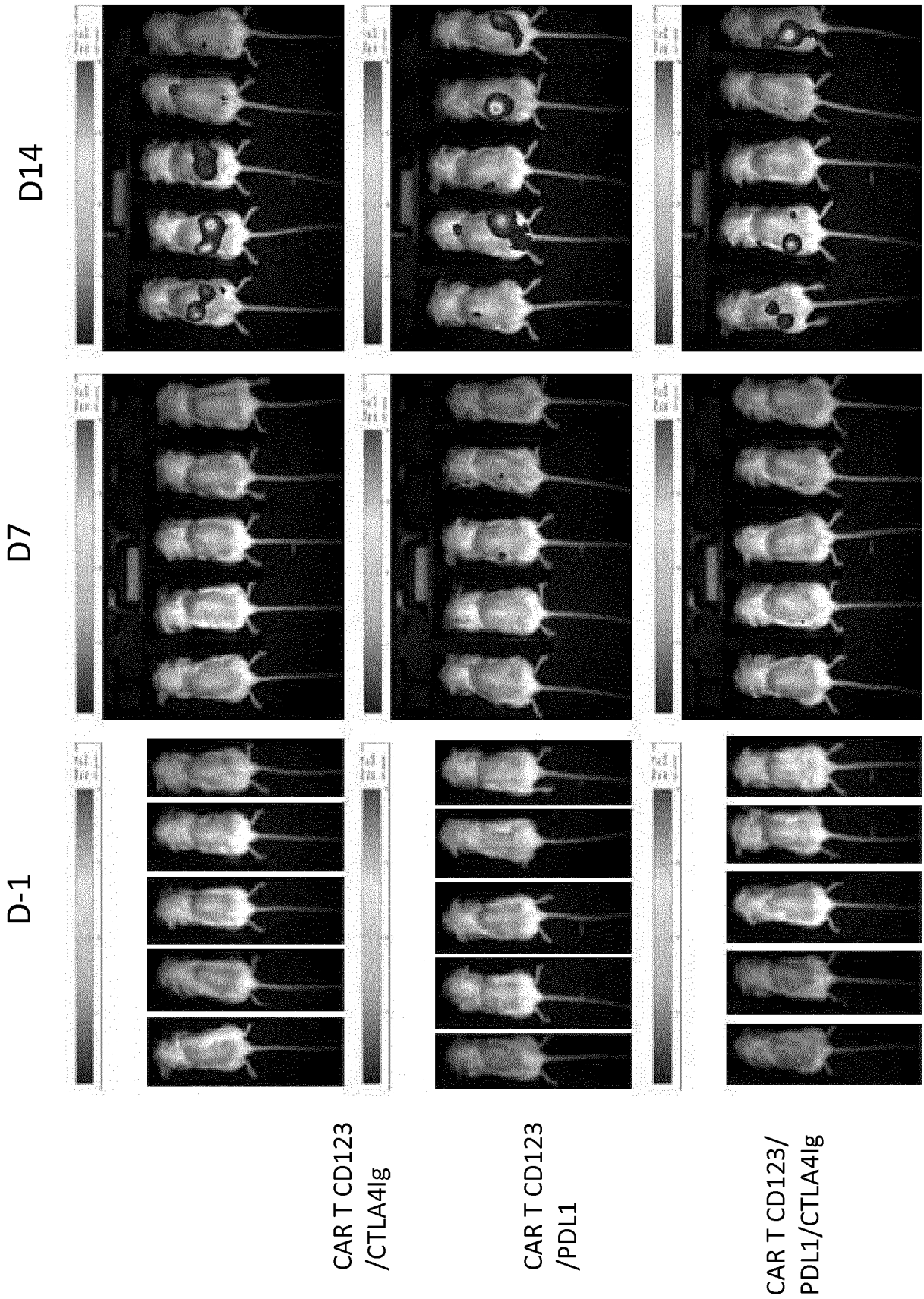

D-1
D7
D14
CAR T CD123 /CTLA4Ig
CAR T CD123 /PDL1
CAR T CD123/ PDL1/CTLA4Ig

Figure 23B

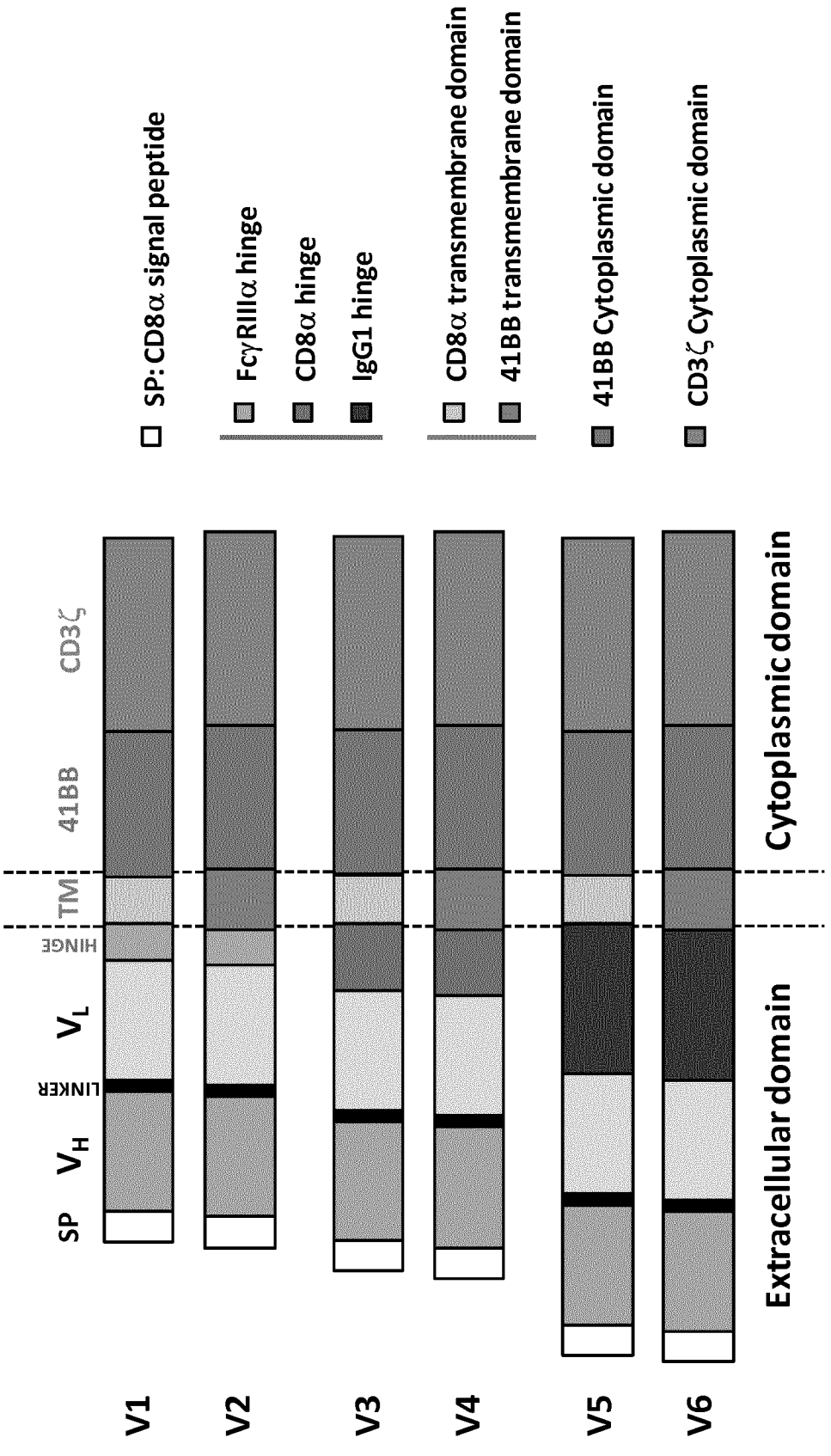

SP: CD8α signal peptide
FcγRIIIα hinge
CD8α hinge
IgG1 hinge
CD8α transmembrane domain
41BB transmembrane domain
41BB Cytoplasmic domain
CD3ζ Cytoplasmic domain
SP
VH
LINKER
VL
HINGE
TM
41BB
CD3ζ
Extracellular domain
Cytoplasmic domain
V1
V2
V3
V4
V5
V6

Figure 24

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/055332

A. CLASSIFICATION OF SUBJECT MATTER
INV. C07K14/74 C12N5/0783 C12N15/85
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C07K C12N

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

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

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 95/17911 A1 (CELL GENESYS INC [US]) 6 July 1995 (1995-07-06) abstract page 10 - page 11 page 22 - page 23 page 33, paragraph 1 -----	1-41
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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
20 May 2016	31/05/2016

Name and mailing address of the ISA/	Authorized officer
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