(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
25 September 2014 (25.09.2014)

WIPO | PCT

(10) International Publication Number
WO 2014/153470 A2

(51) International Patent Classification:
A61K 35/26 (2006.01) *C12N 15/87* (2006.01)

(21) International Application Number:
PCT/US2014/031360

(22) International Filing Date:
20 March 2014 (20.03.2014)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/804,076 21 March 2013 (21.03.2013) US

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*

(54) Title: TARGETED DISRUPTION OF T CELL RECEPTOR GENES USING ENGINEERED ZINC FINGER PROTEIN NUCLEASES

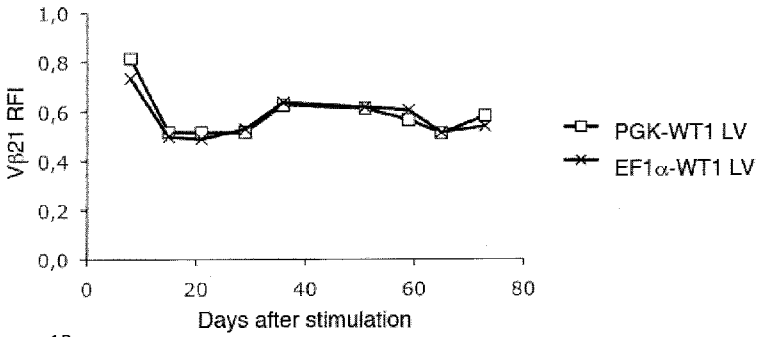

Figure 1B

(57) Abstract: Disclosed herein are methods and compositions for modifying TCR genes, using nucleases (zinc finger nucleases or TAL nucleases) to modify TCR genes.

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TARGETED DISRUPTION OF T CELL RECEPTOR GENES USING ENGINEERED ZINC FINGER PROTEIN NUCLEASES

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims the benefit of U.S. Provisional Application No. 61/804,076, filed March 21, 2013, the disclosure of which is hereby incorporated by reference in its entirety.

TECHNICAL FIELD

[0002] The present disclosure is in the field of genome modification of human cells, including lymphocytes and stem cells.

BACKGROUND

[0003] Various methods and compositions for targeted cleavage of genomic DNA have been described. Such targeted cleavage events can be used, for example, to induce targeted mutagenesis, induce targeted deletions of cellular DNA sequences, and facilitate targeted recombination at a predetermined chromosomal locus. *See*, for example, U.S. Patent Nos. 7,888,121; 7,972,854; 7,914,796; 7,951,925; 8,110,379; 8,409,861; 8,586,526; U.S. Patent Publications 20030232410; 20050208489; 20050026157; 20050064474; 20060063231; 201000218264; 20120017290; 20110265198; 20130137104; 20130122591; 20130177983 and 20130177960 and U.S. Provisional Application No. 61/823,689, the disclosures of which are incorporated by reference in their entireties for all purposes. These methods often involve the use of engineered cleavage systems to induce a double strand break (DSB) or a nick in a target DNA sequence such that repair of the break by an error born process such as non-homologous end joining (NHEJ) or repair using a repair template (homology directed repair or HDR) can result in the knock out of a gene or the insertion of a sequence of interest (targeted integration). Cleavage can occur through the use of specific nucleases such as engineered zinc finger nucleases (ZFN), transcription-activator like effector nucleases (TALENs), or using the CRISPR/Cas system with an engineered crRNA/tracr RNA ('single guide RNA') to guide specific cleavage.

[0004] The T cell receptor (TCR) is an essential part of the selective activation of T cells. Bearing some resemblance to an antibody, the TCR is typically made from two chains, α and β, which co-assemble to form a heterodimer. The antibody resemblance lies in the manner in which a single gene encoding a TCR chain is put together. TCR chains are composed of two regions, a C-terminal constant region and an N-terminal variable region. The genomic loci that encode the TCR chains resemble antibody encoding loci in that the TCR α gene comprises V and J segments, while the β chain locus comprises D segments in addition to V and J segments. During T cell development, the various segments recombine such that each T cell has a unique TCR structure, and the body has a large repertoire of T cells which, due to their unique TCR structures, are capable of interacting with unique antigens displayed by antigen presenting cells. Additionally, the TCR complex makes up part of the CD3 antigen complex on T cells.

[0005] During T cell activation, the TCR interacts with antigens displayed as peptides on the major histocompatability complex (MHC) of an antigen presenting cell. Recognition of the antigen-MHC complex by the TCR leads to T cell stimulation, which in turn leads to differentiation of both T helper cells (CD4+) and cytotoxic T lymphocytes (CD8+) in memory and effector lymphocytes. These cells then can expand in a clonal manner to give an activated subpopulation within the whole T cell population capable of reacting to one particular antigen.

[0006] Cytotoxic T lymphocytes (CTLs) are thought to be essential in killing tumor cells. These cells typically are able to induce apoptosis in cancer cells when the cancer cell displays some antigen on its surface that was previously displayed on the MHC by an antigen presenting cell. Normally, following action against target cells, CTLs will apoptose when the cellular threat is cleared, with a subset of lymphocytes remaining that will further differentiate into memory T cells to persist in case the body is exposed to the antigen again. The pool of memory lymphocytes is possibly highly heterogeneous. Recently, two types of memory T-cells have been identified: effector memory T-cells (CD45RA- CCR7-, CD62L-) and central memory T-cells that are CD45RA negative cells characterized by the expression of CCR7 and CD62L, two molecules required for homing in T-cell areas of secondary lymphoid organs. Upon antigenic stimulation, central memory T-cells produce low levels of effector cytokines such as IL-4 and IFN-γ, but high levels of IL-2, which is able to

sustain their rapid and consistent proliferation. Upon antigen encounter central memory T-cells undergo: 1) proliferation, resulting in an auto-regenerative process, aimed at increasing their pool, and 2) differentiation, resulting in the generation of effector memory T-cells, which are characterized by a low proliferative potential but are able to migrate to inflamed non-lymphoid tissues and mediate the effector phase of the immune response. Protocols enabling gene transfer into T lymphocytes, while preserving their central memory functional phenotype have been developed (see European Patent Publication No EP1956080, Kaneko *et al.*, 2009 *Blood* 113(5):1006-15).

[0007] However, some tumor cells are able to escape surveillance by the immune system, perhaps through mechanisms such as poor clonal expansion of certain CTL subsets expressing the relevant TCR, and localized immune suppression by cancer cells (see Boon *et al*, (2006) *Annu Rev Immunol.* 24:175-208). The notion of a cancer vaccine is built upon the idea of using these cancer specific antigens to stimulate and expand the CTLs that express the appropriate TCR *in vivo*, in an attempt to overcome immune escape, however, these cancer vaccines have yet to show any marked success. In fact, an analysis done in 2004 examined 765 metastatic cancer patients that had been treated in over 35 different cancer vaccine trials, where an overall response was observed in only 3.8% of patients (see Rosenberg *et al* (2004) *Nat. Med.* 10(9): 909–915).

[0008] Adoptive immunotherapy is the practice of achieving highly specific T cell stimulation of a certain subpopulation of CTLs that possess a high-avidity TCR to the tumor antigen, stimulating and expanding them *ex vivo*, and then introducing them into the patient. Adoptive immunotherapy is particularly effective if native lymphocytes are removed from the patient before the infusion of tumor-specific cells. The idea behind this type of therapy is that if the introduced high-avidity CTLs are successful, once the tumor has been cleared, some of these cells will remain as memory T cells and will persist in the patient in case the cancer reappears. In 2002, a study was completed demonstrating regression of metastatic melanoma in patients that were treated under a regime of adoptive immunotherapy following immunodepletion with cyclophosphamide and fludarabine (Dudley *et al*, (2002) *Science*, 298(5594): 850-854). Response rate was even higher if adoptive immunotherapy was preceded by total body irradiation (Dudley *et al* 2008 *J Clin Oncol.* 26(32):5233-9).

[0009] However, adoptive immunotherapy cannot be performed when the T cells of interest containing high avidity TCRs cannot be readily expanded. In addition, it is often difficult to identify and isolate T cells with therapeutic value from cancer patients because tumor antigens are often self-antigens, against which the patient's immune system is made tolerant through mechanisms of deletion or anergy of those T cell clones with the highest avidity. Thus, transfer of genes encoding high avidity TCRs into patient derived T cells has been proposed and demonstrated (see Rubenstein *et al*, (2003) *J of Immunology* 170: 1209-1217). More recently, using a mouse model of malignant melanoma, a statistically significant decrease in tumor mass was found following introduction of normal lymphocytes that had been transduced with retroviral vectors carrying human TCR genes specific for the gp-100 melanoma antigen (Abad *et al*, (2008) *J Immunother.* 31(1): 1–6). TCR gene therapy is also described in Morgan *et al.* (2006) *Science* 314(5796):126-9 and Burns *et al*, 2009 *Blood* 114(14):2888-99.

[0010] However, transfer of any TCR transgenes into host T cells carries with it the caveats associated with most gene transfer methods, namely, unregulated and unpredictable insertion of the TCR transgene expression cassette into the genome, often at a low level. Such poorly controlled insertion of the desired transgene can result in effects of the transgene on surrounding genes as well as silencing of the transgene due to effects from the neighboring genes. In addition, the endogenous TCR genes that are co-expressed in the T cell engineered with the introduced TCR transgene could cause undesired stimulation of the T cell by the antigen recognized by the endogenous TCR, undesired stimulation of the T cell by unintended antigens due to the mispairing of the TCR transgene with the endogenous TCR subunits creating a novel TCR complex with novel recognition properties, or can lead to suboptimal stimulation against the antigen of interest by the creation of inactive TCRs due to heterodimerization of the transgene encoded TCR subunits with the endogenous TCR proteins. In fact, the risk of severe autoimmune toxicity resulting from the formation of self-reactive TCR from mispairing of endogenous and exogenous chains has been recently highlighted in a murine model (Bendle *et al.*, (2010) Nature Medicine 16:565-570) and in human cells (van Loenen *et al.*, (2010) *Proc Natl Acad Sci U S A* 107:10972-7). Additionally, the tumor-specific TCR may be expressed at suboptimal levels on the cell surface, due to competition with the endogenous and mispaired TCR

for the CD3 molecules, required to express the complex on the cell surface. Low TCR expression affects the avidity and efficacy of the transgenic T cell.

[0011] Wilms tumor antigen (WT1 antigen) is a transcription factor normally expressed in embryonic cells. After birth, its expression is limited to only a few cell types including hematopoietic stem cells. However, it has been found to be overexpressed in many types of leukemias and solid tumors (*see* Inoue *et al* (1997) *Blood* 89: 1405-1412) and may contribute to a lack of growth control in these cells. Due to the low expression of WT1 in normal tissues, its expression on cancer cells makes it an attractive target for T-cell mediated therapy. TCR variants with increased avidity to WT1 containing a modified cysteine to discourage mispairing between the endogenous TCR subunits and the transgene TCRs have been transduced into primary T cells and tested for functionality (Kuball *et al* (2007) *Blood* 109(6):2331-8). The data demonstrated that while T cells that had been freshly transduced with the WT1-TCR variants had an increased antigen response as compared to those transduced with a wildtype TCR domain, after several rounds of stimulation with the WT1 antigen, this improved antigen responsiveness was lost (*see* Thomas *et al* (2007) *J of Immunol* 179 (9): 5803-5810). It was concluded that even with the transgene-specific cysteine modification, mispairing with the endogenous TCR peptides may play a role in reducing anti-WT1 avidity seen in cells transduced with the WT1-specific TCRs. *See, also,* U.S. Patent Publication No. 20110158957.

[0012] Another tumor antigen is NY-ESO1. It is a member of the so-called 'CT' set of tumor antigens, meaning that it is expressed on cancer cells and in the testis. Originally identified from expression on an esophageal tumor, NY-ESO1 has now been found to be expressed on several tumor types, including bladder, breast, colorectal, gastric, hepatocarcinoma, head and neck, multiple myeloma, melanoma, non-small cell lung cancer, ovarian, pancreatic, prostate, sarcomas and synovial sarcoma (see Gnjatic *et al* (2006) *Advances in Cancer Research* p. 1), often when those tumors are in advanced stages. Because of its apparent lack of expression on most tissues, NY-ESO1 has been considered for use in a cancer vaccine. Thus, both full length NY-ESO1 protein and peptides derived from the sequence have been and are being used in clinical trials. It appears however that the vaccination method may have limited usefulness, perhaps due to the production of T cells that have limited avidity to the antigen. In addition, many cancer patients harboring NY-ESO1 positive tumors have detectable anti-NY-ESO1 antibodies in their blood, but their tumors are

still able to evade the immune response. One potential solution may be the development of high affinity TCRs against the NY-ESO1 antigen. A study carried out using standard TCR transfer of NY-ESO1 specific TCRs made by three different T cell priming techniques into host T cells (see Sommermeyer *et al* (2012) *Int. J. Cancer* 132: 1360-1367) found that developing a robust TCR for adoptive immunotherapy will require overcoming a number of issues. There are also additional reports of NY-ESO1 specific TCRs that have been produced (see US8367804 and EP2016102B1 for specific examples). A clinical trial has also been carried out where NY-ESO1+ metastatic melanoma or metastatic synovial cell sarcoma patients were treated with autologous lymphocytes harvested from peripheral blood that had been transduced with a NY-ESO1 TCR. Clinical response was seen in 5 of 11 melanoma patients and 4 of 6 synovial cell sarcoma patients (Robbins *et al*, (2011) *J. Clin Oncol* 29(7) : 917).

[0013] Thus, there remains a need for compositions that can introduce desired TCR transgenes into a known chromosomal locus. In addition, there is a need for methods and compositions that can selectively knock out endogenous TCR genes.

SUMMARY

[0014] Disclosed herein are compositions and methods for partial or complete inactivation or disruption of an endogenous TCR gene and compositions and methods for introducing and expressing to desired levels of exogenous TCR transgenes into T-lymphocytes, after or simultaneously with the disruption of the endogenous TCR gene.

[0015] In one aspect, provided herein are zinc finger nucleases (ZFNs), TALENs or a CRISPR/Cas system with an engineered single guide RNA that cleaves a TCR gene. In certain embodiments, the ZFNs, TALENs or CRIPSR/Cas nucleases bind to target sites in a human TCR α gene and/or target sites in a human TCR β gene. In some embodiments, cleavage within the TCR gene(s) with these nucleases results in permanent disruption (*e.g.*, mutation/inactivation) of the TCR α and/or β gene(s).

[0016] In certain embodiments, the nuclease comprises a zinc finger protein. The zinc finger proteins may include 1, 2, 3, 4, 5, 6 or more zinc fingers, each zinc finger having a recognition helix that binds to a target subsite in the target gene. In certain embodiments, the zinc finger proteins comprise 4 or 5 or 6 fingers (designated F1, F2, F3, F4, F5 and F6 and ordered F1 to F4 or F5 or F6 from N-terminus to C-

terminus) and the fingers comprise the amino acid sequence of the recognition regions shown in Table 4 and Table 5 and/or recognize the target sites shown in Tables 4 and 5. In other embodiments, the nucleases are TALENs that may comprise engineered repeat units with canonical or non-canonical repeat variable diresidues (RVDs), for example TRAC and TRBC-specific TALENs as shown in Table 14 operably linked to a nuclease domain (*e.g.,* Type IIS Restriction endonuclease and/or meganuclease). The TALENs include a C-cap sequence, for example a C-terminal region that is less full-length of a wild-type TAL C-terminal sequence (*e.g.,* a +17 or +63 C-cap). C-cap sequences are described in U.S. Patent No. 8,586,526. Additional embodiments comprise use of the CRIPSR/Cas nuclease system where a single guide RNA has been made to target the nuclease to the target site in the TCR α and/or TCR β sequence.

[0017] Any of the nucleases described herein may further comprise a cleavage domain and/or a cleavage half-domain (*e.g.,* a wild-type or engineered *Fok*I cleavage half-domain or meganuclease domain with cleavage activity). Thus, in any of the nucleases described herein, the nuclease domain may comprise a wild-type nuclease domain or nuclease half-domain (*e.g.,* a *Fok*I cleavage half domain). In other embodiments, the nucleases (*e.g.,* ZFNs and/or TALENs) comprise engineered nuclease domains or half-domains, for example engineered *Fok*I cleavage half domains that form obligate heterodimers. *See, e.g.*, U.S. Patent Nos. 7,914,796; 8034,598 and U.S. Patent Publication No. 20080131962.

[0018] In another aspect, the disclosure provides a polynucleotide encoding any of the nucleases described herein. Any of the polynucleotides described herein may also comprise exogenous sequences (donor or patch sequences) for targeted insertion into the TCR α and/or the TCR β gene. In certain embodiments, the donor sequence comprises tumor antigen specific TCR transgene wherein the TCR transgene is a TCR α transgene, a TCR β transgene and combinations thereof. In certain embodiments, the transgene comprises a NY-ESO1-specific transgene where the NY-ESO1-specific transgene is a TCR α transgene, a TCR β transgene and combinations thereof..

[0019] In yet another aspect, a gene delivery vector comprising one or more of the polynucleotides described herein is provided (*e.g.,* donor and/or nuclease(s)). In certain embodiments, the vector is an adenoviral vector (*e.g.,* an Ad5/F35 vector) or a lentiviral vector (LV) including integration competent or integration-defective lentiviral vectors. Thus, also provided herein are adenoviral (Ad) vectors or LVs

comprising a sequence encoding at least one zinc finger nuclease (ZFN), TALEN or CRISPR/Cas nucleases and single guide RNA and/or a donor sequence for targeted integration into a target gene. In certain embodiments, the Ad vector is a chimeric Ad vector, for example an Ad5/F35 vector. In certain embodiments, the lentiviral vector is an integrase-defective lentiviral vector (IDLV) or an integration competent lentiviral vector. In certain embodiments the vector is pseudo-typed with a VSV-G envelope, or with other envelopes. In additional embodiments, the target gene is the human TCR α gene. In certain embodiments, the target gene is the human TCR β gene. The vectors described herein may also comprise donor sequences. In additional embodiments, the donor sequences comprise human TCR genes that are specific for an MHC/antigen complex of interest. In some embodiments, the donor sequences may comprise the human TCR α and/or the human TCR β genes that are specific for an MHC/antigen complex of interest. In certain embodiments, a single vector comprises sequences encoding one or more ZFNs, TALENs or CRISPR/Cas nuclease complex and the donor sequence(s). In other embodiments, the donor sequence(s) are contained in a first vector and the ZFN-, TALEN- or CRISPR/Cas encoding sequences are present in a second vector. In further embodiments, the ZFN-, TALEN-, or CRISPR/Cas-encoding sequences are present in a first vector and the TCR α gene of interest is present in a second vector and the TCR β gene of interest is present in a third vector. In some embodiments, the TCR genes of interest are inserted into the location of the endogenous TCR genes, and in other embodiments the TCR genes of interest are inserted into randomly selected loci, or into a separate locus after genome-wide delivery. In some embodiments, the separate locus for TCR transgene insertion is the PPP1R12C locus (also known as AAVS1, see U.S Patent Number 8,110,379). In other embodiments, the TCR transgene is inserted into a CCR-5 locus. *See,* U.S. Patent No. 7,951,925.

[0020] In yet another aspect, the disclosure provides an isolated T-lymphocyte comprising an exogenous sequence stably integrated into the genome of the T-lymphocyte and in which an endogenous TCR gene is partially or completely inactivated by a zinc finger nuclease or C-cap TALEN (TALEN with a C-terminal truncation). In certain embodiments, the cell comprises any of the proteins, polynucleotides and/or vectors described herein. In certain embodiments, the cell is selected from the group consisting of a stem/progenitor cell, a T-cell (*e.g.,* $CD4^+$ T-cell). In a still further aspect, the disclosure provides a cell or cell line which is

descended from a cell or line as described herein, namely a cell or cell line descended (*e.g.*, in culture) from a cell in which TCR has been inactivated by one or more ZFNs, TALENs or specific CRISPR/Cas nucleases and/or in which a TCR-encoding donor polynucleotide has been stably integrated into the genome of the cell. Thus, descendants of cells as described herein may not themselves comprise the proteins, polynucleotides and/or vectors described herein, but, in these cells, a TCR gene is inactivated and/or a TCR-encoding donor polynucleotide is integrated into the genome and/or expressed.

[0021] In another aspect, described herein are methods of inactivating a TCR gene in a cell by introducing one or more proteins, polynucleotides and/or vectors into the cell as described herein. In any of the methods described herein the ZFNs, TALENs or specific CRISPR/Cas nucleases may induce targeted mutagenesis, targeted deletions of cellular DNA sequences, and/or facilitate targeted recombination at a predetermined chromosomal locus. Thus, in certain embodiments, the ZFNs, TALENs or specific CRISPR/Cas nucleases delete or insert one or more nucleotides of the target gene. In some embodiments the TCR gene is inactivated by ZFN, TALEN or specific CRISPR/Cas nuclease cleavage followed by non-homologous end joining. In other embodiments, a genomic sequence in the target gene is replaced, for example using a ZFN, TALEs or specific CRISPR/Cas nuclease (or vector encoding said ZFN, TALEN or specific CRISPR/Cas nuclease) as described herein and a "donor" sequence that is inserted into the gene following targeted cleavage with the ZFN, TALEN or specific CRISPR/Cas nuclease. In certain embodiments, the donor sequence comprises a NY-ESO1 sequence. The donor sequence may be present in the ZFN, TALEN or specific CRISPR/Cas nuclease vector, present in a separate vector (*e.g.*, Ad or LV vector) or, alternatively, may be introduced into the cell using a different nucleic acid delivery mechanism.

[0022] In another aspect, methods of using the zinc finger proteins, TALENs or specific CRISPR/Cas nucleases and fusions thereof for mutating a TCR gene and/or inactivating TCR function in a cell or cell line are provided. Thus, a method for inactivating a TCR gene in a human cell is provided, the method comprising administering to the cell any of the proteins or polynucleotides described herein.

[0023] In yet another aspect, the disclosure provides a method for treating or preventing cancer, infections, autoimmune disorders, and/or graft-versus-host disease (GVHD) in a subject, the method comprising: (a) introducing, into a cell (*e.g.*,

lymphocyte, stem cell, progenitor cell, etc.), a first nucleic acid encoding a first polypeptide, wherein the first polypeptide comprises: (i) a zinc finger or TALE DNA-binding domain that is engineered to bind to a first target site in a TCR gene; and (ii) a cleavage domain; under conditions such that the polypeptide is expressed in the cell, whereby the polypeptide binds to the target site and cleaves the endogenous TCR gene; and (b) introducing, into the cell, a second nucleic acid encoding a second polypeptide, wherein the second polypeptide comprises: (i) a zinc finger or TALE DNA-binding domain that is engineered to bind to a second target site in a TCR gene; and (ii) a cleavage domain; under conditions such that the polypeptide is expressed in the cell, whereby the polypeptide binds to the target site and cleaves the endogenous TCR gene; and (c) introducing into the cell a third nucleic acid comprising a nucleic acid encoding a TCR gene or TCR genes, specific for a tumor specific antigen in an MHC complex, such that the third nucleic acid is introduced into the endogenous TCR gene and the cell with the introduced third nucleic acid treats or prevents cancer, infections, autoimmune disorders, and/or graft-versus-host disease (GVHD) in the subject. In certain embodiments, steps (a)-(c) are performed *ex vivo* and the method further comprises, following step (c), the step of introducing the cell into the subject. In certain embodiments, the third nucleic acid encoding the TCR gene(s) is expressed under the control of bi-directional promoters (*e.g.,* PGK, EF1α, etc.). In other embodiments, the TCR gene(s) are expressed from bicistronic cassettes (*e.g.,* using viral 2A peptides or an IRES sequence) or by multiple LVs expressing different TCR genes under monodirectional promoters. In certain embodiments, the cell is selected from the group consisting of a stem/progenitor cell, or a T-cell. In any of the methods describes herein, the first nucleic acid may further encode a second polypeptide, wherein the second polypeptide comprises: (i) a zinc finger or TALE DNA-binding domain that is engineered to bind to a second target site in the TCR gene; and (ii) a cleavage domain; such that the second polypeptide is expressed in the cell, whereby the first and second polypeptides bind to their respective target sites and cleave the TCR gene.

[0024] In another aspect, the disclosure also provides a method for treating or preventing cancer in a subject, the method comprising: (a) introducing, into a cell, a first nucleic acid encoding a first polypeptide, wherein the first polypeptide comprises: (i) a zinc finger or TALE DNA-binding domain that is engineered to bind to a first target site in a TCR gene; and (ii) a cleavage domain; under conditions such

that the polypeptide is expressed in the cell, whereby the polypeptide binds to the target site and cleaves the endogenous TCR; and (b) introducing, into a cell, a second nucleic acid encoding a second polypeptide, wherein the second polypeptide comprises: (i) a zinc finger or TALE DNA-binding domain that is engineered to bind to a first target site in a safe harbor locus (*e.g.,* PPP1R12C, CCR5); and (ii) a cleavage domain; under conditions such that the polypeptide is expressed in the cell, whereby the polypeptide binds to the target site and cleaves in the safe harbor locus (*e.g.,* PPP1R12C, CCR5)and (c) introducing into the cell a third nucleic acid comprising a donor nucleic acid encoding a TCR gene or TCR genes specific for a tumor specific antigen in an MHC complex; and (d) introducing the cell into the subject. The nucleic acids comprising the TCR specific ZFN, TALEN or CRISPR/Cas nuclease system may be introduced simultaneously with the ZFN, TALEN or CRISPR/Cas nuclease system specific for the safe-harbor locus and the donor nucleic acid molecule, or the nucleic acid encoding the TCR-specific ZFN, TALEN or CRISPR/Cas nuclease system may be introduced into the cell in a first step, and then the safe harbor locus (*e.g.,* PPP1R12C, CCR5) -specific ZFNs, TALENs or CRISPR/Cas nuclease system and the donor nucleic acid molecule may be introduced in a second step. In certain embodiments, the donor nucleic acid molecule encodes a tumor antigen such as NY-ESO1.

[0025] The disclosure also provides a method of preventing or treating a cancer in a subject comprising introducing, into a subject, a viral delivery particle wherein the viral delivery particle comprises (a) a first nucleic acid encoding a first polypeptide, wherein the first polypeptide comprises: (i) a zinc finger or TALE DNA-binding domain that is engineered to bind to a first target site in a TCR gene; and (ii) a cleavage domain; under conditions such that the polypeptide is expressed in the cell, whereby the polypeptide binds to the target site and cleaves the endogenous TCR; and (b) a second nucleic acid encoding a second polypeptide, wherein the second polypeptide comprises: (i) a zinc finger or TALE DNA-binding domain that is engineered to bind to a first target site in a safe harbor locus (*e.g.,* AAVS1, CCR5, albumin, HPRT etc. (see co-owned US Patent Nos. U.S. 8,110,379 and 7,951,925, and Patent Application Nos.13/624,193 and 13/660,821)); and (ii) a cleavage domain; under conditions such that the polypeptide is expressed in the cell, whereby the polypeptide binds to the target site and cleaves the safe harbor locus (*e.g.,* AAVS1, CCR5, albumin, HPRT); and (c) a third nucleic acid encoding a third polypeptide,

wherein the third polypeptide comprises: (i) a zinc finger or TALE DNA-binding domain that is engineered to bind to a second target site in a safe harbor locus (*e.g.*, AAVS1, CCR5, albumin, HPRT); and (ii) a cleavage domain; under conditions such that the polypeptide is expressed in the cell, whereby the polypeptide binds to the target site and cleaves at the safe harbor locus (*e.g.*, AAVS1, CCR5, albumin, HPRT); and (d) a third nucleic acid comprising a donor nucleic acid encoding a TCR gene or TCR genes specific for a tumor specific antigen in an MHC complex; such that the endogenous TCR gene is cleaved and rendered inactive, and the safe harbor gene (*e.g.*, AAVS1, CCR5, albumin, HPRT) is cleaved and the TCR gene specific for a tumor specific antigen in an MHC complex becomes inserted into the endogenous TCR gene. In certain embodiments, the method further comprises, following step (d), the step of introducing the cell into the subject. In certain embodiments, the donor nucleic acid molecule encodes a tumor antigen such as NY-ESO1.

[0026] In any of the methods described herein, a viral delivery particle can be used to deliver one or more of the polynucleotides (ZFN- or TALEN-encoding and/or donor polynucleotides). Furthermore, in any of the methods and compositions described herein, the cell can be, for example, a stem/progenitor cell (*e.g.*, a $CD34^+$ cell), or a T-cell (*e.g.*, a $CD4^+$ cell).

[0027] Furthermore, any of the methods described herein can be practiced *in vitro, in vivo* and/or *ex vivo*. In certain embodiments, the methods are practiced *ex vivo,* for example to modify PBMCs, *e.g.,* T-cells, to make them specific for a tumor antigen/MHC complex of interest to treat a tumor in a subject. Non-limiting examples of cancers that can be treated and/or prevented include lung carcinomas, pancreatic cancers, liver cancers, bone cancers, breast cancers, colorectal cancers, leukemias, ovarian cancers, lymphomas, brain cancers and the like.

BRIEF DESCRIPTION OF THE DRAWINGS

[0028] **Figure 1, panels A and B,** depict construction and expression of a Wilms tumor antigen (WT1) specific lentiviral vector. Figure 1A depicts a diagram of the genes encoding a codon-optimized, cysteine-modified TCR specific for an HLA-A2-restricted peptide from the Wilms tumor antigen 1 (WT1) cloned into a third generation lentiviral vector (LV) under the control of a bi-directional PGK or EF1α

promoter. *See,* Amendola *et al* (2005) *Nature Biotechnology* 23(1): 108-116 and U.S. Patent Publication No US2006200869. Figure 1B is a graph depicting a time course of Vβ21 TCR expression in lentivirus transduced $CD8^+$ cells cultured in the presence of 5 ng/ml of IL7 and IL15. Vβ21 relative fluorescence intensity (RFI) was calculated as the ratio of the mean fluorescence intensity (MFI) of Vβ21 measured in PGK-WT1 (open squares) or EF1α-WT1 ("X") genetically modified lymphocytes/the MFI of Vβ21 measured in T cells naturally expressingVβ21.

[0029] **Figure 2, panels A to C**, are graphs depicting results of cells transduced with TCR constructs. Figure 2A depicts induction of γIFN production by stimulation of the cells with WT1+HLA-A2+ and WT1+/HLA A2- cells (the indicated primary AML or K562 cells (right most bar in graph)) transduced with vectors expressing the transgenic TCRs either from the PGK/mCMV dual promoter combination (left side group of 4 bars) or the EF1α/mCMV dual promoter (right side group of 4 bars) following exposure to WT1+HLA-A2+ or WT1+HLA-A2- (negative control) primary leukemic blasts from AML patients (designated as AML1 (left most bar), AML2 (second bar from the left) and AML3 (third bar from the left)). Figures 2B and 2C demonstrate the percent killing of the leukemic blasts from AML1 and AML2 (solid lines, closed circles) by the TCR modified cells. Dotted lines represent residual killing of the leukemic blasts by the TCR modified cells in the presence of an excess of cold (not labeled) HLA-A2 target cells, loaded with the WT1 proper peptide.

[0030] **Figure 3, panels A and B**, are graphs depicting GFP expression following introduction of ZFNs targeted to a safe harbor locus together with a GFP donor. Figures 3A and 3B demonstrate the increase in the percentage of GFP positive cells in relation to the amount of Ad5/F35 CCR5-specific ZFN (Fig. 3A) or Ad5/F35 AAVS1-specific ZFN (Fig. 3B) and –IDLV GFP donor DNA cassette used.

[0031] **Figure 4, panels A and B**, depict diagrams of exemplary TCR-α and TCR-β donor molecules (Figure 4A) and the TCR-β genes (Figure 4B). Figure 4A depicts a cassette containing WT1-specific TCR-α and TCR-β donor molecules and shows the regions of homology to the CCR5 integration site. Figure 4B depicts the genomic arrangement of the two TCR-β constant regions in K562 cells (TRBC1 and TRBC2).

[0032] **Figure 5** depicts the percent modification for several pairs of TCR-β-specific ZFNs in K562 cells as measured by a Cel-I Surveyor™ mismatch assay ("Cel-I assay" Transgenomic) The cells were incubated initially at 30°C following transfection, with either 0.1 or 0.4 μg of ZFN plasmid. Percent modification is shown at the bottom of the lanes.

[0033] **Figure 6, panels A and B,** depict percent modification for TCR-α specific ZFNs in K562 cells as measured by a Cel-I assay. Figure 6A depicts the results from a Cel-I assay on cells where the ZFNs were targeted to Exon 1. Figure 6B depicts the results of a Cel I mismatch assay where the ZFNs were targeted to Exon 3. "GFP" indicates cells tranduced with GFP only vectors. Percent alteration (NHEJ) is indicated at the bottom of the lanes. As shown, sorted CD3- lymphocytes survive in the presence of IL7 and IL15.

[0034] **Figure 7, panels A to F**, depict ZFN-mediated cleavage of TCR-β. Untransduced and transduced Jurkat cells using the TCR-β specific ZFN pair 16783 and 16787 at two concentrations of vector demonstrated the loss of CD3 signal at the cell surface (from 2.7% CD3(-) to 20.2% CD3(-) (see Example 6). Figures 7A and 7B show the results of Cel-I assays at the TRBC1 (Fig. 7A) and the TRBC2 (Fig. 7B) locus in Jurkat cells and demonstrate that cleavage has occurred. The measured % gene modification is indicated at the bottom of each lane. Figure 7C is a graph depicting that sorted CD3- primary human lymphocytes can survive in the presence of IL7 and IL15. "UT" indicates untreated cells. Figure 7D shows the percent modification (NHEJ), as assayed by the Cel-I assay observed in the primary T-cell cell pools treated with TCR-beta specific ZFNs. "Bulk" indicates percent of NHEJ observed for the ZFN treated cell pool, while CD3+ or CD3- shows the NHEJ observed for cells that were sorted either as CD3+ or CD3-. "UT" indicates cells that were not treated. The percent NHEJ detected by the assay is indicated at the bottom of the lanes. Figure 7E is a graph depicting percent CD3- cells and demonstrates the persistence of CD3- cells over time (percent of CD3- cells stays fairly constant even up to 45 days) in cells treated with increasing concentrations of ZFNs. Figure 7F is a graph depicting that CD3- cells have lost TCR/CD3 functionality since they do not appear to divide in response to non-specific mitogens. As shown, CD3- cells survive and are stable in culture in the presence of IL7 and IL15 for more than 40 days, do not respond to polyclonal mitogens, and maintain a TCM phenotype.

[0035] **Figure 8** depicts the experimental outline and the FACS results for editing of the TCR-β locus in primary T lymphocytes and the re-introduction of a specific TCR transgene. Cells used were either untreated primary T cells lymphocytes or lymphocytes pre-treated with the TCR-β-specific ZFNs carried by an IDLV and then sorted for CD3(-) primary T cells. Gene transfer was achieved after stimulation of T cells with cell-sized beads coated with antibodies directed to CD3 and to CD28, and cell culture in the presence of IL7 (5ng/ml) and IL15 (5ng/ml) to facilitate the generation of genetically modified central memory lymphocytes, according to European Patent Publication No EP1956080. As shown, cells that were sorted for being CD3(-) after treatment with the TCR-β specific ZFNs and then have the WT1-TCRβ V21.3 and WT1.TCRα transgenes randomly integrated into the genome using a lentiviral vector, show an increase in staining for both CD3 and for Vβ21.3, indicating primary T lymphocytes can undergo endogenous TCR disruption via NHEJ using the TCR-β-specific ZFNs and then be re-targeted to recognize a specific antigen via the introduction of a new TCR encoded by a transgene cassette (PGK-WT1). As a control, UT cells also had the PGK-WT1 cassette inserted and showed a smaller percentage of cells expressing Vβ 21.3 (26%) as compared to the ZFN-treated, CD3(-) population (46%, 92% after polyclonal stimulation), indicating the disruption of the endogenous TCR may improve the cell-surface expression and functionality of the TCR expressed from the transgene.

[0036] **Figure 9, panels A to C,** depict expression of Vβ21 TCR. Figure 9A depicts Vβ21 TCR expression (upper histogram) and $WT1_{126\text{-}134}$ pentamer binding (lower histogram) in $CD8^+$ TCR β chain disrupted and WT1 transduced cells (TCR-β-edited), unedited WT1 LV transduced cells (TCR-transferred), and untransduced lymphocytes treated with the same culture conditions. Figure 9B shows a time course of surface expression of Vβ21 TCR. Average + SD (n=2) of Vβ21 RFI is represented. RFI is calculated from the ratio of the MFI of Vβ21 measured in $CD8^+$ TCR-edited (open triangle) or TCR-transferred (dark circle) lymphocytes/the MFI of Vβ21 measured in $CD8^+$ T cells naturally expressingVβ21. Figure 9C depicts the results of a cytotoxicity assay with TCR-edited and TCR-transferred cells. Functional activity is measured by a ^{51}Chromium release assay for lysis of labeled T2 cells pulsed with increasing concentrations of the $WT1_{126\text{-}134}$ HLA-A2 restricted peptide, or with the irrelevant CMV-derived $pp65_{495\text{-}503}$ HLA-A2 restricted peptide (10 μM) as negative

control, at an Effector/Target (E/T) ratio of 12. Results are represented as average + SD of % of lysis (**, $p<0.01$, *, $p<0.05$ with Mann-Whitney test, TCR-edited n=6, TCR-transferred n=4).

[0037] **Figure 10, panels A and B,** depict the functional activity of WT1 TCR-positive T cell clones as tested by γIFN ELISpot assay. Clones were exposed to T2 cells pulsed with 10nM of the $WT1_{126-134}$ HLA-A2 restricted peptide (10A), or to allogeneic PBMC (10B) at a stimulator/responder ratio of 1:1. The number of specific spots (open triangles and dark circles) observed is shown on the y axis as number of spots produced in presence of stimulators minus the number of spots produced by effectors alone. The results show that the TCR- β edited clones exhibit a higher degree of antigen specificity than the TCR transferred cells which contain both the endogenous and the exogenous TCR genes.

[0038] **Figure 11** depicts Vβ21 expression in ZFN-edited and unedited cells. CD3(-) cells sorted from *TRBC*-disrupted lymphocytes and unedited cells and were transduced at increasing MOI by LV encoding the Vβ21 gene of the WT1-specific TCR and the ΔLNGFR gene (see diagram at the top of figure showing dual expression of the Vβ21 gene and the ΔLNGFR gene) . Transduction efficiency was assessed as % of $\Delta LNGFR^{pos}$ lymphocytes and is shown. Vβ21 expression was measured on $\Delta LNGFR^{pos}$ cells and demonstrates that the transduced Vβ21 gene can be expressed and form active CD3 complexes with the endogenous TCR α chain. The mean fluorescence intensity (MFI) of Vβ21 is shown.

[0039] **Figure 12, panels A to C,** depict CD3 expression in primary lymphocytes treated with ZFN targeting TCR α genes. Figure 12A depicts a diagram of the human locus encoding the TCR α, total length 18 kb; TRAV, variable region genes, TRAD, diversity region genes, TRAC, constant region gene. Displayed above the scheme of the locus are the genomic DNA sequences in TRAC targeted by each TRAC-ZFN. Figure 12A discloses the protein sequence as SEQ ID NO: 109 and the DNA sequence as SEQ ID NO: 108. Figure 12B depicts down-regulation of cell surface CD3 expression measured by flow cytometry in primary human lymphocytes stimulated with baCD3/CD28, cultured with 5ng/ml IL-7, 5ng/ml IL-15, and exposed to TRAC-ZFN IDLVs. The percent of CD3(-) cells is plotted. UT, Untransduced cells. Sorted CD3(-) cells were transduced with WT1-α OFP-LV resulting in expression of CD3 on transduced lymphocytes. Figure 12C depicts a gel showing the

level of targeted gene disruption measured by the Cel-I assay in primary lymphocytes exposed to TRAC-ZFN. The higher migrating product indicating wild type (w/t) gene is shown. Lower migrating products (NHEJ) indicate ZFN-directed gene disruption. "UT" refers to untransduced cells.

[0040] **Figure 13** depicts partial sequence of the genomic *TRAC* ZFN target site in ZFN-treated human lymphocytes was amplified, cloned and sequenced to confirm ZFN-induced modification. Sequence alignment revealed several ZFN-induced deletions and insertions (indels) within the target region. The left column indicates the number of clones retrieved while the right column indicates the number of deleted or inserted nucleotides. Figure 13 discloses SEQ ID NOs: 110-143, respectively, in order of appearance.

[0041] **Figure 14** depicts expression of CD3 in following ZFN editing. Upper panels shows results of studies in which activated T lymphocytes were treated with TRAC-ZFN-AdV (MOI 1000), and CD3(-) lymphocytes were sorted and transduced with 3 μg-p24/10^6cells of PGK-WT1-α LV and CD3(+) cells were sorted. Surface expression of CD3 in transduced cells is shown. After one cycle of polyclonal stimulation, α- edited lymphocytes were treated with TRBC-ZFN-AdV (MOI 10^4) and CD3(-) cells sorted and transduced with 3 μg-p24/10^6cells of PGK-WT1-β LV. Surface expression of Vβ21 TCR and CD3 is shown on transduced cells before and after one cycle of polyclonal stimulation. Percent of events measured in each quadrant are shown, and the experimental timeline is shown on the bottom.

[0042] **Figure 15** depicts Vβ21 TCR expression (upper histogram) and $WT1_{126\text{-}134}$ pentamer binding (lower histogram) are shown in CD8(+) T cells with TCR α/β chains disrupted via introduction of ZFNs and sorting for CD3- cells followed by transduction with the WT1 TCR chains and sorting for CD3+ cells (TCR-edited), unedited WT1 LV transduced cells (TCR-transferred), and untransduced lymphocytes treated with the same culture conditions. The data show that the TCR edited cells have a higher level of Vβ21 expression than those clones wherein both the endogenous and the exogenous TCR genes are present. It also demonstrates that the TCR edited cells display higher binding to the WT1 peptide than those cells that have both sets of TCR genes.

[0043] **Figure 16, panels A to C,** are graphs depicting functional activity of genetically modified lymphocytes was tested by the γIFN ELISpot assay. Three

weeks after polyclonal stimulation, TCR-α/β-edited and TCR transferred lymphocytes were exposed to either i) T2 cells pulsed with increasing concentrations of the $WT1_{126-134}$ HLA-A2 restricted peptide, or with the irrelevant CMV-derived $pp65_{495-503}$ HLA-A2 peptide (see Figure 16A, right side of figure) or ii) $WT1^{+}$ HLA-A2(+) (black in Figure 16B) or HLA-A2(-) (grey) leukemic cells harvested from AML patients with (dashed symbols) or without (full symbols) pulsing with $WT1_{126-134}$ peptide (50nM). Figure 16C shows similar results where allogenic PBMC were used as target. All assays were performed at a stimulator/responder ratio of 1:1. The number of specific spots is shown on the y axis as the number of spots produced in the presence of stimulators minus the number of spots produced by effectors alone. * = $p<0.05$, ** = $p<0.01$, *** = $p<0.001$.

[0044] **Figure 17** depicts analysis for off-target cleavage by TRAC-specific ZFNs. The 15 most likely potential off target sites for the TRAC-specific ZFNs (identified by *in silicio* analysis) were analyzed following ZFN treatment for cleavage by the Cel-I mismatch assay. Each potential off target site was analyzed in 5 samples: untransduced samples (UT), samples that were TRAC negative following TRAC-specific ZFN treatment and sorting (TRAC neg), cells that were TRAC and TRBC negative following sequential treatment with TRAC-specific ZFNs, the TRAC transgene and TRBC-specific ZFNs with sequential rounds of sorting (Double Neg), cells that were negative for the endogenous TRAC and TRBC loci following ZFN treatment as well as modified to comprise non-wild type TRAC and TRBC transgenes (Complete Edited), or TRBC negative following treatment with TRBC-specific ZFNs alone and sorting (TRBC Neg). Potential off target sites are as labeled in Table 12.

[0045] **Figure 18** depicts analysis for off-target cleavage by TRBC-specific ZFNs. 15 potential off target sites for the TRBC-specific ZNS (identified by *in silicio* analysis) were analyzed following ZFN treatment for cleavage by the Cel-I mismatch assay. Each potential off target site was analyzed in 5 samples: untransduced samples (UT), samples that were TRAC negative following TRAC-specific ZFN treatment and sorting (TRAC neg), cells that were TRAC and TRBC negative following sequential treatment with TRAC-specific ZFNs, the TRAC transgene and TRBC-specific ZFNs with sequential rounds of sorting (Double Neg), cells that were negative for the endogenous TRAC and TRBC loci following ZFN treatment as well as modified to comprise non-wild type TRAC and TRBC transgenes (Complete Edited), or TRBC

negative following treatment with TRBC-specific ZFNs alone and sorting (TRBC Neg). Off target sites are as labeled in Table 13. TRBC depicts modification of the intended target site in these samples.

[0046] **Figure 19, panels A to C,** demonstrate expression of the NY-ESO1-specific TCR and binding to the appropriate target. Figure 19A shows a comparison of T cells transduced with the NY-ESO1 specific TCR ("Transfer") to cells that prior to TCR transduction, were first treated with TRAC-specific ZFNs to knock out the endogenous TCR-alpha chain ("SE") or cells that had both the TCR-alpha and the TCR-beta chains knocked out prior ("CE") prior to NY-ESO1 specific TCR transduction. Expression of the specific TCR is shown in the three T cell populations. Figure 19B shows the binding affinity for a dextramer comprised of NY-ESO1 peptides, and shows that the cell population that has had all the endogenous TCR chains deleted (CE) has the highest binding affinity of the T cell populations tested. Figure 19C is a graph depicting the average of 3 consecutive experiments from 3 different donors. The left most bar shows "Transfer" results, the middle bar shows "SE" results and the right most bar shows "CE" results.

[0047] **Figure 20, panels A to D,** depict binding and activity of the T cell populations described in Figure 19. Figure 20A depicts the binding of the different T cell groups against a peptide derived from the NY-ESO1 target, while Figure 20B depicts binding of the T cells to myeloma cell lines that are either HLA-A2$^-$, NY-ESO1$^-$ (MM1S, "A2-ESO-") or are HLA-A2+, NY-ESO1+ (U266, "A2+ESO+"). Binding to the MM1S cell line was nearly undetectable. The T cells were then tested for their ability to cause lysis of the proper cell target as analysed by a ^{51}chromium release assay, (Figures 20C and 20D) and increased lysis of the relevant target cell was observed in comparison with the irrelevant cells by the edited TCR T cells. For Figures 20A, C and D, untreated cells (UT) are are shown by ●; Transfer cells are shown by squares; SE cells are shown by ▲; CE cells are shown by ▼.

[0048] **Figure 21, panels A and B,** depict growth inhibition of cells in a co-culture experiment by the different T cell populations. Figure 21A depicts growth inhibition of the irrevelant MM1S cells that are HLA-A2-, NY-ESO1- in comparison with the U266 HLA-A2+, NY-ESO1+ cells. Figure 21B demonstrates that the edited T cells expand of 2 folds in the presence of the U266 HLA-A2+, NY-ESO1+ target.

[0049] **Figure 22, panels A to D,** are graphs depicting the alloreactivity of NY-ESO1 T-cells. Figure 22A shows the percent of cell lysis in the indicated cell

types at the effector/target ratio of 50:1. Figure 22B shows results of an γ-interferon (γ-IFN) ELISPOT assay in the indicated cells. Figure 22C shows the percent cell lysis in the indicated cells pulsed with the NY-ESO-1 specific peptide while Figure 22D shows the lysis detected when the cells are not pulsed.

[0050] **Figure 23, panels A and B,** depict the percent of human Multiple Myeloma CD138+ cells (hCD138+) in bone marrow of mice treated with the indicated cells (Figure 23A) and the pathological score (hCD3+ infiltration) in mice treated with the indicated cells (Figure 23B).

[0051] **Figure 24, panels A to C,** depict TCR editing by ZFN-encoding mRNA electroporation. Figure 24A depicts ZFN-induced TCR disruption at the indicated dosages at day 5 or 20 days after ZFN electroporation into lymphocytes using either TRAC-specific ZFN mRNAs (left graph) or TRBC-specific ZFN mRNAs (right graph). Figure 24B depicts the fold increase in the number of treated cells. TRAC-specific ZFN treated cells are shown in the left most graph; TRBC-ZFN treated cells are shown in the middle graph and controls are shown in the right most graph. "UT" refers to untreated cells; "UT+E" refers to mock electroporated cells. Figure 24C shows the percentage of the indicated surface phenotypes at day 18 after stimulation. TRAC-ZFN treated cells are shown in the left most graph; TRBC-ZFN treated cells are shown in the middle graph and controls are shown in the right most graph. T stem memory cells (TSCM) are defined as CD62L+ CD45RA+; T central memory (TCM) as CD62L+ CD45RA-; T effector memory (TEM) as CD62L- CD45RA- and terminal effectors (TEMRA) as CD62L- CD45RA+. UT: untreated cells; UT+E: mock electroporated cells; GFP: cells electroporated with GFP encoding mRNA.

[0052] **Figure 25, panels A to D,** depict double TCR editing by ZFN mRNA electroporation. Figure 25A shows a representative analysis to quantify the amount of complete, TCR-alpha and TCR-beta edited cells in the CD3 negative fraction of the co-treated cells. The fraction of single TCR-alpha or TCR-beta edited cells (shown in the squares on right) was measured as percentage of transduced cells that restore surface expression of the CD3 upon complementation with an exogenous TCR alpha or beta gene. The amount of complete edited cells in the total CD3 negative population is then calculated by subtracting the two percentages of single edited cells. Figure 25B is a histogram showing the percentages of CD3 negative cells upon co-electroporation of TRAC- and TRBC-specific ZFN encoding mRNAs containing the

obligated heterodimeric FokI domains (ELD and KKR) or their respective orthologous version (RDD and DRR) (left panel). The percentages of viable cells (indicated on top of the histogram) were calculated as percentages of 7-Amino-actinomycin D (7-AAD) negative cells gated on singlets. 7-AAD intercalates into double-stranded nucleic acids. It is excluded by viable cells but can penetrate cell membranes of dying or dead cells. The right panel of Figure 25B shows composition of the edited cells in the CD3 negative fraction calculated using the LV reporter strategy described above. The top portion of each bar shows the percent of completely edited (30%, 40% and 49% from left to right); the middle portion of each bar shows the percentage of beta edited cells (6%, 44% and 21% from left to right bars); and the lower portion of each bar shows the percent of TCR-alpha edited cells (64%, 16% and 30% from left to right bars). Figure 25C shows the surface phenotype of T cells from at day 18 after stimulation. Four phenotypes are shown: the bottom most portion of each bar shows stem memory cells (TSCM) defined as CD62L+ CD45RA+; the portion second from the bottom on each bar shows T central memory (TCM) as CD62L+ CD45RA-; the portion second from the top of each bar shows T effector memory (TEM) as CD62L- CD45RA- and the top most portion of each bar shows terminal effectors (TEMRA) as CD62L- CD45RA+. "UT" refers to untreated cells. Figure 25D shows growth curves of T cells co-electroporated with the indicated doses of TRAC- and TRBC-specific ZFN mRNAs.

DETAILED DESCRIPTION

[0053] Disclosed herein are zinc finger nucleases (ZFNs) and TALENs targeting a TCR gene (TCR-ZFNs and TCR-TALENs). These nucleases efficiently generate a double strand break (DSB), for example at a predetermined site in a TCR coding region. ZFN- or TALEN-mediated introduction of a site-specific double strand break (DSB) in genes that encode for the TCR gene can result in the specific and permanent disruption of the endogenous TCR complex in human cells, including human T cells. These cells can be selected from a pool by selecting for CD3(-) cells, and culturing them on IL7 and IL15. In addition, disclosed herein are methods and compositions for the replacement of the endogenous TCR genes with TCR transgenes of one's choice, either via random integration or by site directed targeted integration.

General

[0054] Practice of the methods, as well as preparation and use of the compositions disclosed herein employ, unless otherwise indicated, conventional techniques in molecular biology, biochemistry, chromatin structure and analysis, computational chemistry, cell culture, recombinant DNA and related fields as are within the skill of the art. These techniques are fully explained in the literature. *See*, for example, Sambrook *et al.* MOLECULAR CLONING: A LABORATORY MANUAL, Second edition, Cold Spring Harbor Laboratory Press, 1989 and Third edition, 2001; Ausubel *et al.*, CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley & Sons, New York, 1987 and periodic updates; the series METHODS IN ENZYMOLOGY, Academic Press, San Diego; Wolffe, CHROMATIN STRUCTURE AND FUNCTION, Third edition, Academic Press, San Diego, 1998; METHODS IN ENZYMOLOGY, Vol. 304, "Chromatin" (P.M. Wassarman and A. P. Wolffe, eds.), Academic Press, San Diego, 1999; and METHODS IN MOLECULAR BIOLOGY, Vol. 119, "Chromatin Protocols" (P.B. Becker, ed.) Humana Press, Totowa, 1999.

Definitions

[0055] The terms "nucleic acid," "polynucleotide," and "oligonucleotide" are used interchangeably and refer to a deoxyribonucleotide or ribonucleotide polymer, in linear or circular conformation, and in either single- or double-stranded form. For the purposes of the present disclosure, these terms are not to be construed as limiting with respect to the length of a polymer. The terms can encompass known analogues of natural nucleotides, as well as nucleotides that are modified in the base, sugar and/or phosphate moieties (*e.g.*, phosphorothioate backbones). In general, an analogue of a particular nucleotide has the same base-pairing specificity; *i.e.*, an analogue of A will base-pair with T.

[0056] The terms "polypeptide," "peptide" and "protein" are used interchangeably to refer to a polymer of amino acid residues. The term also applies to amino acid polymers in which one or more amino acids are chemical analogues or modified derivatives of a corresponding naturally-occurring amino acids.

[0057] "Binding" refers to a sequence-specific, non-covalent interaction between macromolecules (*e.g.*, between a protein and a nucleic acid). Not all components of a binding interaction need be sequence-specific (*e.g.*, contacts with phosphate residues in a DNA backbone), as long as the interaction as a whole is sequence-specific. Such interactions are generally characterized by a dissociation

constant (K_d) of 10^{-6} M^{-1} or lower. "Affinity" refers to the strength of binding: increased binding affinity being correlated with a lower K_d.

[0058] A "binding protein" is a protein that is able to bind non-covalently to another molecule. A binding protein can bind to, for example, a DNA molecule (a DNA-binding protein), an RNA molecule (an RNA-binding protein) and/or a protein molecule (a protein-binding protein). In the case of a protein-binding protein, it can bind to itself (to form homodimers, homotrimers, *etc.*) and/or it can bind to one or more molecules of a different protein or proteins. A binding protein can have more than one type of binding activity. For example, zinc finger proteins have DNA-binding, RNA-binding and protein-binding activity.

[0059] A "zinc finger DNA binding protein" (or binding domain) is a protein, or a domain within a larger protein, that binds DNA in a sequence-specific manner through one or more zinc fingers, which are regions of amino acid sequence within the binding domain whose structure is stabilized through coordination of a zinc ion. The term zinc finger DNA binding protein is often abbreviated as zinc finger protein or ZFP.

[0060] A "TALE DNA binding domain" or "TALE" is a polypeptide comprising one or more TALE repeat domains/units. The repeat domains are involved in binding of the TALE to its cognate target DNA sequence. A single "repeat unit" (also referred to as a "repeat") is typically 33-35 amino acids in length and exhibits at least some sequence homology with other TALE repeat sequences within a naturally occurring TALE protein. TALENs preferably include C-terminal and/or N-terminal truncations (*e.g.,* C-cap and/or N-cap). *See, e.g.,* U.S. Patent No. 8,586,526, incorporated by reference herein in its entirety.

[0061] Zinc finger and TALE binding domains can be "engineered" to bind to a predetermined nucleotide sequence, for example via engineering (altering one or more amino acids) of the recognition helix region of a naturally occurring zinc finger or TALE protein. Therefore, engineered DNA binding proteins (zinc fingers or TALEs) are proteins that are non-naturally occurring. Non-limiting examples of methods for engineering DNA-binding proteins are design and selection. A designed DNA binding protein is a protein not occurring in nature whose design/composition results principally from rational criteria. Rational criteria for design include application of substitution rules and computerized algorithms for processing information in a database storing information of existing ZFP and/or TALE designs and binding data. See, for example, U.S. Patent Nos. 8,586,526; 6,140,081;

6,453,242; and 6,534,261; see also WO 98/53058; WO 98/53059; WO 98/53060; WO 02/016536 and WO 03/016496.

[0062] A "selected" zinc finger protein or TALE is a protein not found in nature whose production results primarily from an empirical process such as phage display, interaction trap or hybrid selection. See *e.g.*, U.S. 8,586,526; 5,789,538; US 5,925,523; US 6,007,988; US 6,013,453; US 6,200,759; WO 95/19431; WO 96/06166; WO 98/53057; WO 98/54311; WO 00/27878; WO 01/60970 WO 01/88197; WO 02/099084.

[0063] The term "sequence" refers to a nucleotide sequence of any length, which can be DNA or RNA; can be linear, circular or branched and can be either single-stranded or double stranded. The term "donor sequence" refers to a nucleotide sequence that is inserted into a genome. A donor sequence can be of any length, for example between 2 and 10,000 nucleotides in length (or any integer value therebetween or thereabove), preferably between about 100 and 1,000 nucleotides in length (or any integer therebetween), more preferably between about 200 and 500 nucleotides in length.

[0064] A "homologous, non-identical sequence" refers to a first sequence which shares a degree of sequence identity with a second sequence, but whose sequence is not identical to that of the second sequence. For example, a polynucleotide comprising the wild-type sequence of a mutant gene is homologous and non-identical to the sequence of the mutant gene. In certain embodiments, the degree of homology between the two sequences is sufficient to allow homologous recombination therebetween, utilizing normal cellular mechanisms. Two homologous non-identical sequences can be any length and their degree of non-homology can be as small as a single nucleotide (*e.g.*, for correction of a genomic point mutation by targeted homologous recombination) or as large as 10 or more kilobases (*e.g.*, for insertion of a gene at a predetermined ectopic site in a chromosome). Two polynucleotides comprising the homologous non-identical sequences need not be the same length. For example, an exogenous polynucleotide (*i.e.*, donor polynucleotide) of between 20 and 10,000 nucleotides or nucleotide pairs can be used.

[0065] Techniques for determining nucleic acid and amino acid sequence identity are known in the art. Typically, such techniques include determining the nucleotide sequence of the mRNA for a gene and/or determining the amino acid sequence encoded thereby, and comparing these sequences to a second nucleotide or

amino acid sequence. Genomic sequences can also be determined and compared in this fashion. In general, identity refers to an exact nucleotide-to-nucleotide or amino acid-to-amino acid correspondence of two polynucleotides or polypeptide sequences, respectively. Two or more sequences (polynucleotide or amino acid) can be compared by determining their percent identity. The percent identity of two sequences, whether nucleic acid or amino acid sequences, is the number of exact matches between two aligned sequences divided by the length of the shorter sequences and multiplied by 100. An approximate alignment for nucleic acid sequences is provided by the local homology algorithm of Smith and Waterman, Advances in Applied Mathematics 2:482-489 (1981). This algorithm can be applied to amino acid sequences by using the scoring matrix developed by Dayhoff, Atlas of Protein Sequences and Structure, M.O. Dayhoff ed., 5 suppl. 3:353-358, National Biomedical Research Foundation, Washington, D.C., USA, and normalized by Gribskov, Nucl. Acids Res. 14(6):6745-6763 (1986). An exemplary implementation of this algorithm to determine percent identity of a sequence is provided by the Genetics Computer Group (Madison, WI) in the "BestFit" utility application. The default parameters for this method are described in the Wisconsin Sequence Analysis Package Program Manual, Version 8 (1995) (available from Genetics Computer Group, Madison, WI). A preferred method of establishing percent identity in the context of the present disclosure is to use the MPSRCH package of programs copyrighted by the University of Edinburgh, developed by John F. Collins and Shane S. Sturrok, and distributed by IntelliGenetics, Inc. (Mountain View, CA). From this suite of packages the Smith-Waterman algorithm can be employed where default parameters are used for the scoring table (for example, gap open penalty of 12, gap extension penalty of one, and a gap of six). From the data generated the "Match" value reflects sequence identity. Other suitable programs for calculating the percent identity or similarity between sequences are generally known in the art, for example, another alignment program is BLAST, used with default parameters. For example, BLASTN and BLASTP can be used using the following default parameters: genetic code = standard; filter = none; strand = both; cutoff = 60; expect = 10; Matrix = BLOSUM62; Descriptions = 50 sequences; sort by = HIGH SCORE; Databases = non-redundant, GenBank + EMBL + DDBJ + PDB + GenBank CDS translations + Swiss protein + Spupdate + PIR. Details of these programs can be found at the following internet address: http://www.ncbi.nlm.gov/cgi-bin/BLAST. With respect to

sequences described herein, the range of desired degrees of sequence identity is approximately 80% to 100% and any integer value therebetween. Typically the percent identities between sequences are at least 70-75%, preferably 80-82%, more preferably 85-90%, even more preferably 92%, still more preferably 95%, and most preferably 98% sequence identity.

[0066] Alternatively, the degree of sequence similarity between polynucleotides can be determined by hybridization of polynucleotides under conditions that allow formation of stable duplexes between homologous regions, followed by digestion with single-stranded-specific nuclease(s), and size determination of the digested fragments. Two nucleic acid, or two polypeptide sequences are substantially homologous to each other when the sequences exhibit at least about 70%-75%, preferably 80%-82%, more preferably 85%-90%, even more preferably 92%, still more preferably 95%, and most preferably 98% sequence identity over a defined length of the molecules, as determined using the methods above. As used herein, substantially homologous also refers to sequences showing complete identity to a specified DNA or polypeptide sequence. DNA sequences that are substantially homologous can be identified in a Southern hybridization experiment under, for example, stringent conditions, as defined for that particular system. Defining appropriate hybridization conditions is within the skill of the art. See, *e.g.*, Sambrook et al., *supra*; Nucleic Acid Hybridization: A Practical Approach, editors B.D. Hames and S.J. Higgins, (1985) Oxford; Washington, DC; IRL Press).

[0067] Selective hybridization of two nucleic acid fragments can be determined as follows. The degree of sequence identity between two nucleic acid molecules affects the efficiency and strength of hybridization events between such molecules. A partially identical nucleic acid sequence will at least partially inhibit the hybridization of a completely identical sequence to a target molecule. Inhibition of hybridization of the completely identical sequence can be assessed using hybridization assays that are well known in the art (*e.g.*, Southern (DNA) blot, Northern (RNA) blot, solution hybridization, or the like, see Sambrook, et al., *Molecular Cloning: A Laboratory Manual*, Second Edition, (1989) Cold Spring Harbor, N.Y.). Such assays can be conducted using varying degrees of selectivity, for example, using conditions varying from low to high stringency. If conditions of low stringency are employed, the absence of non-specific binding can be assessed using a secondary probe that lacks even a partial degree of sequence identity (for example, a

probe having less than about 30% sequence identity with the target molecule), such that, in the absence of non-specific binding events, the secondary probe will not hybridize to the target.

[0068] When utilizing a hybridization-based detection system, a nucleic acid probe is chosen that is complementary to a reference nucleic acid sequence, and then by selection of appropriate conditions the probe and the reference sequence selectively hybridize, or bind, to each other to form a duplex molecule. A nucleic acid molecule that is capable of hybridizing selectively to a reference sequence under moderately stringent hybridization conditions typically hybridizes under conditions that allow detection of a target nucleic acid sequence of at least about 10-14 nucleotides in length having at least approximately 70% sequence identity with the sequence of the selected nucleic acid probe. Stringent hybridization conditions typically allow detection of target nucleic acid sequences of at least about 10-14 nucleotides in length having a sequence identity of greater than about 90-95% with the sequence of the selected nucleic acid probe. Hybridization conditions useful for probe/reference sequence hybridization, where the probe and reference sequence have a specific degree of sequence identity, can be determined as is known in the art (see, for example, Nucleic Acid Hybridization: A Practical Approach, editors B.D. Hames and S.J. Higgins, (1985) Oxford; Washington, DC; IRL Press).

[0069] Conditions for hybridization are well-known to those of skill in the art. Hybridization stringency refers to the degree to which hybridization conditions disfavor the formation of hybrids containing mismatched nucleotides, with higher stringency correlated with a lower tolerance for mismatched hybrids. Factors that affect the stringency of hybridization are well-known to those of skill in the art and include, but are not limited to, temperature, pH, ionic strength, and concentration of organic solvents such as, for example, formamide and dimethylsulfoxide. As is known to those of skill in the art, hybridization stringency is increased by higher temperatures, lower ionic strength and lower solvent concentrations.

[0070] With respect to stringency conditions for hybridization, it is well known in the art that numerous equivalent conditions can be employed to establish a particular stringency by varying, for example, the following factors: the length and nature of the sequences, base composition of the various sequences, concentrations of salts and other hybridization solution components, the presence or absence of blocking agents in the hybridization solutions (*e.g.*, dextran sulfate, and polyethylene

glycol), hybridization reaction temperature and time parameters, as well as, varying wash conditions. The selection of a particular set of hybridization conditions is selected following standard methods in the art (see, for example, Sambrook, *et al.*, Molecular Cloning: A Laboratory Manual, Second Edition, (1989) Cold Spring Harbor, N.Y.).

[0071] "Recombination" refers to a process of exchange of genetic information between two polynucleotides. For the purposes of this disclosure, "homologous recombination (HR)" refers to the specialized form of such exchange that takes place, for example, during repair of double-strand breaks in cells. This process requires nucleotide sequence homology, uses a "donor" molecule to template repair of a "target" molecule (*i.e.*, the one that experienced the double-strand break), and is variously known as "non-crossover gene conversion" or "short tract gene conversion," because it leads to the transfer of genetic information from the donor to the target. Without wishing to be bound by any particular theory, such transfer can involve mismatch correction of heteroduplex DNA that forms between the broken target and the donor, and/or "synthesis-dependent strand annealing," in which the donor is used to resynthesize genetic information that will become part of the target, and/or related processes. Such specialized HR often results in an alteration of the sequence of the target molecule such that part or all of the sequence of the donor polynucleotide is incorporated into the target polynucleotide.

[0072] "Cleavage" refers to the breakage of the covalent backbone of a DNA molecule. 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. DNA cleavage can result in the production of either blunt ends or staggered ends. In certain embodiments, fusion polypeptides are used for targeted double-stranded DNA cleavage.

[0073] A "cleavage half-domain" is a polypeptide sequence which, in conjunction with a second polypeptide (either identical or different) forms a complex having cleavage activity (preferably double-strand cleavage activity). The terms "first and second cleavage half-domains;" "+ and – cleavage half-domains" and "right and left cleavage half-domains" are used interchangeably to refer to pairs of cleavage half-domains that dimerize.

[0074] An "engineered cleavage half-domain" is a cleavage half-domain that has been modified so as to form obligate heterodimers with another cleavage half-domain (*e.g.,* another engineered cleavage half-domain). *See, also,* U.S. Patent Nos. 7,888,121; 7,914,796; 8,034,598; 8,623,618 and U.S. Patent Publication No. 2011/0201055, incorporated herein by reference in their entireties.

[0075] The term "sequence" refers to a nucleotide sequence of any length, which can be DNA or RNA; can be linear, circular or branched and can be either single-stranded or double stranded. The term "donor sequence" refers to a nucleotide sequence that is inserted into a genome. A donor sequence can be of any length, for example between 2 and 10,000 nucleotides in length (or any integer value there between or there above), preferably between about 100 and 1,000 nucleotides in length (or any integer there between), more preferably between about 200 and 500 nucleotides in length.

[0076] "Chromatin" is the nucleoprotein structure comprising the cellular genome. Cellular chromatin comprises nucleic acid, primarily DNA, and protein, including histones and non-histone chromosomal proteins. The majority of eukaryotic cellular chromatin exists in the form of nucleosomes, wherein a nucleosome core comprises approximately 150 base pairs of DNA associated with an octamer comprising two each of histones H2A, H2B, H3 and H4; and linker DNA (of variable length depending on the organism) extends between nucleosome cores. A molecule of histone H1 is generally associated with the linker DNA. For the purposes of the present disclosure, the term "chromatin" is meant to encompass all types of cellular nucleoprotein, both prokaryotic and eukaryotic. Cellular chromatin includes both chromosomal and episomal chromatin.

[0077] A "chromosome," is a chromatin complex comprising all or a portion of the genome of a cell. The genome of a cell is often characterized by its karyotype, which is the collection of all the chromosomes that comprise the genome of the cell. The genome of a cell can comprise one or more chromosomes.

[0078] An "episome" is a replicating nucleic acid, nucleoprotein complex or other structure comprising a nucleic acid that is not part of the chromosomal karyotype of a cell. Examples of episomes include plasmids and certain viral genomes.

[0079] An "accessible region" is a site in cellular chromatin in which a target site present in the nucleic acid can be bound by an exogenous molecule which

recognizes the target site. Without wishing to be bound by any particular theory, it is believed that an accessible region is one that is not packaged into a nucleosomal structure. The distinct structure of an accessible region can often be detected by its sensitivity to chemical and enzymatic probes, for example, nucleases.

[0080] A "target site" or "target sequence" is a nucleic acid sequence that defines a portion of a nucleic acid to which a binding molecule will bind, provided sufficient conditions for binding exist. For example, the sequence 5'-GAATTC-3' is a target site for the Eco RI restriction endonuclease.

[0081] A "safe harbor locus" is a location within a genome that can be used for integrating exogenous nucleic acids. The addition of exogenous nucleic acids into these safe harbor loci does not cause any significant effect on the growth of the host cell by the addition of the DNA alone. Non-limiting examples of safe harbor genes include, for example, a CCR5 gene, a CXCR4 gene, a PPP1R12C (also known as AAVS1) gene, an albumin gene or a *Rosa* gene. *See, e.g.,* U.S. Patent Nos. 7,951,925 and 8,110,379; U.S. Publication Nos. 201000218264; 20100291048; 20120017290; 20110265198; 20130137104; 20130122591; 20130177983 and 20130177960.

[0082] An "exogenous" molecule is a molecule that is not normally present in a cell, but can be introduced into a cell by one or more genetic, biochemical or other methods. "Normal presence in the cell" is determined with respect to the particular developmental stage and environmental conditions of the cell. Thus, for example, a molecule that is present only during embryonic development of muscle is an exogenous molecule with respect to an adult muscle cell. Similarly, a molecule induced by heat shock is an exogenous molecule with respect to a non-heat-shocked cell. An exogenous molecule can comprise, for example, a functioning version of a malfunctioning endogenous molecule or a malfunctioning version of a normally-functioning endogenous molecule.

[0083] An exogenous molecule can be, among other things, a small molecule, such as is generated by a combinatorial chemistry process, or a macromolecule such as a protein, nucleic acid, carbohydrate, lipid, glycoprotein, lipoprotein, polysaccharide, any modified derivative of the above molecules, or any complex comprising one or more of the above molecules. Nucleic acids include DNA and RNA, can be single- or double-stranded; can be linear, branched or circular; and can be of any length. Nucleic acids include those capable of forming duplexes, as well as triplex-forming nucleic acids. See, for example, U.S. Patent Nos. 5,176,996 and

5,422,251. Proteins include, but are not limited to, DNA-binding proteins, transcription factors, chromatin remodeling factors, methylated DNA binding proteins, polymerases, methylases, demethylases, acetylases, deacetylases, kinases, phosphatases, integrases, recombinases, ligases, topoisomerases, gyrases and helicases.

[0084] An exogenous molecule can be the same type of molecule as an endogenous molecule, *e.g.*, an exogenous protein or nucleic acid. For example, an exogenous nucleic acid can comprise an infecting viral genome, a plasmid or episome introduced into a cell, or a chromosome that is not normally present in the cell. Methods for the introduction of exogenous molecules into cells are known to those of skill in the art and include, but are not limited to, lipid-mediated transfer (*i.e.*, liposomes, including neutral and cationic lipids), electroporation, direct injection, cell fusion, particle bombardment, calcium phosphate co-precipitation, DEAE-dextran-mediated transfer and viral vector-mediated transfer.

[0085] By contrast, an "endogenous" molecule is one that is normally present in a particular cell at a particular developmental stage under particular environmental conditions. For example, an endogenous nucleic acid can comprise a chromosome, the genome of a mitochondrion, chloroplast or other organelle, or a naturally-occurring episomal nucleic acid. Additional endogenous molecules can include proteins, for example, transcription factors and enzymes.

[0086] A "fusion" molecule is a molecule in which two or more subunit molecules are linked, preferably covalently. The subunit molecules can be the same chemical type of molecule, or can be different chemical types of molecules. Examples of the first type of fusion molecule include, but are not limited to, fusion proteins (for example, a fusion between a ZFP or TALE DNA-binding domain and a cleavage domain) and fusion nucleic acids (for example, a nucleic acid encoding the fusion protein described *supra*). Examples of the second type of fusion molecule include, but are not limited to, a fusion between a triplex-forming nucleic acid and a polypeptide, and a fusion between a minor groove binder and a nucleic acid.

[0087] Expression of a fusion protein in a cell can result from delivery of the fusion protein to the cell or by delivery of a polynucleotide encoding the fusion protein to a cell, wherein the polynucleotide is transcribed, and the transcript is translated, to generate the fusion protein. Trans-splicing, polypeptide cleavage and polypeptide ligation can also be involved in expression of a protein in a cell. Methods

for polynucleotide and polypeptide delivery to cells are presented elsewhere in this disclosure.

[0088] A "gene," for the purposes of the present disclosure, includes a DNA region encoding a gene product (see *infra*), as well as all DNA regions which regulate the production of the gene product, whether or not such regulatory sequences are adjacent to coding and/or transcribed sequences. Accordingly, a gene includes, but is not necessarily limited to, promoter sequences, terminators, translational regulatory sequences such as ribosome binding sites and internal ribosome entry sites, enhancers, silencers, insulators, boundary elements, replication origins, matrix attachment sites and locus control regions.

[0089] "Gene expression" refers to the conversion of the information, contained in a gene, into a gene product. A gene product can be the direct transcriptional product of a gene (*e.g.*, mRNA, tRNA, rRNA, antisense RNA, ribozyme, structural RNA or any other type of RNA) or a protein produced by translation of a mRNA. Gene products also include RNAs which are modified, by processes such as capping, polyadenylation, methylation, and editing, and proteins modified by, for example, methylation, acetylation, phosphorylation, ubiquitination, ADP-ribosylation, myristilation, and glycosylation.

[0090] "Modulation" of gene expression refers to a change in the activity of a gene. Modulation of expression can include, but is not limited to, gene activation and gene repression. Modulation may also be complete, *i.e.*, wherein gene expression is totally inactivated or is activated to wild-type levels or beyond; or it may be partial, wherein gene expression is partially reduced, or partially activated to some fraction of wildtype levels.

[0091] "Eucaryotic" cells include, but are not limited to, fungal cells (such as yeast), plant cells, animal cells, mammalian cells and human cells (*e.g.*, T-cells).

[0092] A "region of interest" is any region of cellular chromatin, such as, for example, a gene or a non-coding sequence within or adjacent to a gene, in which it is desirable to bind an exogenous molecule. Binding can be for the purposes of targeted DNA cleavage and/or targeted recombination. A region of interest can be present in a chromosome, an episome, an organellar genome (*e.g.*, mitochondrial, chloroplast), or an infecting viral genome, for example. A region of interest can be within the coding region of a gene, within transcribed non-coding regions such as, for example, leader sequences, trailer sequences or introns, or within non-transcribed regions, either

upstream or downstream of the coding region. A region of interest can be as small as a single nucleotide pair or up to 2,000 nucleotide pairs in length, or any integral value of nucleotide pairs.

[0093] The terms "operative linkage" and "operatively linked" (or "operably linked") are used interchangeably with reference to a juxtaposition of two or more components (such as sequence elements), in which the components are arranged such that both components function normally and allow the possibility that at least one of the components can mediate a function that is exerted upon at least one of the other components. By way of illustration, a transcriptional regulatory sequence, such as a promoter, is operatively linked to a coding sequence if the transcriptional regulatory sequence controls the level of transcription of the coding sequence in response to the presence or absence of one or more transcriptional regulatory factors. A transcriptional regulatory sequence is generally operatively linked in *cis* with a coding sequence, but need not be directly adjacent to it. For example, an enhancer is a transcriptional regulatory sequence that is operatively linked to a coding sequence, even though they are not contiguous.

[0094] With respect to fusion polypeptides, the term "operatively linked" can refer to the fact that each of the components performs the same function in linkage to the other component as it would if it were not so linked. For example, with respect to a fusion polypeptide in which a DNA-binding domain (ZFP, TALE) is fused to a cleavage domain (*e.g.,* endonuclease domain such as FokI, meganuclease domain, etc.), the DNA-binding domain and the cleavage domain are in operative linkage if, in the fusion polypeptide, the DNA-binding domain portion is able to bind its target site and/or its binding site, while the cleavage (nuclease) domain is able to cleave DNA in the vicinity of the target site. The nuclease domain may also exhibit DNA-binding capability (*e.g.*, a nuclease fused to a ZFP or TALE domain that also can bind to DNA). Similarly, with respect to a fusion polypeptide in which a DNA-binding domain is fused to an activation or repression domain, the DNA-binding domain and the activation or repression domain are in operative linkage if, in the fusion polypeptide, the DNA-binding domain portion is able to bind its target site and/or its binding site, while the activation domain is able to upregulate gene expression or the repression domain is able to downregulate gene expression.

[0095] A "functional fragment" of a protein, polypeptide or nucleic acid is a protein, polypeptide or nucleic acid whose sequence is not identical to the full-length

protein, polypeptide or nucleic acid, yet retains the same function as the full-length protein, polypeptide or nucleic acid. A functional fragment can possess more, fewer, or the same number of residues as the corresponding native molecule, and/or can contain one ore more amino acid or nucleotide substitutions. Methods for determining the function of a nucleic acid (*e.g.*, coding function, ability to hybridize to another nucleic acid) are well-known in the art. Similarly, methods for determining protein function are well-known. For example, the DNA-binding function of a polypeptide can be determined, for example, by filter-binding, electrophoretic mobility-shift, or immunoprecipitation assays. DNA cleavage can be assayed by gel electrophoresis. See Ausubel *et al., supra.* The ability of a protein to interact with another protein can be determined, for example, by co-immunoprecipitation, two-hybrid assays or complementation, both genetic and biochemical. See, for example, Fields *et al.* (1989) *Nature* **340:**245-246; U.S. Patent No. 5,585,245 and PCT WO 98/44350.

[0096] A "vector" is capable of transferring gene sequences to target cells. Typically, "vector construct," "expression vector," and "gene transfer vector," mean any nucleic acid construct capable of directing the expression of a gene of interest and which can transfer gene sequences to target cells. Thus, the term includes cloning, and expression vehicles, as well as integrating vectors.

Nucleases

[0097] Described herein are nucleases (*e,g.,*ZFNs or TALE nucleases) that can be used for inactivation of a TCR gene The nuclease may be naturally occurring or may be a chimera of a DNA-binding domain and a cleavage domain. It will be apparent that within the chimera, the component DNA-binding and cleavage domains may both be naturally occurring, may both be non-naturally occurring or one may be naturally occurring and the other may be non-naturally occurring.

[0098] Thus, any nuclease can be used in the methods disclosed herein. For example, naturally-occurring homing endonucleases and meganucleases have very long recognition sequences, some of which are likely to be present, on a statistical basis, once in a human-sized genome. Exemplary homing endonucleases include I-*Sce*I, I-*Ceu*I, PI-*Psp*I, PI-*Sce*, I-*Sce*IV, I-*Csm*I, I-*Pan*I, I-*Sce*II, I-*Ppo*I, I-*Sce*III, I-*Cre*I, I-*Tev*I, I-*Tev*II and I-*Tev*III. Their recognition sequences are known. *See* also U.S. Patent No. 5,420,032; U.S. Patent No. 6,833,252; Belfort *et al.* (1997) *Nucleic*

Acids Res. **25**:3379–3388; Dujon *et al.* (1989) *Gene* **82**:115–118; Perler *et al.* (1994) Nucleic Acids Res. 22, 1125–1127; Jasin (1996) *Trends Genet.* **12**:224–228; Gimble *et al.* (1996) *J. Mol. Biol.* **263**:163–180; Argast *et al.* (1998) *J. Mol. Biol.* **280**:345–353 and the New England Biolabs catalogue.

[0099] It has also been reported that the specificity of homing endonucleases and meganucleases can be engineered to bind non-natural target sites. *See*, for example, Chevalier *et al.* (2002) *Molec. Cell* **10**:895-905; Epinat *et al.* (2003) *Nucleic Acids Res.* **31**:2952-2962; Ashworth *et al.* (2006) *Nature* **441**:656-659; Paques *et al.* (2007) *Current Gene Therapy* **7**:49-66. The DNA-binding domains of the homing endonucleases and meganucleases may be altered in the context of the nuclease as a whole (*i.e.,* such that the nuclease includes the cognate cleavage domain) or may be fused to a heterologous DNA-binding domain (*e.g.,* zinc finger protein or TALE) or to a heterologous cleavage domain. DNA-binding domains derived from meganucleases may also exhibit DNA-binding activity.

[0100] In certain embodiments, the nuclease comprises a zinc finger DNA-binding domain and a restriction endonuclease nuclease domain, also referred to as as zinc finger nucleases (ZFNs).

[0101] In other embodiments, the nuclease comprises an engineered TALE DNA-binding domain and a nuclease domain (*e.g.,* endonuclease and/or meganuclease domain), also referred to as TALENs. Methods and compositions for engineering these TALEN proteins for robust, site specific interaction with the target sequence of the user's choosing have been published (see U.S. Patent No. 8,586,526). In some embodiments, the TALEN comprises a endonuclease (*e.g.,* FokI) cleavage domain or cleavage half-domain. In other embodiments, the TALE-nuclease is a mega TAL. These mega TAL nucleases are fusion proteins comprising a TALE DNA binding domain and a meganuclease cleavage domain. The meganuclease cleavage domain is active as a monomer and does not require dimerization for activity. (See Boissel *et al.*, (2013) *Nucl Acid Res*: 1-13, doi: 10.1093/nar/gkt1224). In addition, the nuclease domain may also exhibit DNA-binding functionality.

[0102] In still further embodiments, the nuclease comprises a compact TALEN (cTALEN). These are single chain fusion proteins linking a TALE DNA binding domain to a TevI nuclease domain. The fusion protein can act as either a nickase localized by the TALE region, or can create a double strand break, depending upon where the TALE DNA binding domain is located with respect to the TevI

nuclease domain (see Beurdeley *et al* (2013) *Nat Comm*: 1-8 DOI: 10.1038/ncomms2782). Any TALENs may be used in combination with additional TALENs (*e.g.,* one or more TALENs (cTALENs or FokI-TALENs) with one or more mega-TALs).

[0103] Thus, any naturally occurring or engineered nuclease having a unique target site can be used in the methods described herein.

A. DNA-Binding domains

[0104] The nucleases described herein typically include a DNA-binding domain and a cleavage domain. Any DNA-binding domain can be used in the practice of the present invention, including but not limited to a zinc finger DNA-binding domain, a TALE DNA binding domain, or a DNA-binding domain from a meganuclease.

[0105] In certain embodiments, zinc finger binding domains that are engineered to bind to a sequence of choice are employed. *See*, for example, Beerli *et al.* (2002) *Nature Biotechnol.* **20:**135-141; Pabo *et al.* (2001) *Ann. Rev. Biochem.* **70:**313-340; Isalan *et al.* (2001) *Nature Biotechnol.* **19:**656-660; Segal *et al.* (2001) *Curr. Opin. Biotechnol.* **12:**632-637; Choo *et al.* (2000) *Curr. Opin. Struct. Biol.* **10:**411-416. Similarly, a TALE DNA-binding domain can be engineered to bind to a sequence of choice. *See, e.g.,* 8,586,526. Engineered zinc finger or TALE DNA binding domains can have a novel binding specificity, compared to a naturally-occurring zinc finger or TALE protein. Engineering methods include, but are not limited to, rational design and various types of selection. Rational design includes, for example, using databases comprising triplet (or quadruplet) nucleotide sequences and individual zinc finger amino acid sequences, in which each triplet or quadruplet nucleotide sequence is associated with one or more amino acid sequences of zinc fingers which bind the particular triplet or quadruplet sequence. See, for example, co-owned U.S. Patents 8,586,526; 6,453,242 and 6,534,261, incorporated by reference herein in their entireties.

[0106] Exemplary selection methods, including phage display and two-hybrid systems, are disclosed in US Patents 5,789,538; 5,925,523; 6,007,988; 6,013,453; 6,410,248; 6,140,466; 6,200,759; and 6,242,568; as well as WO 98/37186; WO 98/53057; WO 00/27878; WO 01/88197 and GB 2,338,237.Enhancement of

binding specificity for zinc finger binding domains has been described, for example, in co-owned WO 02/077227.

[0107] In other embodiments, the DNA binding domain comprises a TALE DNA binding domain (*see*, co-owned U.S. Patent No. 8,586,526, incorporated by reference in its entirety herein). A TALE DNA-binding domain comprises one or more TALE "repeat units." A single "repeat unit" (also referred to as a "repeat") is typically 33-35 amino acids in length, where positions 12 and/or 13 (referred to as hypervariable diresidue region or "RVD") are involved in binding to a DNA nucleotide. An "atypical" RVD is an RVD sequence (positions 12 and 13) that occurs infrequently or never in nature, for example, in less than 5% of naturally occurring TALE proteins, preferably in less than 2% of naturally occurring TALE proteins and even more preferably less than 1% of naturally occurring TALE proteins. An atypical RVD can be non-naturally occurring. The TALE DNA-binding domains preferably include a C-cap sequence and, optionally, an N-cap sequence. The "cap" sequences are preferably a fragment (truncation) of a polypeptide found in full-length TALE proteins, for example any truncation of a C- and/or N-terminal region flanking the TALE repeat domain in a naturally occurring TALE protein. The C-cap may be, for example, truncations as compared to a wild-type C-terminal TALE protein (which is numbered as starting at C-20) including but not limited, C-19, C-18, C-17, C-16, C-15, C-14, C-13, C-12, C-11, C-10, C-9, C-8, C-7, C-6, C-5, C-4, C-3, C-2, C-1, increments to C+1, and then increments to C+2, C+3, etc. towards the C-terminus of the polypeptide (*e.g.,* C+63, which is 83 amino acids in length as it extends from C-20 to C+63).

[0108] In addition, as disclosed in these and other references, zinc finger domains and/or multi-fingered zinc finger proteins or TALEs may be linked together using any suitable linker sequences, including for example, linkers of 5 or more amino acids in length. See, also, U.S. Patent Nos. 6,479,626; 6,903,185; and 7,153,949 for exemplary linker sequences 6 or more amino acids in length. The proteins described herein may include any combination of suitable linkers between the individual zinc fingers of the protein. In addition, enhancement of binding specificity for zinc finger binding domains has been described, for example, in U.S. Patent No. 6,794,136. .

[0109] Selection of target sites; ZFPs or TALEs and methods for design and construction of fusion proteins (and polynucleotides encoding same) are known to those of skill in the art and described in detail in U.S. Patent Nos. 6,140,081; 5,789,538; 6,453,242; 6,534,261; 5,925,523; 6,007,988; 6,013,453; 6,200,759;

WO 95/19431; WO 96/06166; WO 98/53057; WO 98/54311; WO 00/27878; WO 01/60970 WO 01/88197; WO 02/099084; WO 98/53058; WO 98/53059; WO 98/53060; WO 02/016536 and WO 03/016496, the disclosures of which are incorporated by reference in their entireties for all purposes.

[0110] In addition, as disclosed in these and other references, zinc finger domains and/or multi-fingered zinc finger proteins may be linked together using any suitable linker sequences, including for example, linkers of 5 or more amino acids in length. See, also, U.S. Patent Nos. 6,479,626; 6,903,185; and 7,153,949 for exemplary linker sequences 6 or more amino acids in length. The proteins described herein may include any combination of suitable linkers between the individual zinc fingers of the protein.

[0111] Alternatively, the DNA-binding domain may be derived from a nuclease. For example, the recognition sequences of homing endonucleases and meganucleases such as I-*Sce*I, I-*Ceu*I, PI-*Psp*I, PI-*Sce*, I-*Sce*IV, I-*Csm*I, I-*Pan*I, I-*Sce*II, I-*Ppo*I, I-*Sce*III, I-*Cre*I, I-*Tev*I, I-*Tev*II and I-*Tev*III are known. *See* also U.S. Patent No. 5,420,032; U.S. Patent No. 6,833,252; Belfort *et al.*(1997) *Nucleic Acids Res.* **25:**3379–3388; Dujon *et al.* (1989) *Gene* **82:**115–118; Perler *et al.* (1994) Nucleic Acids Res. 22, 1125–1127; Jasin (1996) *Trends Genet.* **12:**224–228; Gimble *et al.* (1996) *J. Mol. Biol.* **263:**163–180; Argast*et al.* (1998) *J. Mol. Biol.* **280:**345–353 and the New England Biolabs catalogue. In addition, the DNA-binding specificity of homing endonucleases and meganucleases can be engineered to bind non-natural target sites. *See*, for example, Chevalier *et al.* (2002) *Molec. Cell* 10:895-905; Epinat *et al.* (2003) *Nucleic Acids Res.* 31:2952-2962; Ashworth *et al.* (2006) *Nature* 441:656-659; Pâques *et al.* (2007) *Current Gene Therapy* 7:49-66; U.S. Patent Publication No. 20070117128.

[0112] In certain embodiments, the DNA binding domain is an engineered zinc finger protein that typically includes at least one zinc finger but can include a plurality of zinc fingers (*e.g.*, 2, 3, 4, 5, 6 or more fingers). Usually, the ZFPs include at least three fingers. Certain of the ZFPs include four, five or six fingers. The ZFPs that include three fingers typically recognize a target site that includes 9 or 10 nucleotides; ZFPs that include four fingers typically recognize a target site that includes 12 to 14 nucleotides; while ZFPs having six fingers can recognize target sites that include 18 to 21 nucleotides. The ZFPs can also be fusion proteins that include

one or more regulatory domains, wherein these regulatory domains can be transcriptional activation or repression domains.

[0113] In other embodiments, the DNA-binding domain comprises a naturally occurring or engineered (non-naturally occurring) TAL effector DNA binding domain. *See, e.g.,* U.S. Patent No. 8,586,526, incorporated by reference in its entirety herein. The plant pathogenic bacteria of the genus *Xanthomonas* are known to cause many diseases in important crop plants. Pathogenicity of *Xanthomonas* depends on a conserved type III secretion (T3S) system which injects more than 25 different effector proteins into the plant cell. Among these injected proteins are transcription activator-like effectors (TALE) which mimic plant transcriptional activators and manipulate the plant transcriptome (see Kay *et al* (2007) *Science*318:648-651 and U.S. Patent Publication No. 20110239315). These proteins contain a DNA binding domain and a transcriptional activation domain. One of the most well characterized TALEs is AvrBs3 from *Xanthomonas campestgris* pv. *Vesicatoria* (see Bonas *et al* (1989) *Mol Gen Genet* 218: 127-136 and WO2010079430). TALEs contain a centralized domain of tandem repeats, each repeat containing approximately 34 amino acids, which are key to the DNA binding specificity of these proteins. In addition, they contain a nuclear localization sequence and an acidic transcriptional activation domain (for a review see Schornack S, *et al* (2006) *J Plant Physiol* 163(3): 256-272). In addition, in the phytopathogenic bacteria *Ralstonia solanacearum* two genes, designated brg11 and hpx17 have been found that are homologous to the AvrBs3 family of *Xanthomonas* in the *R. solanacearum* biovar 1 strain GMI1000 and in the biovar 4 strain RS1000 (*See* Heuer *et al* (2007) *Appl and Envir Micro* 73(13): 4379-4384). These genes are 98.9% identical in nucleotide sequence to each other but differ by a deletion of 1,575 bp in the repeat domain of hpx17. However, both gene products have less than 40% sequence identity with AvrBs3 family proteins of *Xanthomonas*. The zinc finger nucleases described herein bind in a TCR gene. Tables 5 and 6 (see Example 4) describe a number of zinc finger binding domains that have been engineered to bind to nucleotide sequences in the human TCR gene. Each row describes a separate zinc finger DNA-binding domain. The DNA target sequence for each domain is shown in the first column (DNA target sites indicated in uppercase letters; non-contacted nucleotides indicated in lowercase), and the second through fifth columns show the amino acid sequence of the recognition region (amino acids -1 through +6, with respect to the start of the helix) of each of the zinc fingers (F1

through F4 or F5 or F6) in the protein. Also provided in the first column is an identification number for each protein.

[0114] Also described are TALENs that bind in a TCR gene. Table 14 (see, Example 10) describe TALENs that have been engineered to bind to a nucleotide sequence in a human TCR gene. Each row describes a separate TALE DNA binding protein with the indicated number of RVD-containing domains. The DNA target sequence for each domain is shown in the first column (DNA target sites indicated in uppercase letters; non-contacted nucleotides are in lowercase). Also provided in the first column is an identification number for each protein.

[0115] As described below, in certain embodiments, a four- or five-finger binding domain as shown in Tables 5 and 6, or a TALE DNA binding domain as shown in Table 14 is fused to a cleavage half-domain, such as, for example, the cleavage domain of a Type IIs restriction endonuclease such as *Fok*I. A pair of such zinc finger or TALE/nuclease half-domain fusions are used for targeted cleavage, as disclosed, for example, in U.S. Patent No. 8,586,526 and U.S. Publication No. 20050064474.

[0116] For targeted cleavage, the near edges of the binding sites can separated by 5 or more nucleotide pairs, and each of the fusion proteins can bind to an opposite strand of the DNA target.

[0117] In addition, domains from these naturally occurring or engineered nucleases can also be isolated and used in various combinations. For example, the DNA-binding domain from a naturally occurring or engineered homing endonucleases or meganuclease can be fused to a heterologous cleavage domain or half domain (*e.g.,* from another homing endonuclease, meganuclease or TypeIIS endonuclease). These fusion proteins can also be used in combination with zinc finger nucleases described above.

[0118] The nucleases described herein can be targeted to any sequence in any TCR genomic sequence.

B. Cleavage Domains

[0119] The nuclease may comprise heterologous DNA-binding and cleavage domains (*e.g.*, zinc finger nucleases; TALENs, meganuclease DNA-binding domains with heterologous cleavage domains) or, alternatively, the DNA-binding domain of a naturally-occurring nuclease may be altered to bind to a selected target site (*e.g.,* a

meganuclease that has been engineered to bind to site different than the cognate binding site). In certain embodiments, the nuclease is a meganuclease (homing endonuclease). Naturally-occurring meganucleases recognize 15-40 base-pair cleavage sites and are commonly grouped into four families: the LAGLIDADG family, the GIY-YIG family, the His-Cyst box family and the HNH family. Exemplary homing endonucleases include I-*Sce*I, I-*Ceu*I, PI-*Psp*I, PI-*Sce*, I-*Sce*IV, I-*Csm*I, I-*Pan*I, I-*Sce*II, I-*Ppo*I, I-*Sce*III, I-*Cre*I, I-*Tev*I, I-*Tev*II and I-*Tev*III. Their recognition sequences are known. *See* also U.S. Patent No. 5,420,032; U.S. Patent No. 6,833,252; Belfort*et al.*(1997) *Nucleic Acids Res.***25:**3379–3388; Dujon *et al.* (1989) *Gene***82:**115–118; Perler *et al.* (1994) Nucleic Acids Res. 22, 1125–1127; Jasin (1996) *Trends Genet.***12:**224–228; Gimble *et al.* (1996) *J. Mol. Biol.***263:**163–180; Argast*et al.* (1998) *J. Mol. Biol.***280:**345–353 and the New England Biolabs catalogue.

[0120] DNA-binding domains from naturally-occurring meganucleases, primarily from the LAGLIDADG family, have been used to promote site-specific genome modification in plants, yeast, Drosophila, mammalian cells and mice, but this approach has been limited to the modification of either homologous genes that conserve the meganuclease recognition sequence (Monet *et al.* (1999), *Biochem. Biophysics. Res. Common.* 255: 88-93) or to pre-engineered genomes into which a recognition sequence has been introduced (Route *et al.* (1994), *Mol. Cell. Biol.* 14: 8096-106; Chilton *et al.* (2003), *Plant Physiology.* 133: 956-65; Puchta*et al.* (1996), *Proc. Natl. Acad. Sci. USA* 93: 5055-60; Rong *et al.* (2002), *Genes Dev.* 16: 1568-81; Gouble *et al.* (2006), *J. Gene Med.* 8(5):616-622). Accordingly, attempts have been made to engineer meganucleases to exhibit novel binding specificity at medically or biotechnologically relevant sites (Porteus *et al.* (2005), *Nat. Biotechnol.* 23: 967-73; Sussman *et al.* (2004), *J. Mol. Biol.* 342: 31-41; Epinat *et al.* (2003), *Nucleic Acids Res.* 31: 2952-62; Chevalier *et al.* (2002) *Molec. Cell*10:895-905; Ashworth *et al.* (2006) *Nature* 441:656-659; Pâques *et al.* (2007) *Current Gene Therapy* 7:49-66; U.S. Patent Publication Nos. 20070117128; 20060206949; 20060153826; 20060078552; and 20040002092). In addition, naturally-occurring or engineered DNA-binding domains from meganucleases have also been operably linked with a cleavage domain from a heterologous nuclease (*e.g.*, *Fok*I).

[0121] In other embodiments, the nuclease is a zinc finger nuclease (ZFN). ZFNs comprise a zinc finger protein that has been engineered to bind to a target site in a gene of choice and cleavage domain or a cleavage half-domain.

[0122] As noted above, zinc finger binding domains can be engineered to bind to a sequence of choice. *See*, for example, Beerli *et al.* (2002) *Nature Biotechnol.* 20:135-141; Pabo *et al.* (2001) *Ann. Rev. Biochem.*70:313-340; Isalan *et al.*(2001) *Nature Biotechnol.* 19:656-660; Segal *et al.*(2001) *Curr. Opin. Biotechnol.*12:632-637; Choo *et al.* (2000) *Curr. Opin. Struct. Biol.***10:**411-416. An engineered zinc finger binding domain can have a novel binding specificity, compared to a naturally-occurring zinc finger protein. Engineering methods include, but are not limited to, rational design and various types of selection. Rational design includes, for example, using databases comprising triplet (or quadruplet) nucleotide sequences and individual zinc finger amino acid sequences, in which each triplet or quadruplet nucleotide sequence is associated with one or more amino acid sequences of zinc fingers which bind the particular triplet or quadruplet sequence. See, for example, U.S. Patents 6,453,242 and 6,534,261, incorporated by reference herein in their entireties.

[0123] Exemplary selection methods, including phage display and two-hybrid systems, are disclosed in US Patents 5,789,538; 5,925,523; 6,007,988; 6,013,453; 6,410,248; 6,140,466; 6,200,759; and 6,242,568; as well as WO 98/37186; WO 98/53057; WO 00/27878; WO 01/88197 and GB 2,338,237. In addition, enhancement of binding specificity for zinc finger binding domains has been described, for example, in U.S. Patent No. 6,794,136.

[0124] Selection of target sites; ZFNs and methods for design and construction of fusion proteins (and polynucleotides encoding same) are known to those of skill in the art and described in detail in U.S. Patent Nos. 7,888,121 and 8,409,861, incorporated by reference in their entireties herein.

[0125] In addition, as disclosed in these and other references, zinc finger domains and/or multi-fingered zinc finger proteins may be linked together using any suitable linker sequences, including for example, linkers of 5 or more amino acids in length. See, *e.g.*, U.S. Patent Nos. 6,479,626; 6,903,185; and 7,153,949 for exemplary linker sequences 6 or more amino acids in length. The proteins described herein may include any combination of suitable linkers between the individual zinc fingers of the protein.

[0126] In some embodiments, the nuclease is an engineered TALEN. Methods and compositions for engineering these proteins for robust, site specific interaction with the target sequence of the user's choosing have been published (see U.S. Patent No. 8,586,526).

[0127] Nucleases such as ZFNs, TALENs and/or meganucleases also comprise a nuclease (cleavage domain, cleavage half-domain). As noted above, the cleavage domain may be heterologous to the DNA-binding domain, for example a zinc finger or TALE DNA-binding domain and a cleavage domain from a nuclease or a meganuclease DNA-binding domain and cleavage domain from a different nuclease. Heterologous cleavage domains can be obtained from any endonuclease or exonuclease. Exemplary endonucleases from which a cleavage domain can be derived include, but are not limited to, restriction endonucleases and homing endonucleases. *See*, for example, 2002-2003 Catalogue, New England Biolabs, Beverly, MA; and Belfort *et al.* (1997) *Nucleic Acids Res.* 25:3379-3388. Additional enzymes which cleave DNA are known (*e.g.*, S1 Nuclease; mung bean nuclease; pancreatic DNase I; micrococcal nuclease; yeast HO endonuclease; *see* also Linn *et al.* (eds.) *Nucleases*, Cold Spring Harbor Laboratory Press,1993). One or more of these enzymes (or functional fragments thereof) can be used as a source of cleavage domains and cleavage half-domains.

[0128] Similarly, a cleavage half-domain can be derived from any nuclease or portion thereof, as set forth above, that requires dimerization for cleavage activity. In general, two fusion proteins are required for cleavage if the fusion proteins comprise cleavage half-domains. Alternatively, a single protein comprising two cleavage half-domains can be used. The two cleavage half-domains can be derived from the same endonuclease (or functional fragments thereof), or each cleavage half-domain can be derived from a different endonuclease (or functional fragments thereof). In addition, the target sites for the two fusion proteins are preferably disposed, with respect to each other, such that binding of the two fusion proteins to their respective target sites places the cleavage half-domains in a spatial orientation to each other that allows the cleavage half-domains to form a functional cleavage domain, *e.g.*, by dimerizing. Thus, in certain embodiments, the near edges of the target sites are separated by 5-8 nucleotides or by 15-18 nucleotides. However any integral number of nucleotides or nucleotide pairs can intervene between two target sites (*e.g.*, from 2 to 50 nucleotide pairs or more). In general, the site of cleavage lies between the target sites.

[0129] One or more of these enzymes (or functional fragments thereof) can be used as a source of cleavage domains and cleavage half-domains. Similarly, a cleavage half-domain can be derived from any nuclease or portion thereof, as set forth above, that requires dimerization for cleavage activity. In general, two fusion proteins are required for cleavage if the fusion proteins comprise cleavage half-domains. Alternatively, a single protein comprising two cleavage half-domains can be used. The two cleavage half-domains can be derived from the same endonuclease (or functional fragments thereof), or each cleavage half-domain can be derived from a different endonuclease (or functional fragments thereof). In addition, the target sites for the two fusion proteins are preferably disposed, with respect to each other, such that binding of the two fusion proteins to their respective target sites places the cleavage half-domains in a spatial orientation to each other that allows the cleavage half-domains to form a functional cleavage domain, *e.g.*, by dimerizing. Thus, in certain embodiments, the near edges of the target sites are separated by 5-8 nucleotides or by 15-18 nucleotides. However any integral number of nucleotides or nucleotide pairs can intervene between two target sites (*e.g.*, from 2 to 50 nucleotide pairs or more). In general, the site of cleavage lies between the target sites.

[0130] Restriction endonucleases (restriction enzymes) are present in many species and are capable of sequence-specific binding to DNA (at a recognition site), and cleaving DNA at or near the site of binding. Certain restriction enzymes (*e.g.*, Type IIS) cleave DNA at sites removed from the recognition site and have separable binding and cleavage domains. For example, the Type IIS enzyme *Fok* I catalyzes double-stranded cleavage of DNA, at 9 nucleotides from its recognition site on one strand and 13 nucleotides from its recognition site on the other. See, for example, U.S. Patents 5,356,802; 5,436,150 and 5,487,994; as well as Li *et al.* (1992) *Proc. Natl. Acad. Sci. USA* **89:**4275-4279; Li *et al.* (1993) *Proc. Natl. Acad. Sci. USA* **90:**2764-2768; Kim *et al.* (1994a) *Proc. Natl. Acad. Sci. USA* **91:**883-887; Kim *et al.* (1994b) *J. Biol. Chem.* **269:**31,978-31,982. Thus, in one embodiment, fusion proteins comprise the cleavage domain (or cleavage half-domain) from at least one Type IIS restriction enzyme and one or more zinc finger binding domains, which may or may not be engineered.

[0131] An exemplary Type IIS restriction enzyme, whose cleavage domain is separable from the binding domain, is *Fok* I. This particular enzyme is active as a dimer. Bitinaite *et al.* (1998) *Proc. Natl. Acad. Sci. USA* **95:** 10,570-10,575.

Accordingly, for the purposes of the present disclosure, the portion of the Fok I enzyme used in the disclosed fusion proteins is considered a cleavage half-domain. Thus, for targeted double-stranded cleavage and/or targeted replacement of cellular sequences using zinc finger-*Fok* I fusions, two fusion proteins, each comprising a *Fok*I cleavage half-domain, can be used to reconstitute a catalytically active cleavage domain. Alternatively, a single polypeptide molecule containing a zinc finger binding domain and two *Fok* I cleavage half-domains can also be used. Parameters for targeted cleavage and targeted sequence alteration using zinc finger-*Fok* I fusions are provided elsewhere in this disclosure.

[0132] A cleavage domain or cleavage half-domain can be any portion of a protein that retains cleavage activity, or that retains the ability to multimerize (*e.g.*, dimerize) to form a functional cleavage domain.

[0133] Exemplary Type IIS restriction enzymes are described in U.S. Patent Publication No. 20070134796, incorporated herein in its entirety. Additional restriction enzymes also contain separable binding and cleavage domains, and these are contemplated by the present disclosure. See, for example, Roberts *et al.* (2003) *Nucleic Acids Res.* **31**:418-420.

[0134] In certain embodiments, the cleavage domain comprises one or more engineered cleavage half-domain (also referred to as dimerization domain mutants) that minimize or prevent homodimerization, as described, for example, in U.S. Patent Nos. 7,888,121; 8,409,861; and U.S. Patent Publication No. 20080131962, the disclosures of all of which are incorporated by reference in their entireties herein. Amino acid residues at positions 446, 447, 479, 483, 484, 486, 487, 490, 491, 496, 498, 499, 500, 531, 534, 537, and 538 of *Fok* I are all targets for influencing dimerization of the *Fok* I cleavage half-domains.

[0135] Exemplary engineered cleavage half-domains of *Fok* I that form obligate heterodimers include a pair in which a first cleavage half-domain includes mutations at amino acid residues at positions 490 and 538 of *Fok* I and a second cleavage half-domain includes mutations at amino acid residues 486 and 499.

[0136] Thus, in certain embodiments, the mutation at 490 replaces Glu (E) with Lys (K); the mutation at 538 replaces Iso (I) with Lys (K); the mutation at 486 replaced Gln (Q) with Glu (E); and the mutation at position 499 replaces Iso (I) with Lys (K). Specifically, the engineered cleavage half-domains described herein were prepared by mutating positions 490 (E→K) and 538 (I→K) in one cleavage half-

domain to produce an engineered cleavage half-domain designated "E490K:I538K" and by mutating positions 486 (Q→E) and 499 (I→L) in another cleavage half-domain to produce an engineered cleavage half-domain designated "Q486E:I499L". The engineered cleavage half-domains described herein are obligate heterodimer mutants in which aberrant cleavage is minimized or abolished. *See, e.g.,* U.S. Patent No. 7,888,121, the disclosure of which is incorporated by reference in its entirety for all purposes.

[0137] Engineered cleavage half-domains described herein can be prepared using any suitable method, for example, by site-directed mutagenesis of wild-type cleavage half-domains (*Fok* I) as described in U.S. Patent No. 7,888,121.

[0138] The engineered cleavage half-domains described herein may be obligate heterodimer mutants in which aberrant cleavage is minimized or abolished. *See, e.g.,* Example 1 of WO 07/139898. In certain embodiments, the engineered cleavage half-domain comprises mutations at positions 486, 499 and 496 (numbered relative to wild-type *Fok*I), for instance mutations that replace the wild type Gln (Q) residue at position 486 with a Glu (E) residue, the wild type Iso (I) residue at position 499 with a Leu (L) residue and the wild-type Asn (N) residue at position 496 with an Asp (D) or Glu (E) residue (also referred to as a "ELD" and "ELE" domains, respectively). In other embodiments, the engineered cleavage half-domain comprises mutations at positions 490, 538 and 537 (numbered relative to wild-type *Fok*I), for instance mutations that replace the wild type Glu (E) residue at position 490 with a Lys (K) residue, the wild type Iso (I) residue at position 538 with a Lys (K) residue, and the wild-type His (H) residue at position 537 with a Lys (K) residue or a Arg (R) residue (also referred to as "KKK" and "KKR" domains, respectively). In other embodiments, the engineered cleavage half-domain comprises mutations at positions 490 and 537 (numbered relative to wild-type *Fok*I), for instance mutations that replace the wild type Glu (E) residue at position 490 with a Lys (K) residue and the wild-type His (H) residue at position 537 with a Lys (K) residue or a Arg (R) residue (also referred to as "KIK" and "KIR" domains, respectively). Engineered cleavage half-domains described herein can be prepared using any suitable method, for example, by site-directed mutagenesis of wild-type cleavage half-domains (*Fok* I) as described in U.S. Patent Nos. 7,888,121; and U.S. Patent Publication Nos. 20080131962; and 20110201055.

[0139] Alternatively, nucleases may be assembled *in vivo* at the nucleic acid target site using so-called "split-enzyme" technology (*see e.g.* U.S. Patent Publication No. 20090068164). Components of such split enzymes may be expressed either on separate expression constructs, or can be linked in one open reading frame where the individual components are separated, for example, by a self-cleaving 2A peptide or IRES sequence. Components may be individual zinc finger binding domains or domains of a meganuclease nucleic acid binding domain.

[0140] Alternatively, the *Fok*I nuclease domain variant known as "Sharkey" may be used (see Guo *et al*, (2010) *J. Mol. Biol.* doi:10.1016/j.jmb.2010.04.060).

[0141] Nuclease expression constructs can be readily designed using methods known in the art. See, *e.g.*, United States Patent Nos. 7,888,121 and 8,409,861 and U.S. Patent Publication Nos. 20030232410; 20050208489; 20050026157; 20060063231; and 20070134796. In certain embodiments, expression of the nuclease is under the control of an inducible promoter, for example the galactokinase promoter which is activated (de-repressed) in the presence of raffinose and/or galactose and repressed in presence of glucose. In particular, the galactokinase promoter is induced and the nuclease(s) expressed upon successive changes in the carbon source (*e.g.*, from glucose to raffinose to galactose). Other non-limiting examples of inducible promoters include *CUP1*, *MET15*, *PHO5*, and tet-responsive promoters.

[0142] The CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)/Cas (CRISPR Associated) nuclease system is a recently engineered nuclease system based on a bacterial system that can be used for genome engineering. It is based on part of the adaptive immune response of many bacteria and archea. When a virus or plasmid invades a bacterium, segments of the invader's DNA are converted into CRISPR RNAs (crRNA) by the 'immune' response. This crRNA then associates, through a region of partial complementarity, with another type of RNA called tracrRNA to guide the Cas9 nuclease to a region homologous to the crRNA in the target DNA called a "protospacer". Cas9 cleaves the DNA to generate blunt ends at the DSB at sites specified by a 20-nucleotide guide sequence contained within the crRNA transcript. Cas9 requires both the crRNA and the tracrRNA for site specific DNA recognition and cleavage. This system has now been engineered such that the crRNA and tracrRNA can be combined into one molecule (the "single guide RNA"), and the crRNA equivalent portion of the single guide RNA can be engineered to guide the Cas9 nuclease to target any desired sequence (see Jinek *et al* (2012) *Science* 337,

p. 816-821, Jinek *et al*, (2013), *eLife* 2:e00471, and David Segal, (2013) *eLife* 2:e00563). Thus, the CRISPR/Cas system can be engineered to create a DSB at a desired target in a genome, and repair of the DSB can be influenced by the use of repair inhibitors to cause an increase in error prone repair.

A. Target Sites

[0143] As described in detail above, DNA domains in ZFNs and TALENs can be engineered to bind to any sequence of choice in a locus. An engineered DNA-binding domain can have a novel binding specificity, compared to a naturally-occurring DNA-binding domain. Engineering methods include, but are not limited to, rational design and various types of selection. Rational design includes, for example, using databases comprising triplet (or quadruplet) nucleotide sequences and individual (*e.g.,* zinc finger) amino acid sequences, in which each triplet or quadruplet nucleotide sequence is associated with one or more amino acid sequences of DNA binding domain which bind the particular triplet or quadruplet sequence. *See*, for example, U.S. Patents 8,586,526; 6,453,242 and 6,534,261, incorporated by reference herein in their entireties. Rational design of TAL-effector domains can also be performed. *See, e.g.,* U.S. Patent No. 8,586,526.

[0144] Exemplary selection methods applicable to DNA-binding domains, including phage display and two-hybrid systems, are disclosed in US Patents 5,789,538; 5,925,523; 6,007,988; 6,013,453; 6,410,248; 6,140,466; 6,200,759; and 6,242,568; as well as WO 98/37186; WO 98/53057; WO 00/27878; and WO 01/88197.

[0145] Selection of target sites; nucleases and methods for design and construction of fusion proteins (and polynucleotides encoding same) are known to those of skill in the art and described in detail in U.S. Patent Nos. 7,888,121 and 8,409,861, incorporated by reference in their entireties herein.

[0146] In addition, as disclosed in these and other references, DNA-binding domains (*e.g.,* multi-fingered zinc finger proteins) may be linked together using any suitable linker sequences, including for example, linkers of 5 or more amino acids. *See, e.g.*, U.S. Patent Nos. 6,479,626; 6,903,185; and 7,153,949 for exemplary linker sequences 6 or more amino acids in length. The proteins described herein may include any combination of suitable linkers between the individual DNA-binding domains of the protein. *See, also*, U.S. Patent No. 8,586,526.

[0147] Additionally, single guide RNAs can be engineered to bind to a target of choice in a genome by commonly known methods known in the art for creating specific RNA sequences. These single guide RNAs are designed to guide the Cas9 to any chosen target site.

Donors

[0148] As noted above, insertion of an exogenous sequence (also called a "donor sequence" or "donor"), for example for correction of a mutant gene or for increased expression of a wild-type gene also can be carried out. It will be readily apparent that the donor sequence is typically not identical to the genomic sequence where it is placed. A donor sequence can contain a non-homologous sequence flanked by two regions of homology to allow for efficient HDR at the location of interest. Additionally, donor sequences can comprise a vector molecule containing sequences that are not homologous to the region of interest in cellular chromatin. A donor molecule can contain several, discontinuous regions of homology to cellular chromatin. For example, for targeted insertion of sequences not normally present in a region of interest, said sequences can be present in a donor nucleic acid molecule and flanked by regions of homology to sequence in the region of interest. Alternatively, a donor molecule may be integrated into a cleaved target locus via non-homologous end joining (NHEJ) mechanisms. *See, e.g.,* U.S. Patent Publication Nos. 20110207221 and 20130326645.

[0149] The donor polynucleotide can be DNA or RNA, single-stranded or double-stranded and can be introduced into a cell in linear or circular form. *See, e.g.,* U.S. Patent Publication Nos. 20100047805; 20110281361; and 20110207221. If introduced in linear form, the ends of the donor sequence can be protected (*e.g.*, from exonucleolytic degradation) by methods known to those of skill in the art. For example, one or more dideoxynucleotide residues are added to the 3' terminus of a linear molecule and/or self-complementary oligonucleotides are ligated to one or both ends. *See*, for example, Chang *et al.* (1987) *Proc. Natl. Acad. Sci. USA*84:4959-4963; Nehls *et al.* (1996) *Science* 272:886-889. Additional methods for protecting exogenous polynucleotides from degradation include, but are not limited to, addition of terminal amino group(s) and the use of modified internucleotide linkages such as, for example, phosphorothioates, phosphoramidates, and O-methyl ribose or deoxyribose residues.

[0150] A polynucleotide can be introduced into a cell as part of a vector molecule having additional sequences such as, for example, replication origins, promoters and genes encoding antibiotic resistance. Moreover, donor polynucleotides can be introduced as naked nucleic acid, as nucleic acid complexed with an agent such as a liposome or poloxamer, or can be delivered by viruses (*e.g.*, adenovirus, AAV, herpesvirus, retrovirus, lentivirus and integrase defective lentivirus (IDLV)).

[0151] The donor is generally inserted so that its expression is driven by the endogenous promoter at the integration site, namely the promoter that drives expression of the endogenous gene into which the donor is inserted (*e.g.*, AAVS1, CCR5, albumin, HPRT etc. However, it will be apparent that the donor may comprise a promoter and/or enhancer, for example a constitutive promoter or an inducible or tissue specific promoter.

[0152] The donor molecule may be inserted into an endogenous gene such that all, some or none of the endogenous gene is expressed. For example, a transgene as described herein may be inserted into an endogenous locus such that some (N-terminal and/or C-terminal to the transgene) or none of the endogenous sequences are expressed, for example as a fusion with the transgene. In other embodiments, the transgene (*e.g.*, with or without additional coding sequences such as for the endogenous gene) is integrated into any endogenous locus, for example a safe-harbor locus.

[0153] When endogenous sequences (endogenous or part of the transgene) are expressed with the transgene, the endogenous sequences may be full-length sequences (wild-type or mutant) or partial sequences. Preferably the endogenous sequences are functional. Non-limiting examples of the function of these full length or partial sequences include increasing the serum half-life of the polypeptide expressed by the transgene (*e.g.*, therapeutic gene) and/or acting as a carrier.

[0154] Furthermore, although not required for expression, exogenous sequences may also include transcriptional or translational regulatory sequences, for example, promoters, enhancers, insulators, internal ribosome entry sites, sequences encoding 2A peptides and/or polyadenylation signals.

Delivery

[0155] The compositions (*e.g.* ZFPs, TALEs, CRISPR/Cas), polynucleotides encoding same, any donor polynucleotides described herein may be delivered to a

target cell containing a TCR gene by any suitable means. Methods of delivering the compositions comprising DNA-binding domains are described, for example, in U.S. Patent Nos. 6,453,242; 6,503,717; 6,534,261; 6,599,692; 6,607,882; 6,689,558; 6,824,978; 6,933,113; 6,979,539; 7,013,219; and 7,163,824, the disclosures of all of which are incorporated by reference herein in their entireties.

[0156] Zinc finger, TALE or CRISPR/Cas proteins as described herein may also be delivered using vectors containing sequences encoding one or more of the zinc finger, TALE or CRISPR/Cas protein(s). Donor encoding polynucleotides may be similarly delivered. Any vector systems may be used including, but not limited to, plasmid vectors, retroviral vectors, lentiviral vectors, adenovirus vectors, poxvirus vectors; herpesvirus vectors and adeno-associated virus vectors, etc. *See, also,* U.S. Patent Nos. 6,534,261; 6,607,882; 6,824,978; 6,933,113; 6,979,539; 7,013,219; and 7,163,824, incorporated by reference herein in their entireties. Furthermore, it will be apparent that any of these vectors may comprise one or more zinc finger protein-encoding sequences, one or more CRISPR/Cas-encoding sequences or one or more TALE-encoding sequences. Thus, when one or more nucleases or nuclease systems and/or donors are introduced into the cell, the nucleases or nuclease systems and/or donors may be carried on the same vector or on different vectors. When multiple vectors are used, each vector may comprise a sequence encoding one or multiple ZFPs, TALEs, CRISPR/Cas system and/or donors.

[0157] Conventional viral and non-viral based gene transfer methods can be used to introduce nucleic acids encoding engineered ZFPs,TALEs, CRISPR/Cas and/or donors in cells (*e.g.*, mammalian cells) and target tissues. Such methods can also be used to administer nucleic acids encoding ZFPs, TALES, CRISPR/Cas and/or donors to cells *in vitro*. In certain embodiments, nucleic acids encoding ZFPs,TALEs, CRISPR/Cas and/or donors are administered for *in vivo* or *ex vivo* gene therapy uses. Non-viral vector delivery systems include DNA plasmids, naked nucleic acid, and nucleic acid complexed with a delivery vehicle such as a liposome or poloxamer. Viral vector delivery systems include DNA and RNA viruses, which have either episomal or integrated genomes after delivery to the cell. For a review of gene therapy procedures, see Anderson, *Science* 256:808-813 (1992); Nabel&Felgner, *TIBTECH* 11:211-217 (1993); Mitani&Caskey, *TIBTECH* 11:162-166 (1993); Dillon, *TIBTECH* 11:167-175 (1993); Miller, *Nature* 357:455-460 (1992); Van Brunt, *Biotechnology* 6(10):1149-1154 (1988); Vigne, *Restorative Neurology and*

Neuroscience 8:35-36 (1995); Kremer &Perricaudet, *British Medical Bulletin* 51(1):31-44 (1995); Haddada*et al.*, in *Current Topics in Microbiology and Immunology* Doerfler and Böhm (eds.) (1995); and Yu *et al., Gene Therapy* 1:13-26 (1994).

[0158] Methods of non-viral delivery of nucleic acids include electroporation, lipofection, microinjection, biolistics, virosomes, liposomes, immunoliposomes, polycation or lipid:nucleic acid conjugates, naked DNA, mRNA, artificial virions, and agent-enhanced uptake of DNA. Sonoporation using, *e.g.*, the Sonitron 2000 system (Rich-Mar) can also be used for delivery of nucleic acids. In a preferred embodiment, one or more nucleic acids are delivered as mRNA. Also preferred is the use of capped mRNAs to increase translational efficiency and/or mRNA stability. Especially preferred are ARCA (anti-reverse cap analog) caps or variants thereof. See US patents US7074596 and US8153773, incorporated by reference herein.

[0159] Additional exemplary nucleic acid delivery systems include those provided by Amaxa® Biosystems (Cologne, Germany), Maxcyte, Inc. (Rockville, Maryland), BTX Molecular Delivery Systems (Holliston, MA) and Copernicus Therapeutics Inc, (*see* for example US6008336). Lipofection is described in *e.g.*, US 5,049,386, US 4,946,787; and US 4,897,355) and lipofection reagents are sold commercially (*e.g.*, Transfectam™,Lipofectin™ and Lipofectamine™ RNAiMAX). Cationic and neutral lipids that are suitable for efficient receptor-recognition lipofection of polynucleotides include those of Felgner, WO 91/17424, WO 91/16024. Delivery can be to cells (*ex vivo* administration) or target tissues (*in vivo* administration).

[0160] The preparation of lipid:nucleic acid complexes, including targeted liposomes such as immunolipid complexes, is well known to one of skill in the art (*see, e.g.*, Crystal, *Science* 270:404-410 (1995); Blaese *et al., Cancer Gene Ther.* 2:291-297 (1995); Behr *et al., Bioconjugate Chem.* 5:382-389 (1994); Remy *et al., Bioconjugate Chem.* 5:647-654 (1994); Gao *et al., Gene Therapy* 2:710-722 (1995); Ahmad *et al., Cancer Res.* 52:4817-4820 (1992); U.S. Pat. Nos. 4,186,183, 4,217,344, 4,235,871, 4,261,975, 4,485,054, 4,501,728, 4,774,085, 4,837,028, and 4,946,787).

[0161] Additional methods of delivery include the use of packaging the nucleic acids to be delivered into EnGeneIC delivery vehicles (EDVs). These EDVs are specifically delivered to target tissues using bispecific antibodies where one arm

of the antibody has specificity for the target tissue and the other has specificity for the EDV. The antibody brings the EDVs to the target cell surface and then the EDV is brought into the cell by endocytosis. Once in the cell, the contents are released (*see* MacDiarmid *et al* (2009) *Nature Biotechnology* 27(7) p. 643).

[0162] The use of RNA or DNA viral based systems for the delivery of nucleic acids encoding engineered ZFPs, TALEs and/or donors take advantage of highly evolved processes for targeting a virus to specific cells in the body and trafficking the viral payload to the nucleus. Viral vectors can be administered directly to patients (*in vivo*) or they can be used to treat cells *in vitro* and the modified cells are administered to patients (*ex vivo*). Conventional viral based systems for the delivery of ZFPs include, but are not limited to, retroviral, lentivirus, adenoviral, adeno-associated, vaccinia and herpes simplex virus vectors for gene transfer. Integration in the host genome is possible with the retrovirus, lentivirus, and adeno-associated virus gene transfer methods, often resulting in long term expression of the inserted transgene. Additionally, high transduction efficiencies have been observed in many different cell types and target tissues.

[0163] The tropism of a retrovirus can be altered by incorporating foreign envelope proteins, expanding the potential target population of target cells. Lentiviral vectors are retroviral vectors that are able to transduce or infect non-dividing cells and typically produce high viral titers. Selection of a retroviral gene transfer system depends on the target tissue. Retroviral vectors are comprised of *cis*-acting long terminal repeats with packaging capacity for up to 6-10 kb of foreign sequence. The minimum *cis*-acting LTRs are sufficient for replication and packaging of the vectors, which are then used to integrate the therapeutic gene into the target cell to provide permanent transgene expression. Widely used retroviral vectors include those based upon murine leukemia virus (MuLV), gibbon ape leukemia virus (GaLV), Simian Immunodeficiency virus (SIV), human immunodeficiency virus (HIV), and combinations thereof (*see, e.g.,* Buchscher *et al., J. Virol.* 66:2731-2739 (1992); Johann *et al., J. Virol.* 66:1635-1640 (1992); Sommerfelt *et al., Virol.* 176:58-59 (1990); Wilson *et al., J. Virol.*63:2374-2378 (1989); Miller *et al., J. Virol.* 65:2220-2224 (1991); PCT/US94/05700).In applications in which transient expression is preferred, adenoviral based systems can be used. Adenoviral based vectors are capable of very high transduction efficiency in many cell types and do not require cell division. With such vectors, high titer and high levels of expression have been

obtained. This vector can be produced in large quantities in a relatively simple system. Adeno-associated virus ("AAV") vectors are also used to transduce cells with target nucleic acids, *e.g.*, in the *in vitro* production of nucleic acids and peptides, and for *in vivo* and *ex vivo* gene therapy procedures (*see, e.g.*, West *et al.*, *Virology* 160:38-47 (1987); U.S. Patent No. 4,797,368; WO 93/24641; Kotin, *Human Gene Therapy* 5:793-801 (1994); Muzyczka, *J. Clin. Invest.* 94:1351 (1994). Construction of recombinant AAV vectors is described in a number of publications, including U.S. Pat. No. 5,173,414; Tratschin *et al.*, *Mol. Cell. Biol.* 5:3251-3260 (1985); Tratschin, *et al.*, *Mol. Cell. Biol.* 4:2072-2081 (1984); Hermonat & Muzyczka, *PNAS* 81:6466-6470 (1984); and Samulski *et al.*, *J. Virol.*63:03822-3828 (1989).

[0164] At least six viral vector approaches are currently available for gene transfer in clinical trials, which utilize approaches that involve complementation of defective vectors by genes inserted into helper cell lines to generate the transducing agent.

[0165] pLASN and MFG-S are examples of retroviral vectors that have been used in clinical trials (Dunbar *et al., Blood* 85:3048-305 (1995); Kohn *et al., Nat. Med.* 1:1017-102 (1995); Malech *et al., PNAS* 94:22 12133-12138 (1997)). PA317/pLASN was the first therapeutic vector used in a gene therapy trial. (Blaese *et al., Science* 270:475-480 (1995)). Transduction efficiencies of 50% or greater have been observed for MFG-S packaged vectors. (Ellem *et al., Immunol Immunother.* 44(1):10-20 (1997); Dranoff *et al., Hum. Gene Ther.* 1:111-2 (1997).

[0166] Vectors suitable for introduction of polynucleotides described herein also include non-integrating lentivirus vectors (IDLV). *See*, for example, Ory *et al.* (1996) *Proc. Natl. Acad. Sci. USA* **93:**11382-11388; Dull *et al.* (1998) *J. Virol.***72:**8463-8471; Zufferyet *al.* (1998) *J. Virol.***72:**9873-9880; Follenzi *et al.* (2000) *Nature Genetics***25:**217-222; U.S. Patent Publication No 20090117617.

[0167] Recombinant adeno-associated virus vectors (rAAV) are a promising alternative gene delivery systems based on the defective and nonpathogenic parvovirus adeno-associated type virus. The vectors are derived from a plasmid that retains only the AAV 145 bp inverted terminal repeats flanking the transgene expression cassette. Efficient gene transfer and stable transgene delivery due to integration into the genomes of the transduced cell are key features for this vector system. (Wagner *et al., Lancet* 351:9117 1702-3 (1998), Kearns *et al., Gene Ther.* 9:748-55 (1996)). Other AAV serotypes, including AAV1, AAV3, AAV4, AAV5,

AAV6 and AAV8, AAV 8.2, AAV9, and AAV rh10 and pseudotyped AAV such as AAV2/8, AAV2/5 and AAV2/6 can also be used in accordance with the present invention.

[0168] In certain embodiments, the vector is a lentiviral vector. A lentiviral vector, as used herein, is a vector which comprises at least one component part derivable from a lentivirus. A detailed list of lentiviruses may be found in Coffin *et al* (1997) "Retroviruses" Cold Spring Harbour Laboratory Press Eds: JM Coffin, SM Hughes, HE Varmus pp 758-763). Lentiviral vectors can be produced generally by methods well known in the art. See, *e.g.*, U.S. Patent Nos. 5,994,136; 6,165,782; and 6,428,953. Preferably, the lentiviral vector is an integrase deficient lentiviral vector (IDLV). *See, e.g.,* U.S. Patent Publication 2009/0117617. IDLVs may be produced as described, for example using lentivirus vectors that include one or more mutations in the native lentivirus integrase gene, for instance as disclosed in Leavitt *et al.* (1996) *J. Virol.* 70(2):721-728; Philippe *et al.* (2006) *Proc. Natl Acad. Sci USA* 103(47): 17684-17689; and WO 06/010834. In certain embodiments, the IDLV is an HIV lentiviral vector comprising a mutation at position 64 of the integrase protein (D64V), as described in Leavitt *et al.* (1996) *J. Virol.* 70(2):721-728.

[0169] In certain embodiments, the vector is an adenovirus vector. Non-limiting examples of Ad vectors that can be used in the present application include recombinant (such as E1-deleted), conditionally replication competent (such as oncolytic) and/or replication competent Ad vectors derived from human or non-human serotypes (*e.g.,* Ad5, Ad11, Ad35, or porcine adenovirus-3); and/or chimeric Ad vectors (such as Ad5/F35) or tropism-altered Ad vectors with engineered fiber (*e.g.*, knob or shaft) proteins (such as peptide insertions within the HI loop of the knob protein). Also useful are "gutless" Ad vectors, *e.g.,* an Ad vector in which all adenovirus genes have been removed, to reduce immunogenicity and to increase the size of the DNA payload. This allows, for example, simultaneous delivery of sequences encoding ZFNs and a donor sequence. Such gutless vectors are especially useful when the donor sequences include large transgenes to be integrated *via* targeted integration.

[0170] Replication-deficient recombinant adenoviral vectors (Ad) can be produced at high titer, and they readily infect a number of different cell types. Most adenovirus vectors are engineered such that a transgene replaces the Ad E1a, E1b, and/or E3 genes; subsequently the replication defective vector is propagated in cells

that provide one or more of the deleted gene functions in *trans*. For example, human 293 cells supply E1 function. Ad vectors can transduce multiple types of tissues *in vivo*, including non-dividing, differentiated cells such as those found in liver, kidney and muscle. Conventional Ad vectors have a large carrying capacity. An example of the use of an Ad vector in a clinical trial involved polynucleotide therapy for antitumor immunization with intramuscular injection (Sterman et al., *Hum. Gene Ther.* 7:1083-1089 (1998)).

[0171] Packaging cells are used to form virus particles that are capable of infecting a host cell. Such cells include 293 cells, which package adenovirus, AAV, and ψ2 cells or PA317 cells, which package retrovirus. Viral vectors used in gene therapy are usually generated by a producer cell line that packages a nucleic acid vector into a viral particle. The vectors typically contain the minimal viral sequences required for packaging and subsequent integration into a host (if applicable), other viral sequences being replaced by an expression cassette encoding the protein to be expressed. The missing viral functions are supplied in *trans* by the packaging cell line. For example, AAV vectors used in gene therapy typically only possess inverted terminal repeat (ITR) sequences from the AAV genome which are required for packaging and integration into the host genome. Viral DNA is packaged in a cell line, which contains a helper plasmid encoding the other AAV genes, namely *rep* and *cap*, but lacking ITR sequences. The cell line is also infected with adenovirus as a helper. The helper virus promotes replication of the AAV vector and expression of AAV genes from the helper plasmid. The helper plasmid is not packaged in significant amounts due to a lack of ITR sequences. Contamination with adenovirus can be reduced by, *e.g.*, heat treatment to which adenovirus is more sensitive than AAV. Additionally, AAV can be produced at clinical scale using baculovirus systems (see US Patent No. U.S. 7,479,554).

[0172] Additional examples of the use of adenovirus vectors for gene transfer in clinical trials include Rosenecker et al., *Infection* 24:1 5-10 (1996); Welsh et al., *Hum. Gene Ther.* 2:205-18 (1995); Alvarez et al., *Hum. Gene Ther.* 5:597-613 (1997); Topf et al., *Gene Ther.* 5:507-513 (1998).

[0173] In certain embodiments, the Ad vector is a chimeric adenovirus vector, containing sequences from two or more different adenovirus genomes. For example, the Ad vector can be an Ad5/F35 vector. Ad5/F35 is created by replacing one or more of the fiber protein genes (knob, shaft, tail, penton) of Ad5 with the

corresponding fiber protein gene from a B group adenovirus such as, for example, Ad35. The Ad5/F35 vector and characteristics of this vector are described, for example, in Ni *et al.* (2005) "Evaluation of biodistribution and safety of adenovirus vectors containing group B fibers after intravenous injection into baboons," *Hum Gene Ther* 16:664-677; Nilsson *et al.* (2004) "Functionally distinct subpopulations of cord blood CD34+ cells are transduced by adenoviral vectors with serotype 5 or 35 tropism," *Mol Ther* 9:377-388; Nilsson *et al.* (2004) "Development of an adenoviral vector system with adenovirus serotype 35 tropism; efficient transient gene transfer into primary malignant hematopoietic cells," *J Gene Med* 6:631-641; Schroers *et al.* (2004) "Gene transfer into human T lymphocytes and natural killer cells by Ad5/F35 chimeric adenoviral vectors," *Exp Hematol* 32:536-546; Seshidhar *et al.* (2003) "Development of adenovirus serotype 35 as a gene transfer vector," *Virology* 311:384-393; Shayakhmetov et al. (2000) "Efficient gene transfer into human CD34(+) cells by a retargeted adenovirus vector," *J Virol* 74:2567-2583; and Sova *et al.* (2004), "A tumor-targeted and conditionally replicating oncolytic adenovirus vector expressing TRAIL for treatment of liver metastases," *Mol Ther* 9:496-509.As noted above, ZFNs and polynucleotides encoding these ZFNs may be delivered to any target cell. Generally, for inactivating a gene CCR-5, the cell is an immune cell, for example, a lymphocyte (B-cells, T-cells such as T helper (T_H) and T cytotoxic cells (T_C), null cells such as natural killer (NK) cells); a mononuclear cell (monocytes, marcophages); a granulocytic cell (granulocytes, neutrophils, eosinophils, basophils); a mast cell; and/or a dendritic cell (Langerhans cells, interstitial dendritic cells, interdigitating dendritic cells, circulating dendritic cells). Macrophages, B lymphocytes and dendritic cells are exemplary antigen-presenting cells involved in T_H cell activation. In certain embodiments, the target cell is a T_H cell, characterized by expression of CD4 on the surface. The target cell may also be a hematopoietic stem cell, which may give rise to any immune cell.

[0174] In many gene therapy applications, it is desirable that the gene therapy vector be delivered with a high degree of specificity to a particular tissue type. Accordingly, a viral vector can be modified to have specificity for a given cell type by expressing a ligand as a fusion protein with a viral coat protein on the outer surface of the virus. The ligand is chosen to have affinity for a receptor known to be present on the cell type of interest. For example, Han *et al.*, *Proc. Natl. Acad. Sci. USA* 92:9747-9751 (1995), reported that Moloney murine leukemia virus can be modified to express

human heregulin fused to gp70, and the recombinant virus infects certain human breast cancer cells expressing human epidermal growth factor receptor. This principle can be extended to other virus-target cell pairs, in which the target cell expresses a receptor and the virus expresses a fusion protein comprising a ligand for the cell-surface receptor. For example, filamentous phage can be engineered to display antibody fragments (*e.g.*, FAB or Fv) having specific binding affinity for virtually any chosen cellular receptor. Although the above description applies primarily to viral vectors, the same principles can be applied to nonviral vectors. Such vectors can be engineered to contain specific uptake sequences which favor uptake by specific target cells.

[0175] Gene therapy vectors can be delivered *in vivo* by administration to an individual patient, typically by systemic administration (*e.g.*, intravenous, intraperitoneal, intramuscular, subdermal, or intracranial infusion) or topical application, as described below. Alternatively, vectors can be delivered to cells *ex vivo*, such as cells explanted from an individual patient (*e.g.*, lymphocytes, bone marrow aspirates, tissue biopsy) or universal donor hematopoietic stem cells, followed by reimplantation of the cells into a patient, usually after selection for cells which have incorporated the vector.

[0176] *Ex vivo* cell transfection for diagnostics, research, or for gene therapy (*e.g.*, via re-infusion of the transfected cells into the host organism) is well known to those of skill in the art. In a preferred embodiment, cells are isolated from the subject organism, transfected with a ZFP nucleic acid (gene or cDNA), and re-infused back into the subject organism (*e.g.*, patient). Various cell types suitable for *ex vivo* transfection are well known to those of skill in the art (*see, e.g.*, Freshney *et al.*, *Culture of Animal Cells, A Manual of Basic Technique* (3rd ed. 1994)) and the references cited therein for a discussion of how to isolate and culture cells from patients).

[0177] Suitable cells include but not limited to eukaryotic and prokaryotic cells and/or cell lines. Non-limiting examples of such cells or cell lines generated from such cells include COS, CHO (*e.g.*, CHO-S, CHO-K1, CHO-DG44, CHO-DUXB11, CHO-DUKX, CHOK1SV), VERO, MDCK, WI38, V79, B14AF28-G3, BHK, HaK, NS0, SP2/0-Ag14, HeLa, HEK293 (*e.g.*, HEK293-F, HEK293-H, HEK293-T), and perC6 cells as well as insect cells such as *Spodoptera fugiperda* (Sf), or fungal cells such as *Saccharomyces*, *Pichia* and *Schizosaccharomyces*. In certain

embodiments, the cell line is a CHO-K1, MDCK or HEK293 cell line. Additionally, primary cells may be isolated and used *ex vivo* for reintroduction into the subject to be treated following treatment with the nucleases (*e.g.* ZFNs or TALENs) or nuclease systems (*e.g.* CRISPR/Cas). Suitable primary cells include peripheral blood mononuclear cells (PBMC), and other blood cell subsets such as, but not limited to, T-lymphocytes such as CD4+ T cells or CD8+ T cells. Suitable cells also include stem cells such as, by way of example, embryonic stem cells, induced pluripotent stem cells, hematopoietic stem cells (CD34+), neuronal stem cells and mesenchymal stem cells.

[0178] In one embodiment, stem cells are used in *ex vivo* procedures for cell transfection and gene therapy. The advantage to using stem cells is that they can be differentiated into other cell types *in vitro*, or can be introduced into a mammal (such as the donor of the cells) where they will engraft in the bone marrow. Methods for differentiating CD34+ cells *in vitro* into clinically important immune cell types using cytokines such a GM-CSF, IFN-γ and TNF-α are known (*see,* Inaba *et al., J. Exp. Med.* 176:1693-1702 (1992)).

[0179] Stem cells are isolated for transduction and differentiation using known methods. For example, stem cells are isolated from bone marrow cells by panning the bone marrow cells with antibodies which bind unwanted cells, such as CD4+ and CD8+ (T cells), CD45+ (panB cells), GR-1 (granulocytes), and Iad (differentiated antigen presenting cells) (*see* Inaba *et al., J. Exp. Med.* 176:1693-1702 (1992)).

[0180] Stem cells that have been modified may also be used in some embodiments. For example, stem cells that have been made resistant to apoptosis may be used as therapeutic compositions where the stem cells also contain the ZFPs, TALEs, CRISPR/Cas systems and/or donors of the invention. Resistance to apoptosis may come about, for example, by knocking out BAX and/or BAK using BAX- or BAK-specific nucleases (*see*, U.S.Patent Publication No. 2010/0003756) in the stem cells, or those that are disrupted in a caspase, again using caspase-6 specific ZFNs for example. Alternatively, resistance to apoptosis can also be achieved by the the use of caspase inhibitors like Z-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-[O-methyl]-fluoromethylketone).

[0181] Vectors (*e.g.*, retroviruses, adenoviruses, liposomes, etc.) containing therapeutic ZFP, TALE, CRISPR/Cas system and/or donor nucleic acids can also be

administered directly to an organism for transduction of cells *in vivo*. Alternatively, naked DNA or mRNA can be administered. Administration is by any of the routes normally used for introducing a molecule into ultimate contact with blood or tissue cells including, but not limited to, injection, infusion, topical application and electroporation. Suitable methods of administering such nucleic acids are available and well known to those of skill in the art, and, although more than one route can be used to administer a particular composition, a particular route can often provide a more immediate and more effective reaction than another route.

[0182] Methods for introduction of DNA into hematopoietic stem cells are disclosed, for example, in U.S. Patent No. 5,928,638. Vectors useful for introduction of transgenes into hematopoietic stem cells, *e.g.*, $CD34^+$ cells, include adenovirus Type 35.

[0183] Vectors suitable for introduction of transgenes into immune cells (*e.g.*, T-cells) include non-integrating lentivirus vectors. *See*, for example, Ory *et al.* (1996) *Proc. Natl. Acad. Sci. USA* **93:**11382-11388; Dull *et al.* (1998) *J. Virol.***72:**8463-8471; Zuffery *et al.* (1998) *J. Virol.***72:**9873-9880; Follenzi *et al.* (2000) *Nature Genetics* **25:**217-222.

[0184] Pharmaceutically acceptable carriers are determined in part by the particular composition being administered, as well as by the particular method used to administer the composition. Accordingly, there is a wide variety of suitable formulations of pharmaceutical compositions available, as described below (*see, e.g., Remington's Pharmaceutical Sciences*, 17th ed., 1989).

Applications

[0185] The disclosed methods and compositions can be used for inactivation of a TCR genomic sequence. As noted above, inactivation includes partial or complete repression of the endogenous TCR α and/or β gene expression in a cell (*e.g.*, T-lymphocyte). Inactivation of a TCR gene can be achieved, for example, by a single cleavage event, by cleavage followed by non-homologous end joining, by cleavage at two sites followed by joining so as to delete the sequence between the two cleavage sites, by targeted recombination of a missense or nonsense codon into the coding region, by targeted recombination of an irrelevant sequence (*i.e.*, a "stuffer" sequence) or another coding sequence of interest into the gene or its regulatory region, so as to disrupt the gene or regulatory region, or by targeting recombination of a splice

acceptor sequence into an intron to cause mis-splicing of the transcript. Inactivation of an endogenous TCR gene can also be accomplished by targeted recombination of a TCR gene(s) specific for a tumor antigen/MHC complex of interest.

[0186] There are a variety of applications for nuclease-mediated inactivation (knockout or knockdown) of a TCR gene. For example, the methods and compositions described herein allow for the generation and/or modification of cells lines (for therapeutic and non-therapeutic uses). Inactivation of the endogenous TCR gene(s) may be coupled with the insertion of genes encoding high avidity TCRs or chimeric antigen receptors (CARS, see Cartellieri *et al* (2010) *J Biomed and Biotech*, Vol 2010, Article ID 956304) against a known target, and the resultant transgenic cells (or descendants of these cells having the same characteristics) may be used as cellular therapeutics. Alternatively, the re-targeting of the T cell may occur *in vivo*, using viral vectors to deliver both the genes encoding the TCR-specific nucleases and the high avidity TCR on a donor nucleic acid. In either case, the materials and methods of the invention may be used in the treatment of cancer. Cells modified *in vitro* may also be used for modeling studies or for screening to find other types of therapeutics that may also work in concert with the TCR modification. Any type of cancer can be treated, including, but not limited to lung carcinomas, pancreatic cancers, liver cancers, bone cancers, breast cancers, colorectal cancers, ovarian cancers, leukemias, lymphomas, brain cancers and the like. Other diseases that may be treated with the technology of the invention include fungal, bacterial and viral infections as well as autoimmune diseases and graft-versus-host disease (GvHD).

[0187] In addition, the methods and compositions described herein can be used to generate model organisms and cell lines, including the generation of stable knock-out cells in any given organism. While ZFN/TALENs/CRISPR/Cas systems offer the ability to knock-out any given gene in cell lines or model organism, in the absence of selection marker these events however can be very rare. Accordingly, the methods described herein, which significantly increase the rate of targeted gene disruption, can be used to generate cell line with new properties. This includes cell lines used for the production of biologicals like Hamster (CHO) cell lines or cell lines for the production of several AAV serotypes like human HEK 293 cells or insect cells like Sf9 or Sf21.

[0188] The methods and compositions of the invention can also be used in the production of transgenic organisms. Transgenic animals can include those developed

for disease models, as well as animals with desirable traits. Embryos may be treated using the methods and compositions of the invention to develop transgenic animals. In some embodiments, suitable embryos may include embryos from small mammals (*e.g.,* rodents, rabbits, etc.), companion animals, livestock, and primates. Non-limiting examples of rodents may include mice, rats, hamsters, gerbils, and guinea pigs. Non-limiting examples of companion animals may include cats, dogs, rabbits, hedgehogs, and ferrets. Non-limiting examples of livestock may include horses, goats, sheep, swine, llamas, alpacas, and cattle. Non-limiting examples of primates may include capuchin monkeys, chimpanzees, lemurs, macaques, marmosets, tamarins, spider monkeys, squirrel monkeys, and vervet monkeys. In other embodiments, suitable embryos may include embryos from fish, reptiles, amphibians, or birds. Alternatively, suitable embryos may be insect embryos, for instance, a *Drosophila* embryo or a mosquito embryo.

EXAMPLES

Example 1: Expression of an optimized, high affinity WT-1 TCR construct

[0189] Genes encoding for a codon-optimized, cysteine-modified TCR specific for an HLA-A2-restricted peptide from the Wilms tumor antigen 1 (WT1), specifically the $WT1_{126-134}$ peptide (Kuball *et al* (2007) *Blood* 109(6):2331-8) and single α21 or β21 WT1 specific TCR chains were cloned into bidirectional self-inactivating transfer vectors pCCLsin.PPT.ΔLNGFR.minCMV.WPGK.eGFP.Wpre or pCCLsin.cPPT.ΔLNGFR.min.CMV.hEF1a.eGFP.Wpre as described in Amendola *et al* (2005) *Nature Biotechnology* 23(1): 108-116, Thomas *et al* (2007) *J. Immunol* 179 (9): 5803-5810, and U.S. Patent Publication No US2006200869 (see Figure 1A)

[0190] The vectors were packaged using an integrase-competent third generation lentivirus vector system, and pseudotyped by VZV envelope, essentially as described in Follenzi and Naldini (2002) *Methods in Enzymology* 346: 454-465. The lentiviral vectors were then used to transduce cells using standard techniques (see below) and cells were characterized by FACs analysis to determine if the exogenous TCRs were being expressed on the cell surface.

[0191] As shown below in Table 1, the WT-1 specific TCR construct was highly expressed, whether driven from the PGK/mCMV dual promoter combination or the EF1α/mCMV dual promoter construct. Numbers in Table are presented as

percent of total signal present in the quadrant gated for VB21 expression and WT1-HLA-A2 pentamer binding.

Table 1: Expression of WT-1 TCR

Promoter	Day 14	Day 22
PGK	12.1	21.3
EF1α	1.48	5.16
Untransduced= 0.085		

[0192] Transduction of T cells was accomplished by activating the cells with anti-CD3/ anti-CD28 antibody-conjugated magnetic beads (Clin ExVivo CD3/CD28; Invitrogen) (baCD3/CD28) where the cells were cultured in IMDM (GIBCO-BRL), 10% FCS with low dose IL-7/IL-15 as described in European Patent Publication No EP1956080 and Kaneko *et al* (2009) *Blood* 113: 1006-1015. This procedure preserved an early T cell differentiation phenotype (CD45RA-/+CD62L+, CD28+CD27+, IL7Ra+, IL-2+ γIFN-/+), and the cells proliferated indistinguishably from untransduced lymphocytes. In these conditions, the PGK dual promoter proved to be superior to the EF1α dual promoter in sustaining stochiometric expression of WT1-specific TCR chains, suggesting that the PGK bi-directional promoter exerts a higher activity in the antisense direction than the bi-directional EF1α promoter. Both promoters however, when tested in the context of a lentiviral vector, supported TCR expression at levels appropriate for efficient HLA-A2/WT1 pentamer binding (16%), for > 70 days after initial stimulation (see Figure 1B).

[0193] TCR transduced cells were also able to exhibit specific γIFN production and cytotoxic activity against WT1+HLA-A2+ primary leukemic blasts from AML patients. In particular, γIFN production in cells transduced with vectors expressing the transgenic TCRs either from the PGK/mCMV dual promoter combination or the EF1α/mCMV dual promoter was increased (Figure 2A) as was % killing (lysis) by the TCR modified cells (Figs. 2B and 2C). In addition, γIFN production was inhibited in the edited lymphocytes (Fig. 2D), in the presence of unlabelled targets expressing the HLA-restriction element and pulsed with the target peptide.

Example 2: Efficient integration of a transgene into the CCR5 locus of central memory T cells

[0194] To test the idea of integrating the WT-1 specific TCR genes into a central memory T cell, GFP was used first as a donor nucleic acid to monitor transduction efficiency and GFP expression from the site of integration. The CCR5 locus was chosen because it has been shown that CCR5 knockout cells are fully functional (see U.S. Patent No. 7,951,925). In addition, the PPP1R12C (AAVS1) locus was similarly targeted (see US Patent Publication 20080299580) The GFP-encoding donor was transduced into the cell using an IDLV vector and the CCR5-specific ZFNs or AAVS1-specific ZFNs were introduced using an Ad5/F35 vector as described above. GFP expression was measured 20 days following transduction.

[0195] As shown in Figure 3, ZFN-mediated integration of GFP transgenes resulted in increased GFP signals, including in relation to the amount of Ad5/F35 donor used (Figs. 3A and 3B). Table 2 below shows the increase in the percent of GFP positive cells in the presence of donor or donor plus ZFNs.

Table 2 GFP signal, percent positive cells

Insert site	UT	+ donor	donor+ZFN
CCR5	0.038	0.083	6.11
AAVS1	0.015	0.18	4.38

Example 3: Integration of WT-1 specific TCR transgenes into the CCR5 locus of Jurkat TCR β-negative cells

[0196] The WT-1 specific TCR transgene construct was then used for targeted integration into the CCR5 locus of Jurkat cells that are TCR β-negative following treatment with TCR-β specific ZFNs. Cells were transfected using standard techniques with WT-1 TCR construct similar to that described in Example 1.

[0197] As seen in Table 3, after introduction of the WT-1 TCR donor (WT1-TCR IDLV) and the CCR5-specific ZFNs (Ad-ZFNs), there is a marked increase in Vβ21 staining or signal, while without the donor or the ZFNs, only background Vβ21 signal is seen. Thus, ZFN-mediated integration of the WT-1 specific TCR into the CCR5 locus occurred in a substantial percentage of the cells.

Table 3: Percent of total signal from VB21+ expression

WT1-TCR IDLV	+	+	+	-
Ad ZFNs	+	++	-	-
Percent VB21 +	16.6	18.7	2.27	0.81

Example 4: Design of TCR-specific ZFNs

[0198] TCR-specific ZFNs were constructed to enable site specific introduction of double strand breaks at either the TCRα and/or TCRβ genes. ZFNs were designed and incorporated into plasmids or IDLV vectors essentially as described in Urnov *et al.* (2005) *Nature* 435(7042):646-651, Lombardo *et al* (2007) *Nat Biotechnol.* Nov;25(11):1298-306, and U.S. Patent Publication 2008/0131962. The recognition helices for exemplary ZFN pairs as well as the target sequence are shown below in Tables 4 and 5. Target sites of the TCR zinc-finger designs are shown in the first column. Nucleotides in the target site that are contacted by the ZFP recognition helices are indicated in uppercase letters; non-contacted nucleotides indicated in lowercase.

Table 4: TCR-α Zinc-finger Designs

ZFN Name Target sequence	F1	F2	F3	F4	F5	F6
25529 (ex 1) ctATGGACtT CAAGAGCAa cagtgctgt (SEQ ID NO:1)	QSGDLTR (SEQ ID NO:2)	QRTHLKA (SEQ ID NO:3)	QSGDRNK (SEQ ID NO: 4)	DRSNLSR (SEQ ID NO:5)	RSDALTQ (SEQ ID NO:6)	N/A
25528 (ex 1) ctCATGTCTA GcACAGTTtg tctgtga (SEQ ID NO:7)	TSGSLSR (SEQ ID NO:8)	QSSVRNS (SEQ ID NO:9)	RSDNLST (SEQ ID NO:10)	DRSALAR (SEQ ID NO:11)	LKQNLDA (SEQ ID NO:12)	N/A
25535 (ex 1) gtGCTGTGGC CtGGAGCAac aaatctga (SEQ ID NO:13)	DRSALSR (SEQ ID NO:14)	QSGHLSR (SEQ ID NO:15)	DRSDLSR (SEQ ID NO:16)	RSDALSR (SEQ ID NO:17)	DRSDLSR (SEQ ID NO:16)	N/A

25534 (ex 1) ttGCTCTTGA AGTCcATAG ACctcatgt (SEQ ID NO:18)	DRSNLSR (SEQ ID NO:5)	QKTSLQA (SEQ ID NO:19)	DRSALSR (SEQ ID NO:14)	QSGNLAR (SEQ ID NO:20)	GKEELNE (SEQ ID NO:21)	RSSDLSR (SEQ ID NO:22)
25537 (ex 1) gcTGTGGCCT GGAGCAAca aatctgact (SEQ ID NO:23)	GNVDLIE (SEQ ID NO:24)	RSSNLSR (SEQ ID NO:25)	RSDALSV (SEQ ID NO:26)	DSSHRTR (SEQ ID NO:27)	WRSCRSA (SEQ ID NO:28)	N/A
25536 (ex 1) ctGTTGCTcT TGAAGTCCat agacctca (SEQ ID NO:29)	DSSDRKK (SEQ ID NO:30)	RSDNLSV (SEQ ID NO:31)	RRFILRG (SEQ ID NO:32)	QSGDLTR (SEQ ID NO:2)	TSGSLTR (SEQ ID NO:33)	N/A
25538 (ex 1) ctGTGGCCtG GAGCAACAa atctgactt (SEQ ID NO:34)	QSGDLTR (SEQ ID NO:2)	QTSTLSK (SEQ ID NO:35)	QSGHLSR (SEQ ID NO:15)	DRSDLSR (SEQ ID NO:16)	RSDALAR (SEQ ID NO:36)	N/A
25540 (ex 1) ctGACTTTGC ATGTGCAaac gccttcaa (SEQ ID NO:37)	QSGDLTR (SEQ ID NO:2)	WRSSLAS (SEQ ID NO:38)	QSGDLTR (SEQ ID NO:2)	HKWVLRQ (SEQ ID NO:39)	DRSNLTR (SEQ ID NO:40)	N/A
25539 (ex 1) ttGTTGCTcC AGGCCACA GCActgttgc (SEQ ID NO:41)	QSGDLTR (SEQ ID NO:2)	QWGTRYR (SEQ ID NO:42)	ERGTLAR (SEQ ID NO:43)	RSDNLRE (SEQ ID NO:44)	QSGDLTR (SEQ ID NO:2)	TSGSLTR (SEQ ID NO:33)
22199 (ex 3) tgAAAGTGG CCGGGtttaatct gctcat (SEQ ID NO:45)	RSAHLSR (SEQ ID NO:46)	DRSDLSR (SEQ ID NO:16)	RSDHLSV (SEQ ID NO:47)	QNNHRIT (SEQ ID NO:48)	N/A	N/A
22189 (ex 3) agGAGGATT CGGAAcccaat cactgaca (SEQ ID NO:49)	QRSNLVR (SEQ ID NO:50)	RNDDRKK (SEQ ID NO:51)	TSGNLTR (SEQ ID NO:52)	TSANLSR (SEQ ID NO:53)	N/A	N/A
25572 (ex 3) gaGGAGGAtT CGGAACCCa atcactgac (SEQ ID NO:54)	DRSTLRQ (SEQ ID NO:55)	QRSNLVR (SEQ ID NO:50)	RNDDRKK (SEQ ID NO:51)	RSAHLSR (SEQ ID NO:46)	QSGHLSR (SEQ ID NO:15)	N/A

25573 (ex 3) gaGGAGGAtT CGGAAcccaat cactgac (SEQ ID NO:54)	QRSNLVR (SEQ ID NO:50)	RNDDRKK (SEQ ID NO:51)	QSGHLAR (SEQ ID NO:56)	QSGHLSR (SEQ ID NO:15)	N/A	N/A
22199 (ex 3) tgAAAGTGG CCGGGtttaatct gctcat (SEQ ID NO:57)	RSAHLSR (SEQ ID NO:46)	DRSDLSR (SEQ ID NO:16)	RSDHLSV (SEQ ID NO:47)	QNNHRIT (SEQ ID NO:48)	N/A	N/A

Table 5: TCR-β Zinc-finger Designs

ZFN Name Target sequence	F1	F2	F3	F4	F5	F6
16783 ccGTAGAACT GGACTTGaca gcggaagt (SEQ ID NO:58)	RSDVLSA (SEQ ID NO:59)	DRSNRIK (SEQ ID NO:60)	RSDVLSE (SEQ ID NO:61)	QSGNLAR (SEQ ID NO:20)	QSGSLTR (SEQ ID NO:62)	N/A
16787 tcTCGGAGAA TGACGAGTG Gacccagga (SEQ ID NO:63)	RSDHLST (SEQ ID NO:64)	RSDNLTR (SEQ ID NO:65)	DRSNLSR (SEQ ID NO:5)	TSSNRKT (SEQ ID NO:66)	RSANLAR (SEQ ID NO:67)	RNDDRKK (SEQ ID NO:51)
22409 tcTCGGAGAA TGACGAGTG Gacccagga (SEQ ID NO:63)	RSDHLST (SEQ ID NO:64)	RSDNLTR (SEQ ID NO:65)	DRSNLSR (SEQ ID NO:5)	LQFNRNQ (SEQ ID NO:68)	RSANLAR (SEQ ID NO:67)	RNDDRKK (SEQ ID NO:51)
22449 tcTCGGAGAA TGACGAGTG Gacccagga (SEQ ID NO:63)	RSDHLST (SEQ ID NO:64)	RSDNLTR (SEQ ID NO:65)	DSSNLSR (SEQ ID NO:69)	LRFNLSN (SEQ ID NO:70)	RSANLAR (SEQ ID NO:67)	RNDDRKK (SEQ ID NO:51)
22454 tcTCGGAGAA TGACGAGTG Gacccagga (SEQ ID NO:63)	RSDHLST (SEQ ID NO:64)	RSDNLTR (SEQ ID NO:65)	DSSNLSR (SEQ ID NO:69)	LHFQLTG (SEQ ID NO:71)	RSANLAR (SEQ ID NO:67)	RNDDRKK (SEQ ID NO:51)

25814 ccGTAGAACT GGACTTGaca gcggaagt (SEQ ID NO:58)	RSDVLSA (SEQ ID NO:59)	DRSNRIK (SEQ ID NO:60)	RSDVLSE (SEQ ID NO:61)	QSGNLAR (SEQ ID NO:20)	QSGSLTR (SEQ ID NO:62)	N/A
25818 ccGTAGAACT GgaCTTGACa gcggaagt (SEQ ID NO:58)	DRSNLSR (SEQ ID NO:5)	LKFALAN (SEQ ID NO:72)	RSDVLSE (SEQ ID NO:61)	QSGNLAR (SEQ ID NO:20)	QSGSLTR (SEQ ID NO:62)	N/A
25820 ccGTAGAACT GGACTTGaca gcggaagt (SEQ ID NO:58)	RSDVLSA (SEQ ID NO:59)	DRSNRIK (SEQ ID NO:60)	RSDVLSE (SEQ ID NO:61)	QSGNLAR (SEQ ID NO:20)	QSGALAR (SEQ ID NO:73)	N/A
25822 ccGTAGAACT GGACTTGaca gcggaagt (SEQ ID NO:58)	RLSVLTI (SEQ ID NO:74)	DRANLTR (SEQ ID NO:75)	RSDVLSE (SEQ ID NO:61)	QSGNLAR (SEQ ID NO:20)	QSGALAR (SEQ ID NO:73)	N/A

Example 5: ZFN activity *in vitro*

[0199] The ZFNs described in Tables 4 and 5 were used to test nuclease activity in K562 cells. To test cleavage activity, plasmids encoding the pairs of human TCR-specific ZFNs described above were transfected into K562 cells. K562 cells were obtained from the American Type Culture Collection and grown as recommended in RPMI medium (Invitrogen) supplemented with 10% qualified fetal bovine serum (FBS, Cyclone). For transfection, one million K562 cells were mixed with 2μg of the zinc-finger nuclease plasmid and 100μL Amaxa Solution V. Cells were transfected in an Amaxa Nucleofector II™ using program T-16 and recovered into 1.4mL warm RPMI medium + 10% FBS.

[0200] Genomic DNA was harvested and a portion of the TCR locus encompassing the intended cleavage site was PCR amplified using the Accuprime HiFi polymerase from Invitrogen as follows: after an initial 3 minute denaturation at 94 °C, 30 cycles of PCR were performed with a 30 second denaturation step at 94 °C followed by a 30 second annealing step at 58 °C followed by a 30 second extension step at 68 °C. After the completion of 30 cycles, the reaction was incubated at 68 °C for 7 minutes, then at 4 °C indefinitely.

[0201] The genomic DNA from the K562 TCR-specific ZFN-treated cells was examined by the Cel-I assay as described, for example, in U.S. Patent Publication Nos. 20080015164; 20080131962 and 20080159996.

[0202] The TCR beta locus in K562 cells has two functional copies with high sequence similarity (TRBC1 and TRBC2) which are both targeted by TCR beta specific ZFNs. *See,* Figure 4B. Thus, initially, PCR primers that would specifically amplify the regions around the intended ZFN cleavage sites specifically from either the TRBC1 or the TRBC2 genes were used to separately analyze NHEJ activity following ZFN driven cleavage for both genes. Exemplary results are presented in Table 6 below for ZFN pair 16787 and 16783.

Table 6: NHEJ activity for pairs of TCR beta-specific ZFNs: analysis of TRBC1 and TRBC2

NHEJ in TRBC2				NHEJ in TRBC1			
TRBC2	ZFN1	ZFN2	% NHEJ	TRBC1	ZFN1	ZFN2	% NHEJ
	16787	16783	8.68		16787	16783	8.73
	GFP		0.00		21	GFP	0.00
	Mock		0.00		22	Mock	0.00
	Water control		0.00		23	Water control	0.00

[0203] The data presented in Table 6 demonstrate that the ZFNs cleave the TRBC1 and TRBC2 genes essentially equally.

[0204] In addition, we tested persistence of ZFN mediated modification of TRBC in K562 cells by harvesting samples at 3 and 10 days after transfection. Results are presented in Table 7 below and demonstrate that with the ZFN pair, 16787 and 16783, target gene modification is stable 10 days following transfection.

Table 7: TCR beta-specific ZFNs in K562 cells

ZFN 1	ZFN 2	% NHEJ	
22449	16783	20.1	Day 3
22454	16783	17.7	

16787	16783	12.1	
GFP		0.0	
22409	16783	14.7	Day 10
22449	16783	8.1	
22454	16783	12.1	
16787	16783	15.6	
GFP		0.0	

[0205] Several ZFN pairs targeting TRBC were analyzed for NHEJ activity following varying amounts of input ZFN (either 0.4 or 0.1 μg of each ZFN). As shown in Figure 5, all ZFN pairs tested exhibited high activity. In this experiment, the cells were treated with a 30° C incubation period following transduction with the ZFNs (see U.S. Patent Publication No: 20110129898). Following analysis of TCR beta-specific ZFN cleavage in K562 cells, several ZFN pairs were tested in either CD4+ or CD8+ mature T cells. Briefly, CD8+ or CD4+ cells were purchased from AllCells and were cultured in RPMI + 10% FBS + 1% L-Glutamine (30 mg/mL) + IL-2 (30 μg/mL, Sigma) and allowed to rest for 4-24 hours.

[0206] Lentiviral vectors were constructed containing the ZFN pairs of interest. They were generated from the HIV derived self-inactivating vector construct and packaged using an HIV integrase carrying the D64V mutation and pseudotyped with the VSV-G envelope as described above. The Ad5/F35 adenoviral vectors were generated as described previously (Perez *et al*, (2008) *Nature Biotechnology* 26: 808-816) after cloning the two sets of ZFNs using a 2A sequence and a cytomegalovirus internal promoter. *See, e.g.,* Holst J *et al* (2006) *Nat Protoc.* 1(1):406-17. 1e6 cells/nucleofection were used with the Amaxa™ Nucleofection kit as specified by the manufacturer for each transduction. Cells were activated 12- 24 hours post nucleofection with anti-CD3/CD28 beads according to manufacturer's protocol (Invitrogen) and grown in IMDM (GIBCO-BRL), 10% FCS media supplemented with 5 ng/mL of IL-7 and IL-15 (Peprotech).

[0207] Cells were harvested 3 days after nucleofection and gene modification efficiency was determined using a Cel-I assay, performed as described in International Patent Publication WO 07/014275. *See, also,* Oleykowski *et al.* (1998) *Nucleic Acids Res.* **26:**4597-4602; Qui *et al.* (2004) *BioTechniques* **36:**702-707; Yeung *et al.* (2005)

BioTechniques **38:**749-758. Several of the ZFN pairs had good activity as measured by the Cel-I assay (NHEJ from 4- 11.9%).

[0208] TCR-α- specific ZFNs were also tested *in vitro* as described above. The cells were incubated at 37 °C for 1 day following the transduction prior to shifting the incubation temperature to 30 °C as described above. *See*, U.S. Patent Publication No. 20110129898. These ZFNs target the TRAC gene, results of a Cel-I assay performed on K562 cells that received various combinations of these ZFNs as described above showed high activity. *See,* Figure 6.

Example 6: Disruption of TCR-β in cells

[0209] The TCR-β-specific ZFNs were then used in experiments to specifically target the TCR locus. Initial experiments were designed to disrupt the TCR locus in Jurkat cells. TCR-β-specific ZFNS 16783 and 16787 were introduced on integrase-defective lentiviral vectors (IDLV) to transiently express the TRBC-targeting ZFNs. Transductions were performed with 0.25μg or 0.5 μg doses of IDLV, based on measurements of HIV Gag p24 in the vector preparations, 48 hours after activation. Vector infectivity ranged from 1 to $5x10^4$ transducing units/ng p24 by vector DNA titration on 293T cells. Cells were then assayed by FACS analysis for loss of the CD3 marker and CD3(-) cells were enriched using LD columns with anti-CD3 MACS Microbeads (Miltenyi Biotec) according to the manufacturer's instructions.

[0210] As shown below in Table 8, following transduction with the ZFNs, there was a vector dose-dependent abrogation of cell surface expression of the TCR/CD3 complex reaching up to 20% of treated cells.

Table 8: Loss of CD3 signal in Jurkat cells treated with TCR-β specific ZFN IDLVs

	Untransformed	0.25 μg IDLV	0.5 μg IDLV
Percent CD3(-)	2.7	13.4	20.2

[0211] A Cel-I assay was performed and confirmed these results with up to 26% of the TRBC alleles (18% TRBC1 and 8% of TRBC2) disrupted in the ZFN treated cells (see Figs 7A and 7B, "Bulk").

[0212] Next, The TRBC ZFNs (16783 and 16787) were introduced into primary human T lymphocytes, and a similar level of CD3 disruption was observed by FACS, as seen in Jurkat cells. Peripheral blood T cells were harvested from healthy donors and activated with CD3 and CD28 conjugated beads. 48 hours post activation the cells were exposed to increasing doses of IDLVs containing the TRBC-specific ZFNs. The cells were then cultured in the presence of low dose (5 ng/mL) IL-7 and IL-15 to promote cell survival and growth. In the primary lymphocytes, up to 7% of the treated cells were CD3 negative while almost no CD3(-) cells were observed in the untreated control and the data is presented below in Table 9.

Table 9: Loss of CD3 signal in primary human T lymphocytes treated with TCR-β specific ZFN IDLVs

	UT	2.5 μg IDLV	5 μg IDLV	18.5 μg IDLV
Percent CD3 (-)	0.17	2.94	3.26	7.07

[0213] Sorted CD3(-) lymphocytes could be expanded and survived over time in the presence of IL7 and IL15 (see Figures 7C and 7D), where percent modification is indicated in Figure 7D. Figure 7E further demonstrates that the CD3(-) cells persist in the population for at least 45 days and also show that the percent of CD3(-) cells in the population stays fairly constant over that time period. The CD3(-) cells do not appear to respond to non-specific mitogen stimulation since, PHA stimulation results in a decrease in the percent of CD3(-) cells in the pool due to expansion of the CD3(+) lymphocytes (Fig. 7F). This result demonstrates absence of CD3 functional signaling in the CD3(-) cells. No phenotypic differences were observed in the CD3(+) and CD3(-) lymphocytes which displayed a similar CD4/CD8 ratio. CD3(-) cells also maintain a central memory phenotype since they remain positive for CD62L, CD28 and IL-7RA (see Table 10 below).

Table 10: CD3(-) cells maintain a Central Memory Phenotype- percent of total fluorescence

	CD3(-)	CD3(+)
CD62L(+)/CD3(-)	97.6	0
CD62L(+)/CD3(+)	1.25	98.4
CD62L(-)/CD3(-)	1.11	0
CD62L(-)/CD3(+)	0	1.61
CD28(+)/CD27(-)	4.66	3.23

CD28(+)/CD27(+)	93.4	94.7
CD28(-)/CD27(-)	0.87	0.68
CD28(-)/CD27(+)	0.97	1.43
IL-7RA(+)/CD8(-)	38.8	40.7
IL-7RA(+)/CD8(+)	47	47
IL-7RA(-)/CD8(-)	3.83	2.84
IL-7RA(-)/CD8(+)	10.4	9.42

[0214] Memory T lymphocytes are less dependent upon TCR signals for homeostatic proliferation than naive T cells; we thus investigated whether homeostatic cytokines could promote survival and growth of previously activated cells, in the absence of TCR expression. Remarkably, the TRBC-ZFNs treated cells could be expanded in culture by supplementation with low dose IL-7 and IL-15, with the proportion of CD3(-) cells remaining stable for more than 50 days in the absence of TCR triggering. Thus, ZFN exposure was well-tolerated in primary lymphocytes and resulted in the stable disruption of the targeted TRBC gene. Therefore, CD3(-) cells were sorted to near purity and further expanded with IL-7 and IL-15 for more than 3 weeks with growth rates similar to CD3(+) cells, demonstrating that homeostatic cytokines do not require TCR signaling functions to promote survival/proliferation of previously activated cells.

[0215] These data demonstrate the successful generation of a novel population of CD8 T cells with phenotypic characteristics of T_{CM} but with surface expression of the endogenous TCR permanently disrupted.

Example 7: Introduction of aWT-1 specific TCR in cells that had previously had the endogenous TCR permanently disrupted

[0216] CD3(-) T lymphocytes were sorted after treatment with the TCR β-specific ZFNs and a lentivirus used to randomly integrate the WT1-TCR β transgene as described in Figure 1 (49.5 ± 30% mean ± SD transduction efficiency, n=4). Thus, in TCR-β-edited cells, expression of the transferred WT1-TCR from an integrated vector rescued surface translocation of CD3 (Figure 8, 1st row).

[0217] In contrast to unedited TCR-transferred lymphocytes in which there was no inherent growth advantage to expression of the introduced TCR (Figure 8, 2nd

row) with respect to the untransduced cells on polyclonal expansion, TCR β chain disrupted cells containing the WT1-TCR could be enriched to >90% purity by polyclonal stimulation, indicating that surface expression of the transferred TCR/CD3 complex in TCR-β-edited cells was necessary and sufficient to promote TCR-mediated expansion of genetically modified cells (Figure 8, 1st row). The exogenous WT1-TCR Vβ chain (Vβ21) was expressed in TCR-β chain disrupted lymphocytes at approximately 2-fold higher mean levels than in unedited TCR-transferred cells and reached expression levels similar to those of the endogenous Vβ21 chain of control T cells and was stably maintained in culture (Figure 9A and Figure 9B). Accordingly, after transduction with the same dose of PGK-WT1 LV, up to 22% of TCR-β-edited lymphocytes bound the $WT1_{126-134}$ pentamer as compared to only 2.6% of unedited cells. (Figure 9A, lower histogram).

[0218] Thus, in the absence of competition from the endogenous TCR β chain, surface expression of the transgenic TCR β chain reaches physiological levels. To verify the function and avidity of TCR-β-edited lymphocytes, we compared TCR β chain disrupted cells with unedited cells transduced with the same PGK-WT1 LV for the ability to lyse $HLA\text{-}A2^+$ targets pulsed with increasing $WT1_{126-134}$ peptide concentrations (see Figure 9C). This functional assay measures activity by a ^{51}Chromium release assay for lysis of labeled T2 cells pulsed with increasing concentrations of the $WT1_{126-134}$ HLA-A2 restricted peptide, or with the irrelevant CMV-derived $pp65_{494-503}$ HLA-A2 restricted peptide (10μM, Proimmune) as a negative control, at an Effector/Target (E/T) ratio of 12.

[0219] Edited T cells were stimulated and 3 weeks later were tested for recognition of the labeled T2 cells by co-incubation for 5 hours. TCR β chain disrupted cells (denoted TCR-edited in Figure 9C) killed targets more effectively than unedited (denoted TCR-transferred) WT1 LV transduced cells (EC50: edited cells: 90.51 nM, with 95%CI: 48.84-167.7; unedited TCR-transferred cells: 269.1 nM, with 95%CI: 175.1-413.5), likely reflecting the higher frequency and expression level of the transgenic WT1 TCR in the TCR-β edited samples. EC50 was calculated by non-linear regression analysis of ^{51}Chromium release data by using the sigmoidal dose-response equation of the GraphPad Prism Software.

[0220] Results are represented as average SD of % lysis (*= $p < 0.05$, ** = $p < 0.01$, TCR-edited n=6, TCR transferred, n = 4). To assess reactivity at a single cell

level, cell were analyzed for Vβ21 expression (see Table 11 below) which showed that, despite fairly equal copy number of the vectors, Vβ21 expression was greater in the TCR-edited cells.

Table 11: TCR expression and vector copy number/cell in TCR transferred and TCR edited lymphocytes

	Disruption endogenous β chain by *TRBC*-ZFN	PGK-WT1 LV or EF1α-WT1 LV	Vβ21 RFI*	% Vβ21+ cells	CpC§
TCR-transferred	No	EF1α-WT1 LV	0.41	36.7	1.9
		PGK-WT1 LV	0.54	62.7	2.1
TCR-edited	Yes	PGK-WT1 LV	0.91	97.3	1.2
UT	No	None	1	3.2	0

*TCR expression was measured by flow cytometry and was plotted as relative fluorescence intensity (RFI=Vβ21 MFI of transduced cells / Vβ MFI of untransduced cells).

§Vector copy *per* cell (CpC) was measured by quantitative PCR as described (Kessels *et al*, (2001) *Nature Immunol* 2 (10):957-61).

[0221] To assess alloreactivity at a single cell level, clones were isolated and expanded from both TCR-β edited and TCR-transferred cells, previously sorted for $WT1_{126-134}$ pentamer binding to enrich for cells displaying optimal exogenous TCR expression. Clones were exposed to T2 cells pulsed with 10 nM of the $WT1_{125-134}$ HLA-A2 restricted peptide (left panel) or to allogenic PRMC (right panel) at a stimulator/responder ratio of 1. The number of specific spots is shown on the y axis as the number of spots produced in the presence of stimulators minus the number of spots produced by effectors alone (**= $p<0.01$). TCR β-edited clones displayed reduced alloreactivity, compared to TCR-transferred cells (see Figure 10, compare the 10A to 10B), possibly reflecting the reduced risk of TCR mispairing in the absence of one endogenous TCR chain.

[0222] These data demonstrate the functional advantage offered by expression of a tumor specific exogenous TCR in a host CTL with abrogated endogenous TCR-β chain expression.

[0223] Theoretically, surface re-expression of the unedited endogenous TCR α chain may still occur in TCR-β edited cells, following TCR gene transfer. To directly assess the potential for misparing in TCR-β chain disrupted lymphocytes, CD3(-) cells were transduced with a LV encoding only the WT1-specific TCR β chain gene and the ΔLNGFR marker (WT1-β-ΔLNGFR-LV). Transduction efficiency was assessed as a percentage of the $\Delta LNGFR^{pos}$ lymphocytes (see Figure 11). Vβ21 expression was measured on $\Delta LNGFR^{pos}$ cells. The mean fluorescent intensity (MFI) of Vβ21 is indicated. Despite the absence of WT1-specific α chain, Vβ21 expression was detected in up to 83% of $\Delta LNGFR^{pos}$ *TRBC*-disrupted cells, demonstrating that even a cysteine-modified TCR β chain inserted into a cell with a *TRBC* disruption is capable of mispairing with the endogenous TCR α chain.

[0224] Next, CD3(-) lymphocytes are used to introduce the WT1-TCR β donor construct into the endogenous TCR locus. The donor is constructed as described above and used in conjunction with the TCR-β-specific ZFNs to cause integration of the TCR-β transgene at the endogenous locus. The cells become positive for both CD3 and the Vβ 21.3 TCR β chain.

Example 8: Disruption and Targeted integration of the TCR-α chain

[0225] To eliminate the potential for TCR chain mispairing, we designed a pair of ZFNs targeting the constant region of the TCR α chain (*TRAC*) gene (Figure 6) and obtained TCR-α- edited T lymphocytes (see Figure 12A) , following the same protocol described to TCR-β editing)and obtained TCR-α-edited T lymphocytes, following the same protocol described for TCR-β-editing (Figure 12B ,12C, 13). To design a complete α/β TCR editing protocol that permits rapid isolation of engineered cells at each step of chain disruption/replacement, we generated a set of LV carrying a single α or β WT1-specific TCR chain, and used IDLV or adenoviral vectors (AdV) to transiently express *TRBC*- or *TRAC*-targeting ZFNs in lymphocytes (Figure 14 for timeline and representative flow conditions/results for full TCR editing)

[0226] CD3(-) cells were efficiently generated with every ZFN-containing vector tested and sequencing at the site of nuclease cleavage reveals the small insertions and deletions (indels) present after repair by NHEJ (Figure 13). AdVs, which proved more efficient in mediating TCR gene disruption than IDLVs, were selected for the purpose of complete TCR editing. T cells harvested from healthy

donors were first exposed to *TRAC*-ZFN-Ad5/F35 48 hrs post-activation with baCD3/CD28, cultured in the presence of IL-7 and IL-15, and the resulting CD3(-) cells isolated by sorting were transduced (49±29& mean ± SD transduction frequency, n=3) with a LV encoding the WT1-α chain (WT1-α LV).

[0227] Cells with rescued CD3 expression were then sorted, stimulated with baCD3/CD28 for one cycle, and then exposed to *TRBC*-ZFN-Ad5/F35. The second round of ZFN exposure yielded up to 23 ± 4% newly CD3(-) cells, indicating that primary T lymphocytes are permissive to multiple rounds of ZFN manipulation. The CD3(-) cells were sorted and transduced (18 ± 7% mean ± SD transduction efficiency, n= 3) with a WT1 TCR-β chain LV. Expression of the transferred WT1-β chain again rescued surface translocation of CD3, which was now co-expressed in balanced proportion with the WT1-TCR Vβ chain in TCR-edited cells (Figure 14 and Figure 15). In contrast to unedited TCR-transferred lymphocytes, TCR-α/β disrupted cells could be enriched to near purity by polyclonal stimulation following TCR gene transfer, and homogenously expressed the high levels of WT1-specific TCR required to bind the $WT1_{126\text{-}134}$ pentamer (see Figure 15).

[0228] These results indicate that surface expression of the transferred TCR/CD3 complex in TCR-edited cells was necessary and sufficient to promote expansion of the cells with the desired specificity for WT1 (Figure 14, right plot). Disruption of the α and β TCR chains was confirmed in TCR- α/β edited cells by Cel-I analysis. No phenotypic differences were observed in TCR-transferred and TCR α/β-edited lymphocytes, which displayed a T_{CM} surface phenotype, as evidenced by high expression of CD62L, CD27, CD28 and IL-7rα. To verify the function and allogenic response of the fully edited lymphocytes, TCR α/β-edited and TCR transferred lymphocytes were polyclonally stimulated.

[0229] Three weeks after polyclonal stimulation, TCR-α/β-edited and TCR transferred lymphocytes were exposed to either i) T2 cells pulsed with increasing concentrations of the $WT1_{126\text{-}134}$ HLA-A2 restricted peptide, or with the irrelevant CMV-derived $pp65_{495\text{-}503}$ HLA-A2 peptide (see Figure 16A) or ii) $WT1^{+}$ $HLA\text{-}A2^{+}$ (black in Figure 16B) or $HLA\text{-}A2^{-}$ (grey) leukemic cells harvested from AML patients with (dashed symbols) or without (full symbols) pulsing with $WT1_{126\text{-}134}$ peptide (50nM). Figure 16C shows similar results where allogenic PBMC were used as target. All assays were performed at a stimulator/responder ratio of 1. Specific spots

are shown on the y axis as spots produced in presence of stimulators minus spots produced by effectors alone. * = p<0.05, ** = p<0.01, *** = p<0.001.

Example 9: Potential off target cleavage analysis.

[0230] *In silico* analysis was used to identify the most likely potential off-target cleavage sites for both the TRAC- and TRBC-specific ZFN pairs as described in Perez *et al* (*ibid*). Sites were identified that contained up to 10 recognition site mismatches for either heterodimer ZFN pairs or homodimer pairs, although the most likely potential off target sites for these ZFN pairs were all targets for ZFN homodimers. The most likely potential off target sites identified are shown below in Tables 12 (TRAC) and 13 (TRBC).

Table 12: Potential off target sites for TRAC-specific ZFNs

Label	Chromosome	Start site	Sequence	# mismatches	Gene
OT1	20	20683361	AGGCACAaGCAAtGTCAC AAGtACcaTGCtTGTACTT (SEQ ID NO:76)	6	RALGAP A2
OT2	6	10525974	AGGTACAaGtAAAGaCGT ATGaACTTTGCtTGTACTT (SEQ ID NO:77)	5	GCNT2
OT3	X	135000000	AAaTACAaGCcAAGcCAA GGTGgCTTTGCGTGTAaA T (SEQ ID NO:78)	6	-
OT4	18	60239118	ATaTACAattAAAGTCAGC TTTtACTTTGCagtTACTT (SEQ ID NO:79)	8	ZCCHC2
OT5	7	48500931	TAGaACAtcCAAAcTCTGG ACCGACTTTGCaTGTcCA G (SEQ ID NO:80)	6	ABCA13
OT6	7	141000000	ATtCAaACaCAAAGTCCCG TGGAtTTTGCtTtTAaAT (SEQ ID NO:81)	7	-
OT7	8	2463159	ATGCAggaGCAAgGTCAC TCTGACcTTcCtTtgcCTT (SEQ ID NO:82)	10	-
OT8	18	4312947	ATGCACACaCAAAcTCAT TTAagCTTTGCtTtTcCAT (SEQ ID NO:83)	7	-
OT9	11	70854569	CAGCcCAtGgAAtGTCATT CTcACaTTGCtTGTGCTT (SEQ ID NO:84)	7	SHANK2
OT10	13	57970961	AAGCAaAaGaAAAaTCAA TATGACTTgGCtTtgGCTT (SEQ ID NO:85)	8	-
OT11	2	69188623	AAGgtCACtCActGTCTGTG TGGAgTTTGCGTGTcCTC	7	-

			(SEQ ID NO:86)		
OT12	X	78538296	AAGCAggaGCAAAGTCAC ATCTtACaTTGCGgcgGCA T (SEQ ID NO:87)	8	-
OT13	2	108000000	ATGTAattcCAAAGTCCTC CATGACcTgGCtTcTACCT (SEQ ID NO:88)	8	-
OT14	8	28249779	CTaCAaAttCAAtGaCAGTA GAGACTTTGCtTtTACTT (SEQ ID NO:89)	8	-
OT15	9	93810846	ATGCAacaGCAAgagCAGC ATGACTTTGttTtTcCTT (SEQ ID NO:90)	10	-
TRAC	14	23016627	GTGCTGTgGCCTGGaGcA ACAAATCTGACTTTGCaT GTGCAA (SEQ ID NO:91)	4	TRAC

Table 13: Potential off target sites for TRBC-specific ZFNs

Label	chromosome	Start site	Sequence	# mismatches	Gene
C1	1	236659757	CCcAagCCAGggCTACTGCT GGGTgGAACTGGACATGC (SEQ ID NO:92)	6	-
C10	10	90573967	CCcTGTgCgGTTCTgCTTAA CAGTAGAACaGGACActT (SEQ ID NO:93)	7	LIPM
C5	5	165037707	ACATGTCagaTTCTACATG AGGTAGAACTGttCTTGT (SEQ ID NO:94)	5	-
C2	2	71186796	ACAAGggCAGcTCTgtCCA AGGTAGcACTGGgCCTGT (SEQ ID NO:95)	7	ATP6V1B 1
C15	15	75401377	CgATGTCCAGaTgTACCTC AGGaAGgACTGGcCCTGG (SEQ ID NO:96)	6	-
C3	3	159730398	CCAAGTCCtccTCTAgGAA GGGGTAGAACTGGAaTTtG (SEQ ID NO:97)	6	-
C1.2	1	60766812	GaAGGTCCAGTgCaAtGTT GAaTAGAAgTGGACATcT (SEQ ID NO:98)	7	-
C17	17	11136639	AgAGGcCCAcTcCTAgAAG GGGTAGAcCTGGAtCTGG (SEQ ID NO:99)	7	-
C15.2	15	67440002	CCAGGTCCAGTTCTACCA GCCacAGAtgTGagCATGT (SEQ ID NO:100)	6	SMAD3
C2.2	2	120313989	ACAAtTCCAGTTCaAgAAT CTTtTAaAggTGGACATGG (SEQ ID NO:101)	7	PCDP1

C6	6	166419926	GCtGGTgCAGcTCTACACG GATGcAGAgCTGGtCCTcC (SEQ ID NO:102)	7	-
C2.3	2	114733178	CCtGGgCCAGTgCTgCTTGT CcTtGAACcGGgCCTGG (SEQ ID NO:103)	8	-
C7.2	7	4224762	GgAGaTCCAGTgCgACAGT CAGaAGAAggGGACTcGG (SEQ ID NO:104)	8	SDK1
CX	X	73070675	CTaCAaAttCAAtGaCAGTA GAGACTTTGCtTtTACTT (SEQ ID NO:105)	8	XIST
CX.2	X	130414175	CCAGGTCagGTTCcggAAA GAAGTAGAACTtGACCccT (SEQ ID NO:106)	8	AGSF1
TRBC	7	142499011	TCAAGTCCAGTTCTACGG GCTCtCGGAGaATGACGA GTGGA (SEQ ID NO:107)	2	TRBC

[0231] As shown in Figures 17 and 18, there are no additional bands present in the off- site samples that have been treated with the ZFNs as compared to those that have not been transduced with the ZFN expression vectors (also compare with the TRAC and TRBC loci). Thus, it appears that the TRAC- and TRBC- specific ZFNs are specific for their intended targets.

Example 10: TRAC and TRBC-specific TALENs

[0232] TRAC- and TRBC- specific TALENs were developed and assembled essentially as described in U.S. Patent No. 8,586,526. Base recognition was achieved using the canonical RVD-base correspondences (the "TALE code": NI for A, HD for C, NN for G (NK in half repeat), NG for T). The TALENs were constructed in the "+63" C-cap (C-terminal truncation) of the TAL-effector DNA-binding domain within the TALEN backbone as described in U.S. Patent No. 8,586,526. The targets and numeric identifiers for the TALENs tested are shown below in Table 14.

Table 14: TRAC and TRBC- specific TALENs

SBS #	Site (5'-3')	# of RVDs	SEQ ID NO (target site):	TRAC/TRBC
101511	gtGCTGTGGCCTGGAGCaa	15	144	TRAC
101512	gtGCTGTGGCCTGGAGCAac	16	145	TRAC
101513	ctGTGGCCTGGAGCAACaa	15	146	TRAC
101514	ttGAAGGCGTTTGCACATGca	17	147	TRAC
101515	gtTGAAGGCGTTTGCACATgc	17	148	TRAC
101516	gtTGAAGGCGTTTGCACAtg	16	149	TRAC
101536	ttCCGCTGTCAAGTCCAGTtc	17	150	TRBC

101537	ctGTCAAGTCCAGTTCta	14	151	TRBC
101539	ctGGGTCCACTCGTCATTct	16	152	TRBC
101540	ctGGGTCCACTCGTCATtc	15	153	TRBC
101541	atCCTGGGTCCACTCGTCAtt	17	153	TRBC

[0233] The TALENs were then tested in pairs in K562 cells for the ability to induce modifications at the endogenous TRAC and TRBC chromosomal targets, analyzed by the Cel-I assay as described above in Example 5. The results showed that nearly all protein pairs were active, and that the TALENs and ZFNs have activities that are in the same approximate range. Tables 15 and 16 show matrix comparisons of pairs of the TALENs in terms of % NHEJ detected by the Cel 1 assay.

Table 15: TRAC and TRBC- specific TALENs activity

16A- TRAC (% NHEJ)

	101511	101512	101513
101514	3.4	5.3	5.9
101515	5.9	8.9	8.3
101516	5.3	12.0	16.4

Table 16: TRBC (% NHEJ)

	101536	101537
101539	8.5	0.0
101540	9.9	9.6
101541	15.0	9.9

Example 11: NY-ESO-1 TCR modified T cells

[0234] T cells were modified with a NY-ESO-1 specific TCR Vβ13 (see, for example Robbins *et al* (2011) *J Clin Oncol* 29(7): 917-924) and expression of the engineered TCR was monitored. In this experiment, T lymphocytes were isolated from healthy volunteers and were activated with CD3 and CD28-antibody conjugated beads. The cells were then cultured in the presence of 5 ng/mL IL-7 and 5 ng/mL IL-15 according to the method in Kaneko *et al* (*Blood* (2009) 113(5) p. 1006) and in Bondanza *et al* (*Blood* (2011) 117(24) p. 6469). The cells were then treated in one of three ways: Group 1 was transduced with a third generation bi-directional lentiviral vector (see Amendola *et al* (2005) *Nat. Biotechnol* 23:108-116) comprising the NY-ESO1 specific, HLA-A2 restricted α and β TCR chains (TCR-PGK-NYESO1 LV) to

generate TCR 'transferred' "TR" T cells. Group 2 was treated prior to LV transduction with adenvirus comprising ZFNs specific for TRAC (see Example 6, ZFNs 25539 and 25540), and then were sorted for loss of CD3 signal. $CD3^{neg}$ cells were then transduced with the TCR-PGK-NYESO1 LV vector to generate the "single edited" or "SE" population of cells. Group 3 were treated first with adenovirus comprising the TRAC ZFN pair 25539/25540 as above and sorted for CD3 signal. $CD3^{neg}$ cells were then transduced with a LV vector comprising the NY-ESO-1 TCR α chain and sorted again for CD3 signal. In this instance, $CD3^{pos}$ cells were then stimulated with the baCD3/CD28 beads and exposed to adenovirus comprising the TRBC ZFN pair 16787/16783 and cells were sorted for absence of surface translocation of CD3. $CD3^{neg}$ cells were then transduced with a LV vector comprising the NY-ESO-1 TCR β chain. Thus, Group 3 expressed uniquely the NY-ESO-1 specific TCR without any endogenous TCR complex and was termed the "complete edited" or "CE" population.

[0235] The three groups of cells were analyzed for expression of the exogenous Vβ13 TCR by a cytofluorimetric analysis where antibodies against NY-ESO-1 specific V beta 13.1 chain were used to label the protein. Untransduced T cells were used as control, and the data was expressed as the Mean Fluorescence Intensity (MFI) observed in the transduced T cells versus the controls. The complete edited population demonstrated the highest expression (see, Figure 19A).

[0236] The T cell populations were also tested for binding to a MHC HLA-A2-NY-ESO1 dextramer. The MHC Dextramer consists of a dextran polymer backbone carrying an optimized number of MHC and fluorochrome molecules. MHC Dextramer reagents carry more MHC molecules and more fluorochromes than conventional MHC multimers. This increases avidity for the specific T-cell and enhances staining intensity, thereby increasing resolution and the signal-to-noise ratio. For staining, the protocol supplied by the manufacturer (*e.g.* Immudex Cancer-testis Antigen Dextramer® Collection) was followed. Samples were run through a FACS Canto II flow cytometer (BD Biosciences), and data were analyzed by Flow Jo software (Tree star Inc). The results demonstrated that the complete edited population had the greatest affinity to the NY-ESO1 dextramer (see Figure 19B). The data in Figure 19 is expressed as Relative Fluorescence Intensity (RFI), meaning the ration between the Mean Fluorescence Intensity (MFI) observed in the sample population

(transferred, single edited, or complete edited T cells) as compared with untransduced T cells. Three consecutive experiments were conducted using 3 different donors. The results (Figure 19C) demonstrated that the CE population had the highest signal.

[0237] Additionally, the cells were analysed for phenotypic markers by FACS analysis as in Cieri *et al*, ((2013) *Blood* 121 p. 573-584). The analysis demonstrated that a proportion of the modified T cells displayed the phenotype of stem memory T (T_{SCM}) cells, characterized by the co-expression of CD45RA, CD62L and CD95.

[0238] The complete edited population of TCR edited lymphocytes displayed high avidity for the cognate antigen when challenged with increasing doses of the NY-ESO1 157-165 peptide in a γ-IFN ELISpot assay (*e.g.* Human IFN gamma ELISPOT Ready-SET-Go!®, eBioscience®). Effector cells used were untransduced (UT), Transferred, single edited T cells (SE) and complete edited T cells (CE). The results are shown in Figure 20A and demonstrated that the TCR complete edited (CE) population displayed high avidity for the peptide. T2 cells were loaded with increasing concentrations of NY-ESO-1 157-165 peptide, or with the unrelated $WT1_{126-134}$ peptide derived from the Wilms Tumor antigen 1 ("T2-$WT1_{126-134}$").

[0239] NY-ESO1 redirected T cells were then challenged with a NY-ESO1+. HLA-A2+ myeloma cell line (U266), to verify their ability to recognize a tumor cell that naturally expressed the NY-ESO1 antigen. First a gamma-IFN ELISpot (described above) was performed using the U266 or the MM1S cell lines as target (see Figure 20B) and demonstrated that the NY-ESO1 redirected T cells had high avidity for the relevant HLA-A2+, NY-ESO1+ cells in comparison with untransduced T cells, and almost no binding to the MM1S cells was detected. No recognition was observed against a MM1S (HLA-$A2^-$ and NY-$ESO1^-$) irrelevant target cell. Next, a ^{51}chromium release was performed using standard methods as follows: effector T cells were incubated in V-bottom 96-well plates for 5 h with myeloma cell lines (MM1S and U266) which were previously labeled with ^{51}chromium. Specific lysis was expressed according to the following formula: 100 × (average experimental cpm – average spontaneous cpm)/(average maximum cpm – average spontaneous cpm).

[0240] The results (Figure 20C and 20D) demonstrated that the different populations were able to cause lysis of the relevant target cell U266 (Figure 20C) and not the irrelevant target cell MM1S (Figure 20D). The complete edited T cells (CE) showed the greatest ability to lyse the appropriate target cells.

[0241] The NY-ESO1 re-directed T cells were also tested for their ability to specifically kill NY-ESO1$^+$, HLA-A2$^+$ tumor cells in a co-culture experiment (see Figure 21). In this experiment, the effector T cells were co-cultured with the relevant U266 cell line ("A2+ESO+") or with the irrelevant MM1S line ("A2-ESO-") for 4 days at an effector/target ratio of 1:1. The results demonstrate that the re-directed T cell effectors are able to prohibit growth the relevant HLA-A2+, NY-ESO1+ cell line. Figure 21B demonstrates that the edited T cells, expanded by 2 fold in the presence of the U266 HLA-A2+, NY-ESO1+ target, but did not expand in the presence of the irrelevant A2-ESO- control.

Example 12: Alloreactivity of edited T cells

[0242] To compare the alloreactive potential of the three NY-ESO-1 redirected T cell populations, TCR transferred (Transfer), TCR single edited (SE) and TCR complete edited (CE) T cells were separately plated in mixed lymphocyte reactions (MLR) against irradiated allogeneic peripheral blood mononuclear cells (PBMCs). Donor-matched PBMCs and mock-transduced T cells (UT) were used as controls. After two cycles of stimulation (S1 10 days, S2 7 days), effector cells were tested against a PHA cell line obtained by the same allogeneic targets, and against the autologous cells in a ^{51}Cr release and in a γ-Interferon (γ-IFN) Elispot assay.

[0243] Simultaneously, NY-ESO-1 redirected T cells and controls were stimulated against NY-ESO-1157-165 pulsed HLA-A2+ irradiated cells. After two cycles of stimulation (S1 10 days, S2 7 days), effector cells were tested against the HLA-A2+ T2 cell line pulsed (C) with the NY-ESO-1157-165 peptide or unpulsed (D).

[0244] No response was observed against the autologous cells. Furthermore, as shown in Figure 22, lysis of the allogeneic target by Transfer T cells was significantly higher than by both SE and CE T cells ($p=0.05$) (Figure 22A). In addition, γ-IFN Elispot confirmed the statistically significant difference between Transfer and edited T cells in secreting γ-IFN upon allogeneic stimulation (Figure 22B), suggesting that the residual endogenous polyclonal TCRs and possibly mispaired TCRs expressed on the cell surface of TCR-transferred T cells can lead to off-target reactivity, while SE and CE T cells are devoid of such reactivity. NY-ESO-1 redirected T cells (Transfer, SE and CE) were equally able to lyse T2 cells pulsed

with the NY-ESO-1 specific peptide (Figure 22C) with high specificity as compared to unpulsed cells (Figure 22D).

Example 13: *In vivo* experiments

[0245] To compare the efficacy and safety of NY-ESO-1 single edited (SE), complete edited (CE) and TCR transferred (transfer) T cells *in vivo*, we set up a mouse model based on the injection of the Multiple Myeloma (MM) U266 cell line (HLA-A2+, NY-ESO-1+, hCD138+) followed by the administration of T cells in sub-lethally irradiated NSG mice. Briefly, $10x10^6$ U266 cells were injected via tail vein at day 0. At day 3 mice received intravenously either: PBS (U266), or $10x10^6$ NY-ESO-1 transfer, SE, CE T cells, or donor matched PBMC or donor matched mock-transduced T cells (UT) as controls. Finally a group of mice received $10x10^6$ complete edited T cells redirected to the WT1 126-134 peptide, not expressed by U266 (CEWT1). Mice were monitored at least 3 times per week for xenogeneic Graft versus Host Disease (GvHD) signs and sacrificed by day 70 in absence of any pathological signs. Due to the long time required for U266 to engraft in mice, we considered evaluable for the anti-tumor response only animals that were sacrificed at day 70. All mice were considered evaluable for GvHD assessment.

[0246] Results are shown in Figure 23. Figure 23A shows the percent of human CD138+ MM cells identified by cytofluorimetric analysis of cells harvested from the bone marrow of euthanized mice. In mice treated with the NY-ESO1 redirected T cells, no residual disease could be detected in the bone marrow, nor in the spleen (not shown) at the time of sacrifice, demonstrating the in vivo efficacy of the NY-ESO1 redirected cells. By contrast, all mice injected with PBS (U266) or CE WT1 T cells had tumor cells detectable in their bone marrow.

[0247] At sacrifice, all organs were collected, fixed in formalin, stained with hematoxylin/eosin and simultaneously analyzed by immunohistochemistry after counterstaining with monoclonal anti-hCD3 antibodies and peroxidase-conjugated second-step reagent to detect any possible GvHD activity and to examine T cell specificity. Infiltration into the mouse organs would indicate inappropriate homing of the T cells, and potentially, the beginning stages of GvHD. Pathological grading ranged from 0 (no hCD3+ cells infiltration) to 3 (massive and diffuse hCD3+ cells infiltration). Interestingly, human CD3+ T cells were found infiltrating lungs and livers of 3 out of 5 animals infused with conventional TCR transferred T cells

("Transfer"), similarly to what was observed in 4 of 4 mice injected with donor-matched unmanipulated PBMCs ("PBMC") or with untransduced lymphocytes (5 of 5 mice, "UT"). Conversely, no lymphocyte infiltration was detected in organs of mice treated with either NY-ESO-1 SE or CE T cells (Figure 23B).

Example 14: TCR editing by mRNA electroporation

A. Single TCR editing

[0248] TCR editing by mRNA electroporation of nuclease message was evaluated as follows. Briefly, Human T lymphocytes from peripheral blood were stimulated with anti-CD3/CD28 beads and electroporated two days later with decreasing doses of *in vitro* transcribed mRNA encoding for the ZFNs pair specific for the TRAC or the TRBC gene.

[0249] The extent of ZFN- induced TCR disruption upon treatment was measured as percentage of CD3 negative cells at 5 or 20 days after electroporation in lymphocytes treated with the TRAC-ZFNs (left panel of Figure 24A) and TRBC-ZFNs (right panel of Figure 24A). In addition, the fold increase in the number of treated cells during culture was also evaluated in TRAC-ZFN treated cells (left panel of Figure 24B); TRBC-ZFN treated cells (middle panel of Figure 24B); and in control cells (right panel of Figure 24B). Further, the surface phenotype of T cells at day 18 after stimulation was also evaluated. T stem memory cells (TSCM) are defined as CD62L+ CD45RA+ (*See,* Gattinoni et al. (2011) *Nat Med.* 17(10):1290-7; and Cieri (2013) *Blood* 121(4):573-84); T central memory (TCM) as CD62L+ CD45RA-; T effector memory (TEM) as CD62L- CD45RA- and terminal effectors (TEMRA) as CD62L- CD45RA+. UT: untreated cells; UT+E: mock electroporated cells; GFP: cells electroporated with GFP encoding mRNA. No statistically significant differences were found in the phenotype composition of the TRAC-ZFNs and TRBC-ZFNs treated cells at the mRNA doses utilized (two-way Anova).

B. TCR double editing

[0250] Human T lymphocytes from peripheral blood were stimulated with anti-CD3/CD28 beads and co-electroporated two days later with *in vitro* transcribed mRNAs encoding for both the TRAC and TRBC specific ZFNs pairs as described above. Next, an analysis was done to quantify the amount of complete, TCR-alpha and TCR-beta edited cells in the CD3 negative fraction of the co-treated cells. Briefly,

5 days after electroporation, CD3 negative cells were sorted and transduced separately with bi-directional lentiviral vectors (LV) encoding for the alpha or the beta NY-ESO specific TCR chain and a reported gene (LNGFR or GFP, respectively, schematically depicted in Figure 25A). The fraction of single alpha or beta edited cells was measured as percentage of transduced cells that restore surface expression of the CD3 upon complementation with exogenous TCR alpha or beta. The amount of complete edited cells in the total CD3 negative population was then calculated by subtracting the two percentages of single edited cells. Results are shown in Figure 25A, demonstrating that 40% of the complete edited cell populations were disrupted at both the TCR-alpha and TCR-beta genes.

[0251] The percentages of CD3 negative (CD3-) cells upon co-electroporation of TRAC- and TRBC-specific ZFN mRNAs containing the obligated heterodimeric FokI domains (ELD and KKR) or their respective orthologous version (RDD and DRR). The percentages of viable cells were calculated as percentages of 7-AAD negative cells gated on singlets. In addition, the composition of the edited cells in the CD3 negative fraction was calculated using the LV reporter strategy described above. Results are shown in Figure 25B.

[0252] The surface phenotype of T cells as described above was also determined at day 18 after stimulation. T stem memory cells (TSCM) are defined as CD62L+ CD45RA+ (Gattinoni *et al.* (2011), *ibid.*; Cieri *et al.* (2013), *ibid.*), T central memory (TCM) as CD62L+ CD45RA-, T effector memory (TEM) as CD62L- CD45RA- and terminal effectors (TEMRA) as CD62L- CD45RA+. UT: untreated cells. Results are shown in Figure 25C.

[0253] Growth curves of T cells co-electroporated with the indicated doses of TRAC- and TRBC-specific ZFN mRNAs were also determined and showed that following an initial acute phase of cell loss the day after the co-electroporation, the surviving cells continue to expand in culture with similar kinetics compared to untreated (UT) controls (Figure 25D).

[0254] All patents, patent applications and publications mentioned herein are hereby incorporated by reference in their entirety.

[0255] Although disclosure has been provided in some detail by way of illustration and example for the purposes of clarity of understanding, it will be apparent to those skilled in the art that various changes and modifications can be

practiced without departing from the spirit or scope of the disclosure. Accordingly, the foregoing descriptions and examples should not be construed as limiting.

CLAIMS

What is claimed is:

1. An isolated T-lymphocyte comprising a stably integrated exogenous sequence encoding a T-cell receptor (TCR), wherein at least one endogenous TCR gene within the cell is partially or completely inactivated by a zinc finger nuclease or a TALEN the TALEN comprising a TAL-effector DNA-binding domain and a cleavage domain, and further wherein the TAL-effector DNA-binding domain comprises a C-terminal truncation as compared to a wild-type TAL-effector DNA-domain.

2. The isolated T-lymphocyte of claim 1, wherein the endogenous TCR gene is a TCR α or TCR β gene.

3. The isolated T-lymphocyte of claim 1 or claim 2, wherein the zinc finger nuclease comprises a zinc finger protein having the recognition helix regions shown in a single row of Tables 5 or 6.

4. The isolated T-lymphocyte of any of claim 1 or claim 2, wherein the TALEN binds to a target sequence selected from the group consisting of SEQ ID NOs:144-153.

5. The isolated T-lymphocyte of any of claims 1 to 4, wherein a polynucleotide encoding the zinc finger nuclease or TALEN is introduced into the cell using an integrase-defective lentiviral vector (IDLV), AAV, a plasmid or mRNA.

6. The isolated T-lymphocyte of any of claims 1 to 5, wherein the exogenous sequence is introduced into an endogenous TCR gene, a CCR5 gene or an AAVS1 gene.

7. The isolated T-lymphocyte of any of claims 1 to 6, wherein the exogenous sequence is selected from the group consisting of a tumor antigen specific TCR

transgene wherein the TCR transgene is a TCR α transgene, a TCR β transgene and combinations thereof.

8. The isolated T-lymphocyte of claim 7, wherein the tumor antigen comprises NY-ESO1.

9. A pharmaceutical composition comprising the isolated T-lymphocyte of any of claims 1 to 8.

10. A method of generating a T-lymphocyte according to any of claims 1 to 8, the method comprising

inactivating an endogenous TCR gene in the T-lymphocyte using one or more polynucleotides encoding one or more nucleases, wherein the nucleases cleave the endogenous TCR gene; and

stably integrating the exogenous sequence into the genome of the T-lymphocyte.

11. The method of claim 10, wherein the exogenous sequence is introduced into the cell using an integrase-defective lentiviral vector (IDLV), retroviral vector (RV) or lentiviral Vector (LV).

12. Use of an isolated T-lymphocyte according to any of claims 1 to 8 or use of a pharmaceutical composition according to claim 9 for the treatment of a cancer, an infection, an auto-immune disorder or graft-versus-host disease (GVHD).

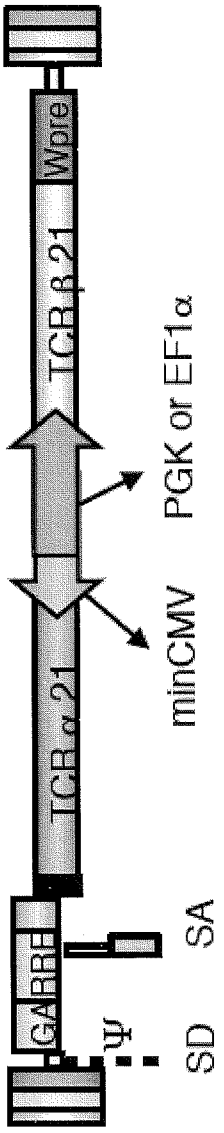


Figure 1A

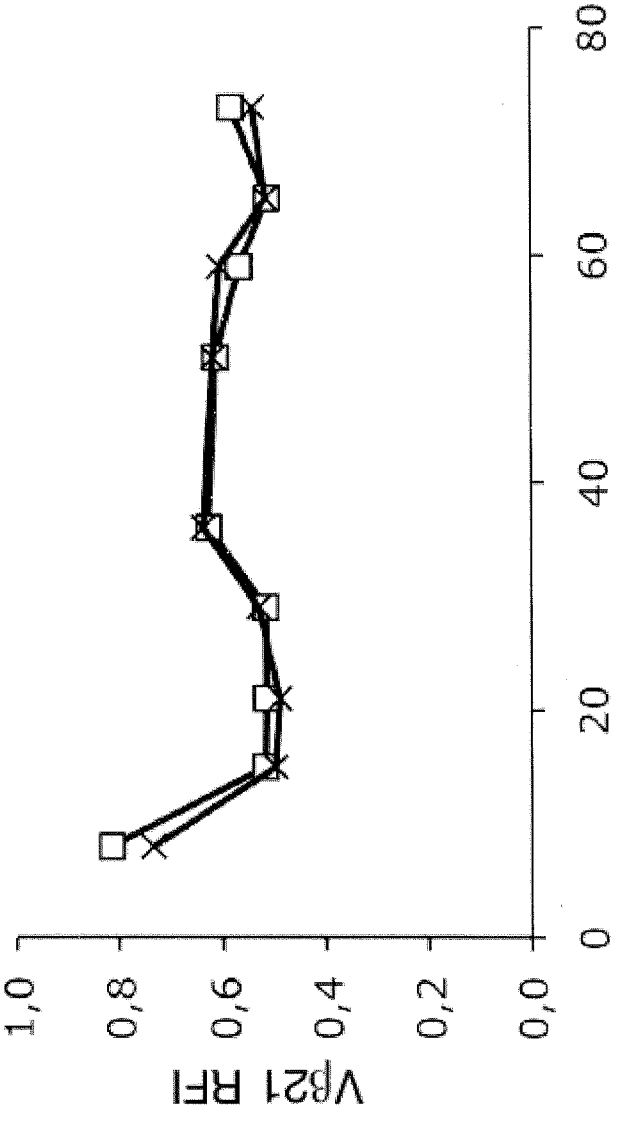


Figure 1B

Figure 2A

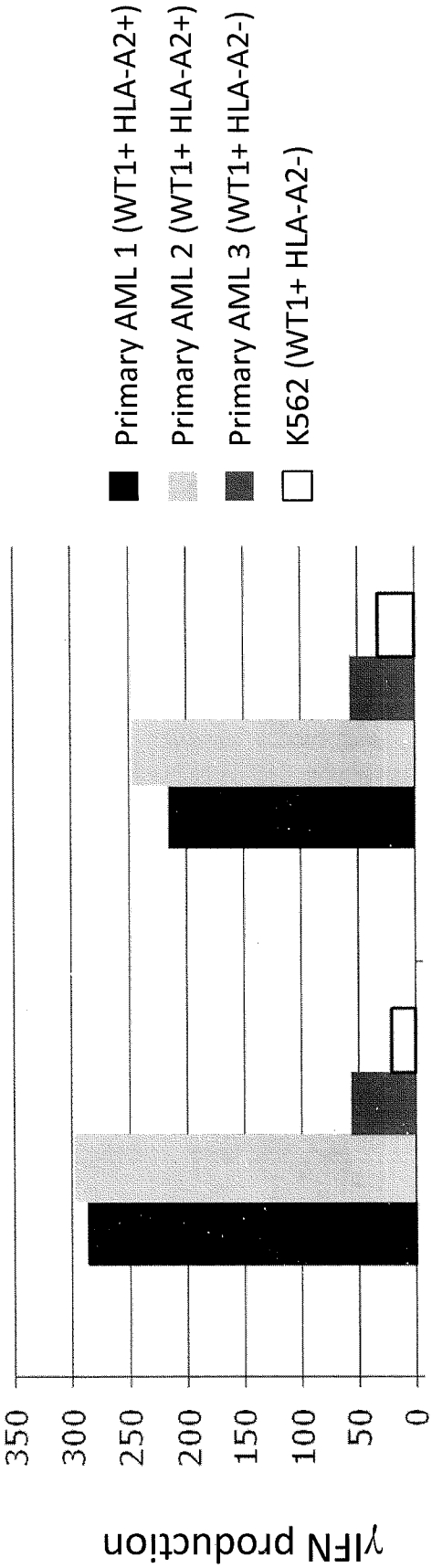


Figure 2B

PGK-WT1

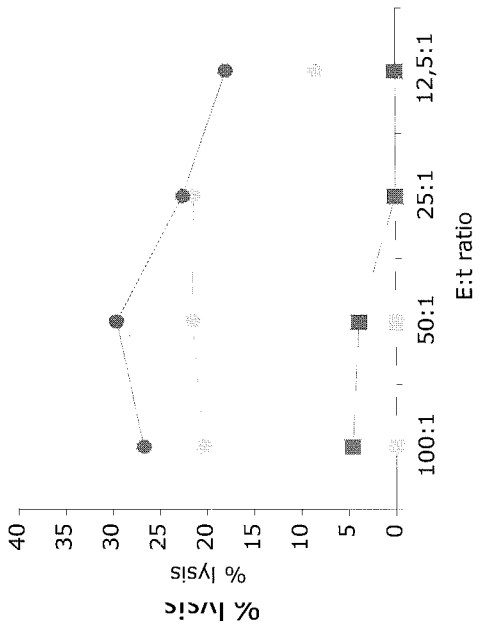
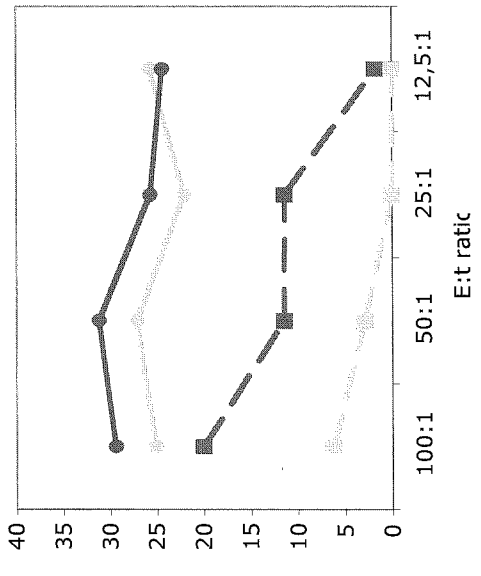


Figure 2C

EF1α-WT1



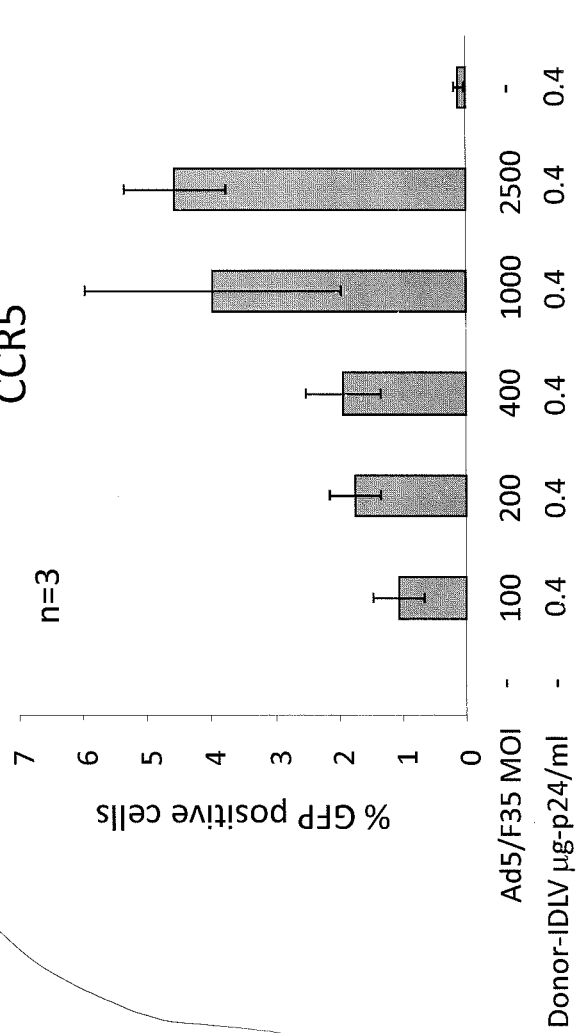


Figure 3A

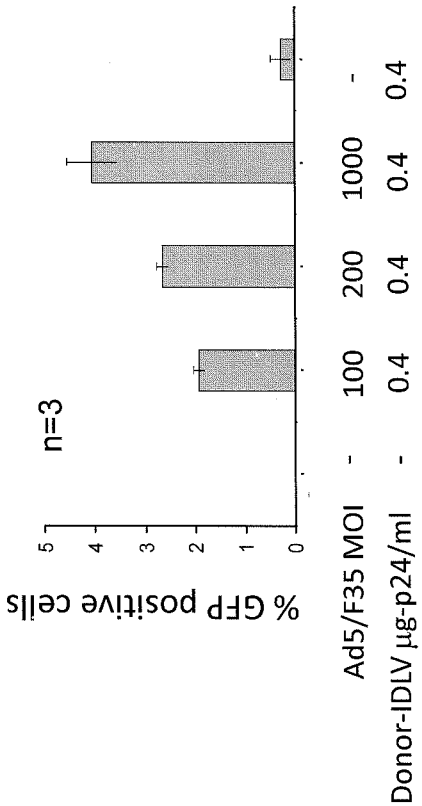
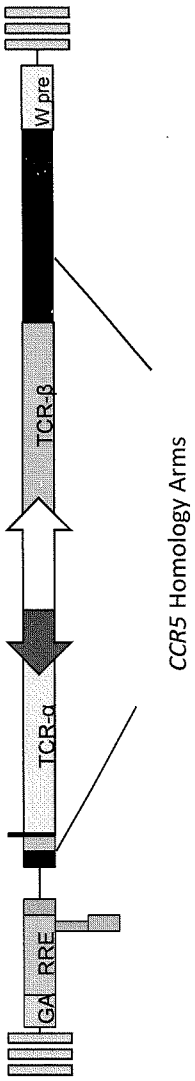


Figure 3B

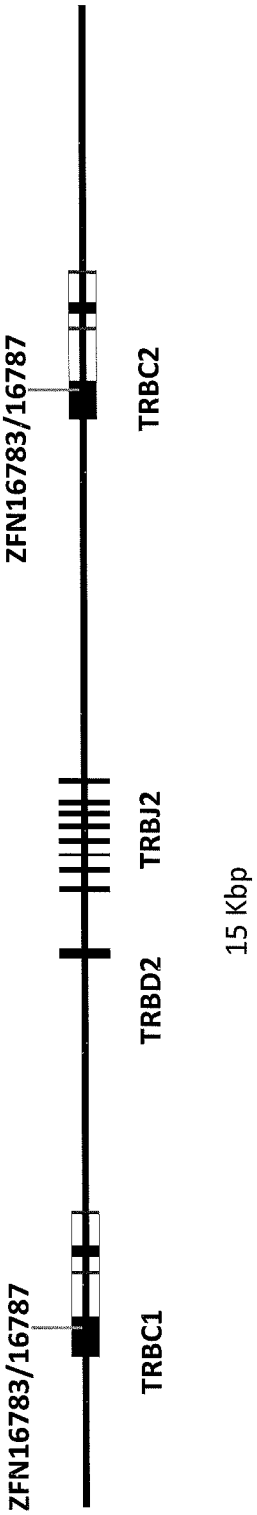
Figure 4

A



B

Arrangement of TCRB1 and TBRC2 genes in K562 cells



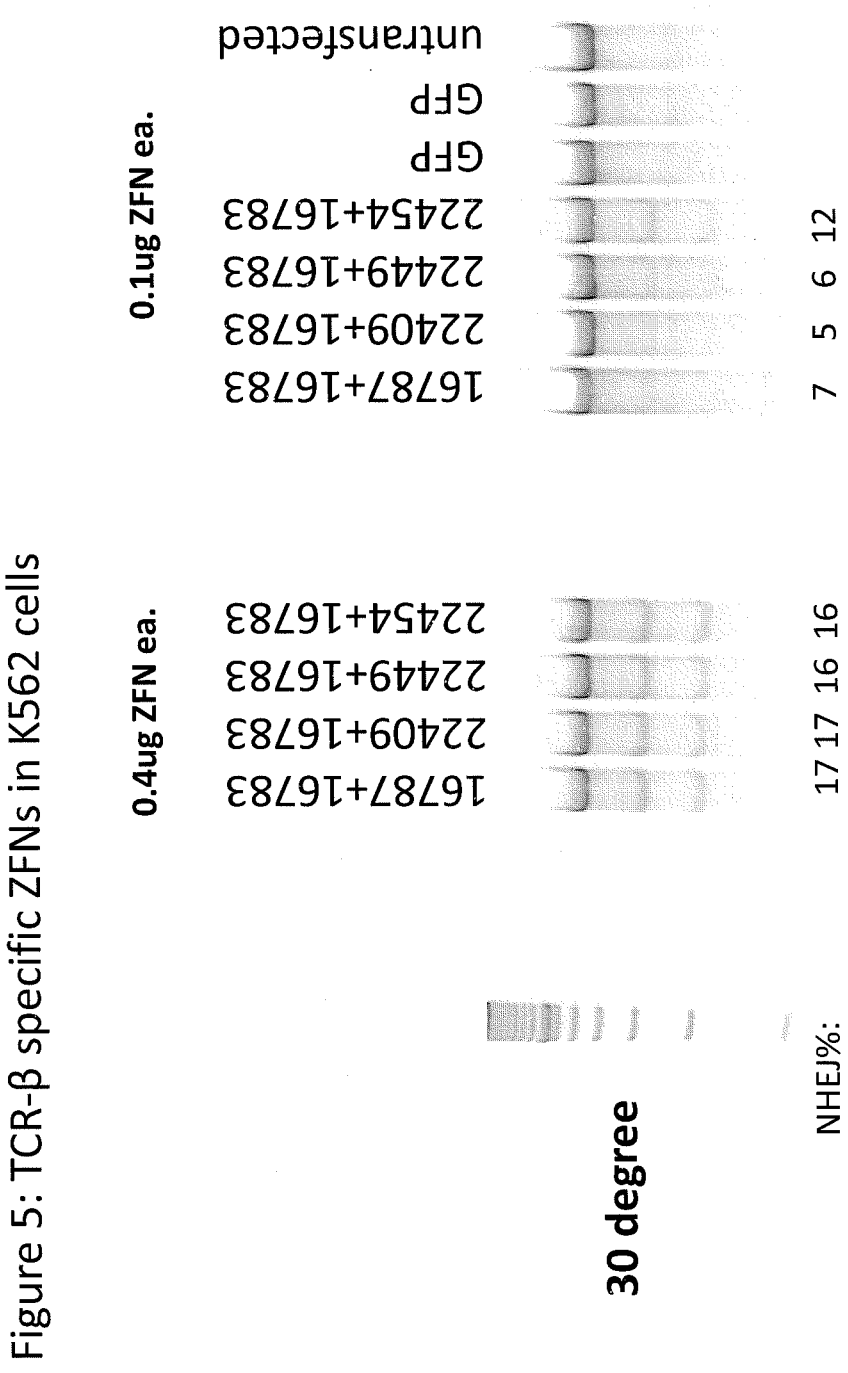
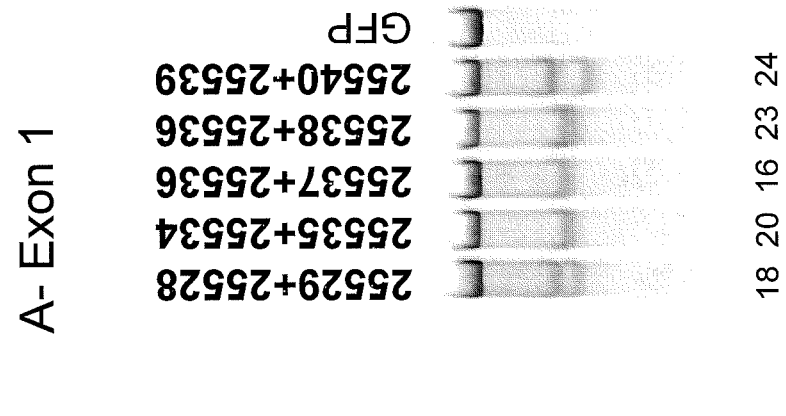


Figure 5: TCR-β specific ZFNs in K562 cells

Figure 6- TCRα specific ZFNs



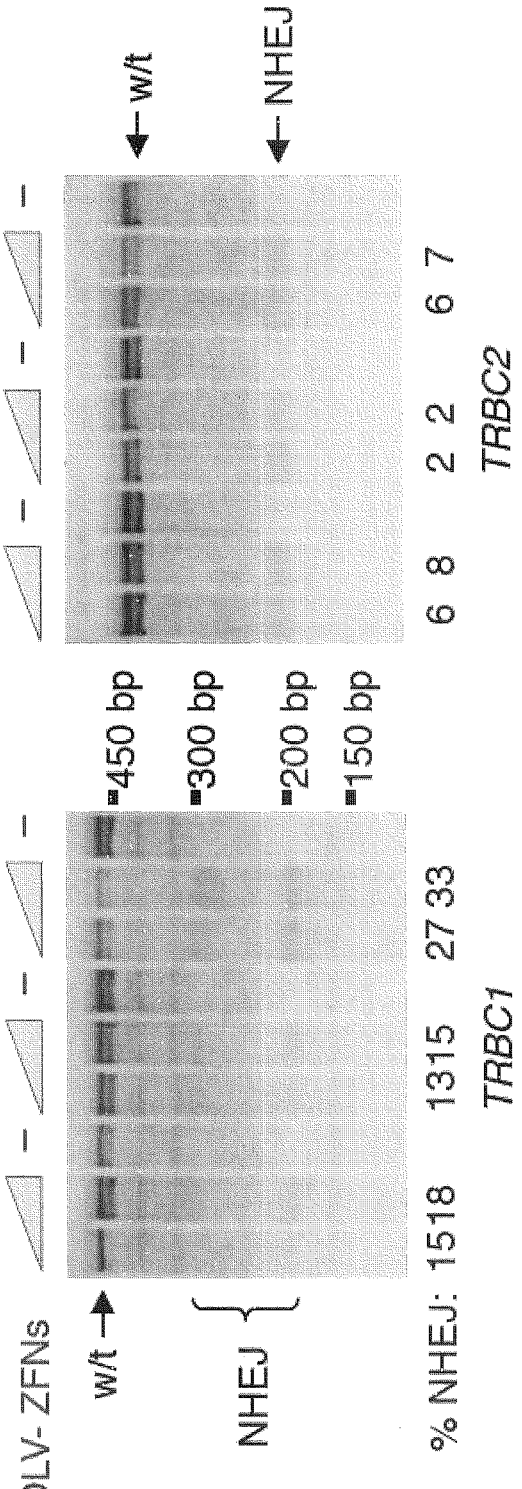

IDLV- ZFNs
w/t
NHEJ
% NHEJ: 15 18
Bulk
CD3 sorted
+
-
13 15
27 33
TRBC1
A
450 bp
300 bp
200 bp
150 bp
Bulk
CD3 sorted
+
-
6 8
2 2
6 7
TRBC2
B
w/t
NHEJ

Figure 7

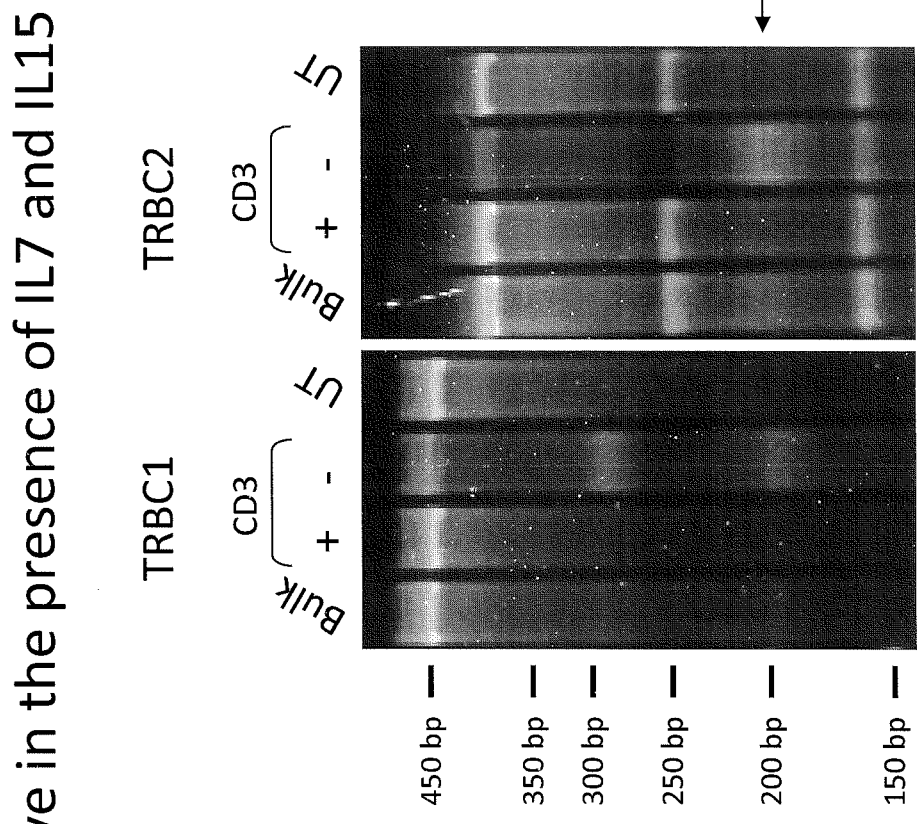


Figure 7D

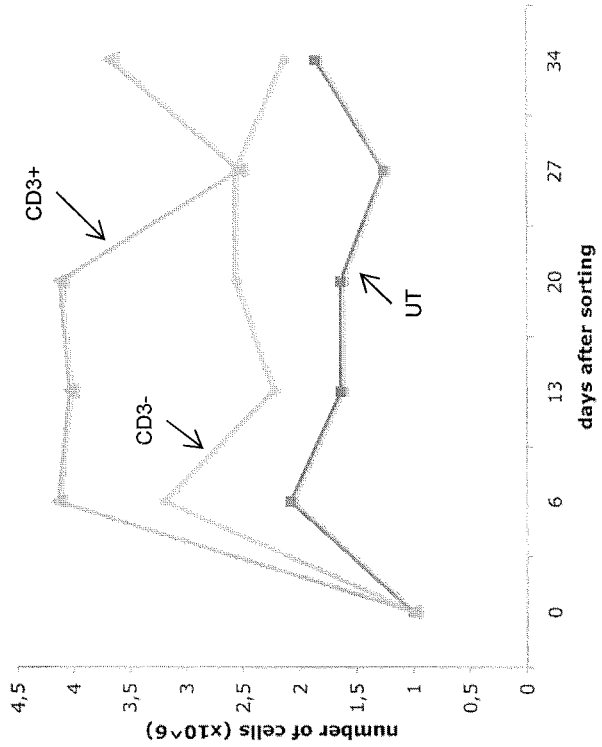


Figure 7C

CD3- cells survive and are stable in culture in the presence of IL7 and IL15 for more than 40 days, do not respond to polyclonal mitogens, maintain a T_{CM} phenotype

Figure 7E

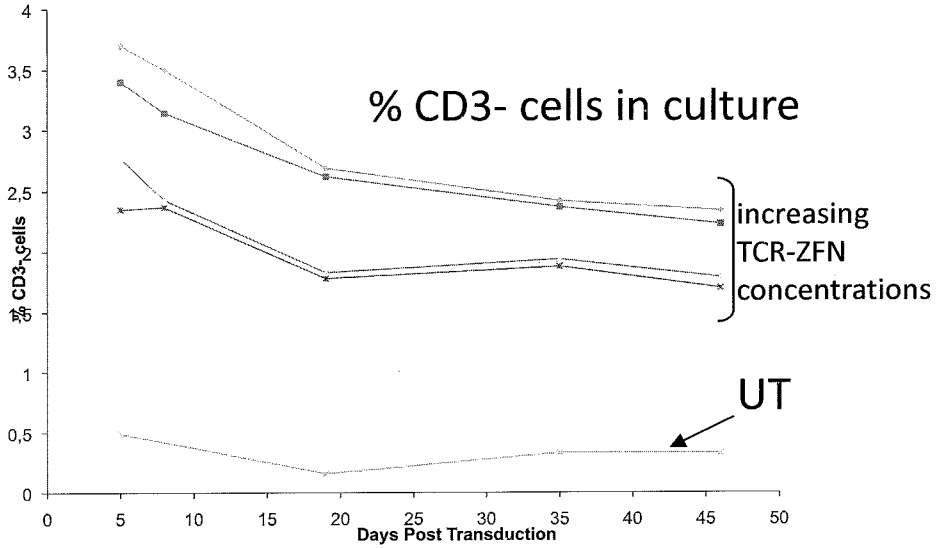


Figure 7F

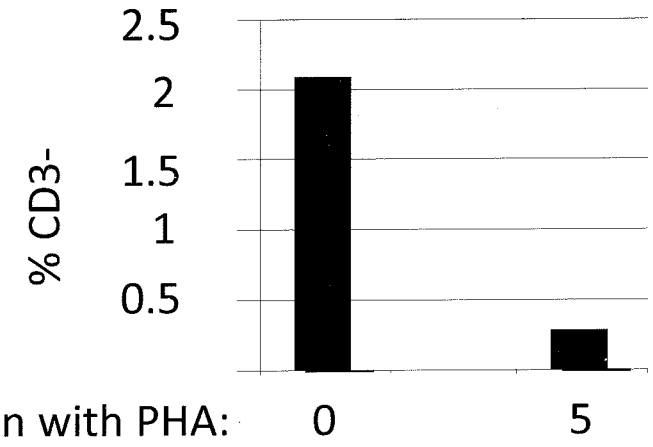
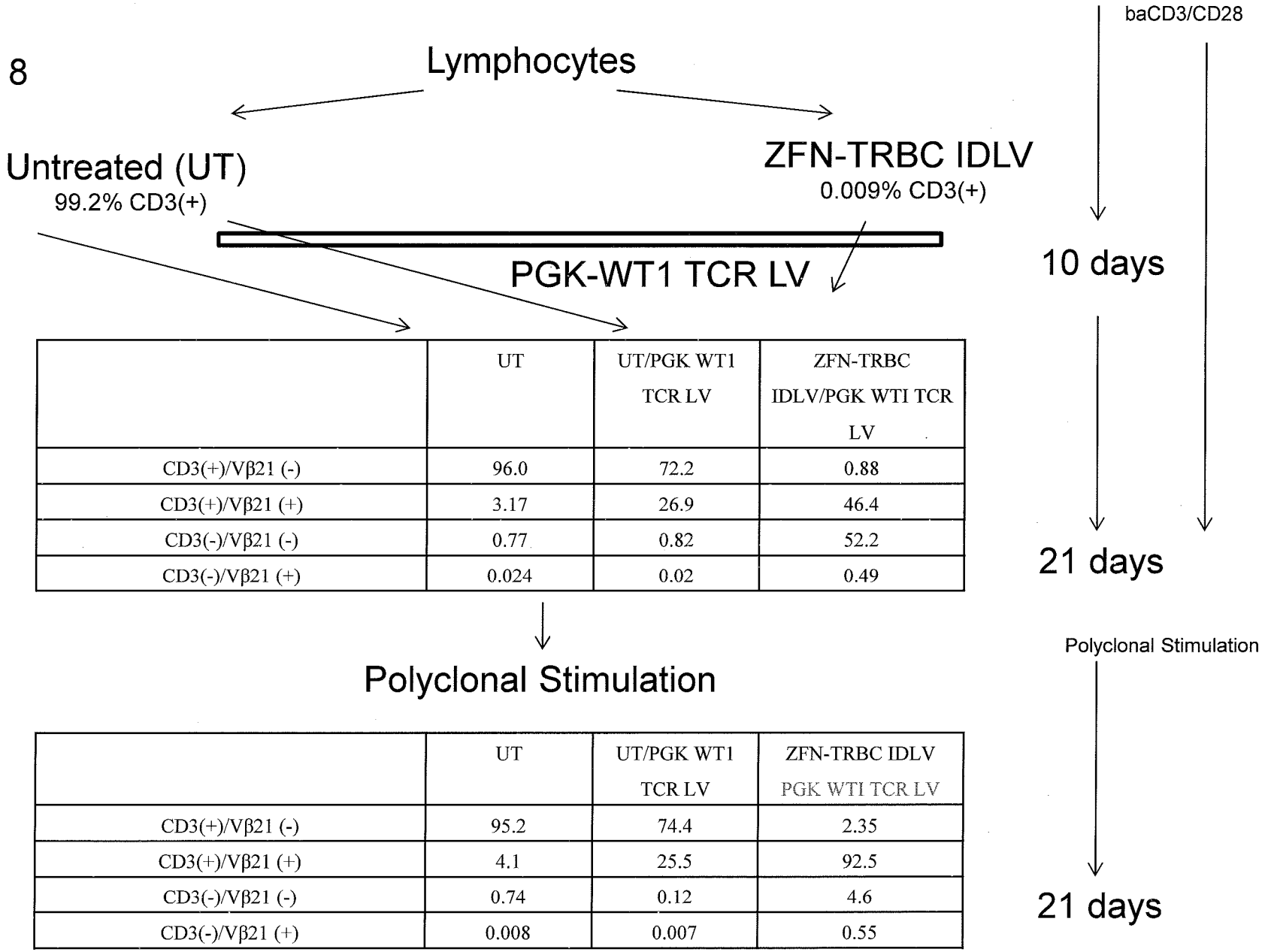


Figure 8



	UT	UT/PGK WT1 TCR LV	ZFN-TRBC IDLV/PGK WTI TCR LV
CD3(+)/Vβ21 (-)	96.0	72.2	0.88
CD3(+)/Vβ21 (+)	3.17	26.9	46.4
CD3(-)/Vβ21 (-)	0.77	0.82	52.2
CD3(-)/Vβ21 (+)	0.024	0.02	0.49

	UT	UT/PGK WT1 TCR LV	ZFN-TRBC IDLV PGK WTI TCR LV
CD3(+)/Vβ21 (-)	95.2	74.4	2.35
CD3(+)/Vβ21 (+)	4.1	25.5	92.5
CD3(-)/Vβ21 (-)	0.74	0.12	4.6
CD3(-)/Vβ21 (+)	0.008	0.007	0.55

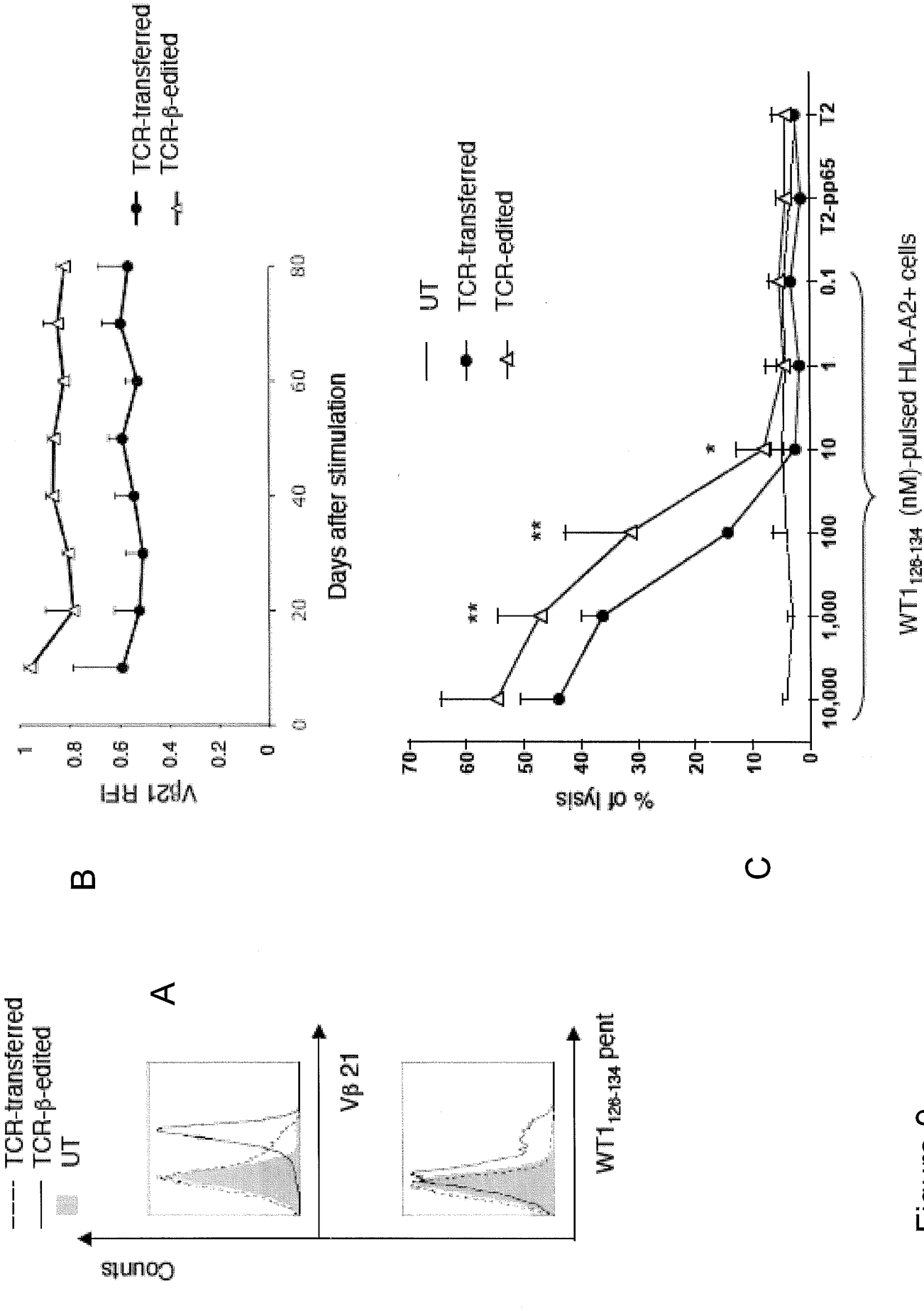
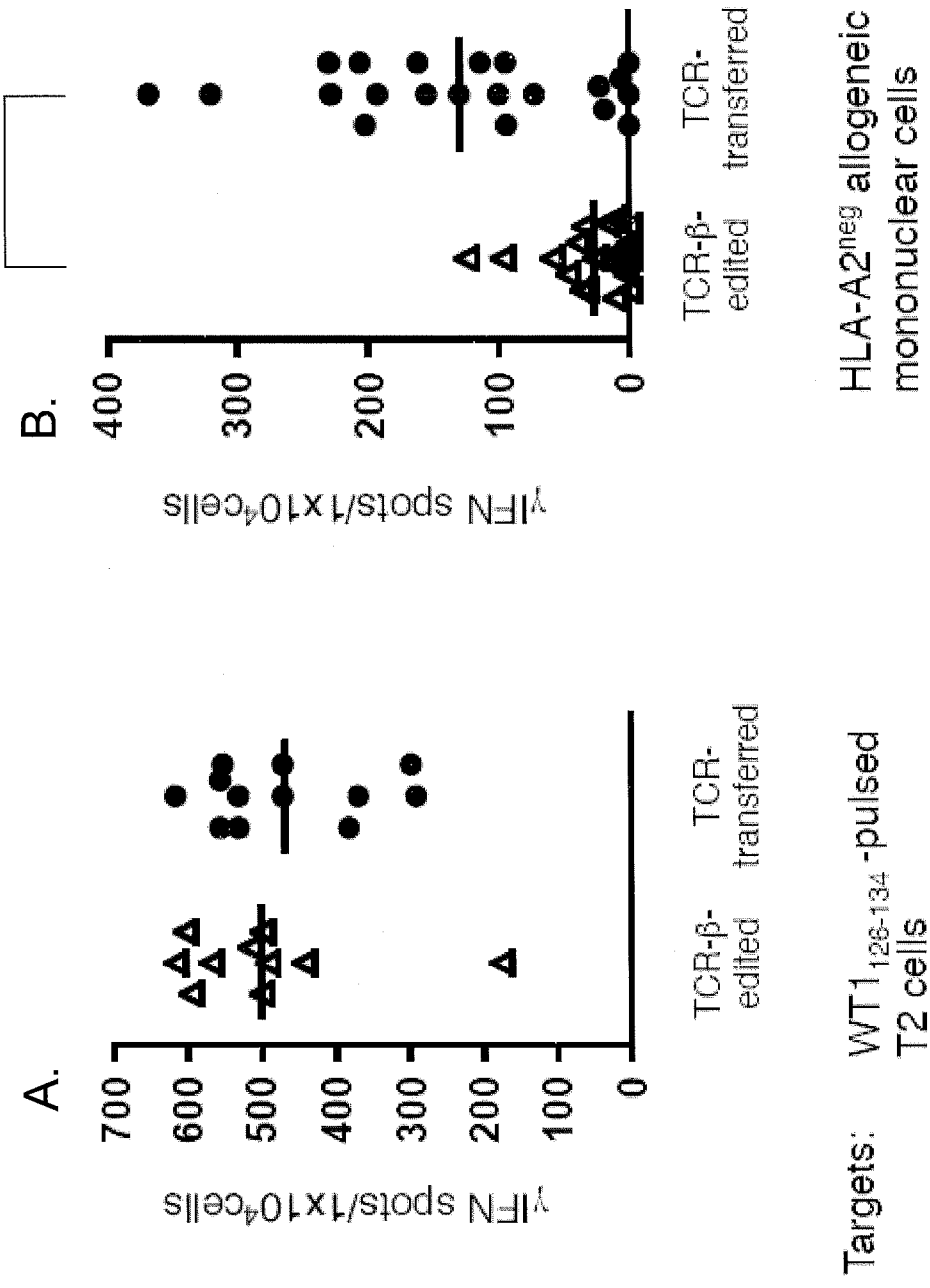
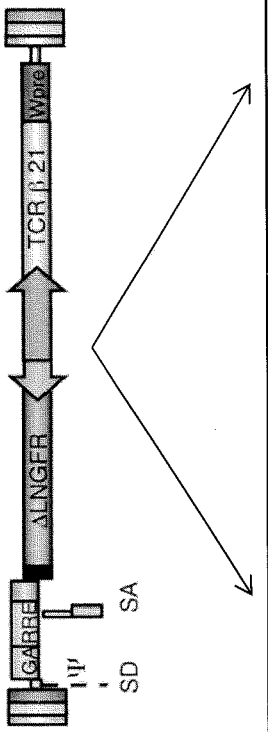

A
TCR-transferred
TCR-β-edited
UT
Counts
Vβ 21
WT1 126-134 pent
B
TCR-transferred
TCR-β-edited
Vβ21 RFI
Days after stimulation
C
UT
TCR-transferred
TCR-edited
% of lysis
WT1 126-134 (nM)-pulsed HLA-A2+ cells
T2-pp65
T2

Figure 9

Figure 10


A.
γIFN spots/1x10^4cells
700
600
500
400
300
200
100
0
TCR-β-edited
TCR-transferred
Targets:
WT1 126-134 -pulsed T2 cells
B.
**
γIFN spots/1x10^4cells
400
300
200
100
0
TCR-β-edited
TCR-transferred
HLA-A2neg allogeneic mononuclear cells



Untreated			TRBC edited CD3(-) cells		
Vector concentration					
+	++	+++	+	++	+++
Percent ΔLNGFR					
20.4%	31.2	53.1	15	21.4	23

Selection of ΔLNGFR (+) cells

Untreated			TRBC edited CD3(-) cells		
Vector concentration					
+	++	+++	+	++	+++
Percent CD3(+)/Vβ21(+)					
28.6%	27.2	30	83.3	83.3	74.5

Figure 11

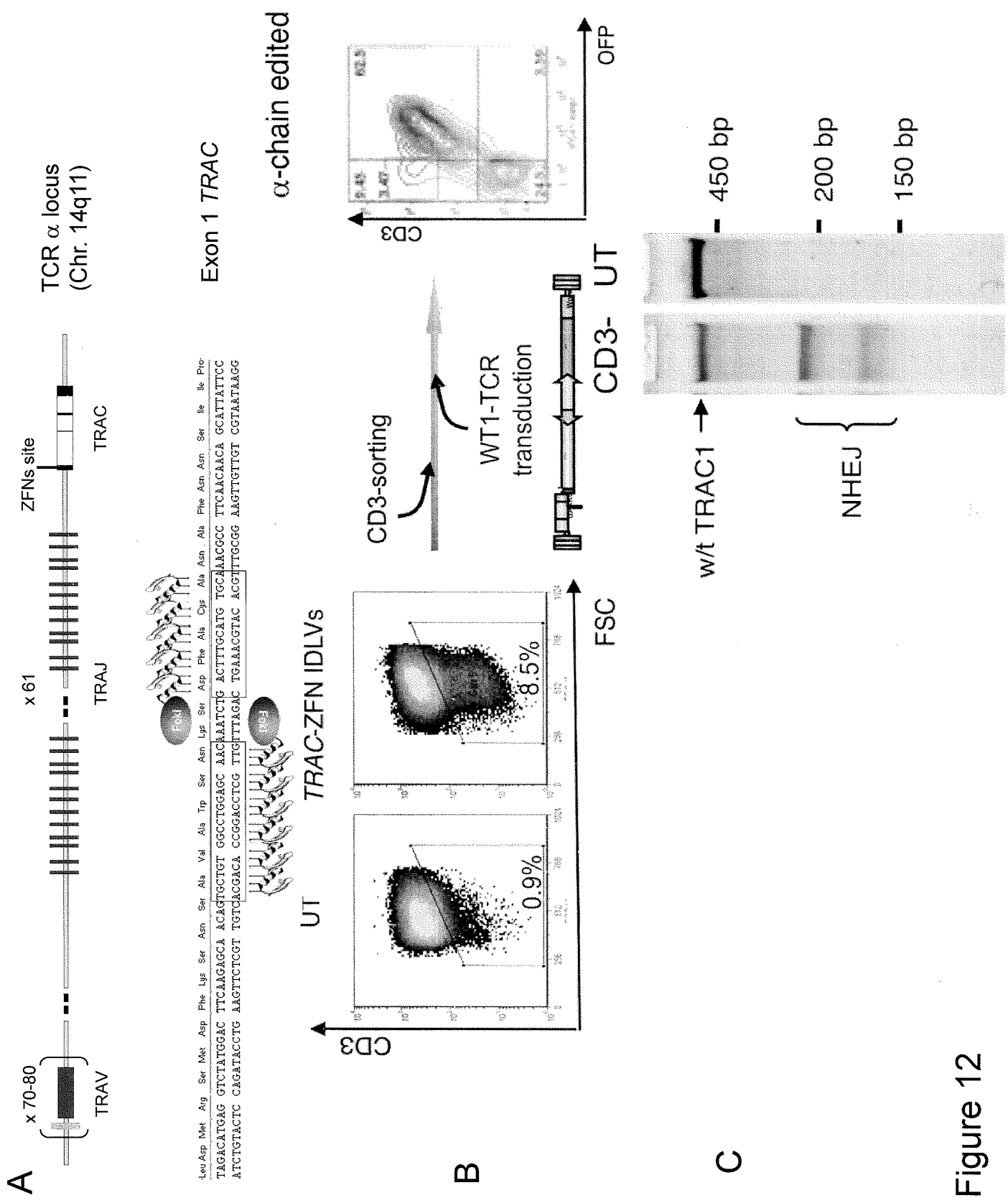

A
TCR α locus
(Chr. 14q11)
ZFNs site
x 61
x 70-80
TRAV
TRAJ
TRAC
Exon 1 TRAC
α-chain edited
B
UT
TRAC-ZFN IDLVs
CD3
FSC
0.9%
8.5%
CD3-sorting
WT1-TCR
transduction
CD3- UT
CD3
OFP
C
w/t TRAC1
NHEJ
450 bp
200 bp
150 bp

Figure 12

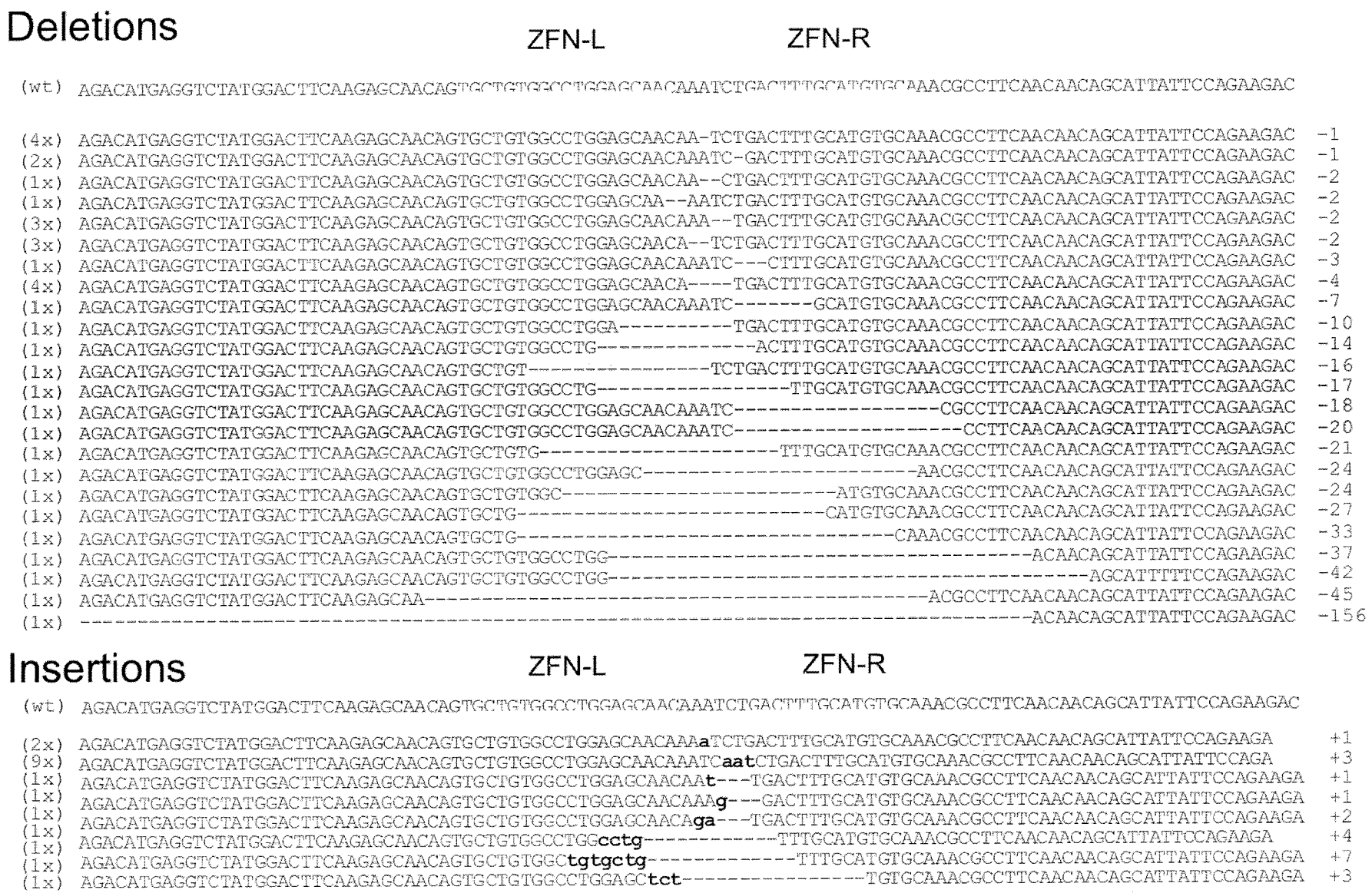

Deletions
ZFN-L
ZFN-R
(wt) AGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCAACAAATCTGACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGAC
(4x) AGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCAACAA-TCTGACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGAC -1
(2x) AGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCAACAAATC-GACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGAC -1
(1x) AGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCAACAA--CTGACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGAC -2
(1x) AGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCAA--AATCTGACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGAC -2
(3x) AGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCAACAAA--TGACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGAC -2
(3x) AGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCAACA--TCTGACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGAC -2
(1x) AGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCAACAAATC---CTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGAC -3
(4x) AGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCAACA----TGACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGAC -4
(1x) AGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCAACAAATC-------GCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGAC -7
(1x) AGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGA----------TGACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGAC -10
(1x) AGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTG----------ACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGAC -14
(1x) AGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGT----------TCTGACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGAC -16
(1x) AGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTG----------TTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGAC -17
(1x) AGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCAACAAATC----------CGCCTTCAACAACAGCATTATTCCAGAAGAC -18
(1x) AGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCAACAAATC----------CCTTCAACAACAGCATTATTCCAGAAGAC -20
(1x) AGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTG----------TTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGAC -21
(1x) AGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGC----------AACGCCTTCAACAACAGCATTATTCCAGAAGAC -24
(1x) AGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGC----------ATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGAC -24
(1x) AGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTG----------CATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGAC -27
(1x) AGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTG----------CAAACGCCTTCAACAACAGCATTATTCCAGAAGAC -33
(1x) AGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGG----------ACAACAGCATTATTCCAGAAGAC -37
(1x) AGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGG----------AGCATTTTTCCAGAAGAC -42
(1x) AGACATGAGGTCTATGGACTTCAAGAGCAA----------ACGCCTTCAACAACAGCATTATTCCAGAAGAC -45
(1x) ----------ACAACAGCATTATTCCAGAAGAC -156
Insertions
ZFN-L
ZFN-R
(wt) AGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCAACAAATCTGACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGAC
(2x) AGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCAACAAAaTCTGACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGA +1
(9x) AGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCAACAAATCaatCTGACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGA +3
(1x) AGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCAACAAt---TGACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGA +1
(1x) AGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCAACAAAg---GACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGA +1
(1x) AGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCAACAga---TGACTTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGA +2
(1x) AGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGG----------TTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGA +4
(1x) AGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGcctg----------TTTGCATGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGA +7
(1x) AGACATGAGGTCTATGGACTTCAAGAGCAACAGTGCTGTGGCCTGGAGCtct----------TGTGCAAACGCCTTCAACAACAGCATTATTCCAGAAGA +3

Figure 13

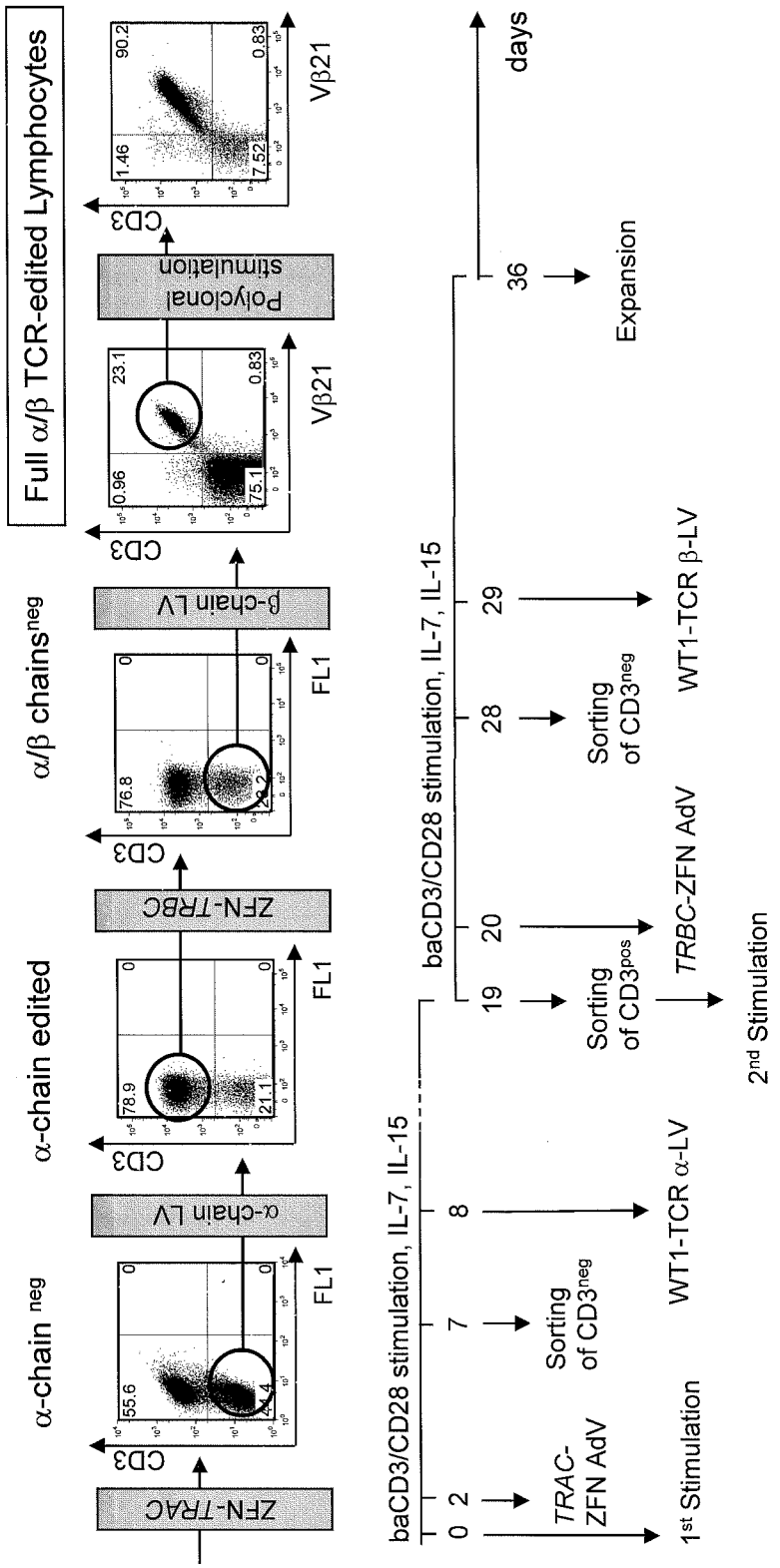

Full α/β TCR-edited Lymphocytes
α-chain neg
α-chain edited
α/β chains neg
ZFN-TRAC
α-chain LV
ZFN-TRBC
β-chain LV
Polyclonal stimulation
CD3
FL1
Vβ21
55.6
21.4
78.9
21.1
76.8
24.2
0.96
75.1
23.1
0.83
1.46
7.52
90.2
0.83
baCD3/CD28 stimulation, IL-7, IL-15
0 2
TRAC-ZFN AdV
1st Stimulation
Sorting of CD3neg
7
8
WT1-TCR α-LV
19
20
Sorting of CD3pos
TRBC-ZFN AdV
2nd Stimulation
baCD3/CD28 stimulation, IL-7, IL-15
28
29
Sorting of CD3neg
WT1-TCR β-LV
36
days
Expansion

Figure 14

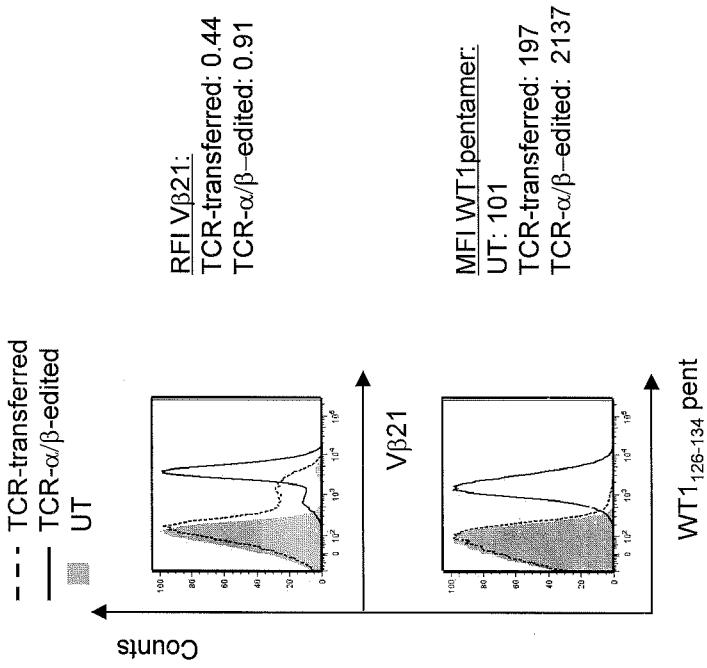

TCR-transferred
TCR-α/β-edited
UT
Counts
RFI Vβ21:
TCR-transferred: 0.44
TCR-α/β–edited: 0.91
Vβ21
MFI WT1pentamer:
UT: 101
TCR-transferred: 197
TCR-α/β–edited: 2137
WT1126-134 pent

Figure 15

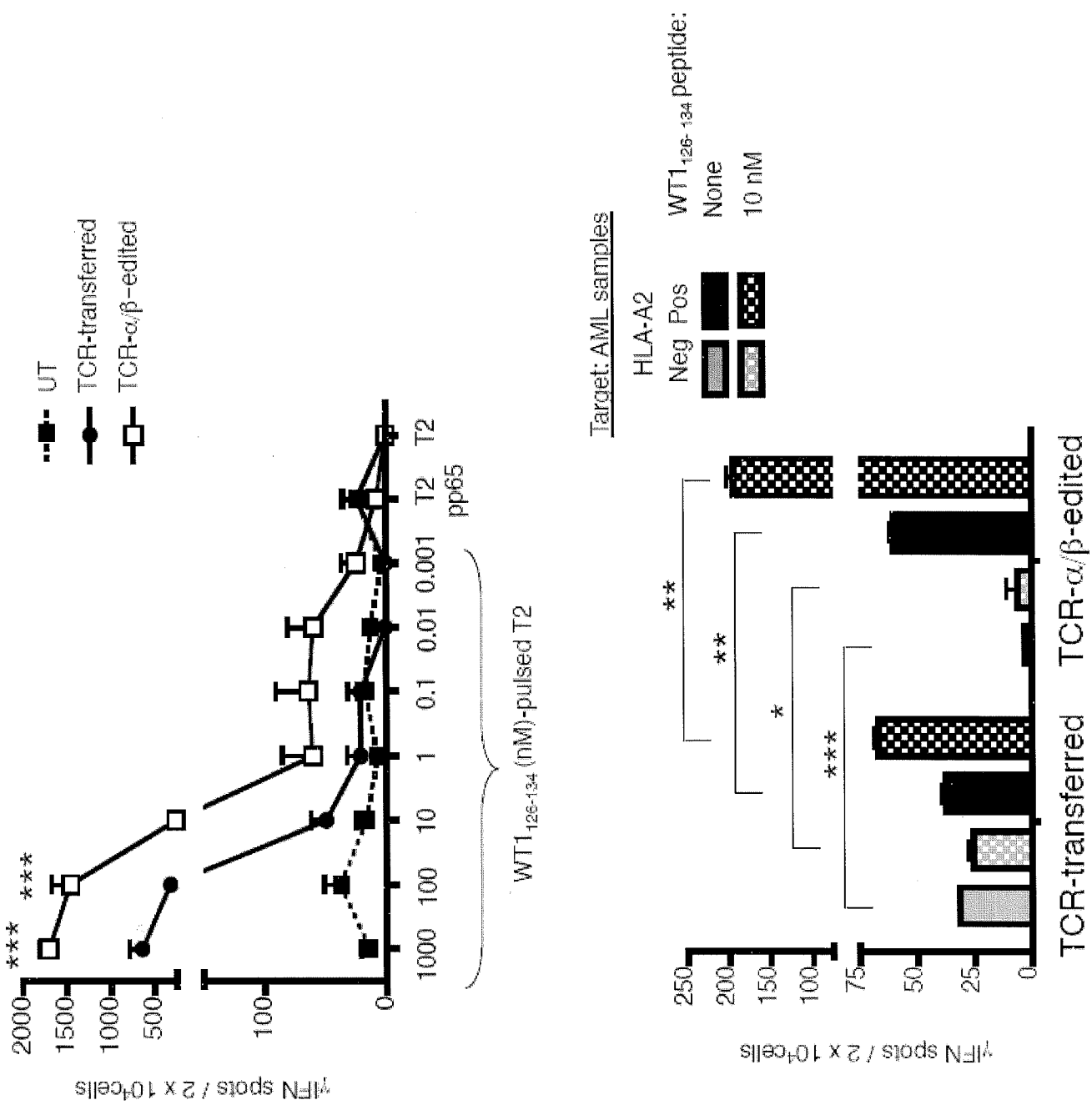

A
UT
TCR-transferred
TCR-α/β-edited
***
***
2000
1500
1000
500
100
0
γIFN spots / 2 x 10^4cells
1000 100 10 1 0.1 0.01 0.001 T2 T2 T2
pp65
WT1126-134 (nM)-pulsed T2
B
Target: AML samples
HLA-A2
Neg Pos
WT1126-134 peptide:
None
10 nM
**
**
*
***
250
200
150
100
75
50
25
0
γIFN spots / 2 x 10^4cells
TCR-transferred
TCR-α/β-edited

Figure 16

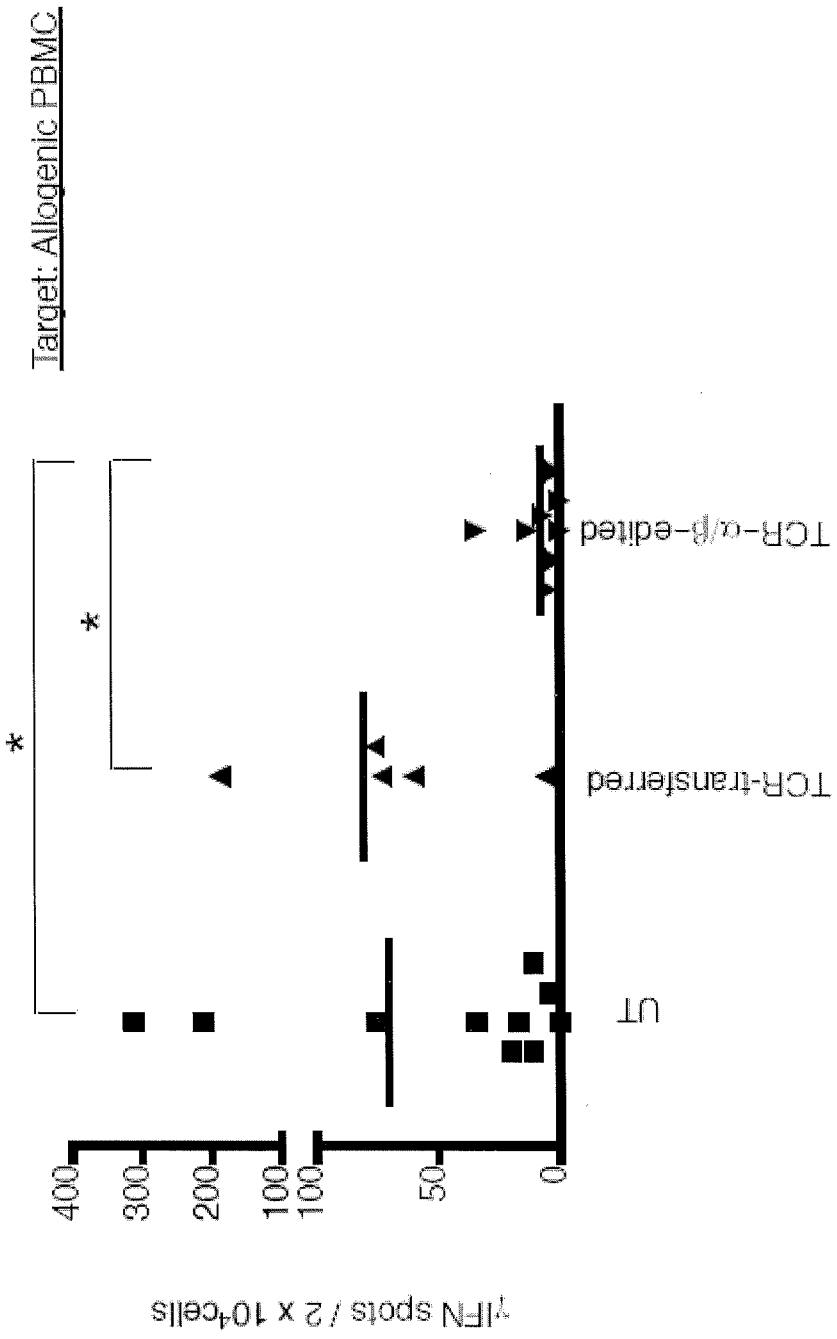
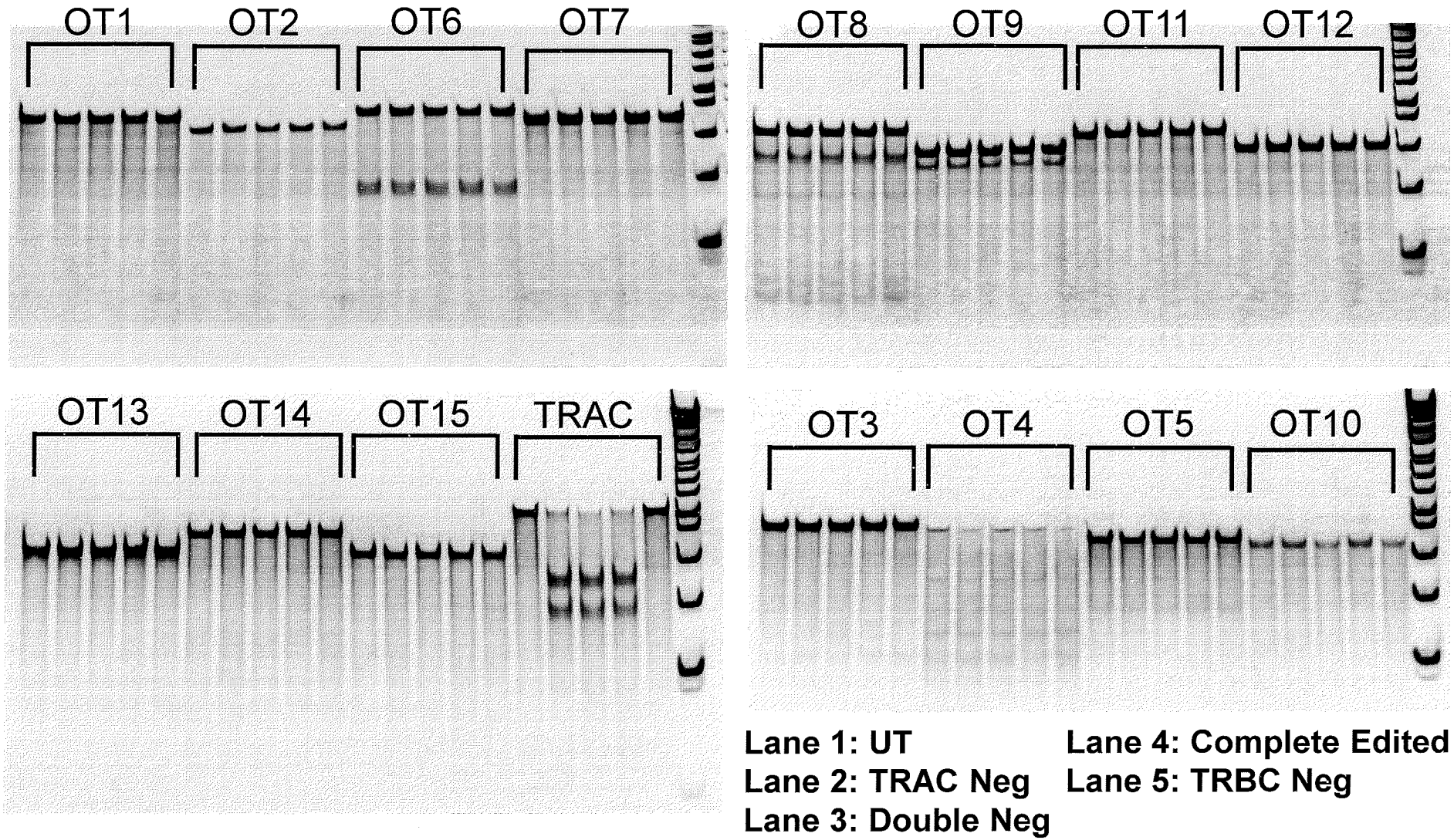

Target: Allogenic PBMC
γIFN spots / 2 x 10⁴cells
400
300
200
100
100
50
0
UT
TCR-transferred
TCR-α/β-edited
*
*

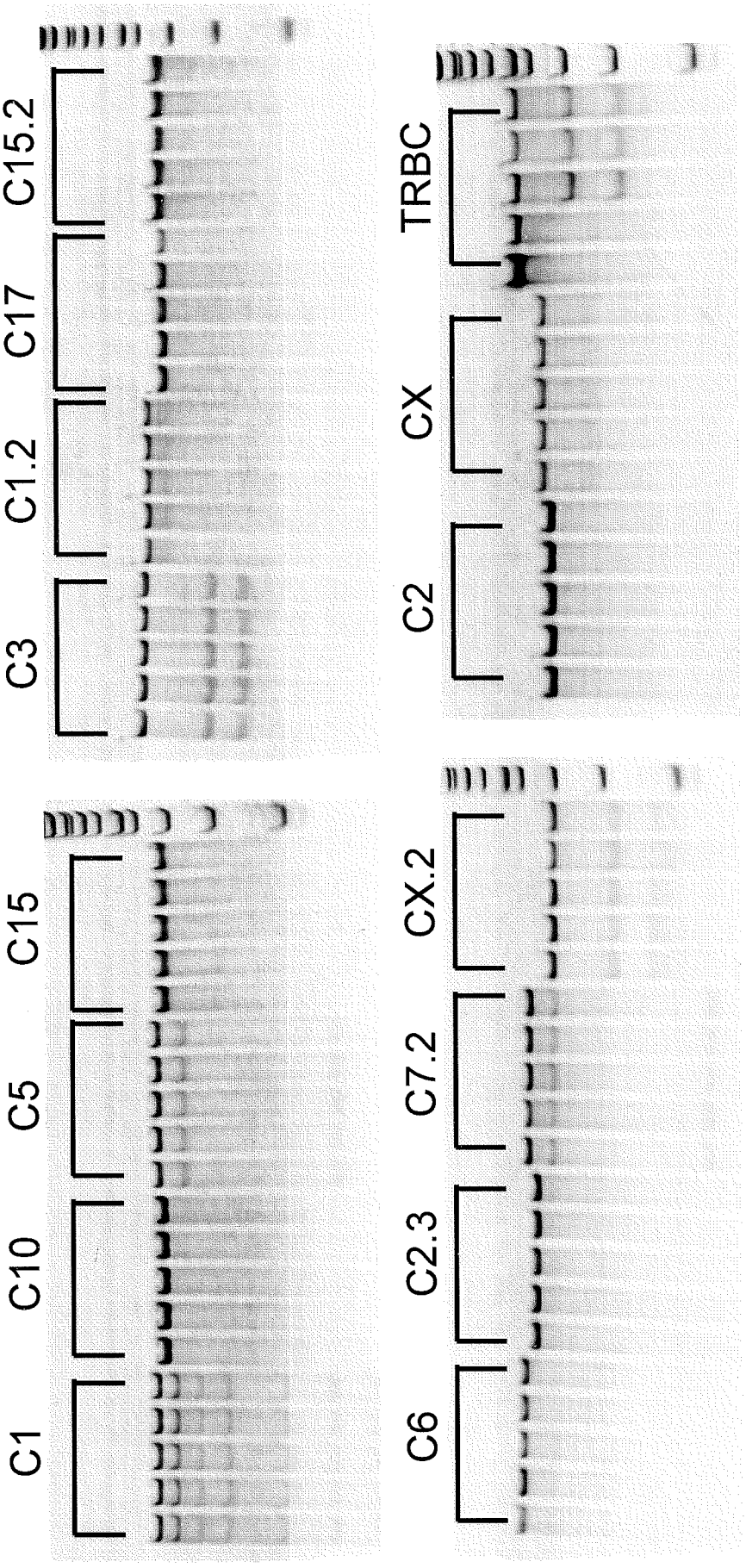
Figure 16C

Figure 17: TRAC Off-Target Analysis



Lane 1: UT
Lane 2: TRAC Neg
Lane 3: Double Neg
Lane 4: Complete Edited
Lane 5: TRBC Neg

Figure 18: TRBC Off-Target Analysis



Lane 1: UT
Lane 2: TRAC Neg
Lane 3: Double Neg
Lane 4: Complete Edited
Lane 5: TRBC Neg

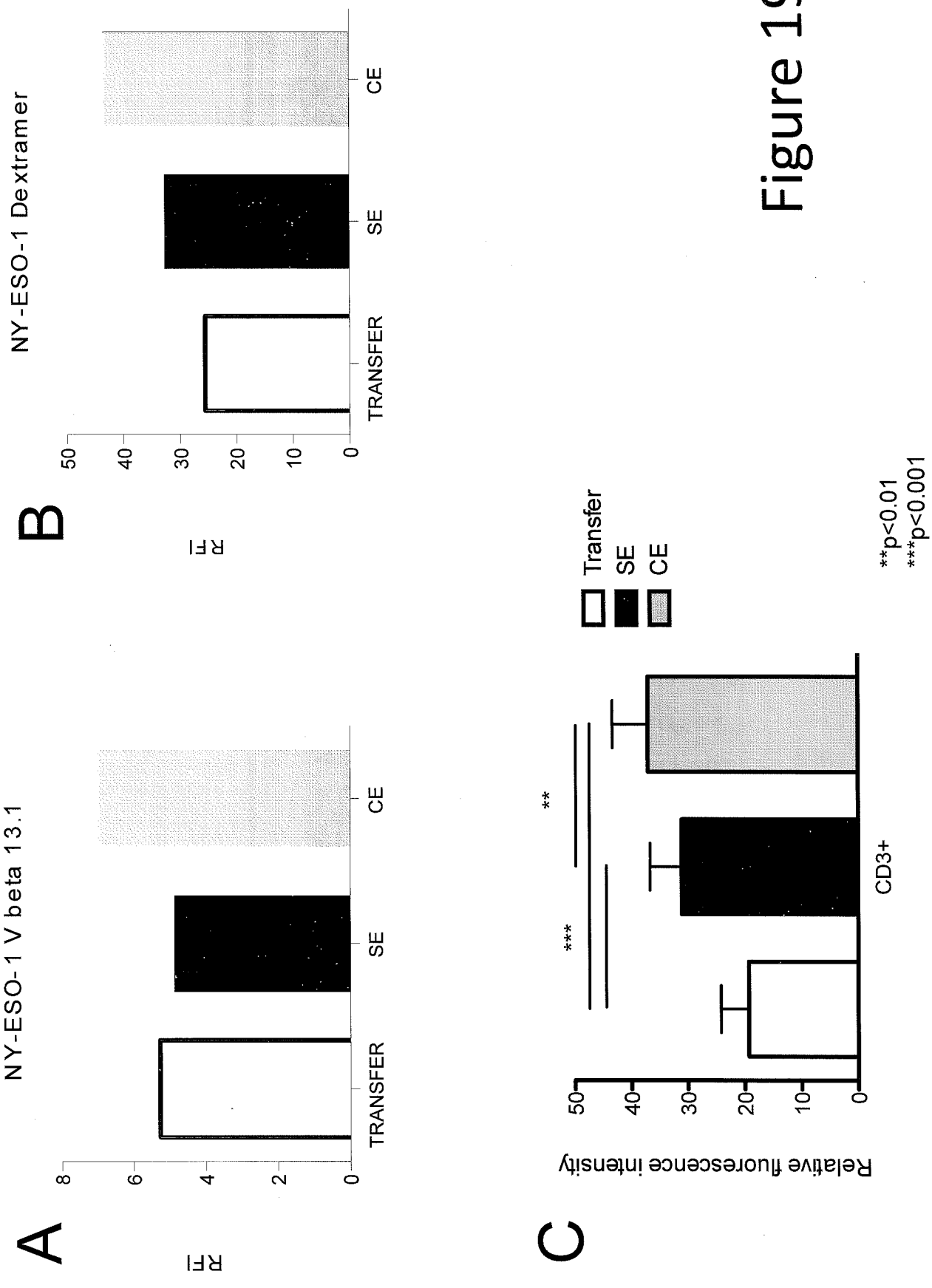

A
NY-ESO-1 V beta 13.1
RFI
0
2
4
6
8
TRANSFER
SE
CE
B
NY-ESO-1 Dextramer
RFI
0
10
20
30
40
50
TRANSFER
SE
CE
C
Relative fluorescence intensity
0
10
20
30
40
50
***
**
CD3+
Transfer
SE
CE
**p<0.01
***p<0.001

Figure 19

Figure 20

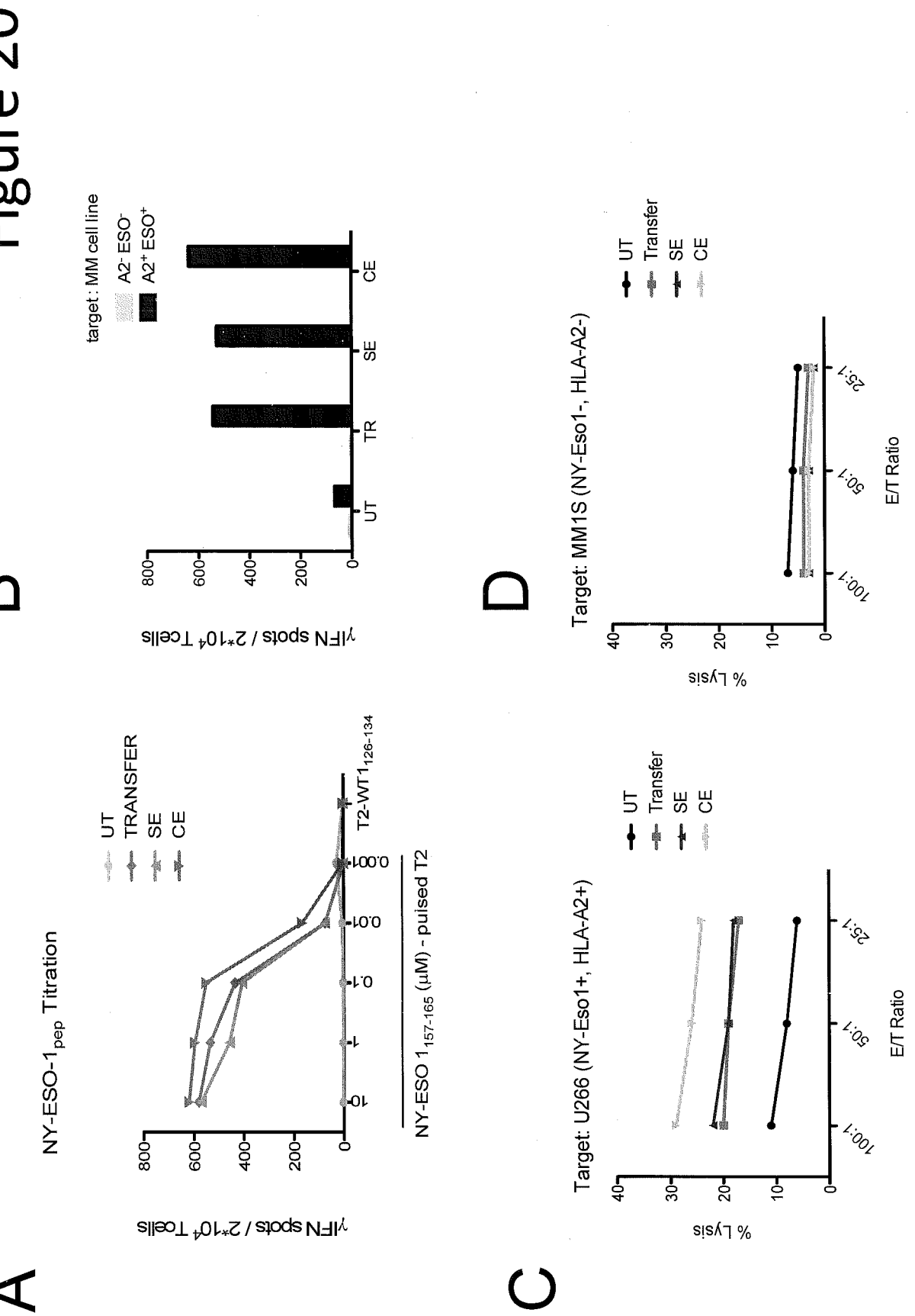
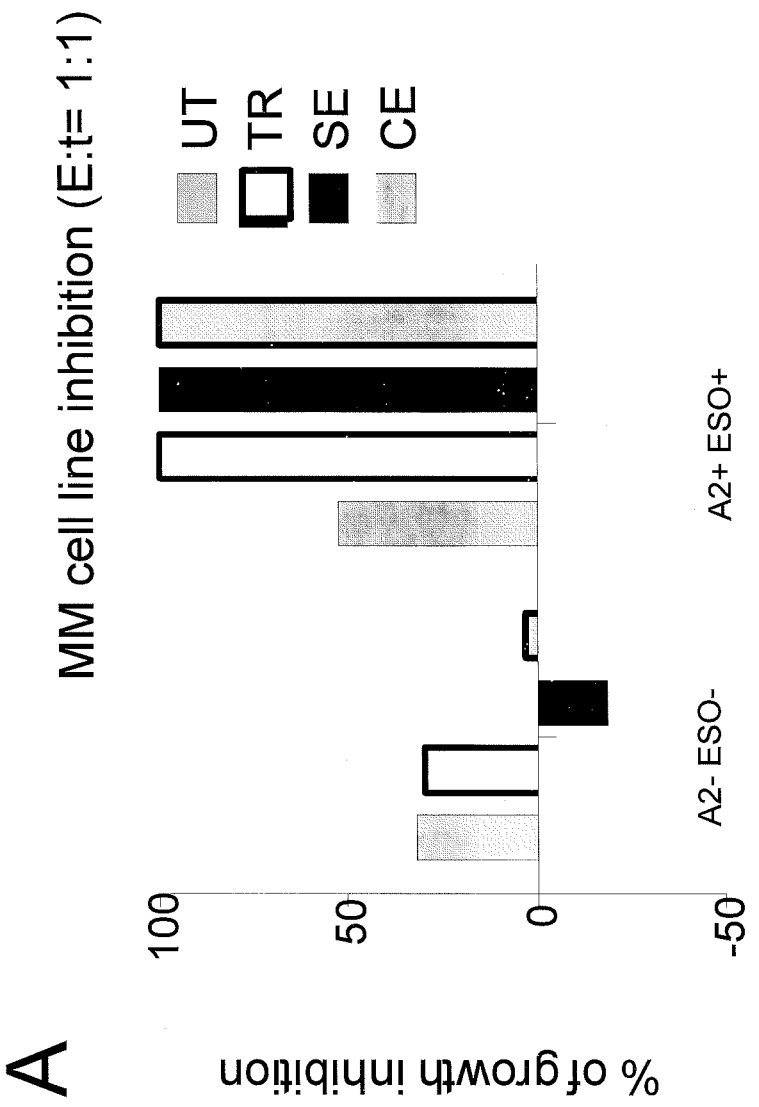

A
NY-ESO-1pep Titration
UT
TRANSFER
SE
CE
γIFN spots / 2*10⁴ Tcells
800
600
400
200
0
10
1
0.1
0.01
0.001
T2-WT1126-134
NY-ESO 1157-165 (μM) - puised T2
B
target : MM cell line
A2- ESO-
A2+ ESO+
γIFN spots / 2*10⁴ Tcells
UT
TR
SE
CE
C
Target: U266 (NY-Eso1+, HLA-A2+)
UT
Transfer
SE
CE
% Lysis
40
30
20
10
0
100:1
50:1
25:1
E/T Ratio
D
Target: MM1S (NY-Eso1-, HLA-A2-)
UT
Transfer
SE
CE
% Lysis
E/T Ratio

Figure 21

A



B

	Fold Increase of T cells in co-culture with myeloma cells			
	UT	TR	SE	CE
HLA-A2$^-$, NY-ESO$^-$	0.99	0.80	0.49	0.68
HLA-A2$^+$, NY-ESO$^+$	0.38	1.96	2.04	1.97

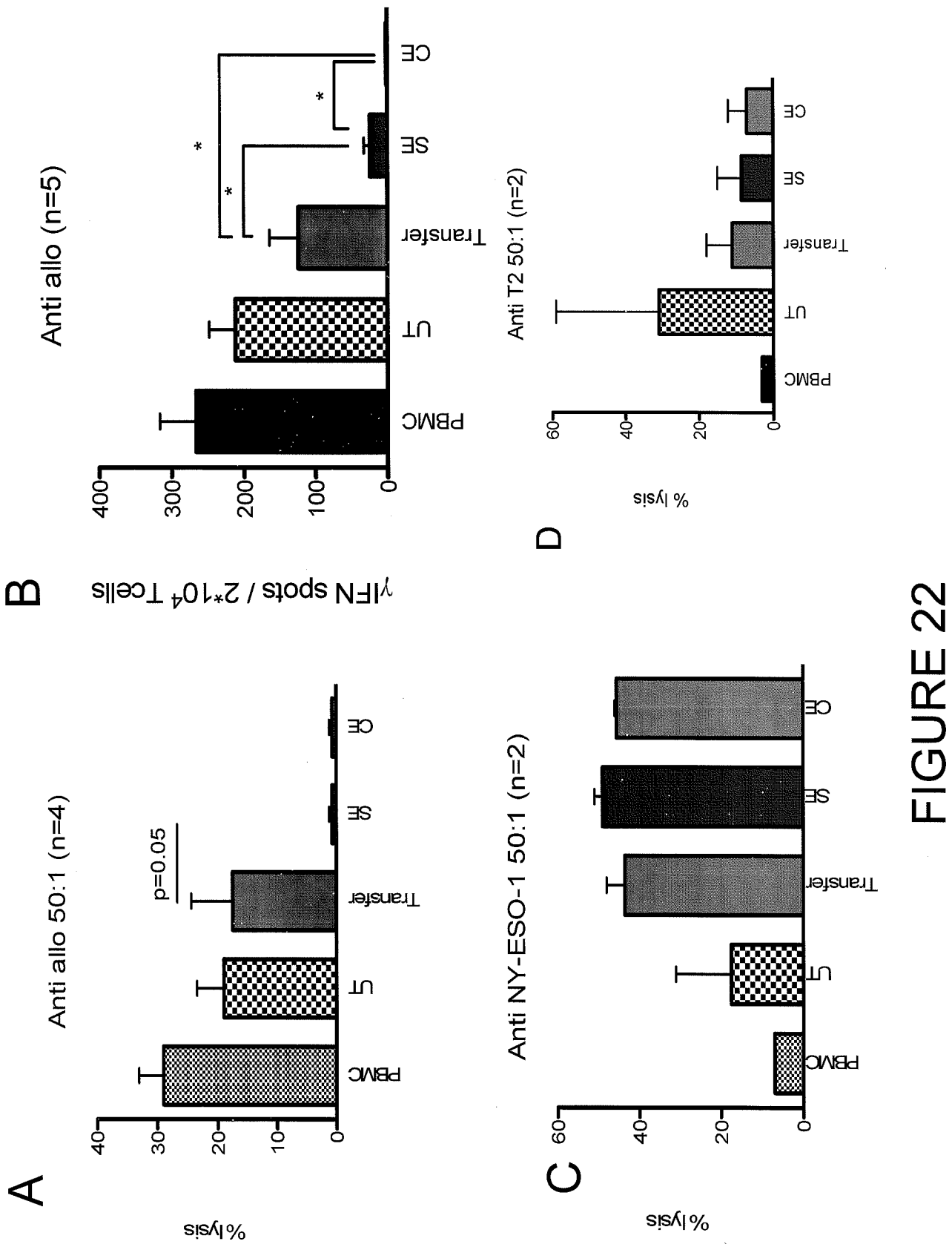

A
Anti allo 50:1 (n=4)
% lysis
0
10
20
30
40
PBMC
UT
Transfer
SE
CE
p=0.05
B
Anti allo (n=5)
γIFN spots / 2*10⁴ Tcells
0
100
200
300
400
PBMC
UT
Transfer
SE
CE
*
*
*
C
Anti NY-ESO-1 50:1 (n=2)
% lysis
0
20
40
60
PBMC
UT
Transfer
SE
CE
D
Anti T2 50:1 (n=2)
% lysis
0
20
40
60
PBMC
UT
Transfer
SE
CE

FIGURE 22

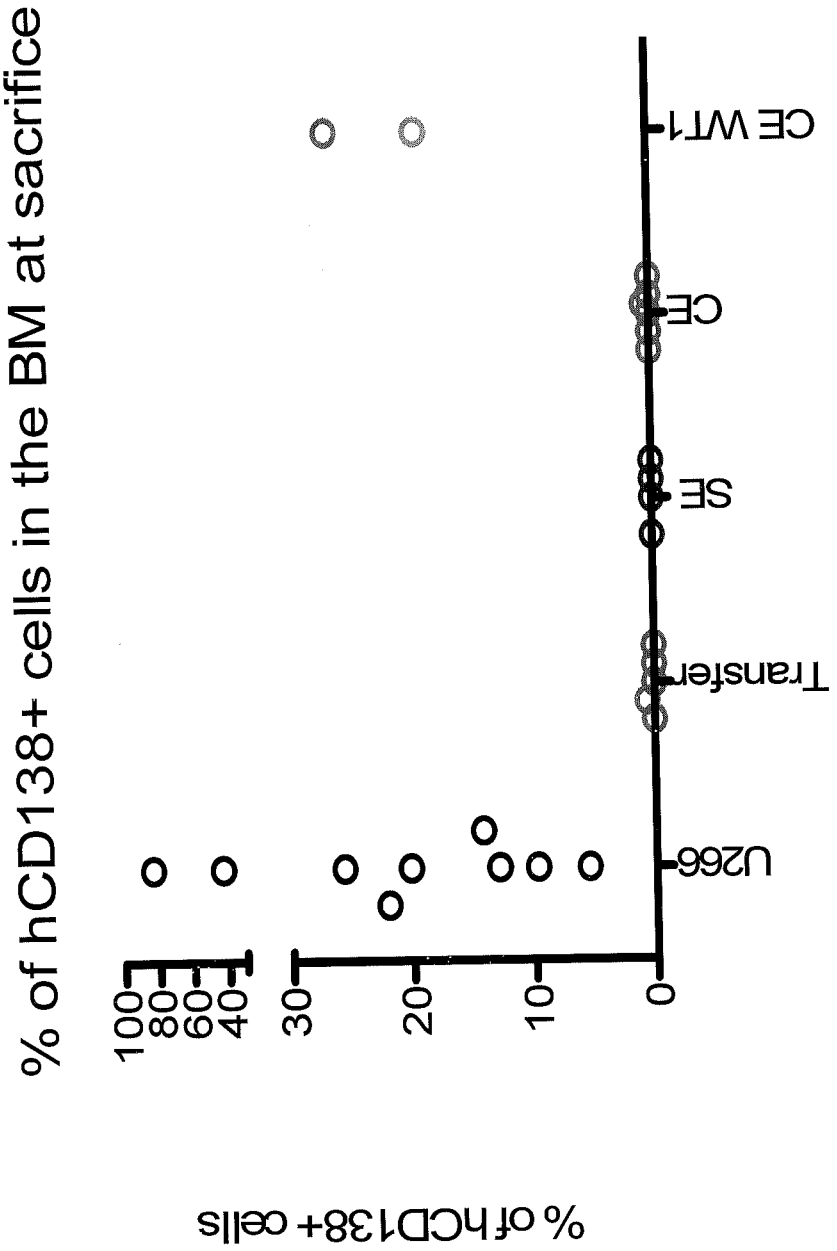

% of hCD138+ cells in the BM at sacrifice
100
80
60
40
30
20
10
0
U266
Transfer
SE
CE
CE WT1
% of hCD138+ cells

FIGURE 23A

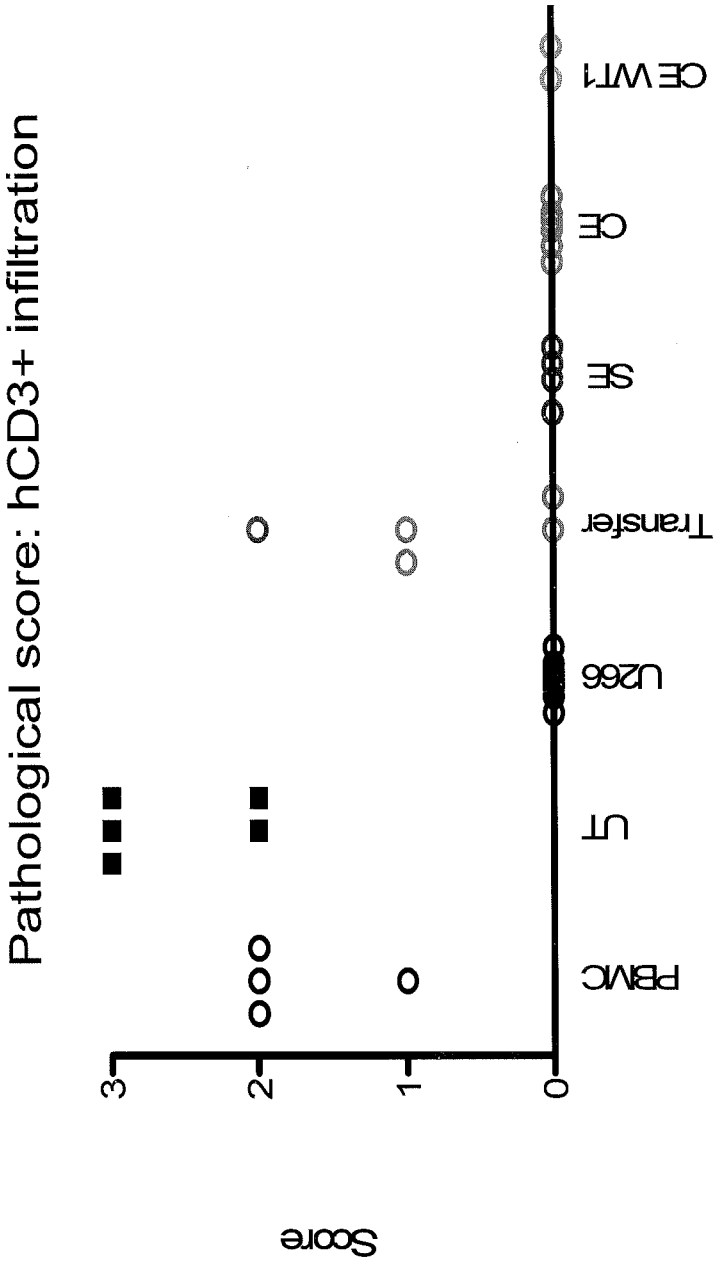

Pathological score: hCD3+ infiltration
Score
0
1
2
3
PBMC
UT
U266
Transfer
SE
CE
CE WT1

FIGURE 23B

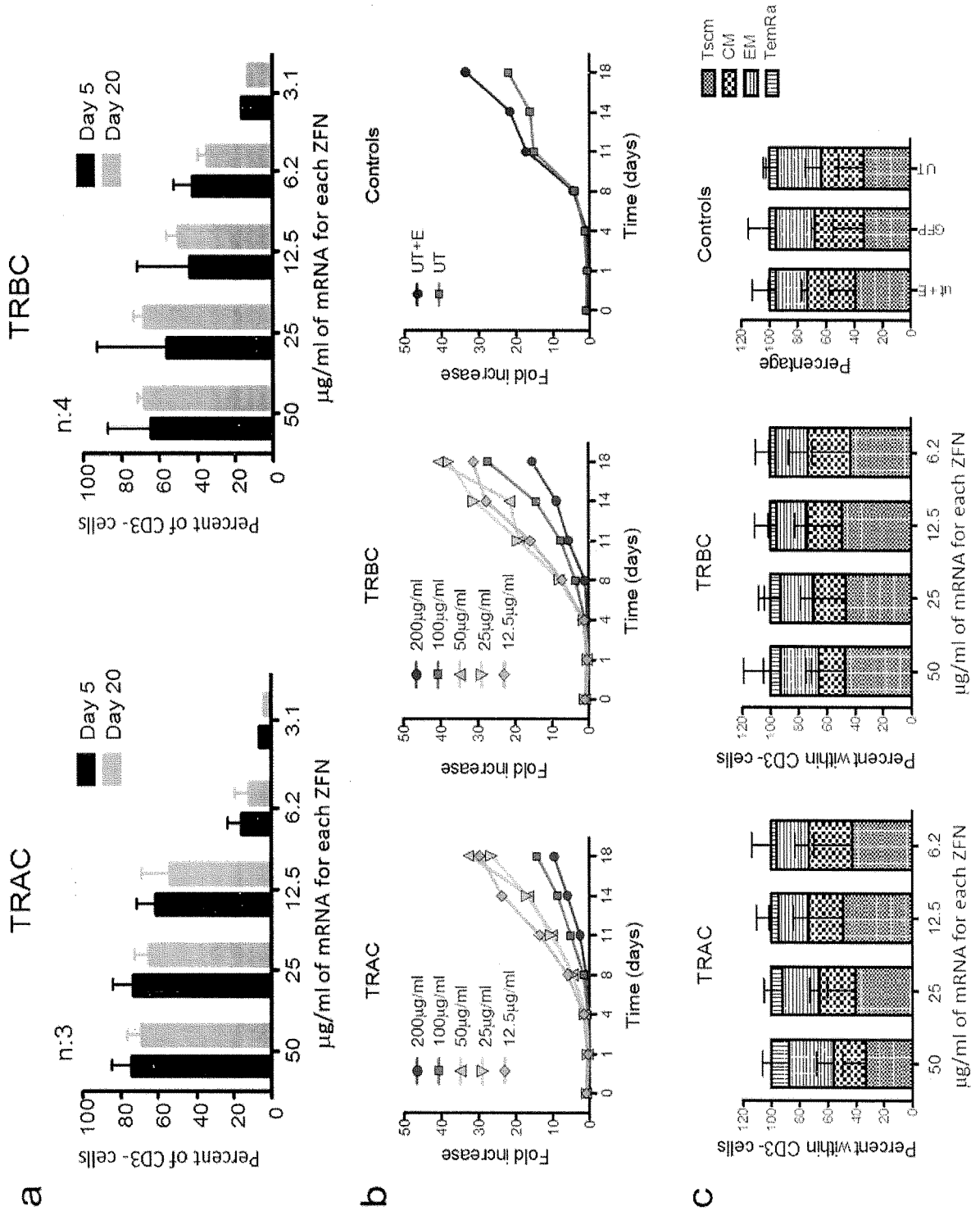
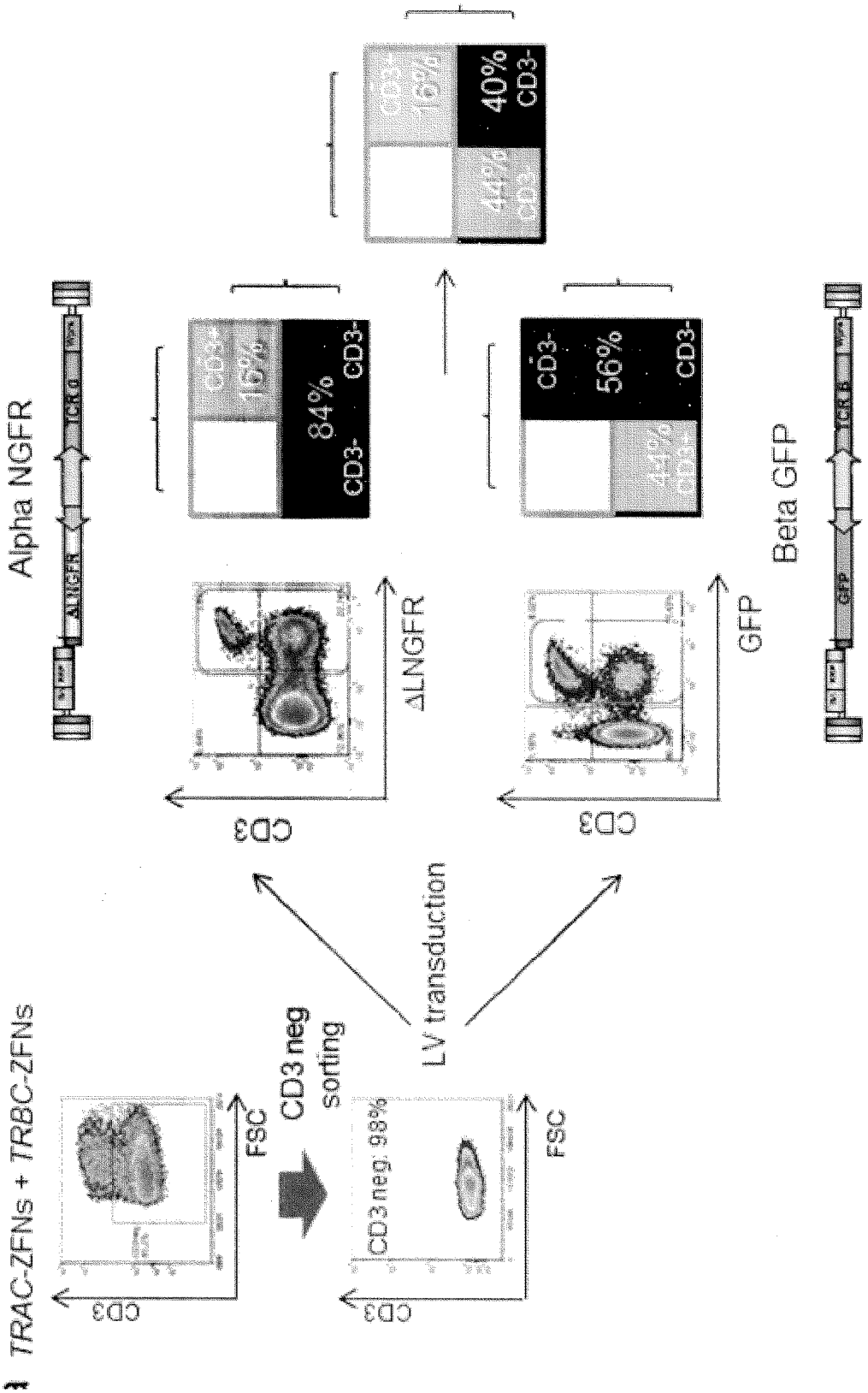

a
TRAC
n:3
Day 5
Day 20
Percent of CD3- cells
µg/ml of mRNA for each ZFN
TRBC
n:4
b
TRAC
TRBC
200µg/ml
100µg/ml
50µg/ml
25µg/ml
12.5µg/ml
Fold increase
Time (days)
Controls
UT+E
UT
c
Tscm
CM
EM
TemRa
Percentage
Percent within CD3- cells

FIGURE 24

FIGURE 25A


TRAC-ZFNs + TRBC-ZFNs
CD3
FSC
CD3 neg sorting
CD3 neg. 98%
LV transduction
Alpha NGFR
ΔLNGFR
TCR α
84%
CD3-
16%
CD3+
40%
CD3-
44%
CD3+
Beta GFP
GFP
TCR β
56%
CD3-
44%
CD3+

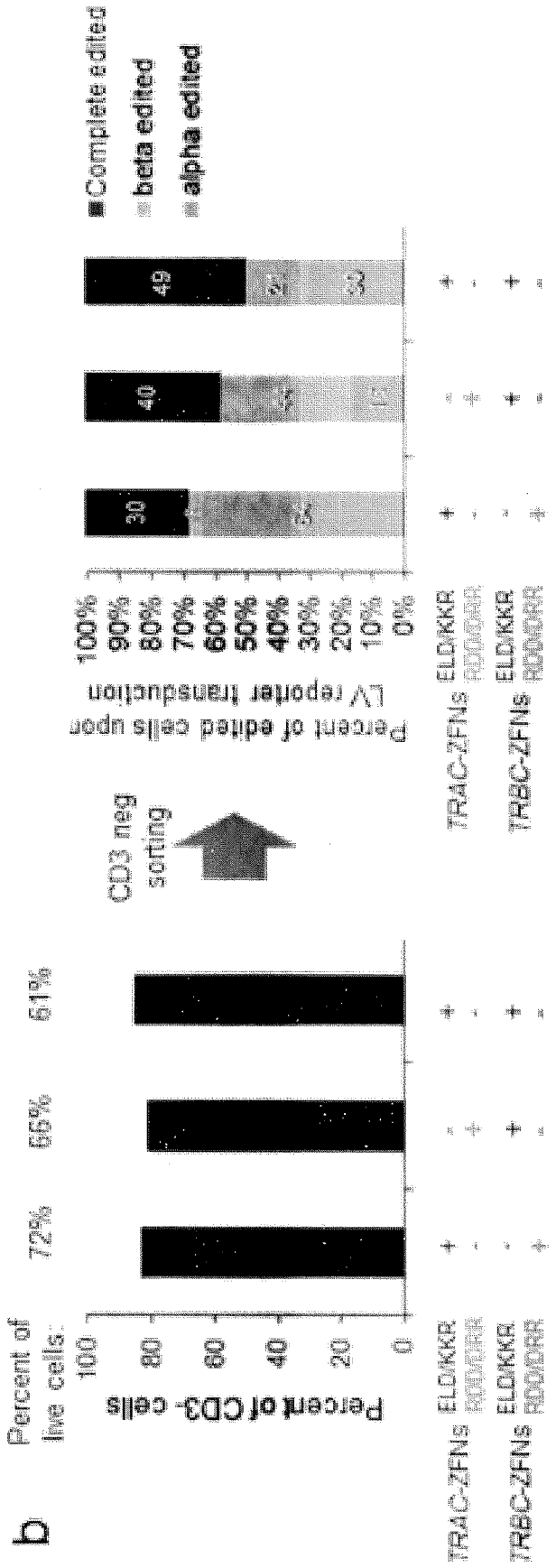

b
Percent of live cells:
72%
66%
61%
Percent of CD3- cells
100
80
60
40
20
0
ELD/KKR
ELD/KKR
TRAC-ZFNs
TRBC-ZFNs
CD3 neg sorting
Percent of edited cells upon LV reporter transduction
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
49
40
30
Complete edited
beta edited
alpha edited

FIGURE 25B

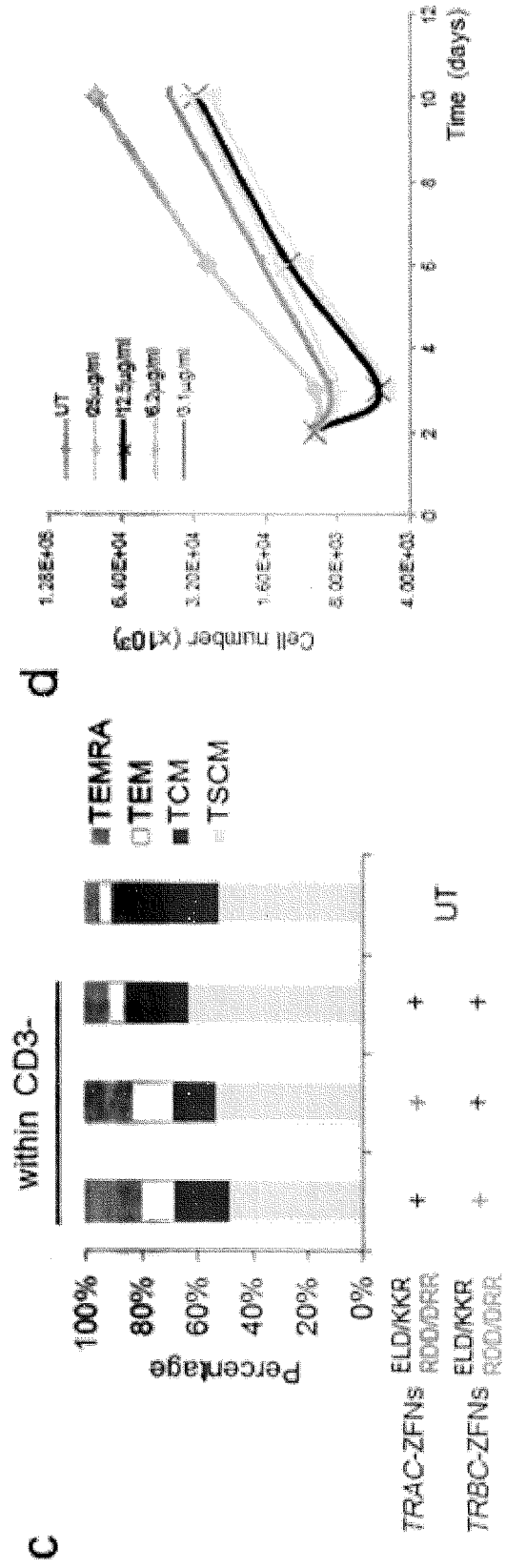

c
within CD3-
Percentage
100%
80%
60%
40%
20%
0%
TEMRA
TEM
TCM
TSCM
TRAC-ZFNs
TRBC-ZFNs
ELD/KKR
ROD/DRR
UT
d
Cell number (x10³)
1.28E+05
6.40E+04
3.20E+04
1.60E+04
8.00E+03
4.00E+03
UT
Time (days)
0
2
4
6
8
10
12

FIGURE 25

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
25 September 2014 (25.09.2014)

WIPO | PCT

(10) International Publication Number
WO 2014/153470 A3

(51) International Patent Classification:
A01N 63/00 (2006.01) *C12N 15/00* (2006.01)
C12N 9/16 (2006.01) *C07H 21/04* (2006.01)

(21) International Application Number:
PCT/US2014/031360

(22) International Filing Date:
20 March 2014 (20.03.2014)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/804,076 21 March 2013 (21.03.2013) US

(71) Applicants: **SANGAMO BIOSCIENCES, INC.** [US/US]; 501 Canal Blvd., Suite A100, Richmond, California 94804 (US). **OSPEDALE SAN RAFFAELE SRL** [IT/IT]; Via Olgettina 60, I-20132 Milan (IT).

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(74) Agent: **PASTERNAK, Dahna S.**; Pasternak Patent Law, 1900 Embarcadero Rd., Suite 211, Palo Alto, California 94303 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

(88) Date of publication of the international search report:
4 December 2014

(54) Title: TARGETED DISRUPTION OF T CELL RECEPTOR GENES USING ENGINEERED ZINC FINGER PROTEIN NUCLEASES

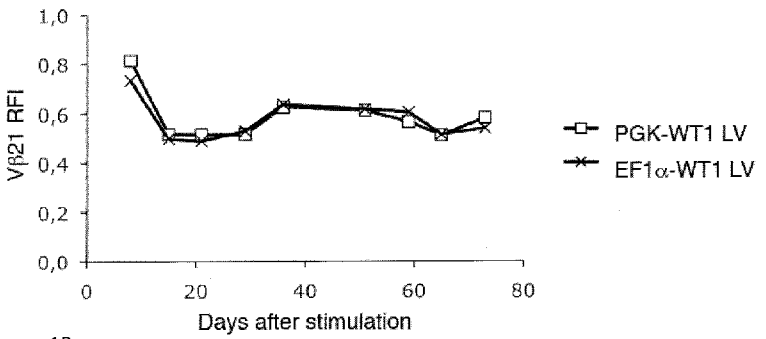

Figure 1B

(57) Abstract: Disclosed herein are methods and compositions for modifying TCR genes, using nucleases (zinc finger nucleases or TAL nucleases) to modify TCR genes.

WO 2014/153470 A3

INTERNATIONAL SEARCH REPORT

14/031360 25.09 2014

International application No.
PCT/US 14/31360

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - A01N 63/00, C12N 9/16, C12N 15/00, C07H 21/04 (2014.01)
CPC - A61K 35/12, C12N 9/16, C12N 15/86

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)
IPC(8) - A01N 63/00, C12N 9/16, C12N 15/00, C07H 21/04 (2014.01)
CPC - A61K 35/12, C12N 9/16, C12N 15/86

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
USPC - 424/93.7; 435/196, 435/455, 435/320.1, 536/23.2, 435/325, 435/375
(keyword limited; terms below)

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
PubWEST; PatBase; Google Scholar
search terms - T-cell receptor, TCR, truncated, transcription-activator like effector nucleases, TALEN, TAL-effector, c-cap, C-terminal truncation, t, cell, lymphocyte, t-cell

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2011/0158957 A1 (BONINI et al.) 30 June 2011 (30.06.2011) para [0014]; [0015]; [0018]; [0022]; [0089]; [0101]; [0125]; [0138]; Table 5; SEQ ID NOs: 13, 41.	1-4
Y	WO 2012/138939 A1 (DUCHATEAU et al.) 11 October 2012 (11.10.2012) abstract; p. 14, para 2-4.	1-4

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
21 August 2014 (21.08.2014)	25 SEP 2014
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 14/31360

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 5-12
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

(19)中华人民共和国国家知识产权局

(12)发明专利申请

(10)申请公布号 CN 105208866 A
(43)申请公布日 2015.12.30

(21)申请号 201480020893.4

(22)申请日 2014.03.20

(30)优先权数据
61/804,076 2013.03.21 US

(85)PCT国际申请进入国家阶段日
2015.10.12

(86)PCT国际申请的申请数据
PCT/US2014/031360 2014.03.20

(87)PCT国际申请的公布数据
WO2014/153470 EN 2014.09.25

(71)申请人 桑格摩生物科学股份有限公司
地址 美国加利福尼亚州
申请人 圣拉斐尔医院有限公司

(72)发明人 P·D·格雷戈里 M·C·霍尔姆斯
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P·格诺韦塞 Z·马格纳尼
S·马斯塔格里奥 L·那尔蒂尼

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代理人 杨昀 沈端

(51)Int.Cl.
A01N 63/00(2006.01)
C12N 9/16(2006.01)
C12N 15/00(2006.01)
C07H 21/04(2006.01)

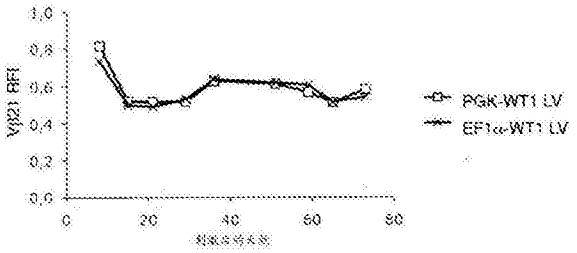
权利要求书1页 说明书55页 附图30页

(54)发明名称

使用工程化锌指蛋白核酸酶靶向断裂T细胞受体基因

(57)摘要

本文公开用于修饰TCR基因的方法和组合物,其使用核酸酶(锌指核酸酶或者TAL核酸酶)来修饰TCR基因。



CN 105208866 A

1. 一种分离的 T 淋巴细胞，其包含稳定整合的、编码 T 细胞受体 (TCR) 的外源序列，其中所述细胞中的至少一个内源 TCR 基因由锌指核酸酶或者 TALEN 部分或者完全地钝化，所述 TALEN 包括 TAL 效应 DNA 结合域和切割域，以及此外其中所述 TAL 效应 DNA 结合域相较于野生型 TAL 效应 DNA 域包含 C 端截断。

2. 如权利要求 1 所述的分离的 T 淋巴细胞，其中所述内源 TCR 基因是 TCRα 基因或者 TCRβ 基因。

3. 如权利要求 1 或权利要求 2 所述的分离的 T 淋巴细胞，其中所述锌指核酸酶包含在表 5 或表 6 的单行中示出的具有识别螺旋区域的锌指蛋白。

4. 如权利要求 1 或权利要求 2 中任一项所述的分离的 T 淋巴细胞，其中所述 TALEN 结合到选自由以下组成的组的靶序列 :SEQ IDNos :144-153。

5. 如权利要求 1 至 4 中任一项所述的分离的 T 淋巴细胞，其中使用整合酶缺陷的慢病毒载体 (IDLV)、AAV、质粒或者 mRNA 将编码所述锌指核酸酶或者 TALEN 的多核苷酸导入所述细胞。

6. 如权利要求 1 至 5 中任一项所述的分离的 T 淋巴细胞，其中将所述外源序列导入内源 TCR 基因、CCR5 基因或者 AAVS1 基因。

7. 如权利要求 1 至 6 中任一项所述的分离的 T 淋巴细胞，其中所述外源序列选自由以下组成的组 :肿瘤抗原特异性 TCR 转基因，其中所述 TCR 转基因是 TCRα 转基因、TCRβ 转基因，以及其组合。

8. 如权利要求 7 所述的分离的 T 淋巴细胞，其中所述肿瘤抗原包括 NY-ESO1。

9. 一种药物组合物，其包含 :如权利要求 1 至 8 中任一项所述分离的 T 淋巴细胞。

10. 一种生成如权利要求 1 至 8 中任一项所述的 T 淋巴细胞的方法，所述方法包括 :

使用编码一种或多种核酸酶的一个或多个多核苷酸钝化 T 淋巴细胞中的内源 TCR 基因，其中所述核酸酶切割所述内源 TCR 基因 ;以及

将所述外源序列稳定地整合到所述 T 淋巴细胞的所述基因组中。

11. 如权利要求 10 所述的方法，其中使用整合酶缺陷的慢病毒载体 (IDLV)、逆转录病毒载体 (RV) 或者慢病毒载体 (LV) 将所述外源序列导入所述细胞。

12. 如权利要求 1 至 8 中任一项所述的分离的 T 淋巴细胞或者如权利要求 9 所述的药物组合物在治疗癌症、传染病、自身免疫性病症和 / 或移植物抗宿主病 (GVHD) 方面的用途。

使用工程化锌指蛋白核酸酶靶向断裂 T 细胞受体基因

[0001] 相关申请的交叉引用

[0002] 本申请要求在 2013 年 3 月 21 日提交的美国临时申请号 61/804,076 的优先权，据此该申请的公开内容通过引用整体并入。

技术领域

[0003] 本公开内容是在人类细胞的基因组修饰领域中，该人类细胞包括淋巴细胞和干细胞。

[0004] 发明背景

[0005] 已经描述用于基因组 DNA 靶向切割的各种方法和组合物。此类靶向切割事件可用于例如诱导靶向诱变、诱导细胞 DNA 序列的靶向缺失，以及促进在预先决定的染色体位点处的靶向重组。参见，例如美国专利号 7,888,121 ;7,972,854 ;7,914,796 ;7,951,925 ;8,110,379 ;8,409,861 ;8,586,526 ; 美国专利公开 20030232410 ;20050208489 ;20050026157 ;20050064474 ;20060063231 ;201000218264 ;20120017290 ;20110265198 ;20130137104 ;20130122591 ;20130177983 和 20130177960，以及美国临时申请号 61/823,689，这些申请的公开内容通过引用整体并入用于所有目的。这些方法往往涉及使用工程化切割系统来诱导靶向 DNA 序列中的双链断裂 (DSB) 或者缺口，以便用诸如非同源端连接 (NHEJ) 的错误倾向过程 (error born process) 对断裂的修复或者使用修复模板 (同源介导的修复或即 HDR) 进行的修复可导致基因敲除或受关注序列的插入 (靶向整合)。可通过使用特异性核酸酶进行切割，如工程化锌指核酸酶 (ZFN)、转录激活因子样效应核酸酶 (TALEN)，或者使用具有工程化 crRNA/tracr RNA(‘单导向 RNA’) 的 CRISPR/Cas 系统来导向特异性切割。

[0006] T 细胞受体 (TCR) 是选择性激活 T 细胞的关键部分。与抗体有些类似，TCR 一般是由两条链，即 α 和 β 组成的，该两条链共同装配而形成杂二聚体。抗体类似处在于编码 TCR 链的单一基因放在一起的方式。TCR 链是由两个区域组成，即 C 端恒定区和 N 端可变区。编码 TCR 链的基因座与编码抗体的基因座的类似处在于 TCR α 基因包括 V 区段和 J 区段，而 β 链基因座除 V 区段和 J 区段之外还包括 D 区段。在 T 细胞发育期间，各种区段重组，以便每一 T 细胞具有唯一的 TCR 结构并且人体具有大量的 T 细胞，T 细胞由于它们唯一的 TCR 结构而能够与由抗原递呈细胞显示的唯一抗原相互作用。另外，TCR 复合物构成 T 细胞上的 CD3 抗原复合物的一部分。

[0007] 在 T 细胞激活期间，TCR 与在抗原递呈细胞的主要组织相容性复合物 (MHC) 上显示为肽的抗原相互作用。TCR 对抗原 -MHC 复合物的识别导致了 T 细胞刺激，该 T 细胞刺激反过来导致记忆淋巴细胞和效应淋巴细胞中 T 辅助细胞 (CD4+) 和细胞毒性 T 淋巴细胞 (CD8+) 的分化。然后，这些细胞可以克隆方式扩增，以在能够对一种特定抗原作出反应的整个 T 细胞群体中提供被激活的亚群。

[0008] 细胞毒性 T 淋巴细胞 (CTL) 被认为在杀伤肿瘤细胞中是关键的。这些细胞一般能够在癌细胞表面上显示出先前由抗原递呈细胞显示在 MHC 上的一些抗原时诱导癌细

胞的凋亡。通常，在对靶细胞作用之后，当细胞威胁被清除时 CTL 将会凋亡，而保留的淋巴细胞的亚组将进一步分化成记忆 T 细胞存留，以防止人体再次暴露于抗原。记忆淋巴细胞池可能是高度异质的。近来，已经鉴别了两种类型的记忆 T 细胞：效应记忆 T 细胞 (CD45RA-CCR7-，CD62L-) 和中枢记忆 T 细胞，中枢记忆 T 细胞是特征为表达 CCR7 和 CD62L 的 CD45RA 阴性细胞，CCR7 和 CD62L 是在次级淋巴器官的 T 细胞区中归巢所需要的两种分子。在抗原刺激时，中枢记忆 T 细胞产生低水平的效应细胞因子如 IL-4 和 IFN-γ，但是高水平的 IL-2，这能够支持中枢记忆 T 细胞的快速和一致的增殖。在遇到抗原时，中枢记忆 T 细胞经历：1) 增殖，导致自动再生过程，目的在于增大中枢记忆 T 细胞池；以及 2) 分化，导致效应记忆 T 细胞的生成，效应记忆 T 细胞特征为低增殖潜能但是能够迁移到发炎的非淋巴组织并且介导免疫反应的效应期。已经研发出能基因转移到 T 淋巴细胞，同时保持基因的中枢记忆功能表型的方案（参见，欧洲专利公开号 EP1956080，Kaneko 等人，2009Blood 113(5)：1006-15)。

[0009] 然而，一些肿瘤细胞能够或许通过诸如表达相关 TCR 的某些 CTL 亚组的不良克隆扩增和由癌细胞局限免疫抑制的机制来逃避免疫系统的监视（参见，Boon 等人 (2006)，Annu Rev Immunol，24：175-208)。癌症疫苗的概念是基于以下理念构建的：使用这些癌症特异性抗原来刺激和扩增在体内表达适当 TCR 的 CTL，以试图克服免疫逃避，然而这些癌症疫苗尚未显示出任何显著的成果。事实上，在 2004 年进行的分析研究 765 位已经在超过 35 个不同的癌症疫苗试验中接受治疗的转移癌患者，其中仅在 3.8％的患者中观察到总反应（参见，Rosenberg 等人 (2004)，Nat.Med.，10(9)：909－915)。

[0010] 过继性免疫疗法是以下实践：实现高度特异性 T 细胞对某些具有针对肿瘤抗原的高亲合力 TCR 的 CTL 亚群的刺激，在体外刺激和扩增亚群，以及然后将亚群导入患者体内。如果在输注肿瘤特异性细胞之前从患者体内去除天然淋巴细胞，则过继性免疫疗法尤其有效。这种类型的疗法背后的理念是：如果所导入的高亲合力 CTL 奏效，则一旦肿瘤被清除，这些细胞中的一些将作为记忆 T 细胞保留并且将存留在患者体内以防癌症复发。在 2002 年完成的一项研究表明在用环磷酰胺和氟达拉滨进行免疫耗竭后，以过继性免疫疗法方案治疗的患者的转移性黑素瘤退变 (Dudley 等人，(2002)Science，298(5594)：850-854)。如果在过继性免疫疗法之间进行全身照射，则应答率会甚至更高 (Dudley 等人，2008，J Clin Oncol，26(32)：5233-9)。

[0011] 然而，当包含高亲合力 TCR 的受关注 T 细胞不能轻易扩增时，无法进行过继性免疫疗法。此外，往往难以从癌症患者鉴别和分离有治疗价值的 T 细胞，这是因为肿瘤抗原往往是自体抗原，针对肿瘤抗原的患者免疫系统通过那些具有最高亲合力的 T 细胞克隆的缺失或者无反应性的机制而变得耐受。因此，已经建议和证明将编码高亲合力的 TCR 的基因转移到来源于患者的 T 细胞中（参见，Rubenstein 等人，(2003)J of Immunology，170：1209-1217)。最近，使用恶性黑素瘤的小鼠模型，在导入已经用携带 gp-100 黑素瘤抗原特异性人类 TCR 基因的逆转录病毒载体转导过的正常淋巴细胞之后，发现具有统计上显著的肿块质量减少 (Abad 等人，(2008)J Immunother，31(1)：1－6)。TCR 基因疗法还在 Morgan 等人 (2006)Science314(5796)：126-9 和 Burns 等人，2009Blood 114(14)：2888-99 中有所描述。

[0012] 然而，任何 TCR 转基因到宿主 T 细胞中的转移带有与大多数的基因转移方法相关

联的警告，即 TCR 转基因表达盒未调节和无法预见的插入到基因组中，插入往往是低水平的。此类控制不良的所需转基因的插入可导致转基因对周围基因的影响，以及由于来自邻近基因的影响导致的转基因沉默。此外，在工程化 T 细胞中与所导入的 TCR 转基因共表达的内源 TCR 基因可引起由内源 TCR 识别的抗原对 T 细胞的非所需刺激、由于 TCR 转基因与内源性 TCR 亚单位错配形成具有新识别性能的新 TCR 复合物导致的非预期抗原对 T 细胞的非所需刺激，或者可导致由于转基因编码的 TCR 亚单位与内源性 TCR 蛋白的异源二聚化而导致的无活性 TCR 的形成从而对受关注抗原的次最优刺激。事实上，因内源链和外源链错配形成自体反应 TCR 而导致严重自体免疫毒性的风险近年来已经在小鼠模型 (Bendle 等人，(2010)Nature Medicine，16 :565-570) 和人类细胞 (van Loenen 等人，(2010)Proc Natl Acad Sci U S A，107 :10972-7) 中凸显。另外，肿瘤特异性 TCR 可以次最优水平在细胞表面上表达，这是由于与在细胞表面上表达复合物所需的 CD3 分子的内源性和错配 TCR 之间的竞争。低 TCR 表达影响转基因 T 细胞的亲合力和效应力。

[0013] Wilms 肿瘤抗原 (WT1 抗原) 是通常在胚细胞中表达的转录因子。在产生之后，抗原的表达仅限于少量细胞类型，包括造血干细胞。然而，已发现该抗原在许多类型的白血病和实体瘤中过表达 (参见，Inoue 等人 (1997)Blood，89 :1405-1412) 并且可促成在这些细胞中缺乏生长控制。由于 WT1 在正常组织中的低表达，WT1 在癌细胞上的表达使得其成为用于 T 细胞介导疗法的有吸引力的靶标。将具有对包含经修饰的半胱氨酸的 WT1 增强的亲合力以防止内源 TCR 亚单位和转基因 TCR 之间错配的 TCR 变体转导到主要 T 细胞中并且测试其功能 (Kuball 等人 (2007)Blood，109(6) :2331-8)。数据表明 :虽然新近用 WT1-TCR 变体转导的 T 细胞与那些用野生型 TCR 域转导的 T 细胞相比具有较强的抗原应答，但是在用 WT1 抗原进行若干轮刺激之后，此种改善的抗原反应性丧失 (参见，Thomas 等人 (2007)J of Immunol 179(9) :5803-5810)。据推断，即使具有转基因特异性半胱氨酸修饰，与内源性 TCR 肽的错配也可在 WT1 特异性 TCR 转导的细胞中所见的抗 WT1 亲合力降低中发挥作用，还参见美国专利公开号 20110158957。

[0014] 另一种肿瘤抗原是 NY-ESO1。NY-ESO1 是所谓的‘CT’的肿瘤抗原组中的一个成员，意味着其在癌细胞上和在睾丸中表达。NY-ESO1 最初被识别为在食管肿瘤上表达，现在其已经被发现在若干肿瘤类型上表达，肿瘤类型包括膀胱肿瘤、乳腺肿瘤、结直肠肿瘤、胃肿瘤、肝癌、头部肿瘤和颈部肿瘤、多发性骨髓瘤、黑素瘤、非小细胞肺癌、卵巢肿瘤、胰腺肿瘤、前列腺肿瘤、肉瘤和滑膜肉瘤 (参见，Gnjatic 等人 (2006)，Advances in Cancer Research，第 1 页)，表达往往是在那些肿瘤处于晚期时。由于在大部分组织上明显缺乏表达，NY-ESO1 已经被考虑用于癌症疫苗。因此，全长 NY-ESO1 蛋白和来源于序列的肽已经并且正被用于临床试验。然而，似乎疫苗接种方法的有效性有限，这也许是由于对抗原具有有限亲合力的 T 细胞的生产。此外，许多有 NY-ESO1 阳性肿瘤的癌症患者血液中可检测出抗 NY-ESO1 抗体，但是他们的肿瘤仍然能够逃避免疫反应。一种潜在的解决方法可为开发对 NY-ESO1 抗原具有高亲合力的 TCR。使用将通过三种不同的 T 细胞启动技术制备的 NY-ESO1 特异性 TCR 标准 TCR 转移到宿主 T 细胞中进行的研究 (参见，Sommermeyer 等人，(2012)Int. J. Cancer 132 :1360-1367) 发现针对过继性免疫疗法研发的稳健 TCR 将需要解决大量的问题。还已经产生关于 NY-ESO1 特异性 TCR 的附加报告 (参见 US8367804 和 EP2016102B1 中的具体实施例)。还已经进行临床试验，在临床试验中用从已经用 NY-ESO1TCR 转导的外周血收获的

自体淋巴细胞来治疗 NY-ESO1+ 转移性黑素瘤或者转移性滑膜细胞肉瘤患者。在 11 名黑素瘤患者中中观察到有 5 名有临床应答，且在 6 名滑膜细胞肉瘤患者中观察到有 4 名有临床应答 (Robbins 等人，(2011) J. Clin Oncol 29(7) :917)。

[0015] 因此，仍然需要能够将所需 TCR 转基因导入已知的染色体座位中的组合物。此外，需要可选择性敲除内源 TCR 基因的方法和组合物。

发明概要

[0016] 本文公开用于部分或者完全钝化或者断裂内源 TCR 基因的组合物和方法，以及用于在断裂内源性 TCR 基因之后或者与断裂内源性 TCR 基因同时地，将外源 TCR 转基因导入 T 淋巴细胞并且表达至所需水平的组合物和方法。

[0017] 在一个方面，本文提供锌指核酸酶 (ZFN)、TALEN，或者具有用于切割 TCR 基因的工程化单导向 RNA 的 CRISPR/Cas 系统。在某些实施方案中，ZFN、TALEN 或者 CRIPSR/Cas 核酸酶结合到人类 TCRα 基因中的靶位点和 / 或人类 TCRβ 基因中的靶位点。在一些实施方案中，用这些核酸酶在 TCR 基因中进行切割会导致 TCRα 和 / 或 β 基因的永久断裂 (例如，突变 / 钝化)。

[0018] 在某些实施方案中，核酸酶包括锌指蛋白。锌指蛋白可包括 1、2、3、4、5、6 或更多个锌指，每一锌指具有结合到靶基因中的靶亚位点的识别螺旋。在某些实施方案中，锌指蛋白包括 4 或者 5 或者 6 个指状结构 (从 N 端到 C 端指定为 F1、F2、F3、F4、F5 和 F6，以及顺序的 F1 到 F4 或者 F5 或者 F6)，并且指状结构包括在表 4 和表 5 中示出的识别区域的氨基酸序列和 / 或识别在表 4 和表 5 中示出的靶位点。在其它实施方案中，核酸酶是可包括具有规范或者不规范的重复序列可变的双氨基酸残基 (repeat variable diresidues, RVD) 的工程化重复单元的 TALEN，例如如表 14 中所示的可操作地连接到核酸酶域 (例如，IIS 型限制性内切酶和 / 或大范围核酸酶) 的 TRAC 和 TRBC 特异性 TALEN。TALEN 包括 C 帽序列，例如比野生型 TAL C 端序列全长更小的 C 端区域 (例如，a+17 或者 +63C 帽)。C 帽序列在美国专利号 8,586,526 中有所描述。另外的实施方案包括 CRIPSR/Cas 核酸酶系统的用途，其中单导向 RNA 已制备用于将核酸酶靶向到 TCRα 序列和 / 或 TCRβ 序列中的靶位点。

[0019] 本文描述的核酸酶中任一个可进一步包括切割域和 / 或切割半域 (例如，野生型或者工程化 FokI 切割半域，或者具有切割活性的大范围核酸酶域)。因此，在本文描述的核酸酶的任一个中，核酸酶域可包括野生型核酸酶域或者核酸酶半域 (例如，FokI 切割半域)。在其它实施方案中，核酸酶 (例如，ZFN 和 / 或 TALEN) 包括工程化核酸酶域或者半域，例如形成专性杂二聚体的工程化 FokI 切割半域。参见，例如美国专利号 7,914,796 ；美国专利号 8034,598 和美国专利公开号 20080131962。

[0020] 在另一方面，本公开内容提供编码本文描述的核酸酶的任一个的多核苷酸。本文描述的多核苷酸的任一个还可包括用于靶向插入到 TCRα 和 / 或 TCRβ 基因中的外源序列 (供体序列或者补丁序列)。在某些实施方案中，供体序列包括肿瘤抗原特异性 TCR 转基因，其中 TCR 转基因是 TCRα 转基因、TCRβ 转基因，以及它们的组合。在某些实施方案中，转基因包括 NY-ESO1 特异性转基因，其中 NY-ESO1 特异性转基因是 TCRα 转基因、TCRβ 转基因，以及它们的组合。

[0021] 在又一个方面，提供包括本文描述的多核苷酸中的一个或多者的基因递送载体

（例如，供体和 / 或核酸酶）。在某些实施方案中，载体是腺病毒载体（例如，Ad5/F35 载体），或者慢病毒载体 (LV)（包括整合型慢病毒载体或者整合缺陷型慢病毒载体）。因此，本文还提供了腺病毒 (Ad) 载体或者 LV，载体包括编码至少一个锌指蛋白核酸酶 (ZFN)、TALEN 或者 CRISPR/Cas 核酸酶和单导向 RNA 的序列和 / 或用于定点整合到靶基因中的供体序列。在某些实施方案中，Ad 载体是嵌合型 Ad 载体，例如 Ad5/F35 载体。在某些实施方案中，慢病毒载体是整合酶缺陷型慢病毒载体 (IDLV) 或者整合型慢病毒载体。在某些实施方案中，载体是具有 VSV-G 包膜或者具有其它包膜的假型载体。在另外的实施方案中，靶基因是人类 TCRα 基因。在某些实施方案中，靶基因是人类 TCRβ 基因。本文描述的载体还可包括供体序列。在另外的实施方案中，供体序列包括特异于受关注 MHC/ 抗原复合物的人类 TCR 基因。在一些实施方案中中，供体序列可包括特异于受关注 MHC/ 抗原复合物的人类 TCRα 基因和 / 或人类 TCRβ 基因。在某些实施方案中，单一载体包括编码一种或多种 ZFN、TALEN 或者 CRISPR/Cas 核酸酶复合物的序列和供体序列。在其它实施方案中，供体序列包含于第一载体中，且 ZFN-、TALEN- 或者 CRISPR/Cas 编码序列存在于第二载体中。在另外的实施方案中，ZFN-、TALEN- 或者 CRISPR/Cas 编码序列存在于第一载体中，且受关注 TCRα 基因存在于第二载体中，以及受关注 TCRβ 基因存在于第三载体中。在一些实施方案中，将受关注 TCR 基因插入内源性 TCR 基因的位置，且在其它实施方案中，将受关注 TCR 基因插入随机选择的基因座，或者在全基因组递送之后插入到单独基因座。在一些实施方案中，用于 TCR 转基因插入的单独基因座是 PPP1R12C 基因座（也被称为 AAVS1，参见美国专利号 8,110,379）。在其它实施方案中，将 TCR 转基因插入到 CCR-5 基因座中。参见，美国专利号 7,951,925。

[0022] 在又一方面，本公开内容提供分离的 T 淋巴细胞，T 淋巴细胞包括稳定地整合到 T 淋巴细胞的基因组中的外源序列，且 T 淋巴细胞中内源的 TCR 基因被用锌指核酸酶或者 C 帽 TALEN（具有 C 端截断的 TALEN）部分地或者完全地钝化。在某些实施方案中，细胞包括本文描述的蛋白质、多核苷酸和 / 或载体的任一个。在某些实施方案中，细胞选自由以下组成的组：干细胞 / 祖细胞，T 细胞（例如，$CD4^+$T 细胞）。在又一方面，本公开内容提供一种从如本文所描述的细胞或者细胞系传代的细胞或者细胞系，即一种从其中 TCR 已经由一个或多个 ZFN、TALEN、或者特异性 CRISPR/Cas 核酸酶钝化和 / 或其中编码 TCR 的供体多核苷酸已经稳定地整合到细胞的基因组中的细胞传代（例如，在培养物中）的细胞或者细胞系。因此，如本文所描述的细胞的子代本身可不包括本文描述的蛋白质、多核苷酸和 / 或载体，但在这些细胞中，TCR 基因被钝化和 / 或编码 TCR 的供体多核苷酸被整合到基因组中和 / 或被表达。

[0023] 在另一方面，本文描述了通过将一个或多个蛋白质、多核苷酸和 / 或载体导入到本文所描述的细胞来钝化 TCR 基因的方法。在本文描述方法的任一个中，ZFN、TALEN 或者特异性 CRISPR/Cas 核酸酶可诱发细胞 DNA 序列的靶向诱变、靶向缺失和 / 或在预先确定的染色体基因座处促进靶向重组。因此，在某些实施方案中，ZFN、TALEN 或者特异性 CRISPR/Cas 核酸酶使靶基因中的一个或多个核苷酸缺失或者插入一个或多个核苷酸到靶基因中。在一些实施方案中，TCR 基因由 ZFN、TALEN 或者特异性 CRISPR/Cas 核酸酶切割钝化，然后进行非同源的端连接。在其它实施方案中，靶基因中的基因组序列被替代，例如使用如本文所描述的 ZFN、TALE 或者特异性 CRISPR/Cas 核酸酶（或者编码 ZFN、TALEN 或者特异性 CRISPR/Cas 核酸酶的载体）以及由 ZFN、TALEN 或者特异性 CRISPR/Cas 核酸酶进行靶向切割后插

入到基因中的“供体”序列。在某些实施方案中，供体序列包括 NY-ESO1 序列。供体序列可存在于 ZFN、TALEN 或者特异性 CRISPR/Cas 核酸酶载体中，存在于单独载体（例如，Ad 载体或者 LV 载体）中，或者替代地可使用不同的核酸递送机制被导入细胞中。

[0024] 在另一方面，提供使用锌指蛋白、TALEN 或者特异性 CRISPR/Cas 核酸酶及其融体来突变 TCR 基因和 / 或钝化细胞或者细胞系中的 TCR 功能的方法。因此，提供一种用于钝化人类细胞中的 TCR 基因的方法，该方法包括向细胞施用本文描述的蛋白质或者多核苷酸的任一个。

[0025] 在又一方面，本发明内容提供一种用于治疗或者预防受试者的癌症、传染病、自身免疫性疾病和 / 或移植物抗宿主病 (GVHD) 的方法，该方法包括 :(a) 将编码第一多肽的第一核酸导入细胞（例如，淋巴细胞、干细胞、祖细胞等），其中第一多肽包括 :(i) 锌指或者 TALE-DNA 结合域，该锌指或者 TALE DNA- 结合域经工程化而结合到 TCR 基因中的第一靶位点 ;以及 (ii) 切割域 ;在使得多肽在细胞中表达的条件下，该多肽藉由该域结合到靶位点并且切割内源的 TCR 基因 ;以及 (b) 将编码第二多肽的第二核酸导入细胞，其中第二多肽包括 :(i) 锌指或者 TALE DNA- 结合域，该锌指或者 TALE DNA- 结合域经工程化而结合到 TCR 基因中的第二靶位点 ;以及 (ii) 切割域 ;在使得多肽在细胞中表达的条件下，该多肽藉由该域结合到靶位点并且切割内源 TCR 基因 ;以及 (c) 将包括编码特异于 MHC 复合物中的肿瘤特异抗原的一或多个 TCR 基因的核酸的第三核酸导入细胞，以便将第三核酸导入到内源 TCR 基因并且具有所导入的第三核酸的细胞治疗或预防受试者的癌症、传染病、自身免疫性疾病和 / 或移植物抗宿主病 (GVHD)。在某些实施方案中，步骤 (a)-(c) 是在体外进行的并且方法在步骤 (c) 之后还包括将细胞导入受试者体内的步骤。在某些实施方案中，编码一个或多个 TCR 基因的第三核酸是在双向启动子（例如，PGK、EF1α 等）的控制下表达的。在其它实施方案中，一个或多个 TCR 基因是在单向启动子的控制下从双顺反盒（例如，使用病毒 2A 肽或者 IRES 序列）或者通过表达不同 TCR 基因的多种 LV 来表达的。在某些实施方案中，该细胞选自由以下组成的组 :干细胞 / 祖细胞，或者 T 细胞。在本文描述的方法中任一个中，第一核酸可进一步编码第二多肽，其中第二多肽包括 :(i) 锌指或者 TALE DNA- 结合域，锌指或者 TALE DNA- 结合域域经工程化而结合到 TCR 基因中的第二靶位点 ;以及 (ii) 切割域 ;以便第二多肽在细胞中表达，第一和第二多肽藉由该域结合到其各自相应的靶位点并且切割 TCR 基因。

[0026] 在另一方面，本发明内容还提供一种用于治疗或者预防受试者的癌症的方法，方法包括 :(a) 将编码第一多肽的第一核酸导入细胞，其中第一多肽包括 :(i) 锌指或者 TALE DNA- 结合域，锌指或者 TALE DNA- 结合域经工程化而结合到 TCR 基因中的第一靶位点 ;以及 (ii) 切割域 ;在使得多肽在细胞中表达的条件下，该多肽藉由该域结合到靶位点并且切割内源 TCR ;以及 (b) 将编码第二多肽的第二核酸导入细胞，其中第二多肽包括 :(i) 锌指或者 TALE DNA- 结合域，锌指或者 TALE DNA- 结合域经工程化而结合到安全港基因座 (safe harbor locus)（例如，PPP1R12C、CCR5）中的第一靶位点 ;以及 (ii) 切割域 ;在使得多肽在细胞中表达的条件下，多肽藉由该域结合到靶位点并且在安全港基因座（例如，PPP1R12C、CCR5）中进行切割 ;以及 (c) 将包括编码特异于 MHC 复合物中的肿瘤特异抗原的一个或多个 TCR 基因的供体核酸的第三核酸导入细胞 ;以及 (d) 将细胞导入受试者体内。可将包括 TCR 特异性 ZFN、TALEN 或者 CRISPR/Cas 核酸酶系统的核酸与特异于安全港基因座的 ZFN、

TALEN 或者 CRISPR/Cas 核酸酶系统以及供体核酸分子同时导入，或者可在第一步骤中将编码 TCR 特异性 ZFN、TALEN 或者 CRISPR/Cas 核酸酶系统的核酸导入细胞，然后可在第二步骤中导入安全港基因座（例如，PPP1R12C、CCR5）特异性 ZFN、TALEN 或者 CRISPR/Cas 核酸酶系统以及供体核酸分子。在某些实施方案中，供体核酸分子编码肿瘤抗原如 NY-ESO1。

[0027] 本发明内容还提供一种用于预防或者治疗受试者的癌症的方法，方法包括将病毒递送颗粒导入受试者体内，其中病毒递送颗粒包括：(a) 编码第一多肽的第一核酸，其中第一多肽包括：(i) 锌指或者 TALE DNA- 结合域，锌指或者 TALE DNA- 结合域经工程化而结合到 TCR 基因中的第一靶位点；以及 (ii) 切割域；在使得多肽在细胞中表达的条件下，多肽藉由该域结合到靶位点并且切割内源 TCR 基因；以及 (b) 编码第二多肽的第二核酸，其中第二多肽包括：(i) 锌指或者 TALE DNA- 结合域，该锌指或者 TALE DNA- 结合域经工程化而结合到安全港基因座（例如，AAVS1、CCR5、白蛋白、HPRT 等（参见共有专利权的美国专利号 U.S.8,110,379 和美国专利号 U.S.7,951,925，以及专利申请号 13/624,193 和号 13/660,821）中的第一靶位点；以及 (ii) 切割域；在使得多肽在细胞中表达的条件下，该多肽藉由该域结合到靶位点并且切割安全港基因座（例如，AAVS1、CCR5、白蛋白、HPRT）；以及 (c) 编码第三多肽的第三核酸，其中第三多肽包括：(i) 锌指或者 TALE DNA- 结合域，该锌指或者 TALE DNA- 结合域经工程化而结合到安全港基因座（例如，AAVS1、CCR5、白蛋白、HPRT）中的第二靶位点；以及 (ii) 切割域；在使得多肽在细胞中表达的条件下，多肽藉由该域结合到靶位点并且在安全港基因座（例如，AAVS1、CCR5、白蛋白、HPRT）处切割；以及 (d) 包括编码特异于 MHC 复合物中的肿瘤特异抗原的一个或多个 TCR 基因的供体核酸的第三核酸；以便切割内源 TCR 基因并使其钝化，以及切割安全港基因（例如，AAVS1、CCR5、白蛋白、HPRT）及将特异于 MHC 复合物中的肿瘤特异抗原的 TCR 基因插入到内源 TCR 基因中。在某些实施方案中，该方法在步骤 (d) 之后还包括将细胞导入受试者体内的步骤。在某些实施方案中，供体核酸分子编码肿瘤抗原如 NY-ESO1。

[0028] 在本文描述的方法的任一个中，病毒递送颗粒可用于递送一个或多个多核苷酸（编码 ZFN 或者 TALEN 的多核苷酸和 / 或供体多核苷酸）。此外，在本文描述的方法和组合物的任一个中，细胞可例如为干细胞 / 祖细胞（例如，$CD34^+$ 细胞），或者 T 细胞（例如，$CD4^+$ 细胞）。

[0029] 另外，本文描述的方法的任一个可在试管内、活体内和 / 或体外实践。在某些实施方案中，该方法是在体外实践的，例如用于修饰 PBMC（例如，T 细胞），以使得 PBMC 特异于受关注肿瘤抗原 /MHC 复合物，从而治疗受试者的肿瘤。可被治疗和 / 或预防的癌症的非限制性实例包括肺癌、胰腺癌、肝癌、骨癌、乳腺癌、结直肠癌、白血病、卵巢癌、淋巴瘤、脑癌等。

[0030] 附图简述

[0031] 图 1，小图 A 和 B 描绘 Wilms 肿瘤抗原 (WT1) 特异性慢病毒载体的构建和表达。图 1A 描绘了在双向 PGK 或者 EF1α 启动子的控制下，将编码特异于来自 Wilms 肿瘤抗原 1(WT1) 的 HLA-A2 限制肽的密码子优化的、用半胱氨酸修饰的 TCR 的基因克隆到第三代慢病毒载体 (LV) 中的图形。参见，Amendola 等人 (2005) Nature Biotechnology，23(1)：108-116 和美国专利公开号 US2006200869。图 1B 是描绘在 5ng/ml 的 IL7 和 IL15 的存在下培养的经慢病毒转导的 $CD8^+$ 细胞中 Vβ21TCR 表达的时程的图解。将 Vβ21 相对荧光强度 (RFI) 计算为在经 PGK-WT1（空心正方形）或者 EF1α-WT1（“X”）基因修饰的淋巴细胞中测量的

Vβ21 的平均荧光强度 (MFI)/ 在天然表达 Vβ21 的 T 细胞中测量的 Vβ21 的 MFI 的比率。

[0032] 图 2，小图 A 到 C，是描绘用 TCR 构建体转导细胞的结果的图解。图 2A 描绘了在暴露于来自 AML 患者（被指定为 AML1（最左侧的柱条）、AML2（左起第二个柱条）、和 AML3（左起第三个柱条））的 WT1+HLA-A2+ 或者 WT1+HLA-A2-（阴性对照）原代白血病细胞之后，用从 PGK/mCMV 双启动子组合（左侧 4 个柱条的组）或者 EF1α/mCMV 双启动子（右侧 4 个柱条的组）表达转基因 TCR 的载体转导的 WT1+HLA-A2+ 和 WT1+/HLA A2- 细胞（所指示的原代 AML 或者 K562 细胞（图中最右侧的柱条））刺激细胞以诱导 γ IFN 产生。图 2B 和图 2C 显示经 TCR 修饰的细胞对来自 AML1 和 AML2（实线、实心圆圈）的白血病原细胞的杀伤百分比。虚线表示在过量的装载有适当 WT1 肽的冷（未标记）HLA-A2 靶细胞的存在下，经 TCR 修饰的细胞对白血病原细胞的剩余杀伤百分比。

[0033] 图 3，小图 A 和 B，是描绘在将靶向到安全港基因座的 ZFN 与 GFP 供体一起导入之后的 GFP 表达的图解。图 3A 和图 3B 显示与所使用的 Ad5/F35CCR5 特异性 ZFN（图 3A）或者 Ad5/F35AAVS1 特异性 ZFN（图 3B）以及 -IDLV GFP 供体 DNA 盒的量相关的 GFP 阳性细胞的百分比增长。

[0034] 图 4，小图 A 和 B，描绘示例性 TCR-α 和 TCR-β 供体分子（图 4A）和 TCR-β 基因（图 4B）的图形。图 4A 描绘了包括 WT1 特异性 TCR-α 和 TCR-β 供体分子的盒并且示出了与 CCR5 整合位点同源的区域。图 4B 描绘了 K562 细胞中两个 TCR-β 恒定区 (TRBC1 和 TRBC2) 的基因组排布。

[0035] 图 5 描绘 K562 细胞中若干对 TCR-β 特异性 ZFN 的修饰百分比，如用 Cel-I Surveyor™错配测定（“Cel-I 测定”，Transgenomic）所测量的。在用 0.1μg 或者 0.4μg 的 ZFN 质粒转染之后，最初将细胞在 30℃孵育。在条带的的底部示出了修饰百分比。

[0036] 图 6，小图 A 和 B，描绘在 K562 细胞中 TCR-α 特异性 ZFN 的修饰百分比，如用 Cel-I 测定所测量的。图 6A 描绘关于其中 ZFN 靶向到外显子 1 的细胞的 Cel-I 测定的结果。图 6B 描绘关于其中 ZFN 靶向到外显子 3 的 Cel-I 错配测定的结果。“GFP”指示用仅表达 GFP 的载体转染的细胞。在条带的底部指示了改变 (NHEJ) 百分比。如图所示，在 IL7 和 IL15 的存在下，分选的 CD3- 淋巴细胞存活。

[0037] 图 7，小图 A 到 F，描绘 ZFN 介导的对 TCR-β 的切割。在两种载体浓度下使用 TCR-β 特异性 ZFN 对 16783 和 16787 的未转导和经转导 Jurkat 细胞显示出细胞表面处的 CD3 信号损失（从 2.7% CD3(-) 到 20.2% CD3(-)（参见实施例 6）。图 7A 和图 7B 示出了 Jurkat 细胞中 TRBC1（图 7A）和 TRBC2（图 7B）基因座处的 Cel-I 测定结果并且显示已经发生切割。在每一条带的底部指示了所测量的基因修饰%。图 7C 是描绘在 IL7 和 IL15 的存在下可存活的分选 CD3- 原代人淋巴细胞的图解。“UT”指示未处理的细胞。图 7D 示出在用 TCR-β 特异性 ZFN 处理过的原代 T 细胞池中观测到的修饰 (NHEJ) 百分比，如用 Cel-I 测定法所测定的。“混合 (Bulk)”指示对用 ZFN 处理过的细胞池所观测到的 NHEJ 百分比，而 CD3+ 或者 CD3- 示出对被分选为 CD3+ 或者 CD3- 的细胞所观测到的 NHEJ。“UT”指示未处理的细胞。在条带的底部指示用测定检测的 NHEJ 百分比。图 7E 是描绘 CD3- 细胞的百分比的图解，并且显示在用浓度不断升高的 ZFN 处理过的细胞中，CD3- 细胞随着时间推移的存活率（甚至至第 45 天，CD3- 细胞的百分比保持相当恒定）。图 7F 是描绘已经丧失 TCR/CD3 功能的 CD3- 细胞的图解，因为细胞不响应于非特异性促细胞分裂剂而出现分裂。如图所示，在 IL7

和 IL15 存在多于 40 天的情况下，CD3- 细胞在培养物中存活并且是稳定的，CD3- 细胞不响应多克隆促细胞分裂剂并且维持 TCM 表型。

[0038] 图 8 描绘编辑原代 T 淋巴细胞中的 TCR-β 基因座并且重新导入特异性 TCR 转基因的实验略图和 FACS 结果。所使用的细胞是未处理的原代 T 淋巴细胞，或者先用 IDLV 携带的 TCR-β 特异性 ZFN 预处理然后分选为 CD3(-) 原代 T 细胞的淋巴细胞。根据欧洲专利公开号 EP1956080，基因转移是在用涂覆有针对 CD3 和 CD28 的抗体的细胞大小的珠粒刺激 T 细胞之后而实现的，并且在 IL7(5ng/ml) 和 IL15(5ng/ml) 的存在下进行细胞培养以促进生成经基因修饰的中枢记忆淋巴细胞。如图所示，在用 TCR-β 特异性 ZFN 处理之后被分选为 CD3(-)，然后使用慢病毒载体将 WT1-TCRβV21.3 和 WT1.TCRα 转基因随机整合到基因组中的细胞示出在针对 CD3 和 Vβ21.3 两者进行染色时增多，这指示原代 T 淋巴细胞可经由使用 TCR-β 特异性 ZFN 进行 NHEJ 而经历内源 TCR 断裂，随后经由导入用转基因盒 (PGK-WT1) 编码的新的 TCR 而重新靶向成识别特异性抗原。作为对照，UT 细胞还具有插入的 PGK-WT1 盒并且相较于经 ZFN 处理的 CD3(-) 群体（在多克隆刺激之后为 46%、92%）表现出较小的表达 Vβ21.3 的细胞的百分比 (26%)，这指示内源 TCR 的断裂可改善细胞表面表达及从转基因表达的 TCR 的功能。

[0039] 图 9，小图 A 到 C，描绘 Vβ21TCR 的表达。图 9A 描绘在 $CD8^+$TCRβ 链断裂并且用 WT1 转导的细胞 (TCR-β 经编辑的）、未编辑的 WT1LV 转导细胞 (TCR 经转移的）和用相同的培养条件处理的未转导淋巴细胞中的 Vβ21TCR 表达（上方直方图）和 $WT1_{126\ 134}$ 五聚体结合（下方直方图）。图 9B 示出了 Vβ21TCR 表面表达的时程。以 Vβ21RFI 的平均值 + 标准偏差 (SD)(n = 2) 表示。RFI 是根据在 $CD8^+$TCR 经编辑（空心三角形）或者 TCR 经转移（黑色圆形）的淋巴细胞中测量的 Vβ21 的 MFI/ 在天然表达 Vβ21 的 $CD8^+$T 细胞中测量的 Vβ21 的 MFI 的比率计算的。图 9C 描绘 TCR 经编辑和 TCR 经转移的细胞的细胞毒性测定结果。功能活性是在效应物 / 靶标 (E/T) 比为 12 时，用 ^{51} 铬释放测定标记的 T2 细胞的细胞溶解来测量的，其中标记的 T2 细胞用不断增加浓度的 $WT1_{126\ 134}$HLA-A2 限制的肽致敏，或者用不相关的 CMV- 来源的 $pp65_{495\ 503}$HLA-A2 限制的肽 (10μM) 致敏以作为阴性对照。结果以细胞溶解 % 的平均值 +SD 来表示 (** 代表 $p<0.01$;* 代表 $p<0.05$，使用曼 - 惠特尼检验，TCR 经编辑的 n = 6，TCR 经转移的 n = 4)。

[0040] 图 10，小图 A 和 B，描绘 WT1TCR- 阳性 T 细胞克隆体的功能活性，如用 γIFN 酶联免疫斑点 (ELISpot) 测定所测试的。将克隆体暴露于用 10nM 的 $WT1_{126\ 134}$HLA-A2 限制的肽致敏的 T2 细胞（小图 10A)，或者以 1 :1 的刺激物 / 应答物比例暴露于同种异体的 PBMC（小图 10B)。所观测到的特异斑点（空心三角形和黑色圆形）的数量在 y 轴上示出为在刺激物的存在下产生的斑点数量减去由效应单独产生的斑点的数量。结果示出 TCR-β 经编辑的克隆体显示出比包含内源和外源 TCR 基因两者的 TCR 经转移的细胞更高程度的抗原特异性。

[0041] 图 11 描绘 ZFN 经编辑的细胞和未经编辑的细胞中的 Vβ21 表达。CD3(-) 细胞是从 TRBC 被断裂的淋巴细胞和未编辑的细胞中分选的，并且在不断增加的 MOI 下经编码 WT1 特异性 TCR 的 Vβ21 基因和 ΔLNGFR 基因的 LV 转导的（参见图顶端处的图形，该图形示出了 Vβ21 基因和 ΔLNGFR 基因的双重表达）。转导效率被评定为 $\Delta LNGFR^{pos}$ 淋巴细胞 % 并且被示出。Vβ21 表达是在 $\Delta LNGFR^{pos}$ 细胞上测量的并且显示经转导的 Vβ21 基因可被表达

并且与内源 TCRα 链一起形成有活性的 CD3 复合物。示出了 Vβ21 的平均荧光强度 (MFI)。

[0042] 图 12，小图 A 到 C，描绘在用靶向 TCR□基因的 ZFN 处理过的原代淋巴细胞中的 CD3 表达。图 12A 描绘编码 TCRα 的人类基因座的图形，基因座全长 18kb ；TRAV，即可变区基因 ；TRAD，即多样性区域基因 ；TRAC，即恒定区基因。基因座方案上方显示的是由每一 TRAC-ZFN 靶向的 TRAC 中的基因组 DNA 序列。图 12A 公开蛋白序列为 SEQ ID NO ：109，以及 DNA 序列为 SEQ ID NO ：108。图 12B 描绘了在用 baCD3/CD28 刺激，以 5ng/ml 的 IL-7、5ng/ml 的 IL-15 培养并且暴露于 TRAC-ZFN IDLV 的原代人淋巴细胞中，用流式细胞术测量的细胞表面 CD3 表达的下调。标绘了 CD3(-) 细胞的百分比。UT 是指未经转导的细胞。经分选的 CD3(-) 细胞用 WT1-αOFP-LV 转导，从而导致 CD3 在经转导的淋巴细胞上表达。图 12C 描绘了凝胶，凝胶示出暴露于 TRAC-ZFN 的原代淋巴细胞中用 Cel-I 测定测量的靶基因断裂水平。示出了指示野生型 (w/t) 基因的较高迁移的产物。较低迁移的产物 (NHEJ) 指示 ZFN 引导的基因断裂。“UT”是指未经转导的细胞。

[0043] 图 13 描绘了用 ZFN 处理的人类淋巴细胞中的基因组 TRAC ZFN 靶位点的部分序列被扩增、克隆以及测序，以确认 ZFN 诱导的修饰。序列比对揭示若干在靶区内 ZFN 诱导的缺失和插入 (indel)。左列指示所检索的克隆数目，而右列指示缺失或者插入的核苷酸的数目。图 13 按照出现次序分别公开 SEQ ID NOs ：110-143。

[0044] 图 14 描绘了 ZFN 编辑之后的 CD3 表达。上方小图示出了其中用 TRAC-ZFN-AdV(MOI 1000) 处理激活的 T 淋巴细胞以分选 CD3(-) 淋巴细胞，然后用 3μg-p24/10^6个细胞的 PGK-WT1-αLV 转导以分选 CD3(+) 细胞的研究的结果。示出了 CD3 在经转导的细胞中的表面表达。在一个周期的多克隆刺激之后，用 TRBC-ZFN-AdV(MOI 10^4) 处理 α 经编辑的淋巴细胞以分选 CD3(-) 细胞，且用 3μg-p24/10^6个细胞的 PGK-WT1-βLV 转导。示出了在一个周期的多克隆刺激前后经转导的细胞上 Vβ21TCR 和 CD3 的表面表达。示出了每一象限中测量的事件百分比，并且在底部上示出了实验的时间线。

[0045] 图 15 描绘了经由导入 ZFN 而断裂 TCRα/β 链以分选 CD3- 细胞，随后用 WT1TCR 链转导以分选 CD3+ 细胞 (TCR 经编辑) 的 CD8(+)T 细胞、未编辑的经 WT1LV 转导的细胞 (TCR 经转移的)，以及用相同培养条件处理过的未转导淋巴细胞中的 Vβ21TCR 表达 (上方直方图) 和 $WT1_{126\ 134}$五聚体结合 (下方直方图)。数据示出 TCR 经编辑的细胞相较于其中存在内源和外源 TCR 基因两者的那些克隆体具有更高水平的 Vβ21 表达。数据还显示 TCR 经编辑的细胞相较于具有两组 TCR 基因的那些细胞展示出对 WT1 肽更高的结合性。

[0046] 图 16，小图 A 到 C，是描绘用 γIFN ELISpot 测定所测试的经基因修饰的淋巴细胞的功能活性的图解。在多克隆刺激三周之后，将 TCR-α/β 经编辑的淋巴细胞和 TCR 经转移的淋巴细胞暴露于 i) 用不断增加浓度的 $WT1_{126\ 134}$HLA-A2 限制的肽致敏，或者用不相关的 CMV 来源的 $pp65_{495\ 503}$HLA-A2 肽致敏的 T2 细胞 (参见图 16A，图右侧) 或者 ii) 从用 $WT1_{126\ 134}$ 肽 (50nM) 致敏 (虚线符号) 或者未用 $WT1_{126\ 134}$肽 (50nM) 致敏 (实线符号) 的 AML 患者收获的 $WT1^+$HLA-A2(+)(图 16B 中的黑色) 或者 HLA-A2(-)(灰色) 白血病细胞。图 16C 示出了其中同种异体的 PBMC 用作靶标的类似结果。所有测定是以 1 ：1 的刺激物 / 应答物比例完成的。特异性斑点的数目在 y 轴上示出为在刺激物的存在下产生的斑点的数目减去由效应物单独产生的斑点的数目。* = $p<0.05$，** = $p<0.01$，*** = $p<0.001$。

[0047] 图 17 描绘了关于用 TRAC 特异性 ZFN 进行脱靶切割的分析。在进行用于切割的

ZFN 处理之后，用 Cel-I 错配测定来分析关于 TRAC 特异性 ZFN（在 silicio 分析中被识别的）的 15 个最可能的潜在脱靶位点。每一潜在脱靶位点在以下 5 种样本中被分析：未转导的样本 (UT)；在 TRAC 特异性 ZFN 处理和分选之后为 TRAC 阴性 (TRAC-) 的样本；在用 TRAC 特异性 ZFN、TRAC 转基因和 TRBC 特异性 ZFN 进行连续处理以及进行连续轮次的分选之后为 TRAC 阴性和 TRBC 阴性的细胞（双重阴性）；在用 ZFN 处理之后对内源 TRAC 和 TRBC 基因座为阴性的，以及被修饰成包括非野生型 TRAC 和 TRBC 转基因的细胞（完全被编辑的）；或者在用 TRBC 特异性 ZFN 单独进行处理并且经分选后为 TRBC 阴性的细胞 (TRBC-)。潜在的脱靶位点是如表 12 中所标记的。

[0048] 图 18 描绘了关于用 TRBC 特异性 ZFN 进行脱靶切割的分析。在进行 ZFN 切割处理之后，用 Cel-I 错配测定来分析关于 TRBC 特异性 ZNS（在 silicio 分析中被识别的）的 15 个潜在脱靶位点。每一潜在脱靶位点在以下 5 种样本中被分析：未转导的样本 (UT)；在 TRAC 特异性 ZFN 处理和分选之后为 TRAC 阴性 (TRAC-) 的样本；在用 TRAC 特异性 ZFN、TRAC 转基因和 TRBC 特异性 ZFN 进行连续处理以及进行连续轮次的分选之后为 TRAC 阴性和 TRBC 阴性的细胞（双重阴性）；在用 ZFN 处理之后对内源性的 TRAC 和 TRBC 基因座为阴性的，以及被修饰成包括非野生型 TRAC 和 TRBC 转基因的细胞（完全被编辑的）；或者在用 TRBC 特异性 ZFN 单独进行处理并且经分选后为 TRBC 阴性的细胞 (TRBC-)。脱靶位点如表 13 中所标记的。TRBC 描绘了对这些样本中预期靶位点的修饰。

[0049] 图 19，小图 A 到 C，显示了 NY-ESO1 特异性 TCR 的表达以及与适当靶标的结合。图 19A 示出了用 NY-ESO1 特异性 TCR 转导的 T 细胞（“转移”）与在 TCR 转导之前首先用 TRAC 特异性 ZFN 处理以敲除内源 TCR-α 链的细胞（“SE”）或者在 NY-ESO1 特异性 TCR 转导之前 TCR-α 和 TCR-β 链两者都被敲除的细胞（“CE”）之间的比较。示出了特异性 TCR 在三种 T 细胞群体中的表达。图 19B 示出了由 NY-ESO1 肽组成的 dextramer 的结合亲合力，并且示出了已经缺失所有的内源 TCR 链的细胞群体 (CE) 具有所测试的 T 细胞群体中最高的结合亲合力。图 19C 是描绘用 3 种不同的供体进行 3 个连续实验的平均值的图解。最左侧的柱条示出“转移”结果，中间的柱条示出“SE”结果，而最右侧的柱条示出“CE”结果。

[0050] 图 20，小图 A 到 D，描绘了在图 19 中描述的 T 细胞群体的结合和活性。图 20A 描绘了不同的 T 细胞群体对来源于 NY-ESO1 靶标的肽的结合，而图 20B 描绘了 T 细胞对 HLA-A2 、NY-ESO1 (MM1S，“A2-ESO-”）骨髓瘤细胞系或者 HLA-A2+、NY-ESO1+(U266，“A2+ESO+”）骨髓瘤细胞系的结合。对 MM1S 细胞系的结合几乎是无法检测到的。然后测试 T 细胞导致适当细胞靶细胞溶解的能力，如用 ^{51}铬释放测定所分析的（图 20C 和图 20D），以及观测到相较于对不相关的细胞，经编辑的 TCR T 细胞对相关靶细胞的细胞溶解增强。关于图 20A、20C 和 20D，未处理的细胞 (UT) 是用●示出的；转移细胞是用正方形示出的；SE 细胞是用▲示出的；CE 细胞是用▼示出的。

[0051] 图 21，小图 A 和 B，描绘了共培养实验中不同的 T 细胞群体对细胞的生长抑制。图 21A 描绘了相较于 U266HLA-A2+、NY-ESO1+ 细胞，HLA-A2-、NY-ESO1- 的不相关 MM1S 细胞的生长抑制。图 21B 显示了在 U266HLA-A2+、NY-ESO1+ 靶标的存在下，经编辑的 T 细胞扩增 2 倍。

[0052] 图 22，小图 A 到 D，是描绘 NY-ESO1T 细胞的同种异体反应性的图解。图 22A 示出了效应物 / 靶标比为 50 :1 时，所指示细胞类型中的细胞溶解百分比。图 22B 示出了所指示细

胞中的 γ-干扰素（γ-IFN)ELISPOT 测定。图 22C 示出了用 NY-ESO-1 特异性肽致敏的所指示细胞中的细胞溶解百分比，而图 22D 示出了当细胞未被致敏时所检测到的细胞溶解。

[0053] 图 23，小图 A 和 B，描绘了用所指示的细胞进行处理的小鼠骨髓中人类多发性骨髓瘤 CD138+ 细胞 (hCD138+) 的百分比（图 23A) 和用所指示的细胞进行处理的小鼠的病理评分 (hCD3+ 渗入）（图 23B)。

[0054] 图 24，小图 A 到 C，描绘了用编码 ZFN 的 mRNA 电穿孔进行的 TCR 编辑。图 24A 描绘了在使用 TRAC 特异性 ZFN mRNA（左图）或者 TRBC 特异性 ZFN mRNA（右图）将 ZFN 电穿孔到淋巴细胞中之后第 5 天或第 20 天时，在所指示剂量处 ZFN 诱导的 TCR 断裂。图 24B 描绘了经处理的细胞的数目增长倍数。用 TRAC 特异性 ZFN 处理的细胞在最左图中示出；用 TRBC-ZFN 处理的细胞在中间图中示出，而对照在最右图中示出。“UT”是指未处理的细胞；“UT+E”是指模拟电穿孔的细胞。图 24C 示出了在刺激之后第 18 天所指示的表面表型的百分比。用 TRAC-ZFN 处理的细胞在最左图中示出；用 TRBC-ZFN 处理的细胞在中间图中示出，而对照在最右图中示出。T 干记忆细胞 (TSCM) 被定义为 CD62L+CD45RA+；T 中枢记忆细胞 (TCM) 被定义为 CD62L+CD45RA-；T 效应记忆细胞 (TEM) 被定义为 CD62L-CD45RA-，且端效应物 (TEMRA) 被定义为 CD62L-CD45RA+。UT：未处理的细胞；UT+E：模拟电穿孔的细胞；GFP：用编码 GFP 的 mRNA 电穿孔的细胞。

[0055] 图 25，小图 A 到 D，描绘了用 ZFN mRNA 电穿孔进行的双重 TCR 编辑。图 25A 示出了用于量化经共处理的细胞的 CD3 阴性部分中 TCR-α 和 TCR-β 完全被编辑的细胞的量的代表性分析。单一 TCR-α 或者 TCR-β 被编辑的细胞（在右图上以正方形示出）的部分被测量为在用外源 TCRα 或者 β 基因进行互补时恢复 CD3 的表面表达的经转导细胞的百分比。CD3 阴性总群体中被完全编辑的细胞的量然后是通过减去两种经单一编辑的细胞的百分比来计算的。图 25B 是示出了在用包含专性的异源二聚体 FokI 域 (ELD 和 KKR) 或者它们的相应直系同源版本 (RDD 和 DRR)、编码 TRAC 特异性 ZFN 和 TRBC 特异性 ZFN 的 mRNA 进行共同电穿孔时 CD3 阴性细胞的百分比的直方图（左小图）。活细胞的百分比（在直方图的顶端上指示）被计算为设门在单线态处的 7-氨基-放线菌素 D(7-AAD) 阴性细胞的百分比。7-AAD 插入到双链核酸中。7-AAD 被活细胞排除，但是能够穿透濒死细胞或者死细胞的细胞膜。图 25B 的右侧小图示出了经编辑的细胞在使用如上所述的 LV 报道基因策略计算的 CD3 阴性部分中的构成。每一柱条的顶部部分示出完全被编辑的百分比（从左至右为 30％、40％和 49％）；每一柱条的中间部分示出 β 链被编辑的细胞的百分比（从左至右的柱条为 6％、44％和 21％）；以及每一柱条的下部部分示出 TCR-α 被编辑的细胞的百分比（从左至右的柱条为 64％、16％和 30％）。图 25C 示出了在刺激之后第 18 天 T 细胞的表面表型。示出了四种表型：每一柱条最底部的部分示出定义为 CD62L+CD45RA+ 的干记忆细胞 (TSCM)；每一柱条上从底部往上第二部分示出被定义为 CD62L+CD45RA- 的 T 中枢记忆细胞 (TCM)；每一柱条上从顶端往下第二部分示出被定义为 CD62L-CD45RA- 的 T 效应记忆细胞 (TEM)；以及每一柱条的最顶部部分示出被定义为 CD62L-CD45RA+ 的端效应细胞 (TEMRA)。“UT”是指未处理的细胞。图 25D 示出用指定剂量的 TRAC- 特异性 ZFN mRNA 和 TRBC 特异性 ZFN mRNA 共同电穿孔的 T 细胞的生长曲线。

具体实施方式

[0056] 本文公开了靶向 TCR 基因的锌指核酸酶 (ZFN) 和 TALEN(TCR-ZFN 和 TCR-TALEN)。这些核酸酶有效地例如在 TCR 编码区中预先确定的位点处导致双链断裂 (DSB)。在编码 TCR 基因的基因中 ZFN 或者 TALEN 介导的位点特异性双链断裂 (DSB) 的导入可导致人类细胞 (包括人类 T 细胞) 中的内源 TCR 复合物的特异性和永久的断裂。这些细胞可通过选择 CD3(-) 细胞,然后在 IL7 和 IL15 上培养细胞而从细胞池中选取。此外,本文公开了用于以所选择的 TCR 转基因,经由随机整合或者定向靶向整合取代内源 TCR 基因的方法和组合物。

[0057] 概要

[0058] 除非另有说明,否则本方法的实践,以及本文公开的组合物的制备与应用采用本领域技术范围内的分子生物学、生物化学、染色质结构和分析、计算化学、细胞培养、重组 DNA 和相关领域中的常规技术。这些技术在文献中已有充分描述。参见,例如,Sambrook 等人,MOLECULAR CLONING :A LABORATORY MANUAL,第二版,Cold Spring Harbor Laboratory Press,1989 和第三版,2001 ;Ausubel 等人, URRENT PROTOCOLS IN MOLECULAR BIOLOGY, John Wiley&Sons,New York,1987 及其定期更新 ;METHODS IN ENZYMOLOGY 系列丛书, Academic Press,San Diego ;Wolffe,CHROMATIN STRUCTURE AND FUNCTION, 第三版, Academic Press,San Diego,1998 ;METHODS IN ENZYMOLOGY,第 304 卷,“Chromatin”(P. M.Wassarman 和 A.P.Wolffe 编辑), Academic Press,San Diego,1999 ; 以及 METHODS IN MOLECULAR BIOLOGY,第 119 卷,“Chromatin Protocols”(P.B.Becker 编辑), Humana Press,Totowa,1999。

[0059] 定义

[0060] 术语“核酸”、“多核苷酸”和“低聚核苷酸”是可互换使用的,并且是指脱氧核糖核苷酸或核糖核甘酸聚合物,可以是直链或环状构型的,且是单链或双链形式的。出于本公开内容的目的,这些术语不构成对聚合物长度的限制。术语可涵盖天然核苷酸的已知类似物,以及在碱基、糖和 / 或磷酸部分中被修饰的核苷酸 (例如,硫代磷酸主链)。一般说来,特定核苷酸的类似物具有相同的碱基配对特异性 ;即 A 的类似物将与 T 进行碱基配对。

[0061] 术语“多肽”、“肽”和“蛋白质”是可互换使用用于指氨基酸残基的聚合物。该术语还适用于其中一个或多个氨基酸是相应的天然存在的化学类似物或者经修饰衍生物的氨基酸聚合物。

[0062] “结合”是指大分子之间 (例如,蛋白质和核酸之间) 的序列特异性、非共价相互作用。不要求结合相互作用的所有组分都是序列特异性 (例如,与 DNA 主链中的磷酸残基接触),只要相互作用整体上是序列特异性即可。此类相互作用一般特征为 $10^{-6}M^{-1}$ 或者更低的电离常数 (K_d)。“亲合力”是指结合强度 ;增大的结合亲合力与降低的 K_d 有关。

[0063] “结合蛋白”是能够非共价地结合到另一分子的蛋白。结合蛋白能够结合到例如 DNA 分子 (DNA 结合蛋白)、RNA 分子 (RNA 结合蛋白) 和 / 或蛋白分子 (蛋白结合蛋白)。在蛋白结合蛋白的情况下,蛋白结合蛋白可结合到其自身 (以形成同源二聚体、同源三聚体等) 和 / 或蛋白结合蛋白可结合到一个或多个不同蛋白的一个或多个分子。结合蛋白可具有一种以上类型的结合活性。例如,锌指蛋白具有 DNA 结合、RNA 结合和蛋白结合活性。

[0064] “锌指 DNA 结合蛋白”(或者结合域) 是蛋白质或者较大蛋白质中的域,其经由一个或多个锌指以序列特异性方式结合 DNA,锌指是其结构经由锌离子的配位作用而稳定化的结合域中的氨基酸序列区域。术语锌指 DNA 结合蛋白往往缩写为锌指蛋白或者 ZFP。

[0065] “TALE DNA-结合域”或者“TALE”是包括一个或多个TALE重复域/单位的多肽。重复域是在TALE结合到其同源靶DNA序列中所涉及的。单一“重复单元”(也被称为“重复”)通常是33-35个氨基酸长,并且表现出与天然存在的TALE蛋白质中的其它TALE重复序列的至少一些序列同源性。TALEN优选地包括C端和/或N端截断(例如,C帽和/或N帽)。参见,例如美国专利号8,586,526,在此通过引用整体并入。

[0066] 锌指结合域和TALE结合域可“经工程化”以结合到预定的核苷酸序列,例如经由天然存在的锌指或者TALE蛋白的识别螺旋区域的工程化(改变一个或多个氨基酸)。因此,工程化DNA结合蛋白(锌指或者TALE)是非天然存在的。工程化DNA结合蛋白的方法的非限制性实例是设计和选择。经设计的DNA结合蛋白是非天然存在的,经设计的DNA结合蛋白的设计/组合物结果主要是根据合理性准则。设计的合理性准则包括取代规则和计算机算法的应用,计算机算法用于处理存储现有ZFP和/或TALE设计和结合数据的数据库中的信息。参见,例如美国专利号8,586,526;6,140,081;6,453,242;和6,534,261;以及参见WO 98/53058;WO 98/53059;WO 98/53060;WO 02/016536和WO 03/016496。

[0067] “被选择的”锌指蛋白或者TALE是在自然界中不存在的蛋白质,蛋白质的产生主要来源于经验过程,诸如噬菌体展示技术、相互作用陷阱或者杂交体选择。参见例如U.S.8,586,526;U.S.5,789,538;US 5,925,523;US 6,007,988;US 6,013,453;US 6,200,759;WO 95/19431;WO 96/06166;WO 98/53057;WO 98/54311;WO 00/27878;WO 01/60970;WO 01/88197;WO 02/099084。

[0068] 术语“序列”是指任何长度的核苷酸序列,该序列可为DNA或者RNA;可为直链的、环状的或者支链的并且可为单链或者双链的。术语“供体序列”是指插入到基因组中的核苷酸序列。供体序列可为任何长度,例如在2个和10,000个核苷酸长度之间(或者范围之间或者高于该范围的任何整数值),优选地在约100个和1,000个核苷酸长度之间(或该范围之间的任何整数),更优选地在约200个和500个核苷酸长度之间。

[0069] “同源但不同一的序列”是指第一序列与第二序列共享一定程度的序列同一性,但是第一序列的序列并不与第二序列的序列同一。例如,包括突变基因的野生型序列的多核苷酸与突变基因的序列是同源但不同一的。在某些实施方案中,两个序列之间的同源性程度足以允许利用正常的细胞机理进行它们之间的同源重组。两个同源但不同一的序列可为任意长度,并且它们的非同源程度可低至单一核苷酸(例如,对于通过靶向同源重组来校正基因组点突变来说)或者高至10kb或者更高(例如,对于将基因插入染色体中预定异常位点来说)。对包含同源但不同一序列的两个多核苷酸不要求为相同长度。例如,可使用20和10,000个之间的核苷酸或核苷酸对的外源多核苷酸(即,供体多核苷酸)。

[0070] 用于确定核酸和氨基酸序列同一性的技术是本领域中已知的。通常,此类技术包括确定某基因mRNA的核苷酸序列和/或确定由其编码的氨基酸序列,并将这些序列与第二核苷酸或氨基酸序列比较。也可以此方式确定并比较基因组序列。一般说来,同一性分别是指两条多核苷酸或多肽序列的核苷酸与核苷酸或氨基酸与氨基酸的确切对应性。可通过测定两个或多个序列(多核苷酸或氨基酸)的同一性百分比来比较这些序列。无论是核酸还是氨基酸序列,两个序列的同一性百分比是两个比对序列之间的确切匹配数目除以较短序列的长度并将结果乘以100。核酸序列的近似比对由Smith和Waterman,Advances in Applied Mathematics,2:482-489(1981)的局部同源算法提供。该算法可通过使用

Dayhoff，Atlas of Protein Sequences and Structure)，M.O.Dayhoff 编，增刊第 5 版，3 :353-358，National Biomedical Research Foundation，Washington，D.C.，USA 开发和通过 Gribskov，Nucl.Acids Res.14(6) :6745-6763(1986) 归一化的评分矩阵应用于氨基酸序列。测定序列同一性百分比的本算法的示例性实施由威斯康星州麦迪逊的遗传计算机公司 (Genetics Computer Group，Madison，WI) 在“BestFit”应用中提供。该方法的默认参数在 WisconsinSequence Analysis Package Program Manual，第 8 版 (1995)(购自威斯康星州麦迪逊的遗传计算机公司）中描述。本公开内容中建立同一性百分比的优选方法是使用由 John F.Collins 和 Shane S.Sturrok 开发，由爱丁堡大学版权所有，并由加州芒廷维尤的智慧遗传公司 (IntelliGenetics，Inc.，Mountain View，CA) 分销的 MPSRCH 程序包。这套程序包可使用 Smith-Waterman 算法，其中评分表使用默认参数（例如，空位开放罚 12 分、空位延伸罚 1 分，以及一个空位罚 6 分）。产生“匹配”值的数据反映了序列同一性。计算序列之间同一性百分比或类似性百分比的其它合适程序一般是本领域已知的，例如另一比对程序是 BLAST，使用默认参数。例如，BLASTN 和 BLASTP 可以使用以下默认参数 :遗传密码＝标准 ;过滤器＝无 ;链＝两条 ;阈值＝ 60 ;预期值＝ 10 ;矩阵＝ BLOSUM62 ;描述＝ 50 个序列 ;分选标准＝高评分 ;数据库＝非冗余，GenBank+EMBL+DDBJ+PDB+GenBank CDS 翻译 +Swiss 蛋白 +Spupdate+PIR。这些程序的细节可在以下互联网网址上找到 :http ://www.ncbi.nlm.gov/cgi-bin/BLAST。考虑本文所述的序列，所需的序列同一性程度的范围为约 80％到 100％以及其中任何整数值。通常，序列之间的同一性百分比为至少 70-75％，优选地 80-82％，更优选地 85-90％，甚至更优选地 92％，更加优选地 95％，以及最优选地 98％的序列同一性。

[0071] 或者，多核苷酸之间的序列相似性程度可以通过多核苷酸在允许同源区域间形成稳定双链体的条件下杂交，然后用单链特异性核酸酶消化并确定消化后片段的大小来确定。当按上述方法确定得到两个核酸或两个多核苷酸序列在分子的限定长度上表现至少约 70％ -75％，优选地 80％ -82％，更优选地 85％ -90％，甚至更优选地 92％，更加优选地 95％，以及最优选地 98％的序列同一性时，序列彼此间基本同源。如本文所用，基本同源也指序列与特定的 DNA 或多肽序列显示完全的同一性。基本同源的 DNA 序列可在 Southern 杂交实验中识别，例如，以在对特定系统限定的严格条件下。本领域技术人员能限定适当的杂交条件。参见例如，Sambrook 等人，同上 ;Nucleic Acid Hybridization :A Practical Approach，B.D.Hames 和 S.J.Higgins 编，(1985)Oxford ;Washington，DC ;IRL Press)。

[0072] 可以如下确定两个核酸片段的选择性杂交。两个核酸分子之间的序列同一性程度影响这两个分子之间杂交事件的效率和强度。部分同一的核酸序列将至少部分抑制与靶分子完全同一的序列的杂交。可使用本领域熟知的杂交测定（例如，Southern(DNA) 印迹，Northern(RNA) 印迹，溶液杂交等，参见 Sambrook 等人，Molecular Cloning :A Laboratory Manual，第二版，(1989)Cold Spring Harbor，N.Y.）评价对完全同一序列的杂交抑制。可使用不同程度的选择性，例如，使用从低到高严格性的不同条件，进行此类测定。如果应用低严格性条件，可使用甚至缺乏部分程度序列同一性的辅助探针（例如，与靶分子具有小于约 30％序列同一性的探针）评价不存在非特异性结合，以使得在不存在非特异性结合事件时，辅助探针将不会与靶标杂交。

[0073] 当利用基于杂交的检测系统时，选择与参考核酸序列互补的核酸探针，然后通过

选择合适条件，探针和参考序列彼此选择性杂交或结合以形成双链分子。能在适当严格杂交条件下选择性与参考序列杂交的核酸分子通常在能检测长度至少约 10-14 核苷酸的靶核酸序列的条件下杂交，靶核酸序列与所选的核酸探针的序列具有至少约 70％的序列同一性。严格杂交条件通常能检测长度至少约 10-14 核苷酸的靶核酸序列，靶核酸序列与所选的核酸探针的序列具有大于约 90-95％的序列同一性。用于探针 / 参考序列杂交的杂交条件可由本领域已知方法确定，其中探针和参考序列具有特定程度的序列同一性（参见例如，Nucleic Acid Hybridization :A Practical Approach，B. D. Hames 和 S. J. Higgins 编，(1985)Oxford ;Washington, DC ;IRL Press)。

[0074] 杂交条件是本领域技术人员熟知的。杂交严格性是指杂交条件不利于包含错配核苷酸的杂交体形成的的程度，同时较高严格性与错配杂交体的较低耐受性相关。影响杂交严格性的因素是本领域技术人员熟知的，其包括但不限于温度、pH、离子强度和有机溶剂如例如甲酰胺和二甲亚砜的浓度。如本领域技术人员已知的，杂交严格性随温度升高、离子强度降低和溶剂浓度降低而增加。

[0075] 关于杂交的严谨性条件，本领域熟知的许多等价条件可以用于通过改变例如下列因素来建立特定严谨性 :序列的长度和特性、不同序列的碱基组成、盐和其它杂交液组分的浓度、杂交液中存在或不存在封闭剂（例如，硫酸葡聚糖和聚乙二醇）、杂交反应温度和时间参数、以及改变洗涤条件。根据本领域的标准方法选择一组特定的杂交条件（参见，例如，Sambrook 等人，Molecular Cloning :A Laboratory Manual，第二版，(1989)Cold Spring Harbor, N. Y.)。

[0076] “重组”是指两个多核苷酸之间交换遗传信息的过程。出于本公开的目的，“同源重组 (HR)”是指发生这种交换的特定形式，例如在修复细胞内双链断裂期间发生。该过程要求核苷酸序列同源性，使用“供体”分子作为模板修复“靶”分子（即，经历双链断裂的分子），且该过程也称作“非交叉基因转化”或“短道基因转化”，因为其导致遗传信息从供体向靶标转移。不希望受限于任何特定理论，此类转移可以涉及断裂的靶标与供体间形成的异源双链 DNA 的错配校正，和 / 或采用供体再合成将成为部分靶的遗传信息的“合成依赖性链退火”，和 / 或相关过程。此类特定 HR 通常导致靶分子的序列改变，而使得供体多核苷酸的部分或全部序列被并入靶多核苷酸。

[0077] “切割”是指 DNA 分子共价主链的断裂。切割可以由多种方法启动，该方法包括但不限于磷酸二酯键的酶促或化学水解。单链切割和双链切割都可行，并且双链切割可以是两次不同的单链切割事件的结果。DNA 切割可导致产生钝端或交错末端。在某些实施方案中，将融合多肽用于靶向双链 DNA 切割。

[0078] “切割半域”是能与第二多肽（两者同一或不同）连接形成具有切割活性（优选双链切割活性）的复合物的多肽序列。术语“第一和第二切割半域”;“+ 和 - 切割半域”和“右和左切割半域”可互换使用以指二聚化的成对切割半域。

[0079] “工程化切割半域”是已经被修饰以便与另一切割半域（例如，另一工程化切割半域）形成专性杂二聚体的切割半域。还参见，美国专利号 7,888,121 ;7,914,796 ;8,034,598 ;8,623,618 和美国专利公开号 2011/0201055，这些专利在此通过引用整体并入。

[0080] 术语“序列”是指任何长度的核苷酸序列，核苷酸序列可为 DNA 或者 RNA ;可为直

链，环状或支链的，以及可为单链或双链。术语“供体序列”是指插入基因组中的核苷酸序列。供体序列可为任何长度，例如在 2 和 10,000 个核苷酸长度之间（或者该范围间或超过该范围的任何整数值），优选地在约 100 和 1,000 个核苷酸长度之间（或其间的任何整数），更优选地在约 200 和 500 个核苷酸长度之间。

[0081] “染色质”是包含细胞基因组的核蛋白结构。细胞染色质包含核酸和蛋白，核酸主要为 DNA，蛋白包括组蛋白和非组蛋白染色体蛋白。大部分真核细胞染色质以核小体形式存在，其中核小体核心包含与八聚体关联的约 150 个碱基对的 DNA，八聚体包含组蛋白 H2A、H2B、H3 和 H4 各两份；以及在核小体核心之间延伸的接头 DNA（长度根据生物体而各有不同）。组蛋白 H1 分子通常与接头 DNA 关联。出于本公开内容的目的，术语“染色质”意在涵盖所有类型的细胞核蛋白，包括原核的与真核的。细胞染色质包括染色体和附加体的染色质。

[0082] “染色体”是包含细胞的全部或部分基因组的染色质复合物。细胞的基因组通常以其核型为特征，该核型是包含细胞基因组的全部染色体的集合。细胞的基因组可包含一个或多个染色体。

[0083] “附加体”是复制的核酸、核蛋白复合物或其它包含并非细胞染色体核型部分的核酸的结构。附加体的实例包括质粒和某些病毒基因组。

[0084] “可进入区”是细胞染色质中的位点，其中核酸中存在的靶位点可被识别靶位点的外源分子结合。不希望受任何具体理论的限制，据信可进入区是不包装在核小体结构中的区域。可进入区的不同结构往往可通过其对化学和酶性探针如核酸酶的敏感度来检测。

[0085] “靶位点”或“靶序列”是限定结合分子将结合的核酸部分的核酸序列，前提是存在结合的充分条件。例如，序列 5’-GAATTC-3’ 是 Eco RI 限制性内切核酸酶的靶位点。

[0086] “安全港基因座”是基因组中可用于整合外源核酸的位置。将外源核酸添加到这些安全港基因座中不会通过单独添加 DNA 而对宿主细胞生长造成任何显著的影响。安全港基因的非限制性实例包括例如 CCR5 基因、CXCR4 基因、PPP1R12C（也被称为 AAVS1）基因、白蛋白基因或者 Rosa 基因。参见例如，美国专利号 7,951,925 和 8,110,379；美国公开号 201000218264；20100291048；20120017290；20110265198；20130137104；20130122591；20130177983 和 20130177960。

[0087] “外源”分子是通常不存在于细胞内的分子，但可通过一种或多种遗传、生化或其它方法导入细胞。“通常存在于细胞内”是对于细胞的具体发育阶段和环境条件而确定的。因此，例如，仅在肌肉的胚胎发育期间存在的分子对于成体肌肉细胞是外源分子。类似地，通过热激诱导的分子对于未热激细胞是外源分子。外源分子可包含例如功能失常的内源分子的功能性形式或者正常功能的内源分子的功能失常形式。

[0088] 除了别的以外，外源分子可以是小分子或大分子等，小分子如由组合化学方法所产生，大分子如蛋白质、核酸、碳水化合物、脂质、糖蛋白、脂蛋白、多糖、上述分子的任何经修饰衍生物，或者是包含一个或多个上述分子的任何复合物。核酸包括 DNA 和 RNA，可以是单链或双链，可以是直链、支链或环状；且可以是任意长度。核酸包括那些能形成双链体以及形成三链体的核酸。参见例如，美国专利号 5,176,996 和 5,422,251。蛋白质包括但不限于 DNA 结合蛋白、转录因子、染色质重塑因子、甲基化 DNA 结合蛋白、聚合酶、甲基化酶、去甲基化酶、乙酰基转移酶、脱乙酰基酶、激酶、磷酸酶、整合酶、重组酶、连接酶、拓扑异构酶、促

旋酶和解旋酶。

[0089] 外源分子可以是与内源分子同一类型的分子，例如外源蛋白或核酸。例如，外源核酸可包含感染性病毒基因组、导入细胞内的质粒或附加体，或包含通常不存在于细胞内的染色体。本领域技术人员已知将外源分子导入细胞内的方法，包括但不限于脂质介导的转移（即脂质体，包括中性和阳离子脂质）、电穿孔、直接注射、细胞融合、粒子轰击、磷酸钙共沉淀、DEAE- 葡聚糖介导的转移和病毒载体介导的转移。

[0090] 相比之下，“内源”分子是通常存在于特定环境条件下特定发育阶段的特定细胞中的分子。例如，内源核酸可包含染色体，线粒体、叶绿体或其它细胞器的基因组，或天然存在的附加体核酸。其它内源分子可包括蛋白质，例如转录因子和酶。

[0091] “融合”分子是其中两个或更多个亚单位分子相连（优选共价相连）的分子。该亚单位分子可以是同一化学类型的分子，或可以是不同化学类型的分子。第一类融合分子的实例包括但不限于融合蛋白（例如，ZFP 或者 TALE DNA- 结合域与切割域之间的融合体）和融合核酸（例如，编码前述融合蛋白的核酸）。第二类融合分子的实例包括但不限于：形成三链体的核酸与多肽之间的融合体，以及小沟结合子与核酸之间的融合体。

[0092] 细胞内融合蛋白的表达可由向细胞递送融合蛋白或通过向细胞递送编码融合蛋白的多核苷酸引起，其中该多核苷酸被转录，且转录物经翻译产生该融合蛋白。细胞内蛋白的表达中也可涉及反式剪接、多肽切割和多肽连接。向细胞递送多核苷酸和多肽的方法在本公开内容中另有描述。

[0093] 出于本公开内容的目的，“基因”包括编码基因产物（见前文）的 DNA 区域，以及调节基因产物生成的所有 DNA 区域，不论这类调节序列是否毗邻编码和 / 或转录序列。因此，基因包括但未必限于：启动子序列，终止子，翻译调节序列如核糖体结合位点和内部核糖体进入位点、增强子、沉默子、隔离子、边界元件、复制起点、基质附着位点和基因座控制区。

[0094] “基因表达”是指基因所含信息转化成基因产物。基因产物可以是基因的直接转录产物（例如，mRNA、tRNA、rRNA、反义 RNA、核酶、结构 RNA、或任何其它类型的 RNA）或由 mRNA 翻译产生的蛋白。基因产物还包括经过修饰的 RNA 和经过修饰的蛋白，RNA 修饰过程如加帽、聚腺苷酸化、甲基化和编辑，蛋白修饰过程如甲基化、乙酰化、磷酸化、泛素化、ADP- 核糖基化、十四烷基化和糖基化。

[0095] 基因表达的“调整”指基因活性的改变。表达的调整可包括但不限于基因激活和基因抑制。调整还可以是完全的，即其中基因表达被完全钝化或者被激活到野生型水平或更高的水平；或者调节是部分的，其中基因表达被部分地减少，或者被部分地激活到野生型水平的一部分。

[0096] “真核”细胞包括但不限于真菌细胞（如酵母）、植物细胞、动物细胞、哺乳动物细胞和人细胞（如 T 细胞）。

[0097] “受关注区域”是需要结合外源分子的细胞染色质的任意区域，例如，基因，或者基因内的非编码序列，或与基因毗邻的非编码序列。结合可以是用于靶向 DNA 切割和 / 或靶向重组的目的。例如，受关注区域可存在于染色体、附加体、细胞器（例如，线粒体、叶绿体）基因组或感染性病毒基因组。例如，受关注区域可以在基因的编码区内，在转录的非编码区如前导序列、尾随序列或内含子内，或在编码区上游或下游的非转录区内。受关注区域可以是小到单个核苷酸对长度或大到 2,000 个核苷酸对长度，或任意整数值的核苷酸对长度。

[0098] 当两个或多个组分（例如序列元件）并置，且该组分排列成组分都可正常发挥作用并允许组分中至少一种能介导至少一种其它组分发挥作用时，术语“操作性连接(operative linkage)”和“操作性相连(operatively linked)”(或“操作性相连(operably linked)”）互换使用。例如，若转录调节序列控制编码序列应答一种或多种转录调节因子存在或缺失时的转录水平，则转录调节序列如启动子与编码序列操作性连接。转录调节序列通常与编码序列顺式操作性连接，但不需要与其直接毗邻。例如，尽管并非毗连，但增强子仍是与编码序列操作性连接的转录调节序列。

[0099] 对于融合多肽，术语“操作性相连”可指各组分在与其它组分的连接中所发挥的功能与其在未相连时的功能相同。例如，对于其中DNA结合域(ZFP，TALE)与切割域（例如，核酸内切酶域诸如FokI，大范围核酸酶域等）融合的融合多肽，若在融合多肽中DNA结合域部分能结合其靶位点和/或其结合位点，而切割（核酸酶）域能切割靶位点附近的DNA，则DNA结合域与切割域操作性连接。核酸酶域还可表现DNA结合能力（例如，融合到ZFP或者TALE域的核酸酶还可结合到DNA)。类似地，对于其中DNA结合域与激活或者抑制域融合的融合多肽，若在融合多肽中DNA结合域部分能结合其靶位点和/或其结合位点，而激活域能上调基因表达或者抑制域能下调基因表达，则DNA结合域与激活或者抑制域操作性连接。

[0100] 蛋白、多肽或核酸的“功能性片段”是序列与全长蛋白、多肽或核酸不同一但保留全长蛋白、多肽或核酸的相同功能的蛋白、多肽或核酸。功能片段可具有比相应的天然分子更多、更少或相同数量的残基，和/或可含有一个或多个氨基酸或核苷酸置换。确定核酸功能（例如，编码功能、与另一核酸杂交的能力）的方法是本领域中熟知的。类似地，确定蛋白质功能的方法也是熟知。例如，可确定多肽的DNA结合功能，例如通过滤膜结合、电泳迁移率改变或免疫沉淀测定。DNA切割可通过凝胶电泳测定。参见Ausubel等人，同上。可确定蛋白与另一蛋白相互作用的能力，例如，通过免疫共沉淀、双杂交测定或互补分析，既可以是遗传的也可以是生化的。参见例如，Fields等人，(1989)Nature，340:245-246；美国专利号5,585,245和PCT WO 98/44350。

[0101] “载体”能转移基因序列到靶细胞。通常，“载体构建体”、“表达载体”、和“基因转移载体”指能指导受关注基因表达和能转移基因序列到靶细胞的任何核酸构建体。因此，该术语包括克隆、和表达工具，以及整合载体。

[0102] 核酸酶

[0103] 本文描述可用于钝化TCR基因的核酸酶（例如，ZFN或者TALE核酸酶）。核酸酶可为天然存在的或者可为DNA结合域和切割域的嵌合体。显而易见的是，在嵌合体中，组成的DNA结合域和切割域可都为天然存在的，可都为非天然存在的，或者其中一个可为天然存在的而另一个可为非天然存在的。

[0104] 因此，在本文公开的方法中可使用任何核酸酶。例如，天然存在的归巢内切核酸酶和大范围核酸酶具有非常长的识别序列，在统计基础上，这些识别序列的一些可能存在于人类大小的基因组中。示例性归巢内切核酸酶包括I-SceI、I-CeuI、PI-PspI、PI-Sce、I-SceIV、I-CsmI、I-PanI、I-SceII、I-PpoI、I-SceIII、I-CreI、I-TevI、I-TevII和I-TevIII。它们的识别序列是已知的。还参见美国专利号5,420,032；美国专利号6,833,252；Belfort等人(1997)Nucleic Acids Res.，25:3379－3388；Dujon等人(1989)Gene，82:115－118；Perler等人(1994)Nucleic Acids Res.，22:1125-1127；Jasin(1996)

Trends Genet.,12:224-228;Gimble 等人(1996)J.Mol.Biol.,263:163-180;Argast 等人(1998)J.Mol.Biol.,280:345-353,以及新英格兰生物实验室目录。

[0105] 还已经报道了归巢内切核酸酶和大范围核酸酶的特异性可被工程化以结合到非天然的靶位点。参见,例如 Chevalier 等人(2002)Molec.Cell,10:895-905;Epinat 等人(2003)NucleicAcids Res.,31:2952-2962;Ashworth 等人(2006)Nature,441:656-659;Paques 等人(2007)Current Gene Therapy,7:49-66。可在作为整体的核酸酶环境中改变归巢内切核酸酶和大范围核酸酶的 DNA 结合域(即,以使得核酸酶包含相关切割域),或者 DNA 结合域可融合到异源 DNA 结合域(例如,锌指蛋白或者 TALE)或者异源切割域。来源于大范围核酸酶的 DNA 结合域还可表现出 DNA 结合活性。

[0106] 在某些实施方案中,核酸酶包含锌指 DNA 结合域和限制性内切核酸酶域,核酸酶也被称为锌指核酸酶(ZFN)。

[0107] 在其它实施方案中,核酸酶包括工程化 TALE DNA-结合域和核酸酶域(例如,内切核酸酶和/或大范围核酸酶域),核酸酶也被称为 TALEN。已经公开了用于工程化这些 TALEN 蛋白质以与使用者选择的靶序列进行稳健的、位点特异性相互作用的方法和组合物(参见美国专利号 8,586,526)。在一些实施方案中,TALEN 包含内切核酸酶(例如,FokI)切割域或者切割半域。在其它实施方案中,TALE 核酸酶是兆 TAL。这些兆 TAL 核酸酶是包含 TALE DNA-结合域和大范围核酸酶切割域的融合蛋白。大范围核酸酶切割域作为单体具有活性的,并且不需要二聚化来获得活性。(参见 Boissel 等人,(2013)Nucl Acid Res:1-13,doi:10.1093/nar/gkt1224)。此外,核酸酶域还可表现出 DNA 结合功能。

[0108] 在其它实施方案中,核酸酶包括紧密的 TALEN(cTALEN)。这些 TALEN 是将 TALE DNA-结合域连接到 TevI 核酸酶域的单链融合蛋白。融合蛋白可用作被 TALE 区域局限的切割酶,或者可造成双链断裂,取决于 TALE DNA-结合域相对于 TevI 核酸酶域的位置(参见 Beurdeley 等人(2013)Nat Comm:1-8DOI:10.1038/ncomms2782)。任何 TALEN 可与另外的 TALEN(例如,具有一个或多个兆 TAL 的一个或多个 TALEN(cTALEN 或者 FokI-TALEN))结合使用。

[0109] 因此,任何具有唯一靶位点的天然存在或者工程化的核酸酶可用于本文描述的方法。

[0110] A.DNA 结合域

[0111] 本文描述的核酸酶通常包含 DNA 结合域和切割域。本发明实践可用任何 DNA 结合域,包括但不限于锌指 DNA 结合域、TALE DNA-结合域,或者来自大范围核酸酶的 DNA 结合域。

[0112] 在某些实施方案中,采用工程化以用于结合到所选择的序列的锌指结合域。参见,例如,Beerli 等人(2002)Nature Biotechnol.,20:135-141;Pabo 等人(2001)Ann.Rev.Biochem.,70:313-340;Isalan 等人(2001)Nature Biotechnol.,19:656-660;Segal 等人(2001)Curr.Opin.Biotechnol.,12:632-637;Choo 等人(2000)Curr.Opin.Struct.Biol.,10:411-416。类似地,TALE DNA-结合域可工程化以用于结合到所选择的序列。参见,例如 8,586,526。与天然存在的锌指或者 TALE 蛋白相比,工程化的锌指或者 TALE DNA-结合域可具有新的结合特异性。工程化方法包括但不限于合理设计与各种选择类型。合理设计包括例如使用包含三体(或四体)核苷酸序列和个别锌指氨基酸序列的数据库,其中各三体

或四体核苷酸序列与一种或多种结合该特定三体或四体序列的锌指氨基酸序列相关联。参见，例如共同拥有的的美国专利 8,586,526 ;6,453,242 和 6,534,261，在此通过引入整体并入。

[0113] 示例性选择方法包括噬菌体展示和双杂交系统，公开于美国专利 5,789,538 ;5,925,523 ;6,007,988 ;6,013,453 ;6,410,248 ;6,140,466 ;6,200,759 和 6,242,568 ;以及 WO 98/37186 ;WO 98/53057 ;WO 00/27878 ;WO 01/88197 和 GB 2,338,237。锌指结合域结合特异性的增强已经在例如共同拥有的 WO 02/077227 中有所描述。

[0114] 在其它实施方案中，DNA 结合域包括 TALE DNA- 结合域（参见，共同拥有的美国专利号 8,586,526，在此通过引入整体并入）。TALE DNA- 结合域包含一个或多个 TALE“重复单元”。单一“重复单元”（也被称为重复）通常是 33-35 个氨基酸长度，其中结合到 DNA 核苷酸涉及 " 重复单元 " 中的 12 位和 / 或 13 位（被称为高变双残基区或者“RVD”）。“非典型”RVD 是在自然界中罕见存在或者从不存在的 RVD 序列（12 位和 13 位），例如在天然存在的 TALE 蛋白中小于 5%，优选地在天然存在的 TALE 蛋白中小于 2%，以及甚至更优选地在天然存在的 TALE 蛋白中小于 1%。非典型的 RVD 可为非天然存在的。TALE DNA- 结合域优选地包含 C- 帽序列和任选地 N- 帽序列。“帽”序列优选地是在全长 TALE 蛋白中存在的多肽的片段（截断），例如对天然存在的 TALE 蛋白中侧接 TALE 重复域的 C 端区域和 / 或 N 端区域的任何截断。C- 帽可为例如相较于野生型 C 端 TALE 蛋白（野生型 C 端 TALE 蛋白编号为起始于 C-20）的截断，该截断包括但不限于，C-19、C-18、C-17、C-16、C-15、C-14、C-13、C-12、C-11、C-10、C-9、C-8、C-7、C-6、C-5、C-4、C-3、C-2、C-1，递增到 C+1，以及然后递增到 C+2、C+3 等，朝向多肽（例如，C+63，多肽从 C-20 延伸到 C+63，为 83 个氨基酸长度）的 C 端。

[0115] 此外，如在这些和其它参考文献中所公开的，锌指域和 / 或多指锌指蛋白或者 TALE 可使用任何合适的接头序列连接在一起，包括例如长度为 5 个或更多个氨基酸的接头。还参见美国专利号 6,479,626 ;6,903,185 ;以及 7,153,949 来了解长度为 6 个或更多个氨基酸的示例性接头序列。本文所述的蛋白可包括该蛋白的个别锌指之间的合适接头的任意组合。此外，锌指结合域结合特异性的增强已经在例如美国专利号 6,794,136 中有所描述。

[0116] 靶位点的选择 ;ZFP 或者 TALE，以及融合蛋白（和编码融合蛋白的多核苷酸）的设计与构建方法是本领域中的技术人员已知的并且在美国专利号 6,140,081 ;5,789,538 ;6,453,242 ;6,534,261 ;5,925,523 ;6,007,988 ;6,013,453 ;6,200,759 ;WO 95/19431 ;WO 96/06166 ;WO 98/53057 ;WO 98/54311 ;WO 00/27878 ;WO 01/60970 ;WO 01/88197 ;WO 02/099084 ;WO 98/53058 ;WO 98/53059 ;WO 98/53060 ;WO 02/016536 和 WO 03/016496 中详细描述，这些专利的公开内容通过引用整体并入用于所有目的。

[0117] 此外，如在这些和其它参考文献中所公开的，锌指域和 / 或多指锌指蛋白可使用任何合适的接头序列连接在一起，包括例如长度为 5 个或更多个氨基酸的接头。还参见美国专利号 6,479,626 ;6,903,185 ;以及 7,153,949 来了解长度为 6 个或更多个氨基酸的示例性接头序列。本文所述的蛋白可包括该蛋白的个别锌指之间的合适接头的任何组合。

[0118] 或者，DNA 结合域可衍生于核酸酶。例如，已知归巢内切核酸酶和大范围核酸酶的识别序列如 I-SceI、I-CeuI、PI-PspI、PI-Sce、I-SceIV、I-CsmI、I-PanI、I-SceII、

I-PpoI、I-SceIII、I-CreI、I-TevI、I-TevII 和 I-TevIII。还参见美国专利号 5,420,032；美国专利号 6,833,252；Belfort 等人 (1997)Nucleic Acids Res.,25:3379-3388；Dujon 等人 (1989)Gene,82:115-118；Perler 等人 (1994)Nucleic Acids Res.,22:1125-1127；Jasin(1996)Trends Genet.,12:224-228；Gimble 等人 (1996)J.Mol.Biol.,263:163-180；Argastet 等人 (1998)J.Mol.Biol.,280:345-353,以及新英格兰生物实验室目录。此外,可将归巢内切核酸酶和大范围核酸酶的 DNA 结合特异性经工程化以结合到非天然的靶位点。参见,例如 Chevalier 等人 (2002)Molec.Cell,10:895-905；Epinat 等人 (2003)Nucleic Acids Res.,31:2952-2962；Ashworth 等人 (2006)Nature,441:656-659；Pâques 等人 (2007)Current Gene Therapy,7:49-66；美国专利公开号 20070117128。

[0119] 在某些实施方案中,DNA 结合域是工程化锌指蛋白,锌指蛋白通常包含至少一个锌指,但是也可包含多个锌指(例如,2、3、4、5、6 个或更多个锌指)。ZFP 经常包含至少三个锌指。某些 ZFP 包含四个、五个或者六个锌指。包含三个锌指的 ZFP 通常识别包含 9 个或者 10 个核苷酸的靶位点；包含四个锌指的 ZFP 通常识别包含 12 个或者 14 个核苷酸的靶位点；而具有六个锌指的 ZFP 能够识别包含 18 个到 21 个核苷酸的靶位点。ZFP 还可为包含一个或多个调节域的融合蛋白,其中这些调节域可为转录激活域或者转录抑制域。

[0120] 在其它实施方案中,DNA 结合域包括天然存在的或者经工程化的(非天然存在的)TAL 效应 DNA 结合域。参见,例如美国专利号 8,586,526,在此通过引用整体并入。已知黄单胞菌属 (genus Xanthomonas) 的植物病原菌在重要农作物中导致很多疾病。黄单胞菌属的病原性取决于保守的 III 型分泌 (T3S) 系统,该系统向植物细胞内注入超过 25 种不同的效应蛋白。这些注入的蛋白中,有模拟植物转录激活物并操纵植物转录组的转录激活样效应 (TALE)(参见 Kay 等人 (2007)Science,318:648-651 和美国专利公开号 20110239315)。这些蛋白含有 DNA 结合域和转录激活域。最为充分表征的 TALE 之一是来自野油菜黄单胞菌辣椒斑点病致病变种 (Xanthomonas campestgris pv.Vesicatoria) 的 AvrBs3(参见 Bonas 等人 (1989)Mol Gen Genet,218:127-136 和 WO2010079430)。TALE 含有串联重复的集中化域,每一重复含有约 34 个氨基酸,它们是这些蛋白的 DNA 结合特异性的关键。此外,它们含有核定位序列和酸性转录激活域(综述参见 Schornack S 等人 (2006)J Plant Physiol,163(3):256-272)。此外,在致植物病细菌烟草青枯菌 (Ralstonia solanacearum) 中,已发现烟草青枯菌生物变型 (biovar)1 菌株 GMI1000 和生物变型 4 菌株 RS1000 中称为 brg11 和 hpx17 的两个基因与黄单胞菌属 (Xanthomonas) 的 AvrBs3 家族同源(参见 Heuer 等人 (2007)Appl and Envir Micro,73(13):4379-4384)。这些基因在核苷酸序列方面彼此 98.9%同一,但区别为 hpx17 重复域中的 1,575bp 缺失。但是,两种基因产物与黄单胞菌属的 AvrBs3 家族蛋白的序列同一性都低于 40%。本文描述的锌指核酸酶结合在 TCR 基因中。表 5 和表 6(参见实施例 4)描述了已经工程化而结合到人类 TCR 基因中的核苷酸序列的大量锌指结合域。每一行描述了单独的锌指 DNA 结合域。在第一列中示出了每一域的 DNA 靶序列 (DNA 靶位点以大写字母指示；未接触的核苷酸以小写字母指示),以及第二到第五列示出该蛋白中每一锌指 (F1 到 F4 或者 F5 或者 F6) 的识别区域的氨基酸序列(根据螺旋的起点,氨基酸 -1 到 +6)。在第一列中还提供了每一蛋白的标识号。

[0121] 还描述结合到 TCR 基因中的 TALEN。表 14(参见实施例 10)描述了已经工程化而结合到人类 TCR 基因中的核苷酸序列的 TALEN。每一行描述具有包含 RVD 的域的标识号的

单独的 TALE DNA- 结合蛋白。在第一列中示出每一域的 DNA 靶序列 (DNA 靶位点以大写字母指示 ;未接触的核苷酸以小写字母指示)。在第一列中还提供每一蛋白的标识号。

[0122] 如下所述,在某些实施方案中,如表 5 和表 6 中所示的四指或五指结合域,或者如表 14 中所示的 TALE DNA- 结合域被融合到切割半域,诸如例如 IIs 型限制性内切核酸酶 (诸如 FokI) 的切割域。一对此类锌指或者 TALE/ 核酸酶半域融合被用于靶向切割,如例如在美国专利号 8,586,526 和美国公开号 20050064474 中所公开的。

[0123] 对于靶向切割,结合位点的近边可以通过 5 个或更多核苷酸对来分开,并且每一融合蛋白质可结合至 DNA 靶标的相反链。

[0124] 此外,来自这些天然存在的或者工程化核酸酶的域还可被分离并且以不同组合使用。例如,来自天然存在的或者工程化归巢内切核酸酶或者大范围核酸酶的 DNA 结合域可融合到异源切割域或者半域 (例如,来自另一种归巢内切核酸酶、大范围核酸酶或者 TypeIIS 内切核酸酶)。这些融合蛋白还可与如上所述的锌指核酸酶结合使用。

[0125] 本文描述的核酸酶可靶向到任何 TCR 基因组序列中的任何序列。

[0126] B. 切割域

[0127] 核酸酶可包含异源 DNA 结合和切割域 (例如,锌指核酸酶 ;TALEN、大范围核酸酶 DNA 结合域和异源切割域),或者可选地天然存在的核酸酶的 DNA 结合域可被改变而结合到所选定的靶位点 (例如,大范围核酸酶已经被工程化而结合到与同源结合位点不同的位点的)。在某些实施方案中,核酸酶是大范围核酸酶 (归巢内切核酸酶)。天然存在的大范围核酸酶识别 15-40 碱基对的切割位点并且通常分为四个家族 :LAGLIDADG 家族、GIY-YIG 家族、His-Cyst 盒家族与 HNH 家族。示例性归巢内切核酸酶包括 I-SceI、I-CeuI、PI-PspI、PI-Sce、I-SceIV、I-CsmI、I-PanI、I-SceII、I-PpoI、I-SceIII、I-CreI、I-TevI、I-TevII 和 I-TevIII。它们的识别序列是已知的。还参见美国专利号 5,420,032 ;美国专利号 6,833,252 ;Belfort 等人 (1997)Nucleic Acids Res.,25 :3379-3388 ;Dujon 等人 (1989) Gene,82 :115-118 ;Perler 等人 (1994)Nucleic Acids Res.,22 :1125-1127 ;Jasin(1996) Trends Genet.,12 :224-228 ;Gimble 等人 (1996)J.Mol.Biol.,263 :163-180 ;Argastet 等人 (1998)J.Mol.Biol.,280 :345-353,以及新英格兰生物实验室目录。

[0128] 来自天然存在的大范围核酸酶的 DNA 结合域,主要是来自 LAGLIDADG 家族的 DNA 结合域,已在植物、酵母、果蝇、哺乳动物细胞和小鼠中用于促进位点特异基因组修饰,但该方法局限于修饰保留大范围核酸酶识别序列的同源基因 (Monet 等人 (1999), Biochem. Biophysics.Res.Common.,255 :88-93) 或已经导入识别序列预先工程化的基因组 (Route 等人 (1994), Mol.Cell.Biol.,14 :8096-106 ;Chilton 等人 (2003), Plant Physiology., 133 :956-65 ;Puchta 等人 (1996), Proc.Natl.Acad.Sci.USA,93 :5055-60 ;Rong 等人 (2002), Genes Dev.,16 :1568-81 ;Gouble 等人 (2006), J.Gene Med.,8(5) :616-622)。因此,已尝试工程化大范围核酸酶使其在医学或生物技术相关位点呈现新型结合特异性 (Porteus 等人 (2005), Nat.Biotechnol.,23 :967-73 ;Sussman 等人 (2004), J.Mol.Biol., 342 :31-41 ;Epinat 等人 (2003), Nucleic Acids Res.,31 :2952-62 ;Chevalier 等人 (2002)Molec.Cell,10 :895-905 ;Ashworth 等人 (2006)Nature,441 :656-659 ; Pâques 等人 (2007)Current Gene Therapy,7 :49-66 ;美国专利公开号 20070117128 ;20060206949 ; 20060153826 ;20060078552 ;和 20040002092)。此外,天然存在或工程化来自大范围核酸酶

的 DNA 结合域也已和来自异源核酸酶（例如，FokI）的切割域操作性连接。

[0129] 在其它实施方案中，核酸酶是锌指核酸酶（ZFN）。ZFN 包含已经经工程化而结合到所选基因中的靶位点的锌指蛋白，以及切割域或者切割半域。

[0130] 如上所述，锌指结合域可经工程化而结合到所选择的序列。参见例如，Beerli 等人（2002）Nature Biotechnol.，20：135-141；Pabo 等人（2001）Ann. Rev. Biochem.，70：313-340；Isalan 等人（2001）Nature Biotechnol.，19：656-660；Segal 等人（2001）Curr. Opin. Biotechnol.，12：632-637；Choo 等人（2000）Curr. Opin. Struct. Biol.，10：411-416。相较于天然存在的锌指蛋白，工程化锌指结合域可具有新型的结合特异性。工程化方法包括但不限于合理设计与各种选择类型。合理设计包括例如利用包含三体（或四体）核苷酸序列和个别锌指氨基酸序列的数据库，其中各三体或四体核苷酸序列与一种或多种结合该特定三体或四体序列的锌指氨基酸序列相关联。参见，例如美国专利 6,453,242 和 6,534,261，在此通过引用整体并入。

[0131] 示例性选择方法包括噬菌体展示和双杂交系统，公开于美国专利 5,789,538；5,925,523；6,007,988；6,013,453；6,410,248；6,140,466；6,200,759 和 6,242,568；以及 WO 98/37186；WO 98/53057；WO 00/27878；WO 01/88197 和 GB 2,338,237。此外，锌指结合域结合特异性的增强已经在例如美国专利号 6,794,136 中有所描述。

[0132] 靶位点的选择；ZFN，以及融合蛋白（和编码融合蛋白的多核苷酸）的设计与构建方法是本领域中的技术人员已知的并且在美国专利号 7,888,121 和 8,409,861 中详细描述，在此通过引用整体并入。

[0133] 此外，如在这些和其它参考文献中所公开的，锌指域和 / 或多指锌指蛋白可使用任何合适的接头序列连接在一起，包括例如长度为 5 个或更多个氨基酸的接头。例如参见美国专利号 6,479,626；6,903,185；以及 7,153,949 来了解长度为 6 个或更多个氨基酸的示例性接头序列。本文所述的蛋白可包括该蛋白的个别锌指之间的合适接头的任何组合。

[0134] 在一些实施方案中，核酸酶是工程化 TALEN。已经公开用于工程化这些蛋白质以与使用者选择的靶序列进行稳健的、位点特异性相互作用的方法和组合物（参见美国专利号 8,586,526）。

[0135] 核酸酶如 ZFN、TALEN 和 / 或大范围核酸酶还包含核酸酶（切割域，切割半域）。如上所述，切割域可以与 DNA 结合域是异源的，例如锌指或者 TALE DNA- 结合域和来自核酸酶的切割域或大范围核酸酶 DNA 结合域，以及来自另一不同的核酸酶的切割域。异源切割域可获自任何内切核酸酶或外切核酸酶。可衍生出切割域的示例性内切核酸酶，包括但不限于限制性内切核酸酶和归巢内切核酸酶。参见例如，马萨诸塞州贝弗利（Beverly，MA）的 NEB 公司的 2002-2003 产品目录；和 Belfort 等人（1997）Nucleic Acids Res.，25：3379-3388。已知切割 DNA 的其它酶（例如，S1 核酸酶；绿豆核酸酶；胰 DNA 酶 I；微球菌核酸酶；酵母 HO 内切核酸酶；还参见 Linn 等人编，Nucleases，Cold Spring Harbor Laboratory Press，1993）。可将这些酶的一种或多种（或其功能性片段）用作切割域和切割半域的来源。

[0136] 类似地，需要二聚化才具有切割活性的切割半域可衍生自任何核酸酶或其部分，如上所述。一般说来，如果融合蛋白包含切割半域，则切割需要两个融合蛋白。或者，可使用包含两个切割半域的单个蛋白。这两个切割半域可衍生于相同内切核酸酶（或其功能性

片段），或各切割半域可衍生自不同的内切核酸酶（或其功能性片段）。此外，优选地将两种融合蛋白的靶位点相对彼此排列，从而这两种融合蛋白与其各自相应的靶位点的结合将切割半域放置为能使切割半域形成功能性切割域（例如，通过二聚化）的彼此空间定位。因此，在某些实施方案中，这些靶位点的近边是用 5-8 个核苷酸或者用 15-18 个核苷酸分离的。然而，两个靶位点间可间插有任何整数数量的核苷酸或核苷酸对（例如，2 至 50 个核苷酸对或更多）。一般说来，切割位点位于靶位点之间。

[0137] 可将一种或多种这些酶（或其功能性片段）用作切割域和切割半域的来源。类似地，需要二聚化才具有切割活性的切割半域可衍生自任何核酸酶或其部分，如上所述。一般说来，如果融合蛋白包含切割半域，则切割需要两个融合蛋白。或者，可使用包含两个切割半域的单个蛋白。这两个切割半域可衍生自相同内切核酸酶（或其功能性片段），或各切割半域可衍生自不同的内切核酸酶（或其功能性片段）。此外，优选地将两种融合蛋白的靶位点相对彼此排列，从而这两种融合蛋白与其各自相应的靶位点的结合将切割半域放置为能使切割半域形成功能性切割域（例如，通过二聚化）的彼此空间定位。因此，在某些实施方案中，这些靶位点的近边是用 5-8 个核苷酸或者用 15-18 个核苷酸分离的。然而，两个靶位点间可间插有任何整数数量的核苷酸或核苷酸对（例如，2 至 50 个核苷酸对或更多）。一般说来，切割位点位于靶位点之间。

[0138] 限制性内切核酸酶（限制性酶）存在于许多物种，并且能够序列特异性结合 DNA（在识别位点处），并在结合位点处或其附近切割 DNA。某些限制性酶（例如，IIS 型）在从远离识别位点的位点处切割 DNA 并且具有可分离的结合与切割域。例如，IIS 型酶 FokI 催化 DNA 的双链切割，在一条链上距其识别位点 9 个核苷酸处切割，而在另一条链上距其识别位点 13 个核苷酸处切割。参见例如，美国专利 5,356,802 ;5,436,150 和 5,487,994 ;以及 Li 等人 (1992)Proc.Natl.Acad.Sci.USA,89 :4275-4279 ;Li 等人 (1993)Proc.Natl.Acad.Sci.USA,90 :2764-2768 ;Kim 等人 (1994a)Proc.Natl.Acad.Sci.USA,91 :883-887 ;Kim 等人 (1994b)J.Biol.Chem.,269 :31,978-31,982。因此，在一个实施方案中，融合蛋白包含来自至少一种 IIS 型限制性酶的切割域（或切割半域）和一个或多个经或未经工程化的锌指结合域。

[0139] FokI 是示例性 IIS 型限制性酶，其切割域可与结合域分离。该特定的酶为二聚体时有活性。Bitinaite 等人 (1998)Proc.Natl.Acad.Sci.USA,95 :10,570-10,575。因此，出于本发明内容的目的，所公开的融合蛋白中所用的 FokI 酶的部分被视为切割半域。因此，对于使用锌指 -FokI 融合体的靶向双链切割和 / 或靶向细胞序列置换，可使用各自包含某 FokI 切割半域的两种融合蛋白来重建催化活性的切割域。或者，也可使用含有锌指结合域和两个 FokI 切割半域的单一多肽分子。使用锌指 -FokI 融合体进行靶向切割和靶向序列改变的参数在本发明内容中另行提供。

[0140] 切割域或切割半域可以是保留切割活性或保留多聚化（例如，二聚化）能力以形成功能性切割域的蛋白的任何部分。

[0141] 示例性 IIS 型限制性酶在美国专利公开号 20070134796 中有所描述，在此通过引用整体并入。其它的限制性酶还含有可分离的结合与切割域，并且这些酶在本公开内容中也有所设想。参见例如，Roberts 等人 (2003)Nucleic Acids Res.,31 :418-420。

[0142] 在某些实施方案中，切割域包含一个或多个工程化切割半域（也称作二聚化域突

变体），其将同源二聚作用降至最小或阻止同源二聚作用，例如，如美国专利号 7,888,121；8,409,861；以及美国专利公开号 20080131962 中所描述，所有该专利的公开内容在此通过引用整体并入。在 FokI 的 446、447、479、483、484、486、487、490、491、496、498、499、500、531、534、537 和 538 位的氨基酸残基都是影响 FokI 切割半域二聚化的靶标。

[0143] 形成专性杂二聚体的示例性工程化 FokI 切割半域包括以下一对：第一切割半域包括 FokI 的 490 和 538 位氨基酸残基处的突变，和第二切割半域包括 486 和 499 氨基酸残基处的突变。

[0144] 因此，在某些实施方案中，490 位的突变将 Glu(E) 替换为 Lys(K)；538 位的突变将 Iso(I) 替换为 Lys(K)；486 位的突变将 Gln(Q) 替换为 Glu(E)；而 499 位的突变将 Iso(I) 替换为 Lys(K)。具体地，制备本文所述工程化切割半域是通过在一个切割半域的 490 位突变 (E → K) 和 538 位突变 (I → K) 来产生名为“E490K：I538K”的工程化切割半域，并通过另一切割半域的 486 位突变 (Q → E) 和 499 位突变 (I → L) 来产生名为“Q486E：I499L”的工程化切割半域。本文所述的工程化切割半域是专性杂二聚体突变体，其异常切割降至最低或被消除。参见例如美国专利号 7,888,121，其公开内容通过引用整体并入用于所有目的。

[0145] 本文所述的工程化切割半域是使用任何合适的方法制备的，例如如美国专利号 7,888,121 中所描述的通过野生型切割半域 (Fok I) 的定点突变。

[0146] 本文所述的工程化切割半域可为专性杂二聚体突变体，其异常切割降至最低或被消除。参见例如，WO 07/139898 的实施例 1。在某些实施方案中，工程化切割半域包含在 486、499 和 496 位（根据野生型 FokI 编号）的突变，比如以下突变：将野生型 486 位的 Gln(Q) 残基替换为 Glu(E) 残基，野生型 499 位的 Iso(I) 残基替换为 Leu(L) 残基，以及野生型 496 位的 Asn(N) 残基替换为 Asp(D) 或 Glu(E) 残基（还分别称作“ELD”和“ELE”域）。在其它实施方案中，工程化切割半域包括在 490、538 和 537 位（根据野生型 FokI 编号）的突变，比如以下突变：将野生型 490 位的 Glu(E) 残基替换为 Lys(K) 残基，野生型 538 位的 Iso(I) 残基替换为 Lys(K) 残基，以及野生型 537 位的 His(H) 残基替换为 Lys(K) 或 Arg(R) 残基（还分别称作“KKK”和“KKR”域）。在其它实施方案中，工程化切割半域包括在 490 和 537 位（根据野生型 FokI 编号）的突变，比如以下突变：将野生型 490 位的 Glu(E) 残基替换为 Lys(K) 残基，野生型 537 位的 His(H) 残基替换为 Lys(K) 或 Arg(R) 残基（还分别称作“KIK”和“KIR”域）。本文所述的工程化切割半域可用任何合适的方法制备，例如通过如美国专利号 7,888,121；和美国专利公开号 20080131962；以及 20110201055 所描述的定点诱变野生型切割半域 (FokI) 来制备。

[0147] 或者，核酸酶可使用所谓的“分裂酶 (split-enzyme)”的技术（参见例如，美国专利公开号 20090068164) 在核酸靶位点处体内组装。这类分裂酶的组分可在单独的表达构建体上表达，或者可以连接于个别的组分相互分开的某一开放读框中，例如，组分由自切割 2A 肽或 IRES 序列分开。组分可以是个别的锌指结合域或大范围核酸酶核酸结合域的域。

[0148] 或者，可使用被称为“Sharkey”的 FokI 核酸酶域变体（参见 Guo 等人，(2010) J.Mol.Biol.doi:10.1016/j.jmb.2010.04.060)。

[0149] 可使用本领域已知的方法容易地设计核酸酶表达构建体。参见例如，美国专利号 7,888,121 和 8,409,861，以及美国专利公开号 20030232410；20050208489；20050026157；

20060063231 ;和 20070134796。在某些实施方案中,核酸酶的表达可以在可诱导的启动子的控制下,例如,半乳糖激酶启动子在棉子糖和 / 或半乳糖的存在下被活化(解除抑制)而在葡萄糖存在下被抑制。特别地,半乳糖激酶启动子在碳源连续改变时(例如,从葡萄糖到棉子糖到半乳糖)被诱导并且表达核酸酶。可诱导启动子的其它非限制性实例包括 CUP1、MET15、PHO5,以及 tet 反应启动子。

[0150] CRISPR(成簇规律间隔短回文重复序列 /Cas(CRISPR 关联的)核酸酶系统是近来基于可用于基因组工程学的细菌系统的工程化核酸酶系统。核酸酶系统是基于许多细菌和古生菌 (archea) 的部分适应性免疫反应。当病毒或者质粒侵入细菌时,侵入物的 DNA 片段通过‘免疫反应’而转变为 CRISPR RNA(crRNA)。此 crRNA 然后经由部分互补的区域而与另一类型的 RNA(被称为 tracrRNA) 关联,从而将 Cas9 核酸酶导向靶 DNA(被称为“前间区序列 (protospacer)”) 中与 crRNA 同源的区域。Cas9 切割 DNA 以在由 crRNA 转录本中含有的 20 个核苷酸的导向序列规定的位点处双链断裂 (DSB) 产生平端。Cas9 需要 crRNA 和 tracrRNA 两者以用于位点特异性 DNA 识别和切割。此系统现已经工程化,而使得 crRNA 和 tracrRNA 可结合成一个分子(“单导向 RNA”),以及单导向 RNA 的 crRNA 等效部分可经工程化以导向 Cas9 核酸酶来靶向任何所需的序列(参见 Jinek 等人 (2012)Science,337 :第 816-821 页 ;Jinek 等人 (2013),eLife,2 :e00471 ;以及 David Segal(2013)eLife,2 :e00563)。因此,CRISPR/Cas 系统可经工程化以在基因组中所需的靶标处引起 DSB,以及 DSB 的修复可受到修复抑制剂使用的影响而导致错误倾向修复增强。

[0151] A. 靶位点

[0152] 如上文详细描述的,ZFN 和 TALEN 中的 DNA 域可经工程化而结合到基因座中任何所选的序列。相较于天然存在的 DNA 结合域,工程化 DNA 结合域可具有新型的结合特异性。工程化方法包括但不限于合理设计与各种选择类型。合理设计包括例如利用包含三体(或四体)核苷酸序列和个别(例如,锌指)氨基酸序列的数据库,其中各三体或四体核苷酸序列与结合该特定三体或四体序列的 DNA 结合域的一种或多种氨基酸序列相关联。参见,例如美国专利 8,586,526 ;6,453,242 和 6,534,261,在此通过引用整体并入。还可进行 TAL 效应域的合理设计。参见,例如美国专利号 8,586,526。

[0153] 适用于 DNA 结合域的示例性选择方法包括噬菌体展示和双杂交系统,公开于美国专利 5,789,538 ;5,925,523 ;6,007,988 ;6,013,453 ;6,410,248 ;6,140,466 ;6,200,759 和 6,242,568 ;以及 WO 98/37186 ;WO 98/53057 ;WO 00/27878 ;和 WO 01/88197。

[0154] 靶位点的选择 ;核酸酶,以及融合蛋白(和编码融合蛋白的多核苷酸)的设计与构建方法是本领域中的技术人员已知的并且在美国专利号 7,888,121 和 8,409,861 中详细描述,在此通过引用整体并入。

[0155] 此外,如在这些和其它参考文献中所公开的,DNA 结合域(例如,多指锌指蛋白)可使用任何合适的接头序列连接在一起,包括例如长度为 5 个或更多个氨基酸的接头。例如参见美国专利号 6,479,626 ;6,903,185 ;以及 7,153,949 来了解长度为 6 个或更多个氨基酸的示例性接头序列。本文所述的蛋白可包括该蛋白的个别 DNA 结合域之间的合适接头的任何组合。还参见,美国专利号 8,586,526。

[0156] 另外,单导向 RNA 可通过本领域熟知的用于产生特异 RNA 序列的方法经工程化以结合到基因组中所选择的靶标。这些单导向 RNA 被设计成将 Cas9 导向到任何选定的靶位

点。

[0157] 供体

[0158] 如上所述，还可进行外源序列（也被称为“供体序列”或“供体”）的插入，以例如用于校正突变基因或者用于增加野生型基因的表达。将容易显而易见的是供体序列通常与其所位于的基因组序列并非同一的。供体序列可包含侧接于两个同源区域的非同源序列，以允许在受关注位置处进行有效 HDR。另外，供体序列可包含含有与细胞染色质中受关注区域并非同源的序列的载体分子。供体分子可包含若干与细胞染色质同源的不连续区域。例如，对于通常在受关注区域中不存在的序列的靶向插入，序列可存在于供体核酸分子中并且侧接于受关注区域中的序列同源的多个区域。或者，供体分子可通过非同源的端连接 (NHEJ) 机制而被整合到经切割的靶基因座中。参见例如，美国专利公开号 20110207221 和 20130326645。

[0159] 供体多核苷酸可为 DNA 或者 RNA，单链或者双链，以及可被以直链或环状形式导入细胞中。参见例如，美国专利公开号 20100047805 ;20110281361 ;以及 20110207221。如果以直链形式导入，则可通过本领域中的技术人员已知的方法来保护供体序列的末端（例如，防止被外切核酸降解）。例如，可将一个或多个双脱氧核苷酸残基添加到直链分子的 3’端和 / 或将自补的低聚核苷酸连接到一个或者两个末端。参见例如，Chang 等人 (1987)Proc. Natl. Acad. Sci. USA，84 :4959-4963 ;Nehls 等人 (1996)Science，272 :886-889。用于保护外源多核苷酸不被降解的其它方法包括但不限于添加端氨基，以及使用经修饰的核苷酸间连接，如例如硫代磷酸、氨基磷酸酯和邻甲基核糖或者脱氧核糖残基。

[0160] 可将多核苷酸作为具有附加序列的载体分子的组成部分而导入细胞中，附加序列为如例如复制起点、启动子和编码抗生素抗性的基因。而且，供体多核苷酸可作为裸核酸导入，作为与药剂如脂质体或者泊洛沙姆复合的核酸导入，或者可用病毒（例如，腺病毒、AAV、疱疹病毒、逆转录病毒、慢病毒，以及整合酶缺陷的慢病毒 (IDLV)）递送。

[0161] 一般插入供体以使得在整合位点处由内源启动子驱动供体的表达，即供体插入到驱动内源基因表达的启动子（例如，AAVS1、CCR5、白蛋白、HPRT 等）。然而，将显而易见的是，供体可包含启动子和 / 或增强子，例如组成型启动子或者可诱导的启动子或者组织特异性启动子。

[0162] 可将供体分子插入内源基因中，以使得所有、一些或者没有内源基因被表达。例如，可将如本文所述的转基因插入内源基因座，以使得一些（连接到转基因的 N 端和 / 或 C 端）或者没有内源序列例如作为与转基因的融合体而被表达。在其它实施方案中，将转基因（例如，有或者没有如编码内源基因的附加编码序列）整合到任何内源基因座例如安全港基因座中。

[0163] 当内源序列（内源的转基因或者作为转基因的组成部分）与转基因一起表达时，内源序列可为全长序列（野生型或者突变型）或者部分序列。优选地内源序列是功能性的。这些全长或者部分序列的功能的非限制性实例包括用转基因（例如，治疗基因）表达的多肽的血清半衰期延长和 / 或充当载体。

[0164] 另外，虽然不要求表达，但是外源序列也可包含转录或者翻译调节序列，例如启动子、增强子、隔离子、内部核糖体进入位点、编码 2A 肽和 / 或多腺苷酸化信号的序列。

[0165] 递送

[0166] 可用任何合适的手段将本文描述的组合物（例如，ZFP、TALE、CRISPR/Cas）、编码组合物的多核苷酸、任何供体多核苷酸递送到包含 TCR 基因的靶细胞中。用于递送包含 DNA 结合域的组合物的方法在例如美国专利号 6,453,242 ;6,503,717 ;6,534,261 ;6,599,692 ;6,607,882 ;6,689,558 ;6,824,978 ;6,933,113 ;6,979,539 ;7,013,219 ;以及 7,163,824 中有所描述，这些公开内容在此通过引用整体并入。

[0167] 还可使用包含编码锌指、TALE 或者 CRISPR/Cas 蛋白中的一种或多种的序列的载体来递送如本文所描述的锌指、TALE 或者 CRISPR/Cas 蛋白。也可类似地递送编码供体的多核苷酸。可使用任意载体系统，包括但不限于质粒载体、逆转录病毒载体、慢病毒载体、腺病毒载体、痘病毒载体 ;疱疹病毒载体以及腺相关病毒载体等。还参见美国专利号 6,534,261 ;6,607,882 ;6,824,978 ;6,933,113 ;6,979,539 ;7,013,219 ; 和 7,163,824，在此通过引用整体并入。另外，将显而易见的是，这些载体中的任一个可包含一种或多种锌指蛋白编码序列、一种或多种 CRISPR/Cas 编码序列或者一种或多种 TALE 编码序列。因此，当将一种或多种核酸酶或者核酸酶系统和 / 或供体导入细胞时，核酸酶或者核酸酶系统和 / 或供体可在相同载体上携带或者在不同载体上携带。当使用多个载体时，每一载体可包含编码一种或多种 ZFP、TALE、CRISPR/Cas 系统和 / 或供体的序列。

[0168] 可以使用传统的基于病毒和非病毒的基因转移方法来将编码工程化 ZFP、TALE、CRISPR/Cas 和 / 或供体的核酸导入细胞（例如，哺乳动物细胞）和靶组织中。此类方法还可用来体外施用编码 ZFP、TALE、CRISPR/Cas 和 / 或供体的核酸于细胞中。在某些实施方案中，编码 ZFP、TALE、CRISPR/Cas 和 / 或供体的核酸可以施用于体内或离体基因治疗用途。非病毒载体递送系统包括 DNA 质粒、裸核酸，以及与递送工具（诸如，脂质体或者泊洛沙姆）复合的核酸。病毒载体递送系统包括 DNA 病毒和 RNA 病毒，这两种病毒在递送到细胞后具有附加体或整合的基因组。关于基因治疗方法的概述参见 Anderson, Science, 256 : 808-813(1992) ;Nabel&Felgner, TIBTECH, 11 :211-217(1993) ;Mitani&Caskey, TIBTECH, 11 :162-166(1993) ;Dillon, TIBTECH, 11 :167-175(1993) ;Miller, Nature, 357 : 455-460(1992) ;Van Brunt, Biotechnology, 6(10) :1149-1154(1988) ;Vigne, Restorative Neurology and Neuroscience, 8 :35-36(1995) ;Kremer&Perricaudet, British Medical Bulletin, 51(1) :31-44(1995) ;Haddada 等人，in Current Topics in Microbiology and Immunology, Doerfler 和 Böhm 编 (1995) ;以及 Yu 等人，Gene Therapy, 1 :13-26(1994)。

[0169] 核酸的非病毒递送方法包括电穿孔、脂转染、微注射、基因枪、病毒体、脂质体、免疫脂质体、聚阳离子或脂质 :核酸共轭物、裸 DNA、mRNA、人工病毒颗粒以及介质增强 DNA 摄取。使用例如 Sonitron2000 系统 (Rich-Mar) 的声孔效应也可以用于核酸递送。在优选实施方案中，一种或多种核酸是作为 mRNA 递送的。使用加帽的 mRNA 来增加翻译效率和 / 或 mRNA 稳定性也是优选的。ARCA（抗 - 反向帽类似物）帽或者其变体是尤其优选的。参见美国专利 US7074596 和 US8153773，该专利在此以引用方式并入。

[0170] 其它示例性的核酸递送系统包括由 Amaxa® Biosystems 公司（德国科隆 (Cologne, Germany)）、Maxcyte 公司（马里兰罗克威尔 (Rockville, Maryland)）、BTX Molecular Delivery Systems 公司（马萨诸塞州霍利斯顿 (Holliston, MA)）以及 CopernicusTherapeutics 公司（参见例如 US6008336）提供的那些。脂转染描述于例如 US 5,049,386、US 4,946,787 ;以及 US 4,897,355）中，并且脂转染试剂市场上有售（例如，

Transfectam™、Lipofectin™和 Lipofectamine™ RNAiMAX)。适用于多核苷酸有效受体识别脂转染的阳离子和中性脂质包括 Felgner,WO 91/17424、WO 91/16024 中描述的那些。递送可以是到细胞(离体施用)或靶组织(体内施用)。

[0171] 脂质:核酸复合物,包括靶向的脂质体如免疫脂质复合物的制备是本领域中的技术人员熟知的(参见,例如 Crystal,Science,第 270:404-410(1995);Blaese 等人,Cancer Gene Ther.,2:291-297(1995);Behr 等人,Bioconjugate Chem.,5:382-389(1994);Remy 等人,Bioconjugate Chem.,5:647-654(1994);Gao 等人,Gene Therapy,2:710-722(1995);Ahmad 等人,Cancer Res.,52:4817-4820(1992);美国专利号 4,186,183、4,217,344、4,235,871、4,261,975、4,485,054、4,501,728、4,774,085、4,837,028 以及 4,946,787)。

[0172] 其它的递送方法包括使用将待递送的核酸包装至 EnGeneIC 递送工具(EDV)中。这些 EDV 使用双特异性抗体特异性地向靶组织递送,其中该抗体的一个臂具有对于靶组织具有特异性,并且另一个臂具有对于 EDV 的特异性。抗体将 EDV 带入靶细胞表面,然后通过胞吞作用将 EDV 带入细胞中。一旦在细胞中,就将释放内含物(参见 MacDiarmid 等人(2009)Nature Biotechnology,27(7):第 643 页)。

[0173] 使用基于 RNA 病毒或者 DNA 病毒的系统来递送编码工程化 ZFP、TALE 和/或供体的核酸利用将病毒靶向到人体中的特异细胞并且将病毒有效负载运输到细胞核中的高度进化过程。可将病毒载体直接施用于患者(体内),或者病毒可用于体外处理细胞,然后将经修饰的细胞施用于患者(离体)。用于递送 ZFP 的传统的基于病毒的系统包括但不限于用于基因转移的逆转录病毒载体、慢病毒载体、腺病毒载体、腺相关病毒载体、牛痘载体和单纯性疱疹病毒载体。可能用逆转录病毒、慢病毒和腺相关病毒基因转移方法整合入宿主基因组,这常常导致插入的转基因的长期表达。另外,在许多不同细胞类型和靶组织中已经观测到高转导效率。

[0174] 可通过引入外来的包膜蛋白、扩展靶细胞的潜在靶群体来改变逆转录病毒向性(tropism)。慢病毒载体是能够转导或感染非分裂细胞的逆转录病毒载体,并且一般产生高病毒效价。逆转录病毒基因转移系统的选择取决于靶组织。逆转录病毒载体由顺式作用长端重复组成,其包装容量高达 6-10kb 的外来序列。最小的顺式作用 LTR 足以复制和包装载体,然后用 LTR 将治疗基因整合入靶细胞,以提供永久的转基因表达。广泛采用的逆转录病毒载体包括基于鼠白血病病毒(MuLV)、长臂猿白血病病毒(GaLV)、猿免疫缺陷病毒(SIV)、人体免疫缺陷病毒(HIV),以及其组合的那些载体(参见例如 Buchscher 等人,J.Virol.,66:2731-2739(1992);Johann 等人,J.Virol.,66:1635-1640(1992);Sommerfelt 等人,Virol.,176:58-59(1990);Wilson 等人,J.Virol.,63:2374-2378(1989);Miller 等人,J.Virol.,65:2220-2224(1991);PCT/US94/05700)。在优选瞬时表达的应用中,可采用基于腺病毒的系统。基于腺病毒的载体能够在许多细胞类型中非常高效地转导,并且不需要细胞分裂。已经用这种载体获得了高效价和高水平表达。可在相对简单的系统中大量产生此类载体。腺相关病毒(“AAV”)载体也用于以靶核酸转导细胞,例如在体外产生核酸和肽,以及用于体内和离体基因治疗方法(参见例如 West 等人,Virology,160:38-47(1987);美国专利 No.4,797,368;WO 93/24641;Kotin,Human Gene Therapy,5:793-801(1994);Muzyczka,J.Clin.Invest.,94:1351(1994)。许多公开,包括美国专利号 5,173,414;

Tratschin 等人,Mol.Cell.Biol.,5 :3251-3260(1985) ;Tratschin 等人,Mol.Cell.Biol.,4 :2072-2081(1984) ;Hermonat 和 Muzyczka, PNAS,81 :6466-6470(1984) ;和 Samulski 等人,J.Virol.,63 :03822-3828(1989) 中描述了重组 AAV 载体的构建。

[0175] 在临床试验中,目前至少有六种病毒载体方式可用于基因转移,它们利用的方法涉及用插入辅助细胞系的基因补充缺陷载体,以产生转导物质。

[0176] pLASN 和 MFG-S 是已经用于临床试验的逆转录病毒载体的实例 (Dunbar 等人,Blood,85 :3048-305(1995) ;Kohn 等 人,Nat.Med.,1 :1017-102(1995) ;Malech 等 人,PNAS,94 :2212133-12138(1997))。PA317/pLASN 是用于基因治疗试验的第一种治疗载体。(Blaese 等人, Science,270 :475-480(1995))。在 MFG-S 包装载体中观察到的转导效率为 50%或更高。(Ellem 等人,Immunol Immunother.,44(1) :10-20(1997) ;Dranoff 等人,Hum. GeneTher.,1 :111-2(1997)。

[0177] 本文描述的适用于导入多核苷酸的载体还包括非整合的慢病毒载体 (IDLV)。参见例如,Ory 等人 (1996)Proc.Natl.Acad.Sci.USA,93 :11382-11388 ;Dull 等人 (1998) J.Virol.,72 :8463-8471 ;Zufferyet 等人 (1998)J.Virol.,72 :9873-9880 ;Follenzi 等人 (2000)Nature Genetics,25 :217-222 ;美国专利公开号 20090117617。

[0178] 重组腺相关病毒载体 (rAAV) 是有希望的可选的基因递送系统,它基于缺陷型和非致病性细小病毒腺相关型病毒。所有载体都来源于仅保留侧接于转基因表达盒的 AAV 145bp 末端反向重复的质粒。由于整合入转导细胞的基因组中,有效的基因转移和稳定的转基因递送是这种载体系统的关键特征。(Wagner 等人, Lancet,351 :91171702-3(1998) ;Kearns 等人, Gene Ther.,9 :748-55(1996))。还可根据本发明使用其它 AAV 血清型,包括 AAV1、AAV3、AAV4、AAV5、AAV6 和 AAV8、AAV8.2、AAV9 和 AAV rh10,以及假病毒 AAV,诸如 AAV2/8、AAV2/5 以及 AAV2/6。

[0179] 在某些实施方案中,载体是慢病毒载体。如本文所用的慢病毒载体是包含来源于慢病毒的至少一个组成部分的载体。慢病毒的详细列表可在 Coffin 等人 (1997)“Retroviruses”Cold Spring Harbour Laboratory Press,JM Coffin、SM Hughes、HE Varmus 编,第 758-763) 中找到。慢病毒载体一般可用本领域中熟知的方法产生。参见例如美国专利号 5,994,136 ;6,165,782 ;和 6,428,953。优选地,慢病毒载体是整合酶缺陷的慢病毒载体 (IDLV)。参见例如美国专利公开 2009/0117617。IDLV 可如所描述的,例如使用包含天然慢病毒整合酶基因中的一个或多个突变的慢病毒载体来产生,例如如在 Leavitt 等人 (1996)J.Virol.,70(2) :721-728 ;Philippe 等人 (2006)Proc.Natl Acad.Sci USA, 103(47) :17684-17689 ;以及 WO 06/010834 中所公开的。在某些实施方案中,IDLV 是包含整合酶蛋白的 64 位处的突变的 HIV 慢病毒载体 (D64V),如在 Leavitt 等人 (1996)J.Virol., 70(2) :721-728 中所描述的。

[0180] 在某些实施方案中,载体是腺病毒载体。可用于本申请案中的 Ad 载体的非限制性实例包括重组 Ad 载体 (诸如 E1 缺失的)、有条件复制能力 Ad 载体 (诸如,溶瘤细胞的) 和 / 或来源于人类或者非人类血清型的复制型 Ad 载体 (例如, Ad5、Ad11、Ad35,或者猪腺病毒 -3) ;和 / 或嵌合的 Ad 载体 (诸如 Ad5/F35),或者趋性改变的 Ad 载体,趋性改变的 Ad 载体具有工程化纤维 (例如,节结或者轴) 蛋白 (诸如,节结蛋白的 HI 环中的肽插入物)。“无病毒基因的”Ad 载体也是可用的,例如其中已经去除所有腺病毒基因以减少免疫原性和

增加DNA有效负载的大小的Ad载体。这允许例如同时递送编码ZFN的序列和供体序列。此类“无病毒基因的”载体在供体序列包括待经由靶向整合来整合的大型转基因时尤其有用。

[0181] 可产生高效价的复制缺陷型重组腺病毒载体(Ad),它们容易感染许多不同细胞类型。将大部分腺病毒载体工程化,以使转基因替代Ad E1a、E1b和/或E3基因;随后在反式提供一种或多种缺失的基因功能的细胞中增殖复制缺陷型载体。例如,人293细胞提供E1功能。Ad载体可体内转导多种组织类型,包括非分裂、分化细胞,如肝、肾和肌肉中存在的那些细胞。常规Ad载体具有大容纳量。临床试验中使用Ad载体的一个实例包括用肌肉内注射进行抗肿瘤免疫的多核苷酸治疗(Sterman等人,Hum.Gene Ther.,7:1083-1089(1998))。

[0182] 使用包装细胞形成能够感染宿主细胞的病毒颗粒。这种细胞包括293细胞、ψ2细胞或PA317细胞,293细胞能包装腺病毒,PA317细胞能包装逆转录病毒。通常用将核酸载体包装到病毒颗粒中的生产细胞系来产生用于基因治疗的病毒载体。载体通常含有用于包装和随后整合入宿主(如果可行)所需的最小病毒序列,其它病毒序列被编码待表达蛋白的表达盒所替代。包装细胞系反式提供丧失的病毒功能。例如,用于基因治疗的AAV载体通常仅具有来自AAV基因组的、包装和整合入宿主基因组所必需的端反向重复(ITR)序列。将病毒DNA包装到含有辅助质粒的细胞系中,该辅助质粒编码其它AAV基因,即rep和cap,但没有ITR序列。也用腺病毒作为辅助者感染该细胞系。辅助病毒促进AAV载体的复制和来自辅助质粒的AAV基因的表达。由于缺少ITR序列,不能大量包装辅助质粒。可通过(例如)热处理降低腺病毒污染,腺病毒对热处理的敏感性高于AAV。另外,可使用杆状病毒系统以临床规模产生AAV(参见美国专利号U.S.7,479,554)。

[0183] 在临床试验中使用腺病毒载体进行基因转移的其它实例包括Rosenecker等人,Infection,24:15-10(1996);Welsh等人,Hum.Gene Ther.,2:205-18(1995);Alvarez等人,Hum.Gene Ther.,5:597-613(1997);Topf等人,Gene Ther.,5:507-513(1998)。

[0184] 在某些实施方案中,Ad载体是嵌合的腺病毒载体,包含来自两种或更多种不同的腺病毒基因组的序列。例如,Ad载体可为Ad5/F35载体。Ad5/F35是通过用来自B亚族腺病毒(诸如,例如Ad35)的相应纤维蛋白质基因替代Ad5的一个或多个纤维蛋白基因(节结,轴、尾部、五邻体)而创建的。Ad5/F35载体和此载体的特性在例如Ni等人,(2005)“Evaluation of biodistribution and safety of adenovirus vectors containing group B fibers after intravenous injection into baboons,”Hum Gene Ther,16:664-677;Nilsson等人(2004)“Functionally distinct subpopulations of cord blood CD34+cells are transduced by adenoviral vectors with serotype 5or 35tropism,”Mol Ther,9:377-388;Nilsson等人(2004)“Development of an adenoviral vector system with adenovirus serotype 35tropism;efficient transient gene transfer into primary malignant hematopoietic cells,”J Gene Med,6:631-641;Schroers等人(2004)“Gene transfer into human T lymphocytes and natural killer cells by Ad5/F35chimeric adenoviral vectors,”Exp Hematol,32:536-546;Seshidhar等人(2003)“Development of adenovirus serotype 35as a gene transfer vector,”Virology,311:384-393;Shayakhmetov等人(2000)“Efficient gene transfer into human CD34(+)cells by a retargeted adenovirus vector,”J Virol,74:2567-2583;以及Sova等人(2004),“A tumor-targeted and conditionally replicating oncolytic adenovirus vector

expressing TRAIL for treatment of liver metastases,"Mol Ther,9:496-509 中有所描述。如上所述,ZFN 和编码这些 ZFN 的多核苷酸可被递送到任何靶细胞。一般来说,为了钝化基因 CCR-5,细胞是免疫细胞,例如淋巴细胞 (B 细胞,T 细胞如 T 辅助细胞 (T_H) 和 T 细胞毒性细胞 (T_C),裸细胞如自然杀伤 (NK) 细胞);单核细胞(大单核细胞、巨噬细胞);粒细胞(粒性白细胞、中性粒细胞、嗜酸性粒细胞、嗜碱性粒细胞);肥大细胞;和 / 或树突状细胞(郎格罕氏细胞、间质树突状细胞、指突状树突细胞、外周血树突细胞)。巨噬细胞、B 淋巴细胞和树突状细胞是 T_H细胞激活中所涉及的示例性抗原递呈细胞。在某些实施方案中,靶细胞是 T_H细胞,特征为 CD4 在细胞表面上的表达。靶细胞还可为造血干细胞,造血干细胞可产生任何免疫细胞。

[0185] 在许多基因治疗应用中,需要以高度特异性将基因治疗载体递送至特定组织类型。因此,可修饰病毒载体,使其通过表达配体而具有对给定细胞类型的特异性,该配体与病毒外表面上的病毒外壳蛋白形成为融合蛋白。选择配体,使其对已知存在于受关注细胞类型上的受体具有亲合力。例如 Han 等人,Proc.Natl.Acad.Sci.USA,92:9747-9751(1995) 报道,可修饰莫洛尼鼠白血病病毒以表达融合于 gp70 的人调蛋白,并且该重组病毒能感染表达人表皮生长因子受体的某些人乳腺癌细胞。这个原理可延用于其它病毒 - 靶细胞对,其中靶细胞表达一种受体,而病毒表达包含该细胞表面受体的配体的融合蛋白。例如,可工程化丝状噬菌体,以呈现对基本上任何所选细胞受体都具有特异性结合亲合力的抗体片段(如 FAB 或 Fv)。虽然上述描述主要应用于病毒载体,但同样的原理也可应用于非病毒载体。可工程化此类载体,使其含有有利于特靶向细胞摄取的特异性摄取序列。

[0186] 可通过给予单个患者,一般是通过全身给药(例如,静脉内、腹膜内、肌内、皮下或颅内输注)或局部应用来体内递送基因治疗载体,如下所述。或者,可将载体离体递送至细胞中,例如,从单个患者体内取出细胞(例如,淋巴细胞、骨髓吸取物、组织活检)或通用供体造血干细胞,然后,通常在选择并入载体的细胞后将细胞再植入患者。

[0187] 本领域技术人员熟知用于诊断、研究或基因治疗的离体细胞转染(如通过将转染细胞再输注到宿主生物体中)。在一个优选实施方案中,从受试者生物体中分离细胞,用 ZFP 核酸(基因或 cDNA) 转染,再输注回受试者生物体(例如,患者)内。本领域技术人员熟知适用于离体转染的各种细胞类型(参见例如 Freshney 等人,Culture of Animal Cells, A Manual of Basic Technique(第 3 版,1994)),其中引用的参考文献是关于如何分离和培养患者细胞的论述)。

[0188] 合适的细胞包括但不限于真核和原核细胞和 / 或细胞系。此类细胞或者从此类细胞产生的细胞系的非限制性实例包括 COS、CHO(例如,CHO-S、CHO-K1、CHO-DG44、CHO-DUXB11、CHO-DUKX、CHOK1SV)、VERO、MDCK、WI38、V79、B14AF28-G3、BHK、HaK、NS0、SP2/0-Ag14、HeLa、HEK293(例如,HEK293-F、HEK293-H、HEK293-T),以及 perC6 细胞,以及昆虫细胞诸如草地贪夜蛾 (Spodoptera fugiperda)(Sf),或真菌细胞诸如酵母属 (Saccharomyces)、毕赤酵母属 (Pichia) 和裂殖酵母属 (Schizosaccharomyces)。在某些实施方案中,细胞系是 CHO-K1、MDCK 或 HEK293 细胞系。另外,可分离和离体使用原代细胞,以在用核酸酶(例如,ZFN 或者 TALEN) 或者核酸酶系统(例如,CRISPR/Cas) 进行处理之后将细胞再导入待治疗的受试者体内。合适的原代细胞包括外周血单核细胞 (PBMC),以及其它的血细胞亚群,诸如但不限于 T 淋巴细胞,如 CD4+T 细胞或者 CD8+T 细胞。合适的细胞还包

括干细胞，诸如，举例来说，胚胎干细胞、诱导性多能干细胞、造血干细胞（CD34+）、神经干细胞和间充质干细胞。

[0189] 在一个实施方案中，干细胞用于离体细胞转染和基因治疗的方法。采用干细胞的优点是它们可在体外分化为其它细胞类型，或者可被导入哺乳动物（诸如细胞供体）中，随后可移入骨髓中。使用细胞因子如GM-CSF、IFN-γ和TNF-α使CD34+细胞体外分化为临床上重要的免疫细胞类型的方法是已知的（参见Inaba等人，J.Exp.Med.，176：1693-1702(1992)）。

[0190] 用已知方法分离用于转导和分化的干细胞。例如，用结合不需要的细胞，如CD4+和CD8+(T细胞）、CD45+(泛B细胞(panB cell))、GR-1(粒细胞）和Iad(分化的抗原递呈细胞）的抗体淘选骨髓细胞，从而从骨髓细胞中分离干细胞（参见Inaba等人，J.Exp.Med.，176：1693-1702(1992)）。

[0191] 在一些实施方案中还可使用已经修饰的干细胞。例如，已经对凋亡具有抗性的干细胞可被用作治疗性组合物，其中干细胞还包含本发明的ZFP、TALE、CRISPR/Cas系统和/或供体。对凋亡的抗性可例如通过以下方式发生：使用BAX-的核酸酶或者BAK-特异性核酸酶（参见，美国专利公开号2010/0003756）敲除干细胞中的BAX和/或BAK，或者干细胞再用例如半胱天冬酶-6特异性ZFN敲除半胱天冬酶中断裂的BAX和/或BAK。或者，还可通过使用半胱天冬酶抑制剂如Z-VAD-FMK(苄氧羰基-缬氨酰-丙氨酰-天冬氨酰[邻甲基]-氟甲基酮）来实现对凋亡的抗性。

[0192] 也可向生物体直接施用含有治疗性ZFP、TALE、CRISPR/Cas系统和/或供体核酸的载体（例如，逆转录病毒、腺病毒、脂质体等）以体内转导细胞。或者，可施用裸DNA或者mRNA。可通过常用于将分子导入成与血液或组织细胞最终接触的任何途径施用，包括但不限于注射、输注、局部施用和电穿孔。施用此类核酸的合适方法可用并且为本领域中的技术人员熟知，并且，虽然可使用一种以上的途径施用特定组合物，但是一种特定途径往往可提供比另一种途径更直接且更有效的反应。

[0193] 例如，在美国专利号5,928,638中公开了将DNA导入造血干细胞中的方法。将转基因导入造血干细胞（例如，$CD34^+$细胞）中有用的载体包括35型腺病毒。

[0194] 将转基因导入免疫细胞（例如，T细胞）中的合适载体包括非整合慢病毒载体。参见，例如，Ory等人(1996)Proc.Natl.Acad.Sci.USA，93：11382-11388；Dull等人(1998)J.Virol.，72：8463-8471；Zuffery等人(1998)J.Virol.，72：9873-9880；Follenzi等人(2000)Nature Genetics，25：217-222。

[0195] 医药学上可接受的载体是部分地由施用的特定组合物，以及用于施用组合物的特定方法所确定的。因此，如下所述，存在多种多样的可用医药组合物的合适制剂（参见例如，Remington's Pharmaceutical Sciences，第17版，1989）。

[0196] 应用

[0197] 所公开的方法和组合物可用于钝化TCR基因组序列。如上所述，钝化包括部分或者完全抑制细胞（例如，T淋巴细胞）中内源TCRα和/或β基因的表达。TCR基因的钝化可通过例如以下方式来实现：通过单一切割事件；通过切割后进行非同源末端连接；通过在两个位点处切割后连接，以便使两个切割位点之间的序列缺失；通过将错义或者无义密码子靶向重组到编码区域中；通过将不相关的序列（即，“填充物”序列）或者另一受关

注编码序列靶向重组到基因或者基因的调节区域，以便使基因或者调节区域断裂；或者通过将剪接受体序列靶向重组到内含子中以造成转录本的错误剪接。内源 TCR 基因的钝化还可通过靶向重组一个或多个特异于受关注肿瘤抗原 /MHC 复合物的 TCR 基因来实现。

[0198] 存在各种用于核酸酶介导的 TCR 基因钝化（敲除或敲减）的应用。例如，本文描述的方法和组合物允许细胞系的生成和 / 或修饰（用于治疗和非治疗用途）。一个或多个内源 TCR 基因的钝化可与编码高亲合力 TCR 或者抗已知靶标的嵌合抗原受体 (CARS，参见 Cartellieri 等人 (2010)J Biomed and Biotech，第 2010 卷，文章编号 956304) 的基因的插入相结合，以及生成的转基因细胞（或者具有相同特性的这些细胞的子代）可用作细胞治疗剂。或者，可使用递送供体核酸上编码 TCR 特异核酸酶和高亲合力 TCR 的基因的病毒载体，在体内进行 T 细胞的重新靶向。不论哪种情况，都可使用本发明的材料和方法来治疗癌症。体外修饰细胞还可用于模型研究或者用于筛选以找到还可与 TCR 修饰协同作用的其它类型的治疗剂。可治疗任何类型的癌症，包括但不限于肺癌、胰腺癌、肝癌、骨癌、乳腺癌、结直肠癌、白血病、卵巢癌、淋巴瘤、脑癌等。可用本发明的技术治疗的其它疾病包括真菌、细菌和病毒感染，以及身免疫性疾病和 / 或移植物抗宿主病 (GvHD)。

[0199] 此外，本文描述的方法和组合物可用于生成模式生物体和细胞系，包括在任何给定的生物体中生成稳定的敲除细胞。虽然 ZFN/TALEN/CRISPR/Cas 系统提供在细胞系或者模式生物体中敲除任何给定基因的能力，但是在不存在选择标记的情况下，这些事件是非常罕见的。因此，本文所描述的方法显著升高了靶基因断裂率，该方法可用于生成具有新特性的细胞系。此方法包括用于产生生物样仓鼠 (CHO) 细胞系的细胞系或者用于产生若干 AAV 血清型样人类 HEK 293 细胞或者昆虫细胞样 Sf9 或者 Sf21 的细胞系。

[0200] 本发明的方法和组合物还可用于产生转基因生物体。转基因动物可包括开发用于疾病模型的那些转基因动物，以及具有所需性状的动物。可使用本发明的方法和组合物处理胚胎，以开发转基因动物。在一些实施方案中，合适的胚胎可包括来自小型哺乳动物（例如，啮齿动物，兔子等）、同伴动物、家畜和灵长类动物的胚胎。啮齿动物的非限制性实例可包括小鼠、大鼠、仓鼠、沙鼠和豚鼠。同伴动物的非限制性实例可包括猫、狗、兔子、刺猬，以及雪貂。家畜的非限制性实例可包括马、山羊、绵羊、猪、骆驼、羊驼，以及牛。灵长类动物的非限制性实例可包括卷尾猴属、黑猩猩、狐猴、猕猴、绒猴、金丝猴、蜘蛛猿、松鼠猴，以及长尾黑颌猴。在其它实施方案中，合适的胚胎可包括来自鱼、爬行动物、两栖动物或者禽类的胚胎。或者，合适的胚胎可为昆虫胚胎，例如果蝇胚胎或者蚊虫胚胎。

[0201] 实施例

[0202] 实施例 1：经优化的、高亲合力 WT-1TCR 构建体的表达

[0203] 将编码特异于 HLA-A2 限制的 Wilms 肿瘤抗原 1(WT1) 肽（具体地，$WT1_{126\ 134}$肽 (Kuball 等人 (2007)Blood，109(6)：2331-8)) 的密码子优化的、用半胱氨酸修饰的 TCR 的基因和 WT1 特异性 TCRα21 或者 β21 单链克隆到双向自钝化转移载体 pCCLsin.PPT.ΔLNGFR.minCMV.WPGK.eGFP.Wpre 或者 pCCLsin.cPPT.ΔLNGFR.min.CMV.h EF1a.eGFP.Wpre，如在 Amendola 等人 (2005)Nature Biotechnology，23(1)：108-116；Thomas 等人 (2007)J.Immunol，179(9)：5803-5810；以及美国专利公开 NoUS2006200869 中所描述的（参见图 1A)。

[0204] 使用整合酶型的第三代慢病毒载体系统包装载体，以及用 VZV 包膜假型化，基本

上如 Follenzi 和 Naldini(2002),Methods in Enzy mology346 :454-465 中所描写的。随后使用标准技术（见下文）来用慢病毒载体转导细胞,并且细胞特征为 FACs 分析以确定外源 TCR 是否正在细胞表面上表达。

[0205] 如下表 1 所示,无论是由 PGK/mCMV 双启动子组合还是由 EF1α/mCMV 双启动子构建体驱动,WT-1 特异性 TCR 构建体都高度表达。表中的数字呈现为在针对 VB21 表达和 WT1-HLA-A2 五聚体结合设门的象限中存在的总信号的百分比。

[0206] 表 1 :WT-1TCR 的表达

[0207]

启动子	第 14 天	第 22 天
PGK	12.1	21.3
EF1α	1.48	5.16
未转导的=0.085		

[0208] T 细胞的转导是通过用抗 CD3/ 抗 CD28 抗体共轭的磁性珠粒 (Clin ExVivo CD3/CD28 ;Invitrogen)(baCD3/CD28) 激活细胞而实现的,其中细胞是在含有低剂量 IL-7/IL-15 和 10％ FCS 的 IMDM(GIBCO-BRL) 培养基中培养的,如在欧洲专利公开 No EP1956080 和 Kaneko 等人 (2009)Blood,113 :1006-1015 中所描述的。此方法保留早期 T 细胞分化表型 (CD45RA-/+CD62L+、CD28+CD27+、IL7Ra+、IL-2+γ IFN-/+),并且细胞从未转导的淋巴细胞无差别地增殖。在这些条件下,PGK 双启动子被证明在支持 WT1 特异性 TCR 链的化学计量表达方面优于 EF1a 双启动子,这表明沿反义方向,PGK 双向启动子比双向 EF1α 启动子发挥更高的活性。然而,当在慢病毒载体的背景下测试时这两种启动子,在初始刺激超过 70 天之后,支持在适合于有效的 HLA-A2/WT1 五聚体结合的水平的 TCR 表达 (16％)(参见图 1B)。

[0209] TCR 转导细胞还能够表现出抗来自 AML 患者的 WT1+HLA-A2+ 原代白血病细胞的特异 γ IFN 产生和细胞毒素活性。特别地,在用以 PGK/mCMV 双启动子组合或者 EF1α/mCMV 双启动子表达转基因 TCR 的载体转导的细胞中 γ IFN 的产量增多（图 2A),γ IFN 的产量被表示为 TCR 修饰的细胞的杀伤（细胞溶解）％（图 2B 和图 2C)。此外,在表达 HLA 限制元件的未标记靶标的存在下,并且用靶肽致敏时,经编辑淋巴细胞中的 γ IFN 产生被抑制（图 2D)。

[0210] 实施例 2 :转基因到中枢记忆 T 细胞的 CCR5 基因座中的有效整合

[0211] 为了测试将 WT-1 特异性 TCR 基因整合到中枢记忆 T 细胞中的想法,首先使用 GFP 作为供体核酸来监测转导效率和整合位点的 GFP 表达。选择 CCR5 基因座是因为已经示出 CCR5 敲除细胞是全功能性的（参见美国专利号 7,951,925)。此外,PPP1R12C(AAVS1) 基因座类似地被靶向的（参见美国专利公开 20080299580)。使用 IDLV 载体将编码 GFP 的供体转导到细胞中,以及使用 Ad5/F35 载体将 CCR5 特异性 ZFN 或者 AAVS1 特异性 ZFN 导入细胞,如上所述。在转导 20 天之后测量 GFP 表达。

[0212] 如图 3 所示,ZFN 介导的 GFP 转基因整合导致 GFP 信号增多,包括涉及与所使用的 Ad5/F35 供体的量（图 3A 和图 3B)。下表 2 示出在供体或者供体加 ZFN 的存在下,GFP 阳性细胞的百分比增加。

[0213] 表 2.GFP 信号，阳性细胞百分比

[0214]

插入位点	UT	+ 供体	供体 +ZFN
CCR5	0.038	0.083	6.11
AAVS1	0.015	0.18	4.38

[0215] 实施例 3 :WT-1 特异性 TCR 转基因到 Jurkat TCRβ - 阴性细胞的 CCR5 基因座中的整合

[0216] 随后将 WT-1 特异性 TCR 转基因构建体靶向整合到用 TCR-β 特异性 ZFN 处理后呈 TCRβ - 阴性的 Jurkat 细胞的 CCR5 基因座中。使用标准技术，用类似于实施例 1 中所描述的 WT-1TCR 构建体转染细胞。

[0217] 如从表 3 可看出的，在导入 WT-1TCR 供体 (WT1-TCR IDLV) 和 CCR5 特异性 ZFN(Ad-ZFN) 之后，存在 Vβ21 染色或者信号显著的增加，而在没有供体或者 ZFN 时，仅看到背景 Vβ21 信号。因此，ZFN 介导的 WT-1 特异 TCR 整合到 CCR5 基因座中，这在相当大百分比的细胞中存在。

[0218] 表 3 :Vβ21+ 表达的总信号的百分比

[0219]

WT1-TCR IDLV	+	+	+	-
Ad ZFN	+	++	-	-
VB21+的百分比	16.6	18.7	2.27	0.81

[0220] 实施例 4 :TCR 特异性 ZFN 的设计

[0221] TCR 特异性 ZFN 被构建成能在 TCRα 和 / 或 TCRβ 基因处双链断裂的位点特异性导入。ZFN 被设计和并入到质粒或 IDLV 载体中，基本上如在 Urnov 等人 (2005)Nature，435(7042) :646-651 ;Lombardo 等人 (2007)Nat Biotechnol.Nov,25(11) :1298-306 ;以及美国专利公开 2008/0131962 中所描述的。示例性 ZFN 对的识别螺旋和靶序列在下表 4 和下表 5 中示出。TCR 锌指设计的靶位点在第一列中示出。与 ZFP 识别螺旋接触的靶位点中的核苷酸以大写字母指示，而未接触的核苷酸以小写字母指示。

[0222] 表 4 :TCR-α 锌指设计

[0223]

ZFN靶标序列	F1	F2	F3	F4	F5	F6
25529 (外显子1) ctATGGACtTCAAGAGCAacagtgctgt (SEQ ID NO:1)	QSGDLTR (SEQ ID NO:2)	QRTHLKA (SEQ ID NO:3)	QSGDRNK (SEQ ID NO:4)	DRSNLSR (SEQ ID NO:5)	RSDALTQ (SEQ ID NO:6)	不适用
25528 (外显子1) ctCATGTCTAGcACAGTTtgtctgtga (SEQ ID NO:7)	TSGSLSR (SEQ ID NO:8)	QSSVRNS (SEQ ID NO:9)	RSDNLST (SEQ ID NO:10)	DRSALAR (SEQ ID NO:11)	LKQNLDA (SEQ ID NO:12)	不适用
25535 (外显子1) gtGCTGTGGCCtGGAGCAacaaatctga (SEQ ID NO:13)	DRSALSR (SEQ ID NO:14)	QSGHLSR (SEQ ID NO:15)	DRSDLSR (SEQ ID NO:16)	RSDALSR (SEQ ID NO:17)	DRSDLSR (SEQ ID NO:16)	不适用

[0224]

25534 (外显子1) ttGCTCTTGA AGTCcATAG ACctcatgt (SEQ ID NO:18)	DRSNLSR (SEQ ID NO:5)	QKTSLQA (SEQ ID NO:19)	DRSALSR (SEQ ID NO:14)	QSGNLAR (SEQ ID NO:20)	GKEELNE (SEQ ID NO:21)	RSSDLSR (SEQ ID NO:22)
25537 (外显子1) gcTGTGGCCT GGAGCAAca aatctgact (SEQ ID NO:23)	GNVDLIE (SEQ ID NO:24)	RSSNLSR (SEQ ID NO:25)	RSDALSV (SEQ ID NO:26)	DSSHRTR (SEQ ID NO:27)	WRSCRSA (SEQ ID NO:28)	不适用
25536 (外显子1) ctGTTGCTcT TGAAGTCCat agacctca (SEQ ID NO:29)	DSSDRKK (SEQ ID NO:30)	RSDNLSV (SEQ ID NO:31)	RRFILRG (SEQ ID NO:32)	QSGDLTR (SEQ ID NO:2)	TSGSLTR (SEQ ID NO:33)	不适用
25538 (外显子1) ctGTGGCCtG GAGCAACAa atctgactt (SEQ ID NO:34)	QSGDLTR (SEQ ID NO:2)	QTSTLSK (SEQ ID NO:35)	QSGHLSR (SEQ ID NO:15)	DRSDLSR (SEQ ID NO:16)	RSDALAR (SEQ ID NO:36)	不适用
25540 (外显子1) ctGACTTTGC ATGTGCAaac gccttcaa (SEQ ID NO:37)	QSGDLTR (SEQ ID NO:2)	WRSSLAS (SEQ ID NO:38)	QSGDLTR (SEQ ID NO:2)	HKWVLRQ (SEQ ID NO:39)	DRSNLTR (SEQ ID NO:40)	不适用
25539 (外显子1) ttGTTGCTcC AGGCCACA GCActgttgc (SEQ ID NO:41)	QSGDLTR (SEQ ID NO:2)	QWGTRYR (SEQ ID NO:42)	ERGTLAR (SEQ ID NO:43)	RSDNLRE (SEQ ID NO:44)	QSGDLTR (SEQ ID NO:2)	TSGSLTR (SEQ ID NO:33)
22199 (外显子3) tgAAAGTGG CCGGGtttaatct gctcat (SEQ ID NO:45)	RSAHLSR (SEQ ID NO:46)	DRSDLSR (SEQ ID NO:16)	RSDHLSV (SEQ ID NO:47)	QNNHRIT (SEQ ID NO:48)	不适用	不适用
22189 (外显子3) agGAGGATT CGGAAcccaat cactgaca (SEQ ID NO:49)	QRSNLVR (SEQ ID NO:50)	RNDDRKK (SEQ ID NO:51)	TSGNLTR (SEQ ID NO:52)	TSANLSR (SEQ ID NO:53)	不适用	不适用
25572 (外显子3) gaGGAGGAtT CGGAACCCa atcactgac (SEQ ID NO:54)	DRSTLRQ (SEQ ID NO:55)	QRSNLVR (SEQ ID NO:50)	RNDDRKK (SEQ ID NO:51)	RSAHLSR (SEQ ID NO:46)	QSGHLSR (SEQ ID NO:15)	不适用

[0225]

25573 (外显子3) gaGGAGGAtTCGGAAccccaatcactgac (SEQ ID NO:54)	QRSNLVR (SEQ ID NO:50)	RNDDRKK (SEQ ID NO:51)	QSGHLAR (SEQ ID NO:56)	QSGHLSR (SEQ ID NO:15)	不适用	不适用
22199 (外显子3) tgAAAGTGGCCGGGtttaatctgctcat (SEQ ID NO:57)	RSAHLSR (SEQ ID NO:46)	DRSDLSR (SEQ ID NO:16)	RSDHLSV (SEQ ID NO:47)	QNNHRIT (SEQ ID NO:48)	不适用	不适用

[0226] 表 5 :TCR-β 锌指设计

[0227]

ZFN名称 靶序列	F1	F2	F3	F4	F5	F6
16783 ccGTAGAACTGGACTTGacagcggaagt (SEQ ID NO:58)	RSDVLSA (SEQ ID NO:59)	DRSNRIK (SEQ ID NO:60)	RSDVLSE (SEQ ID NO:61)	QSGNLAR (SEQ ID NO:20)	QSGSLTR (SEQ ID NO:62)	不适用
16787 tcTCGGAGAATGACGAGTGGacccagga (SEQ ID NO:63)	RSDHLST (SEQ ID NO:64)	RSDNLTR (SEQ ID NO:65)	DRSNLSR (SEQ ID NO:5)	TSSNRKT (SEQ ID NO:66)	RSANLAR (SEQ ID NO:67)	RNDDRKK (SEQ ID NO:51)
22409 tcTCGGAGAATGACGAGTGGacccagga (SEQ ID NO:63)	RSDHLST (SEQ ID NO:64)	RSDNLTR (SEQ ID NO:65)	DRSNLSR (SEQ ID NO:5)	LQFNRNQ (SEQ ID NO:68)	RSANLAR (SEQ ID NO:67)	RNDDRKK (SEQ ID NO:51)
22449 tcTCGGAGAATGACGAGTGGacccagga (SEQ ID NO:63)	RSDHLST (SEQ ID NO:64)	RSDNLTR (SEQ ID NO:65)	DSSNLSR (SEQ ID NO:69)	LRFNLSN (SEQ ID NO:70)	RSANLAR (SEQ ID NO:67)	RNDDRKK (SEQ ID NO:51)
22454 tcTCGGAGAATGACGAGTGGacccagga (SEQ ID NO:63)	RSDHLST (SEQ ID NO:64)	RSDNLTR (SEQ ID NO:65)	DSSNLSR (SEQ ID NO:69)	LHFQLTG (SEQ ID NO:71)	RSANLAR (SEQ ID NO:67)	RNDDRKK (SEQ ID NO:51)

[0228]

25814 ccGTAGAACT GGACTTGaca gcggaagt (SEQ ID NO:58)	RSDVLSA (SEQ ID NO:59)	DRSNRIK (SEQ ID NO:60)	RSDVLSE (SEQ ID NO:61)	QSGNLAR (SEQ ID NO:20)	QSGSLTR (SEQ ID NO:62)	不适用
25818 ccGTAGAACT GgaCTTGACa gcggaagt (SEQ ID NO:58)	DRSNLSR (SEQ ID NO:5)	LKFALAN (SEQ ID NO:72)	RSDVLSE (SEQ ID NO:61)	QSGNLAR (SEQ ID NO:20)	QSGSLTR (SEQ ID NO:62)	不适用
25820 ccGTAGAACT GGACTTGaca gcggaagt (SEQ ID NO:58)	RSDVLSA (SEQ ID NO:59)	DRSNRIK (SEQ ID NO:60)	RSDVLSE (SEQ ID NO:61)	QSGNLAR (SEQ ID NO:20)	QSGALAR (SEQ ID NO:73)	不适用
25822 ccGTAGAACT GGACTTGaca gcggaagt (SEQ ID NO:58)	RLSVLTI (SEQ ID NO:74)	DRANLTR (SEQ ID NO:75)	RSDVLSE (SEQ ID NO:61)	QSGNLAR (SEQ ID NO:20)	QSGALAR (SEQ ID NO:73)	不适用

[0229] 实施例 5 :体外 ZFN 活性

[0230] 使用表 4 和表 5 中描述的 ZFN 来测试 K562 细胞中的核酸酶活性。为了检测切割活性,将编码如上所述的成对人类 TCR 特异 ZFN 的质粒转染到 K562 细胞中。K562 细胞获自美国典型培养物保藏中心并且如所建议的在补充有 10％合格的胎牛血清 (FBS, Cyclone) 的 RPMI 培养基 (Invitrogen) 中生长。对于转染,将一百万个 K562 细胞与 2 □ g 的锌指核酸酶质粒和 100 □ L 的 Amaxa 溶液 V(Amaxa Solution V) 混合。在 Amaxa 核转染仪 II™(Amaxa Nucleofector II™) 中使用程序 T-16 转染细胞,然后将细胞回收到 1.4mL 温热的 RPMI 培养基 +10％ FBS 中。

[0231] 收获基因组 DNA,并且对涵盖预期切割位点的 TCR 基因座部分进行 PCR 扩增,PCR 扩增是使用购自 Invitrogen 的 Accuprime HiFi 聚合酶如下进行的 :在 94℃下最初 3 分钟变性后,经 94℃下 30 秒二次变性步骤,接着是 58℃下 30 秒二次退火步骤,接着是 68℃下 30 秒二次延伸步骤进行 30 个 PCR 循环。完成 30 个循环后,在 68℃下使反应孵育 7 分钟,然后无限期地在 4℃下孵育。

[0232] 通过例如在美国专利公开号 20080015164、20080131962 和 20080159996 中描述的 Cel-I 测定来检查来自经 K562TCR 特异性 ZFN 处理的细胞的基因组 DNA。

[0233] K562 细胞中的 TCR β 基因座具有两个具有高度序列相似性的功能性拷贝 (TRBC1 和 TRBC2),功能性拷贝都由 TCR β 特异 ZFN 靶向。参见图 4B。因此最初,将特异地扩增特异地来自 TRBC1 基因或者 TRBC2 基因的预期 ZFN 切割位点的周围区域的 PCR 引物被用于分别分析对这两种基因进行 ZFN 驱动切割之后的 NHEJ 活性。关于 ZFN 对 16787 和 16783 的示例性结果提供于下表 6 中。

[0234] 表 6 :成对 TCR β - 特异 ZFN 的 NHEJ 活性 :对 TRBC1 和 TRBC2 的分析

[0235]

TRBC2中的NHEJ				TRBC1中的NHEJ			
TRBC2	ZFN1	ZFN2	% NHEJ	TRBC1	ZFN1	ZFN2	% NHEJ
	16787	16783	8.68		16787	16783	8.73
	GFP		0.00		21	GFP	0.00
	Mock		0.00		22	Mock	0.00
	水对照		0.00		23	水对照	0.00

[0236] 在表 6 中提供的数据显示 ZFN 基本上平均地切割 TRBC1 和 TRBC2 基因。

[0237] 此外，我们通过在转染之后 3 天和 10 天时收获样本而测试了 K562 细胞中 ZFN 介导修饰的 TRBC 的存活率。结果提供于下表 7 中，并且显示对于 ZFN 对 16787 和 16783，靶基因修饰在转染后 10 天时是稳定的。

[0238] 表 7 :K562 细胞中的 TCRβ 特异性 ZFN

[0239]

ZFN 1	ZFN 2	% NHEJ	
22449	16783	20.1	3 天
22454	16783	17.7	

[0240]

16787	16783	12.1	
GFP		0.0	
22409	16783	14.7	10 天
22449	16783	8.1	
22454	16783	12.1	
16787	16783	15.6	
GFP		0.0	

[0241] 在输入不同数量的 ZFN（各 ZFN 为 0.4μg 或者 0.1μg）之后，分析靶向 TRBC 的若干 ZFN 对的 NHEJ 活性。如图 5 所示，所有检测的 ZFN 对表现出高活性。在本实验中，细胞在用 ZFN 转导之后，用 30℃的孵育期进行处理（参见美国专利公开号 :20110129898）。在对 K562 细胞中的 TCRβ 特异性 ZFN 切割进行分析之后，在 CD4+ 或者 CD8+ 成熟 T 细胞中检测若干 ZFN 对。简言之，CD8+ 或者 CD4+ 细胞是从 AllCells 购买的，并且在 RPMI+10％ FBS+1％

L- 谷氨酰胺 (30mg/mL)+IL-2(30 μ g/mL，Sigma) 中培养并允许休息 4-24 小时。

[0242] 慢病毒载体被构建成包含受关注 ZFN 对。慢病毒载体是从 HIV 来源的自钝化载体构建体产生的，并且使用携带 D64V 突变体的 HIV 整合酶包装，然后用 VSV-G 包膜假型化，如上所述。Ad5/F35 腺病毒载体是在使用 2A 序列和巨细胞病毒内部启动子克隆两组 ZFN 之后如先前所描述的产生的 (Perez 等人，(2008)，Nature Biotechnology，26 :808-816。参见例如，Holst J 等人 (2006)Nat Protoc.，1(1) :406-17。1e6 细胞 / 核转染是按照制造商的规定与 Amaxa™核转染试剂盒 (Amaxa™ Nucleofection kit) 一起用于每一转导的。在核转染 12-24 小时之后，根据制造商的方案 (Invitrogen 公司) 用抗 CD3/CD28 珠粒激活细胞，然后使细胞在包含补充有的 5ng/mL 的 IL-7 和 IL-15(Peprotech 公司) 的 10％ FCS 的 IMDM(GIBCO-BRL 公司) 培养基中生长。

[0243] 在核转染后 3 天时收获细胞，并且使用 Cel-I 测定确定基因修饰效率，测定如在国际性专利公开 WO 07/014275 中所描述的进行。还参见 Oleykowski 等人 (1998)Nucleic Acids Res.，26 :4597-4602 ；Qui 等 人 (2004)BioTechniques，36 :702-707 ；Yeung 等 人 (2005)BioTechniques，38 :749-758。若干 ZFN 对具有良好的活性，如用 Cel-I 测定所测量的 (NHEJ 为 4-11.9％)。

[0244] 还体外检测 TCR-α 特异性 ZFN，如上。在转导之后，孵育在将孵育温度变换到 30℃之前，在 37℃孵育下孵育细胞 1 天，如上所述。参见，美国专利公开号 20110129898。这些 ZFN 靶向到 TRAC 基因，对接受如上所述的这些 ZFN 的各种组合的 K562 细胞进行的 Cel-I 测定的结果显示出高活性。参见图 6。

[0245] 实施例 6 ：细胞中 TCR-β 的断裂

[0246] 然后在实验中使用 TCR-β 特异性 ZFN 来特异地靶向 TCR 基因座。初始实验被设计用于使 Jurkat 细胞中的 TCR 基因座断裂。将 TCR-β 特异性 ZFNS 16783 和 16787 导入到整合酶缺陷型慢病毒载体 (IDLV) 上，以瞬时表达 TRBC 靶向的 ZFN。在激活后 48 小时，基于对载体制备中的 HIV Gag p24 的测量，用 0.25 μ g 或者 0.5 μ g 剂量的 IDLV 进行转导。在 293T 细胞上用载体 DNA 滴定法测得的载体感染性处在从 1 到 5×10^4 个转导单位 /ng p24 的范围中。然后用 FACS 分析测定细胞中 CD3 标记物的损失，以及根据制造商的说明使用具有抗 CD3MACS 微珠 (Miltenyi Biotec 公司) 的 LD 型细胞分选柱来富集 CD3(-) 细胞。

[0247] 如下表 8 所示，在用 ZFN 转导之后，在高达 20％的经处理细胞中存在对 TCR/CD3 复合物细胞表面表达的载体剂量依赖性抑制。

[0248] 表 8 ：用 TCR-β 特异性 ZFN IDLV 处理过的 Jurkat 细胞中的 CD3 信号损失

[0249]

	未转化的	0.25 μ g IDLV	0.5 μ g IDLV
CD3(-) 百分比	2.7	13.4	20.2

[0250] 进行 Cel-I 测定，以及确认这些结果具有高达 26％的 TRBC 等位基因 (18％的 TRBC1 和 8％的 TRBC2) 在经 ZFN 处理的细胞中断裂 (参见图 7A 和图 7B 中的“混合”)。

[0251] 接着，将 TRBC ZFN(16783 和 16787) 导入原代人类 T 淋巴细胞，并且如在 Jurkat 细胞中所看到的，用 FACS 观测到类似水平的 CD3 断裂。从健康供体收获外周血 T 细胞，以及用 CD3 和 CD28 共轭珠粒激活细胞。在激活后 48 小时，将细胞暴露于剂量不断增加的包

含 TRBC 特异性 ZFN 的 IDLV。然后在低剂量 (5ng/mL) IL-7 和 IL-15 的存在下培养细胞以促进细胞存活和生长。在原代淋巴细胞中高达 7％的经处理细胞是 CD3 阴性的，而在未处理的对照中几乎未观测到 CD3(-) 细胞，这些数据呈现在下表 9 中。

[0252] 表 9 ：用 TCR-β 特异性 ZFN IDLV 处理过的原代人类 T 淋巴细胞中的 CD3 信号损失

[0253]

	UT	2.5μg IDLV	5μg IDLV	18.5μg IDLV
CD3(-) 百分比	0.17	2.94	3.26	7.07

[0254] 在 IL7 和 IL15 的存在下，所分选的 CD3(-) 淋巴细胞可随着时间的推移而扩增和存活 (参见图 7C 和图 7D)，其中修饰百分比在图 7D 中指示。图 7E 还表明 CD3(-) 细胞在群体中存留至少 45 天，并且还示出在时间段期间在群体中保持为比较恒定的 CD3(-) 细胞的百分比。CD3(-) 细胞似乎对非特异性促细胞分裂剂刺激不反应，因为由于 CD3(+) 淋巴细胞的扩增 PHA 刺激使细胞池中 CD3(-) 细胞的百分比减小 (图 7F)。此结果显示 CD3(-) 细胞中不存在 CD3 功能信号。在表现类似 CD4/CD8 比率的 CD3(+) 淋巴细胞和 CD3(-) 淋巴细胞中未观测到表型差异。CD3(-) 细胞还保持中枢记忆表型，因为它们对 CD62L、CD28 和 IL-7RA 仍然是阳性的 (参见下表 10)。

[0255] 表 10 ：保持中枢记忆表型的 CD3(-) 细胞占总荧光强度的百分比

[0256]

	CD3(-)	CD3(+)
CD62L(+)/CD3(-)	97.6	0
CD62L(+)/CD3(+)	1.25	98.4
CD62L(-)/CD3(-)	1.11	0
CD62L(-)/CD3(+)	0	1.61
CD28(+)/CD27(-)	4.66	3.23

[0257]

CD28(+)/CD(+)	93.4	94.7
CD28(-)/CD27(-)	0.87	0.68
CD28(-)/CD27(+)	0.97	1.43
IL-7RA(+)/CD8(-)	38.8	40.7
IL-7RA(+)/CD8(+)	47	47

IL-7RA(-)/CD8(-)	3.83	2.84
IL-7RA(-)/CD8(+)	10.4	9.42

[0258] 相较于初始 T 细胞，记忆 T 淋巴细胞较少取决于稳态增殖的 TCR 信号；因此我们研究在不存在 TCR 表达的情况下，稳态性细胞因子是否可促进先前激活的细胞的存活和生长。明显地，经 TRBC-ZFN 处理的细胞可在补充有低剂量 IL-7 和 IL-15 的培养基中扩增，其中在不存在 TCR 触发的情况下，CD3(-) 细胞的比例保持稳定达 50 天以上。因此，在原代淋巴细胞中 ZFN 暴露是被良好耐受的，并且导致靶向 TRBC 基因的稳定断裂。因此，CD3(-) 细胞被分选成几乎纯的，并且在 IL-7 和 IL-15 存在下，以类似于 CD3(+) 细胞的生长速率进一步扩增 3 周以上，这显示稳态性细胞因子不需要 TCR 信号功能来促进先前激活的细胞的存活 / 增殖。

[0259] 这些数据显示成功产生了具有 T_{CM}的表型特性但是内源 TCR 的表面表达被永久断裂的新型 CD8T 细胞群体。

[0260] 实施例 7：将 WT-1 特异性 TCR 导入到具有先前已永久断裂的内源 TCR 的细胞中

[0261] 在用 TCRβ 特异性 ZFN 和用于随机整合 WT1-TCRβ 转基因的慢病毒处理之后，分选 CD3(-)T 淋巴细胞，如图 1 所述 (49.5±30％平均值 ± 标准偏差的转导效率，n ＝ 4)。因此，在经 TCRβ 编辑的细胞中，来自整合载体的转移 WT1-TCR 的表达挽救 CD3 的表面转位 (图 8，第 1 行)。

[0262] 相较于其中相对于多克隆扩增的未转导细胞没有表达所导入的 TCR 的固有的生长优势的未编辑 TCR 经转移的淋巴细胞 (图 8，第 2 行)，包含 WT1-TCR 的 TCRβ 链被断裂的细胞可通过多克隆刺激而富集到大于 90％的纯度，这指示 TCRβ 经编辑的细胞中转移 TCR/CD3 复合物的表面表达是必需的并且足以促进 TCR 介导的基因修饰细胞扩增 (图 8，第 1 行)。外源 WT1-TCR Vβ 链 (Vβ21) 是在 TCRβ 链被断裂的淋巴细胞中以相较于未编辑 TCR 经转移的细胞高大约 2 倍的平均水平表达的，并且达到了类似于对照 T 细胞的内源 Vβ21 链的表达水平，表达水平在培养期间被稳定地保持 (图 9A 和图 9B)。因此，在用相同剂量的 PGK-WT1LV 转导之后，相较于仅 2.6％的未编辑细胞结合 $WT1_{126\ 134}$五聚体，高达 22％的 TCR-β 经编辑的淋巴细胞结合 $WT1_{126\ 134}$五聚体 (图 9A，下方直方图)。

[0263] 因此，在不存在来自内源 TCRβ 链的竞争的情况下，转基因 TCRβ 链的表面表达达到生理学水平。为了验证 TCR-β 经编辑的淋巴细胞的功能和亲合力，我们比较 TCRβ 链断裂的细胞与用相同 PGK-WT1LV 转导以获得细胞溶解 $HLA\text{-}A2^{+}$靶标的能力的未编辑细胞，$HLA\text{-}A2^{+}$靶标是用不断增加的 $WT1_{126\ 134}$肽浓度致敏的 (参见图 9C)。此功能测定在效应物 / 靶标 (E/T) 比为 12 时，针对标记的 T2 细胞的细胞溶解用 ^{51}铬释放测定来测量活性，其中标记的 T2 细胞用不断增加浓度的 $WT1_{126\ 134}$HLA-A2 限制的肽致敏，或者用不相关的 CMV- 来源的 $pp65_{494\ 503}$HLA-A2 限制的肽 (10μM，Proimmune 公司) 致敏以作为阴性对照。

[0264] 经编辑的 T 细胞被刺激以及在 3 周后进行检测，孵育以通过共孵育 5 小时来识别所标记的 T2 细胞。TCRβ 链断裂的细胞 (在图 9C 中表示为 TCR 经编辑的) 相较于未编辑的 (表示为 TCR 经转移的)WT1LV 经转导的细胞更为有效地杀伤靶标 (EC50：经编辑的细胞：90.51nM，具有 95％ CI：48.84-167.7；未编辑的 TCR 转移细胞：269.1nM，具有 95％ CI；175.1-413.5)，这可能反映了转基因 WT1TCR 在 TCR-β 经编辑的样本中具有较高的频率和

表达水平。EC50 是通过使用 GraphPad Prism 软件的 S 形曲线剂量 - 应答公式对 ^{51}铬释放数据进行非线性回归分析而计算的。

[0265] 结果被表示为细胞溶解%的平均 SD(* = p<0.05,** = p<0.01,TCR 经编辑的 n = 6,TCR 经转移的 n = 4)。为了在单细胞水平评价反应性,分析细胞的 Vβ21 表达(参见下表 11),该表达显示,虽然载体拷贝数完全相等,但是在 TCR 经编辑的细胞中 Vβ21 表达更高。

[0266] 表 11 :TCR 经转移淋巴细胞和 TCR 经编辑淋巴细胞中的 TCR 表达和载体拷贝数 / 细胞

[0267]

	由 *TRBC*-ZFN 进行的内源β链断裂	PGK-WT1 LV 或 EF1α-WT1 LV	Vβ21 RFI*	(Vβ21+ 细胞)%	CpC§
TCR- 经转移	否	EF1α-WT1 LV	0.41	36.7	1.9
		PGK-WT1 LV	0.54	62.7	2.1
TCR经编辑	是	PGK-WT1 LV	0.91	97.3	1.2
UT	否	无	1	3.2	0

[0268] *通过流式细胞计术法测量 TCR 表达并且将其绘制为相对荧光强度 (RFI = 经转导细胞的 Vβ21MFI/ 未转导细胞的 Vβ MFI)。

[0269] §每细胞的载体拷贝 (CpC) 是通过如所描述的定量 PCR 测量的 (Kessels 等人,(2001)Nature Immunol,2(10) :957-61)。

[0270] 为了评价单细胞水平的同种异体反应性,从先前针对 $WT1_{126\ 134}$五聚体结合分选的 TCR-β 经编辑细胞和 TCR 经转移细胞分离和扩增克隆体,以富集展示最优的外源 TCR 表达的细胞。将克隆体暴露于用 10nM 的 $WT1_{125\ 134}$HLA-A2 限制的肽致敏的 T2 细胞(左侧小图),或者以为 1 的刺激物 / 反应物比暴露于异源的 PRMC(右侧小图)。特异性斑点的数目在 y 轴上示出为在刺激物的存在下产生的斑点的数目减去由效应物单独产生的斑点的数目 (** = p<0.01)。相较于 TCR 经转移的细胞,TCRβ 经编辑的克隆体显示出较低的同种异体反应性(参见图 10,比较图 10A 到图 10B),可能反映在不存在一个内源性 TCR 链的情况下 TCR 错配的风险降低。

[0271] 这些数据显示通过在具有受抑制的内源性 TCR-β 链表达的宿主 CTL 中表达肿瘤特异性外源 TCR 提供了功能优势。

[0272] 理论上,在 TCR 基因转移之后,未编辑的内源 TCRα 链的表面重表达可仍然存在于 TCR-β 经编辑的细胞中。为了直接评价 TCR-β 链断裂的淋巴细胞中错配的可能性,用编码仅 WT1 特异性 TCRβ 链基因和 ΔLNGFR 标记物的 LV(WT1-β-ΔLNGFR-LV) 转导 CD3(-) 细

胞。转导效率是以 ΔLNGFR+淋巴细胞的百分比来评价的（参见图 11）。测量 ΔLNGFR+细胞上的 Vβ21 表达。指示 Vβ21 的平均荧光强度 (MFI)。虽然不存在 WT1 特异性 α 链，但是在高达 83％的 ΔLNGFR+TRBC 断裂细胞中检测到 Vβ21 表达，这显示甚至被插入具有 TRBC 断裂的细胞中的经半胱氨酸修饰的 TCRβ 链能够与内源性 TCRα 链错配。

[0273] 接着，使用 CD3(-) 淋巴细胞来将 WT1-TCRβ 供体构建体导入内源 TCR 基因座。供体是如上所述构建的，并且与 TCR-β 特异性 ZFN 结合使用以使得 TCR-β 转基因被整合到内源基因座处。细胞变成对 CD3 和 Vβ21.3TCRβ 链两者是阳性的。

[0274] 实施例 8：TCR-α 链的断裂和靶向整合

[0275] 为了排除 TCR 链错配的可能，我们设计靶向到 TCRα 链基因 (TRAC) 的恒定区的一对 ZFN（图 6），且按照关于 TCR-β 编辑所描述的相同方案）获得了 TCR-α 经编辑的 T 淋巴细胞（参见图 12A），以及按照关于 TCR-β 编辑所描述的相同方案获得 TCR-α 经编辑的 T 淋巴细胞（图 12B、图 12C 和图 13）。为了设计在每一链断裂 / 置换步骤处允许快速分离工程化细胞的完整 α / β TCR 编辑方案，我们产生一组携带 WT1 特异性 TCRα 或 β 单链的 LV，并且使用 IDLV 或者腺病毒载体 (AdV) 来在淋巴细胞中瞬时表达靶向到 TRBC 或者 TRAC 的 ZFN（图 14 示出了完整 TCR 编辑的时间线和代表性流程条件 / 结果）。

[0276] CD3(-) 细胞是由所测试的每一包含 ZFN 的载体有效产生的，并且对核酸酶切割位点处的序列测序揭示在通过 NHEJ 修复之后存在小型插入和缺失 (indel)（图 13）。为了进行完整的 TCR 编辑，选择被证明比 IDLV 更有效地调节 TCR 基因断裂的 AdV。首先在用 baCD3/CD28 激活后，将从健康供体收获的 T 细胞暴露于 TRAC-ZFN-Ad5/F35 达 48 小时，然后在 IL-7 和 IL-15 的存在下培养，以及用编码 WT1-α 链的 LV(WT1-α LV) 来转导通过分选分离所得的 CD3(-) 细胞 (49±29& 平均值 ± 标准偏差的转导频率，n = 3)。

[0277] 然后分选具有所挽救的 CD3 表达的细胞，用 baCD3/CD28 刺激一个周期，以及然后暴露于 TRBC-ZFN-Ad5/F35。第二周期 ZFN 暴露产生了高达 23±4％的新型 CD3(-) 细胞，指示原代 T 淋巴细胞容许多周期 ZFN 调控。分选 CD3(-) 细胞，以及用 WT1TCR-β 链 LV 转导 CD3(-) 细胞 (18±7％平均值 ± 标准偏差的转导效率，n = 3)。经转移的 WT1-β 链的表达再次挽救 CD3 的表面转位，该表达现与 TCR 经编辑细胞中的 WT1-TCRVβ 链以均衡比例共表达（图 14 和图 15）。与未编辑的 TCR 经转移的淋巴细胞相比，TCR-α / β 断裂的细胞可在 TCR 基因转移之后通过多克隆刺激富集成几乎纯的，并且均匀地表达结合 $WT1_{126\ 134}$五聚体所需的高水平 WT1 特异性 TCR（参见图 15）。

[0278] 这些结果指示经转移的 TCR/CD3 复合物在 TCR 经编辑的细胞中的表面表达是必需的并且足以促进具有 WT1 所需特异性的细胞的扩增（图 14，右侧绘图）。通过 Cel-I 分析来确认 TCR-α / β 经编辑的细胞中的 TCRα 和 β 链的断裂。在 TCR 经转移和 TCRα / β 经编辑的淋巴细胞中未观测到表型差异，淋巴细胞呈现 T_{CM}表面表型，由 CD62L、CD27、CD28 和 IL-7rα 的高度表达所证明的。为了验证完全编辑的淋巴细胞的功能和同种异体反应，对 TCRα / β 经编辑的和 TCR 经转移的淋巴细胞进行多克隆刺激。

[0279] 在多克隆刺激三周之后，将 TCR-α / β 经编辑的淋巴细胞和 TCR 经转移的淋巴细胞暴露于 i) 用不断增加浓度的 $WT1_{126\ 134}$HLA-A2 限制的肽致敏，或者用不相关的 CMV 来源的 $pp65_{495\ 503}$HLA-A2 肽致敏的 T2 细胞（参见图 16A，图式的右侧）或者 ii) 从用 $WT1_{126\ 134}$肽 (50nM) 致敏（虚线符号）或者未用 $WT1_{126\ 134}$肽 (50nM) 致敏（实线符号）的 AML 患者收获

的 $WT1^{+}HLA\text{-}A2^{+}$（图 16B 中的黑色）或者 HLA-A2（灰色）白血病细胞。图 16C 示出其中异源 PBMC 用作靶标的类似结果。所有测定是以 1 的刺激物 / 反应物比率进行的。特异性斑点在 y 轴上示出为在刺激物的存在下产生的斑点减去由效应单独产生的斑点。* = $p<0.05$，** = $p<0.01$，*** = $p<0.001$。

[0280] 实施例 9 :潜在的脱靶切割分析。

[0281] 使用计算机模拟分析 (In silico analysis) 来识别 TRAC 特异性 ZFN 和 TRBC 特异性 ZFN 对最可能的潜在脱靶切割位点，如在 Perez 等人（同上）中所描述的。识别杂二聚体 ZFN 对或者同型二聚体对中包含多达 10 个识别位点错配的位点，但是这些 ZFN 对最可能的潜在脱靶位点是 ZFN 同型二聚体的所有靶标。被识别的最可能的潜在脱靶位点示出于下表 12(TRAC) 和下表 13(TRBC) 中。

[0282] 表 12 :TRAC 特异性 ZFN 的潜在脱靶位点

[0283]

标签	染色体	起始位点	序列	错配数	基因
OT1	20	20683361	AGGCACAaGCAAtGTCAC AAGtACcaTGCtTGTACTT (SEQ ID NO:76)	6	RALGAP A2
OT2	6	10525974	AGGTACAaGtAAAGaCGT ATGaACTTTGCtTGTACTT (SEQ ID NO:77)	5	GCNT2
OT3	X	135000000	AAaTACAaGCcAAGcCAA GGTGgCTTTGCGTGTAaA T (SEQ ID NO:78)	6	-
OT4	18	60239118	ATsTACAatAAAGTCAGC TTTtACTTTGCagtTACTT (SEQ ID NO:79)	8	ZCCHC2
OT5	7	48500931	TAGaACAtcCAAAcTCTGG ACCGACTTTGCaTGTcCA G (SEQ ID NO:80)	6	ABCA13
OT6	7	141000000	ATtCAaACaCAAAGTCCCG TGGAtTTTGCtTtTAaAT (SEQ ID NO:81)	7	-
OT7	8	2463159	ATGCAggaGCAAgGTCAC TCTGACcTTcCtTgcCTT (SEQ ID NO:82)	10	-
OT8	18	4312947	ATGCACACaCAAAcTCAT TTAagCTTTGCtTTcCAT (SEQ ID NO:83)	7	-
OT9	11	70854569	CAGCcCAtGgAAtGTCATT CTcACaTTGCtTGTGCTT (SEQ ID NO:84)	7	SHANK2
OT10	13	57970961	AAGCAaAaGaAAAaTCAA TATGACTTgGCtTtgGCTT (SEQ ID NO:85)	8	-
OT11	2	69188623	AAGgtCACtCActGTCTGTG TGGAgTTTGCGTGTcCTC	7	-

[0284]

			(SEQ ID NO:86)		
OT12	X	78538296	AAGCAggaGCAAAGTCAC ATCTtACaTTGCGgcgGCA T (SEQ ID NO:87)	8	-
OT13	2	108000000	ATGTAattcCAAAGTCCTC CATGACcTgGCtTcTACCT (SEQ ID NO:88)	8	-
OT14	8	28249779	CTaCAaAttCAAtGaCAGTA GAGACTTTGCtTtTACTT (SEQ ID NO:89)	8	-
OT15	9	93810846	ATGCAacaGCAAgagCAGC ATGACTTTQttTtTcCTT (SEQ ID NO:90)	10	-
TRAC	14	23016627	GTGCTGTgGCCTGGaGcA ACAAATCTGACTTTGCaT GTGCAA (SEQ ID NO:91)	4	TRAC

[0285] 表 13 :TRBC 特异性 ZFN 的潜在脱靶位点

[0286]

标签	染色体	起始位点	序列	错配数	基因
C1	1	236659757	CCcAagCCAGggCTACTGCT GGGTgGAACTGGACATGC (SEQ ID NO:92)	6	-
C10	10	90573967	CCcTGTgCgGTTCTgCTTAA CAGTAGAACaGGACActT (SEQ ID NO:93)	7	LIPM
C5	5	165037707	ACATGTCagaTTCTACATG AGGTAGAACTGttCTTGT (SEQ ID NO:94)	5	-
C2	2	71186796	ACAAGggCAGcTCTgtCCA AGGTAGcACTGGgCCTGT (SEQ ID NO:95)	7	ATP6V1B 1
C15	15	75401377	CgATGTCCAGaTgTACCTC AGGaAGgACTGGcCCTGG (SEQ ID NO:96)	6	-
C3	3	159730398	CCAAGTCCTccTCTAgGAA GGGGTAGAACTGGAaTTtG (SEQ ID NO:97)	6	-
C12	1	80766812	GaAGGTCCAGTgCaAtGTT GAaTAGAAgTGGACATcT (SEQ ID NO:98)	7	-
C17	17	11136639	AgAGGcCCAcTcCTAgAAG GGGTAGAcCTGGAtCTGG (SEQ ID NO:99)	7	-
C15.2	15	67440002	CCAGGTCCAGTTCTACCA GCCacAGAtgTGagCATGT (SEQ ID NO:100)	6	SMAD3
C2.2	2	120313989	ACAAtTCCAGTTCaAgAAT CTTtTAaAggTGGACATGG (SEQ ID NO:101)	7	PCDP1

[0287]

C6	6	166419926	GCtGGTgCAGcTCTACACG GATGcAGAgCTGGtCCTcC (SEQ ID NO:102)	7	-
C2.3	2	114753178	CCtGGgCCAGTgCTgCTTGT CcTtGAACcGGgCCTGG (SEQ ID NO:103)	8	-
C7.2	7	4224762	GgAGaTCCAGTgCgACAGT CAGaAGAAggGGACTcGG (SEQ ID NO:104)	8	SDK1
CX	X	73070673	CTaCAaAttCAAtGaCAGTA GAGACTTTGCTtTACTT (SEQ ID NO:105)	8	XIST
CX.2	X	130414175	CCAGGTCagGTTCeggAAA GAAGTAGAACTtGACCccT (SEQ ID NO:106)	8	AGSF1
TRBC	7	1424990111	TCAAGTCCAGTTCTACGG GCTCtCGGAGaATGACGA GTGGA (SEQ ID NO:107)	2	TRBC

[0288] 如图 17 和图 18 所示，相较于没有用 ZFN 表达载体转导的那些脱靶位点样本（还相较于 TRAC 和 TRBC 基因座），在已经用 ZFN 处理的脱靶位点样本中不存在附加条带。因此，显而易见 TRAC 特异性 ZFN 和 TRBC 特异性 ZFN 特异于其预期靶标。

[0289] 实施例 10 :TRAC 特异性 TALEN 和 TRBC 特异性 TALEN

[0290] TRAC 特异性 TALEN 和 TRBC 特异性 TALEN 是基本上如美国专利号 8,586,526 所描述进行开发和组装的。碱基识别是使用规范 RVD 碱基对应来实现的（“TALE 编码”:NI 对应 A、HD 对应 C、NN 对应 G(NK 是半重复的)，NG 对应 T)。TALEN 被构建在 TALEN 主链的 TAL 效应 DNA 结合域的“+63”C- 帽（C 端截断）中，如美国专利号 8,586,526 所描述的。所测试的 TALEN 的靶标和数字标识符示出于下表 14 中。

[0291] 表 14 :TRAC 特异性 TALEN 和 TRBC 特异性 TALEN

[0292]

SBS #	位点(5'-3')	RVD的数量	SEQ ID NO (靶位点)	TRAC/TRBC
101511	gtGCTGTGGCCTGGAGCaa	15	144	TRAC
101512	gtGCTGTGGCCTGGAGCAac	16	145	TRAC
101513	ctCTGGCCTGGAGCAACaa	15	146	TRAC
101514	ttGAAGGCGTTTGCACATGca	17	147	TRAC
101515	gtTGAAGGCGTTTGCACATgc	17	148	TRAC
101516	gtTGAAGGCGTTTGCACAtg	16	149	TRAC
101536	ttCCGCTGTCAAGTCCAGTtc	17	150	TRBC

[0293]

101537	ctGTCAAGTCCAGTTCtc	14	151	TRBC
101539	ctGGGTCCACTCCTCATTct	16	152	TRBC
101540	ctGGGTCCACTCCTCATTc	15	153	TRBC
101541	atCCTGGGTCCACTCCTCAtt	17	153	TRBC

[0294] 随后在 K562 细胞中成对地测试 TALEN 诱导内源 TRAC 和 TRBC 染色体靶标修饰的能力，测试是通过如上文在实施例 5 中描述的 Cel-I 测定来分析的。结果示出几乎所有的蛋白对是有活性的，并且 TALEN 和 ZFN 具有在大致相同的范围中的活性。表 15 和表 16 示

出了在用 Cel 1 测定检测的 NHEJ%方面对成对 TALEN 的矩阵比较。

[0295] 表 15 :TRAC 特异性 TALEN 和 TRBC 特异性 TALEN 的活性

[0296] 16A-TRAC(NHEJ%)

[0297]

	101511	101512	101513
101514	3.4	5.3	5.9
101515	5.9	8.9	8.3
101516	5.3	12.0	16.4

[0298] 表 16 :TRBC(NHEJ%)

[0299]

	101536	101537
101539	8.5	0.0
101540	9.9	9.6
101541	15.0	9.9

[0300] 实施例 11 :NY-ESO-1TCR 修饰的 T 细胞

[0301] T细胞是由NY-ESO-1特异性TCR Vβ13所修饰的(参见,例如Robbins等人(2011) JClin Oncol,29(7) :917-924),并且监测工程化 TCR 的表达。在该实验中,从健康志愿者分离 T 淋巴细胞,以及用 CD3 和 CD28 抗体共轭珠粒激活 T 淋巴细胞。随后根据在 Kaneko 等人 (Blood(2009),113(5) :第 1006 页) 和在 Bondanza 等人 (Blood(2011),117(24) :第 6469 页) 中描述的方法,在 5ng/mL 的 IL-7 和 5ng/mL 的 IL-15 的存在下培养细胞。随后用以下三种方式其中一种来处理细胞 :组 1 用包含 NY-ESO1 特异性、HLA-A2 限制的 TCRα 链和 TCRβ 链的第三代双向慢病毒载体 (TCR-PGK-NYESO1LV)(参见 Amendola 等人 (2005)Nat. Biotechnol,23 :108-116) 进行转导,以产生 TCR‘经转移’的“TR”T 细胞。组 2 在 LV 转导之前用包含特异于 TRAC 的 ZFN(参见实施例 6,ZFN 25539 和 25540) 的腺病毒处理,以及随后根据 CD3 信号的损失对组 2 进行分选。随后用 TCR-PGK-NYESO1LV 载体转导 CD3 细胞,以产生“经单一编辑的”或者“SE”细胞群体。组 3 首先用包含如上所述的 TRAC ZFN 对 25539/25540 的腺病毒处理,然后根据 CD3 信号进行分选。CD3 细胞随后用包含 NY-ESO-1TCRα 链的 LV 载体转导,以及再次根据 CD3 信号进行分选。在这种情况下,$CD3^+$细胞随后用 baCD3/CD28 珠粒刺激,并暴露于包含 TRBC ZFN 对 16787/16783 的腺病毒,然后分选不存在 CD3 表面转位的细胞。CD3 细胞随后用包含 NY-ESO-1TCRβ 链的 LV 载体转导。因此,组 3 在没有任何内源的 TCR 复合物的情况下唯一地表达 NY-ESO-1 特异性 TCR,并且被称为“经完全编辑的”或者“CE”群体。

[0302] 用细胞荧光分析法来分析这三组细胞对外源 Vβ13TCR 的表达,其中使用抗 NY-ESO-1 特异性 Vβ13.1 链的抗体来标记蛋白。未转导的 T 细胞用作对照,以及数据可被

表示为在经转导的 T 细胞中观测到的平均荧光强度 (MFI) 比对照中观测到的 MFI。经完全编辑的群体显示最高的表达 (参见,图 19A)。

[0303] 还测试了 T 细胞群体对 MHC HLA-A2-NY-ESO1dextramer 的结合。MHC Dextramer 由携带数量优化的 MHC 和荧光染料分子的右旋糖苷聚合物主链构成。MHC Dextramer 反应物比常规的 MHC 多聚体携带更多的 MHC 分子和更多的荧光染料。这增强对特异性 T 细胞的亲合力并且增强染色强度,从而增大了分辨度和信噪比。按照制造商供应的方案 (例如, Immudex 肿瘤 - 睾丸抗原 Dextramer® 集合) 来进行染色。样本通过 FACS Canto II 流式细胞仪 (BD Biosciences 公司) 跑样,以及用 Flow Jo 软件 (Tree star 公司) 分析数据。结果显示经完全编辑的群体具有对 NY-ESO1dextramer 最大的亲合力 (参见图 19B)。图 19 中的数据被表示为相对荧光强度 (RFI),意思是在样本群体 (经转移、经单一编辑或者经完全编辑的 T 细胞) 中观测到的平均荧光强度 (MFI) 与未转导 T 细胞中观测到的 MFI 之间的比率。使用 3 个不同的供体进行三组连续实验。结果 (图 19C) 显示 CE 群体具有最高的信号。

[0304] 另外,用 FACS 分析来分析细胞的表型标记,如在 Cieri 等人 ((2013)Blood,121 : 第 573-584 页) 中所述。分析显示部分经修饰的 T 细胞表现干记忆 T(T_{SCM}) 细胞的表型,特征为 CD45RA、CD62L 和 CD95 的共表达。

[0305] TCR 经编辑的淋巴细胞的经完全编辑群体当用不断增加剂量的 NY-ESO1 157-165 肽致敏时,在 γ-IFN ELISpot 测定 (例如,人类 IFNγ ELISPOT Ready-SET-Go!®, eBioscience®) 中显示对同源抗原的高亲合力。所使用的效应细胞是未转导的 (UT)、经转移的、经单一编辑的 T 细胞 (SE),以及经完全编辑的 T 细胞 (CE)。结果示出于图 20A,并且显示 TCR 经完全编辑 (CE) 的群体表现对肽的高亲合力。T2 细胞加载有不断增加浓度的 NY-ESO-1 157-165 肽,或者加载有来源于 Wilms 肿瘤抗原 1 的无关 $WT1_{126\ 134}$ 肽 (“T2-$WT1_{126\ 134}$”)。

[0306] NY-ESO1 重定向 T 细胞 1 随后由 NY-ESO1+.HLA-A2+ 骨髓瘤细胞系 (U266) 致敏,以验证其识别天然表达 NY-ESO1 抗原的肿瘤细胞的能力。首先使用 U266 或者 MM1S 细胞系作为靶标来进行 γ-IFN ELISpot(如上所述)(参见图 20B),测定显示相较于未转导的 T 细胞,NY-ESO1 重定向 T 细胞具有对相关 HLA-A2+、NY-ESO1+ 细胞的高亲合力,并且几乎未检测到与 MM1S 细胞的结合。未观测到对抗 MM1S(HLA-A2 和 NY-ESO1) 不相关靶细胞的识别。接着,使用如下标准方法进行 ^{51} 铬释放 :在 V 形底 96 孔培养平板中将效应子 T 细胞与骨髓瘤细胞系 (MM1S 和 U266) 一起孵育 5 小时,骨髓瘤细胞系事先用 ^{51} 铬标记。特异性细胞溶解是根据以下公式表示的 :100×(平均实验溶解 - 平均天然溶解)/(平均最大溶解 - 平均天然溶解)。

[0307] 结果 (图 20C 和图 20D) 显示不同的群体能够引起相关靶细胞 U266 的细胞溶解 (图 20C),但是不能够造成不相关的靶细胞 MM1S 的胞溶 (图 20D)。经完全编辑 T 细胞 (CE) 表现出溶解适当的靶细胞的最大能力。

[0308] 还在共培养实验中检测 NY-ESO1 重定向 T 细胞特异地杀伤 $NY\text{-}ESO1^{+}$、$HLA\text{-}A2^{+}$ 肿瘤细胞的能力 (参见图 21)。在这个实验中,在效应物 / 靶标比为 1 :1 时,将效应 T 细胞与相关的 U266 细胞系 (“A2+ESO+”) 或者不相关的 MM1S 细胞系 (“A2-ESO-”) 共培养 4 天。结果显示重定向 T 细胞效应子能够阻遏相关的 HLA-A2+、NY-ESO1+ 细胞系的生长。图

21B 显示在 U266HLA-A2+、NY-ESO1+ 靶标的存在下经编辑的 T 细胞扩增 2 倍，而在不相关的 A2-ESO- 对照的存在下经编辑的 T 细胞不扩增。

[0309] 实施例 12 :经编辑 T 细胞的同种异体反应性

[0310] 为了比较三个 NY-ESO-1 重定向 T 细胞群体的同种异体反应潜能，在抗经辐射的同种异体外周血单核细胞 (PBMC) 的混合淋巴细胞反应 (MLR) 中分别平板培养 TCR 经转移 (转移)、TCR 经单一编辑 (SE) 和 TCR 经完全编辑 (CE) 的 T 细胞。将供体匹配的 PBMC 和 mock 经转导的 T 细胞 (UT) 用作对照。在两个周期的刺激 (S110 天，S27 天) 之后，在 ^{51}Cr 释放和 γ 干扰素 (γ -IFN)Elispot 测定中检测效应细胞对用以相同同种异体靶标获得的 PHA 细胞系和自体同源细胞的反应。

[0311] 同时，用以 NY-ESO-1157-165 致敏的 HLA-A2+ 经辐射细胞刺激 NY-ESO-1 重定向 T 细胞和对照。在两个周期的刺激 (S1 10 天，S27 天) 之后，测试效应细胞对用以细胞对用以 NY-ESO-1157-165 肽致敏 (C) 或者未致敏 (D) 的 HLA-A2+T2 细胞系的反应。

[0312] 未观测到对自体同源细胞的反应。另外，如图 22 所示，转移 T 细胞对异源靶标的细胞溶解作用显著高于 SE 和 CE T 细胞对异源靶标的细胞溶解作用 ($p = 0.05$)(图 22A)。此外，γ -IFN Elispot 确证当同种异体刺激时转移 T 细胞和经编辑 T 细胞之间在分泌 γ -IFN 方面存在统计上显著的差异，这表明剩余的内源多克隆 TCR 和在 TCR 经转移 T 细胞的细胞表面上表达的可能错配的 TCR 可导致脱靶反应性，而 SE 和 CE T 细胞则没有此类反应性。NY-ESO-1 重定向 T 细胞 (转移、SE 和 CE) 同样能够相较于溶解未致敏细胞 (图 22D) 以高特异性溶解用 NY-ESO-1 特异性肽致敏的 T2 细胞 (图 22C)。

[0313] 实施例 13 :体内实验

[0314] 为了比较 NY-ESO-1 经单一编辑 (SE)、经完全编辑 (CE) 和 TCR 经转移 (转移) 的 T 细胞的体内效力和安全性，我们设置了基于以下步骤的小鼠模型 :注射多发性骨髓瘤 (MM) U266 细胞系 (HLA-A2+、NY-ESO-1+、hCD138+)，然后向经亚致死辐射的 NSG 小鼠施用 T 细胞。简言之，在第 0 天通过尾静脉注射注入 10×10^6 个 U266 细胞。在第 3 天小鼠接受静脉内注射以下物质 :PBS(U266)，或者 10×10^6 NY-ESO-1 转移 T 细胞、SE T 细胞、CE T 细胞，或者供体匹配 PBMC，或者作为对照的供体匹配 mock 转导 T 细胞 (UT)。最终一组小鼠接受重定向到不由 U266 表达的 WT1126-134 肽的 10×10^6 个经完全编辑的 T 细胞 (CEWT1)。每周至少 3 次监测小鼠的异种移植物抗宿主病 (GvHD) 病征，并且在第 70 天没有任何病理性的病征的情况下处死小鼠。由于将 U266 植入小鼠需要较长的时间，所以我们考虑仅评估在第 70 天处死的动物的抗肿瘤反应。所有小鼠可视为是 GvHD 评价可评估的。

[0315] 结果示于图 23。图 23A 示出通过对从安乐死小鼠的骨髓收获的细胞进行细胞荧光分析识别的人类 CD138+MM 细胞的百分比。在用 NY-ESO1 重定向 T 细胞处理的小鼠中，在骨髓中或者在处死时的脾脏 (未示出) 中未检测到残留疾病，这显示 NY-ESO1 重定向细胞具有体内效力。相较而言，注入 PBS(U266) 或者 CE WT1T 细胞的所有小鼠在其骨髓中具有可检测出的肿瘤细胞。

[0316] 在处死时所有器官被收集，用福尔马林固定，用苏木色素 / 伊红染色，并且在用单克隆抗 hCD3 抗体和过氧化物酶共轭的第二步反应物复染之后，通过免疫组织化学法同时分析，以检测任何可能的 GvHD 活性和检查 T 细胞特异性。小鼠器官中的渗透物将指示 T 细胞的不适当归巢，以及潜在地 GvHD 的初始阶段。病理分级在从 0(无 hCD3+ 细胞渗入) 到

3(大量和扩散的 hCD3+ 细胞渗入)的范围内。有趣的是,已发现人类 CD3+T 细胞渗入到注入传统的 TCR 转移 T 细胞的 5 只动物中的 3 只的肺和肝中(“转移”),这类似于在注入供体匹配的未操纵 PBMC(“PBMC”)的 4 只小鼠中的 4 只中或者注入未转导的淋巴细胞 (5 只小鼠中的 5 只,“UT”) 中观测到的渗入。相反地,在用 NY-ESO-1SE 或者 CE T 细胞处理的小鼠的器官中未检测淋巴细胞渗入(图 23B)。

[0317] 实施例 14 :使用 mRNA 电穿孔的 TCR 编辑

[0318] A. 单一 TCR 编辑

[0319] 通过核酸酶信息的 mRNA 电穿孔进行的 TCR 编辑是由如下评估的。简言之,用抗 CD3/CD28 珠粒刺激来自外周血的人类 T 淋巴细胞,并且在两天后用不断减少剂量的体外转录 mRNA 进行电穿孔,mRNA 编码特异于 TRAC 基因或者 TRBC 基因的 ZFN 对。

[0320] 处理时 ZFN 诱导的 TCR 断裂的程度被测量为在用 TRAC-ZFN 处理(图 24A 中的左小图)和用 TRBC-ZFN 处理(图 24A 中的右小图)的淋巴细胞中电穿孔后 5 天或者 20 天时 CD3 阴性细胞的百分比。此外,还评估了在用 TRAC-ZFN 处理过的细胞(图 24B 的左小图)中 ;在用 TRBC-ZFN 处理过的细胞(图 24B 的中间小图)中 ;以及在对照细胞(图 24B 的右小图)中培养期间经处理细胞的数目的成倍增加。另外,还评估刺激后第 18 天时的 T 细胞表面表型。T 干记忆细胞 (TSCM) 被定义为 CD62L+CD45RA+(参见 Gattinoni 等人 (2011) Nat Med.,17(10) :1290-7 ;以及 Cieri(2013)Blood,121(4) :573-84) ;T 中枢记忆 (TCM) 被定义为 CD62L+CD45RA- ;T 效应因子记忆 (TEM) 被定义为 CD62L-CD45RA-,以及端效应因子 (TEMRA) 被定义为 CD62L-CD45RA+。UT :未处理的细胞 ;UT+E :mock 电穿孔细胞 ;GFP :用编码 GFP 的 mRNA 电穿孔的细胞。在所利用的 mRNA 剂量处,用 TRAC ZFN 处理的细胞和用 TRBC ZFN 处理的细胞的表型组成不存在统计上显著的差异(双向方差分析)。

[0321] B. TCR 双重编辑

[0322] 用抗 CD3/CD28 珠粒刺激来自外周血的人类 T 淋巴细胞,并且在两天后用体外转录 mRNA 进行共同电穿孔,mRNA 编码 TRAC 特异性 ZFN 对和 TRBC 特异性 ZFN 对两者,如上所述。接着,进行分析以量化经共处理的细胞中为 CD3 阴性部分的 TCR-α 和 TCR-β 经完全编辑的细胞的量。简言之,在电穿孔后 5 天时,分别用编码 α 或者 β NY-ESO 特异性 TCR 链和报道基因(分别为 LNGFR 或 GFP,在图 25A 中示意性地描绘)的双向慢病毒载体 (LV) 分选和转导 CD3 阴性细胞。单一 α 或者 β 编辑的细胞分数被测量为当与外源 TCRα 或者 β 互补时修复 CD3 表面表达的经转导细胞的百分比。CD3 阴性总群体中经完全编辑的细胞的量随后是通过减去两种单一编辑的细胞的百分比来计算的。结果示出于图 25A 中,显示 40% 的经完全编辑细胞群体在 TCR-α 基因和 TCR-β 基因两者处断裂。

[0323] 在用包含专性的异源二聚体 FokI 域 (ELD 和 KKR) 或者它们的相应直系同源版本 (RDD 和 DRR) 的 TRAC 特异性 ZFN 和 TRBC 特异性 ZFN mRNA 进行共同电穿孔时 CD3 阴性 (CD3-) 细胞的百分比。活细胞的百分比被计算为设门在单线态处的 7-AAD 阴性细胞的百分比。此外,经编辑细胞在 CD3 阴性部分中的组成是使用如上所述的 LV 报道基因策略计算的。结果示出在图 25B 中。

[0324] 还在刺激后第 18 天确定如上所述 T 细胞的表面表型。T 干记忆细胞 (TSCM) 被定义为 CD62L+CD45RA+(参见 Gattinoni 等人 (2011) 同上 ;Cieri 等人 (2013),同上) ;T 中枢记忆 (TCM) 被定义为 CD62L+CD45RA- ;T 效应记忆 (TEM) 被定义为 CD62L-CD45RA-,以及端

效应子 (TEMRA) 被定义为 CD62L-CD45RA+。UT :未处理的细胞。结果示出于图 25C。

[0325] 还确定用所指示剂量的 TRAC 特异性 ZFN mRNA 和 TRBC 特异性 ZFN mRNA 共同电穿孔的 T 细胞的生长曲线,生长曲线示出在共同电穿孔次日细胞消亡的初始急性期之后,存活的细胞以与未处理的 (UT) 对照类似的动力学继续在培养基中扩增 (图 25D)。

[0326] 本文提及的所有专利、专利申请和出版物据此通过引用整体并入。

[0327] 虽然已经出于透彻了解的目的以图示和举例说明的方式相当详细地提供了本公开内容,但是对本领域中的技术人员将显而易见的是在不脱离本公开的精神或者范围的情况下可实现各种改变和修改。因此,上述描述和实施例不应被理解为限制。

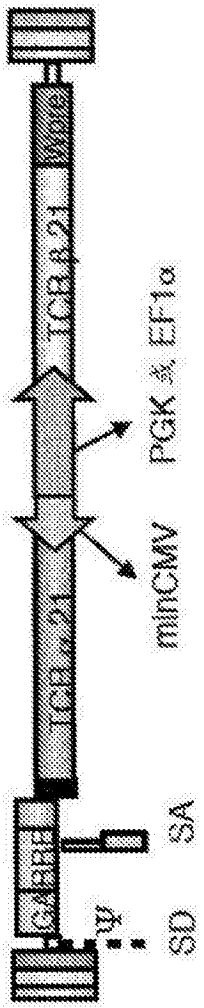

TCRA21
TCRB21
minCMV
PGK & EF1α
SA
SD
Ψ
CβBR

图 1A

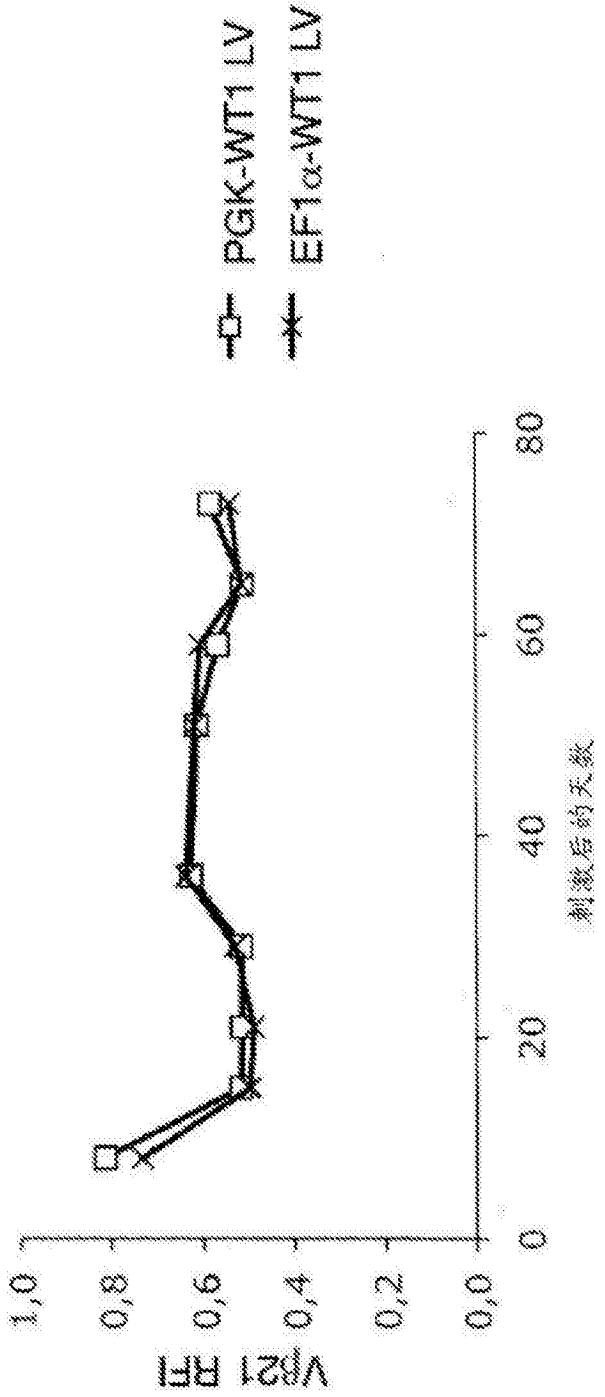

PGK-WT1 LV
EF1α-WT1 LV
Vβ21 RFI
1,0
0,8
0,6
0,4
0,2
0,0
0
20
40
60
80
刺激后的天数

图 1B

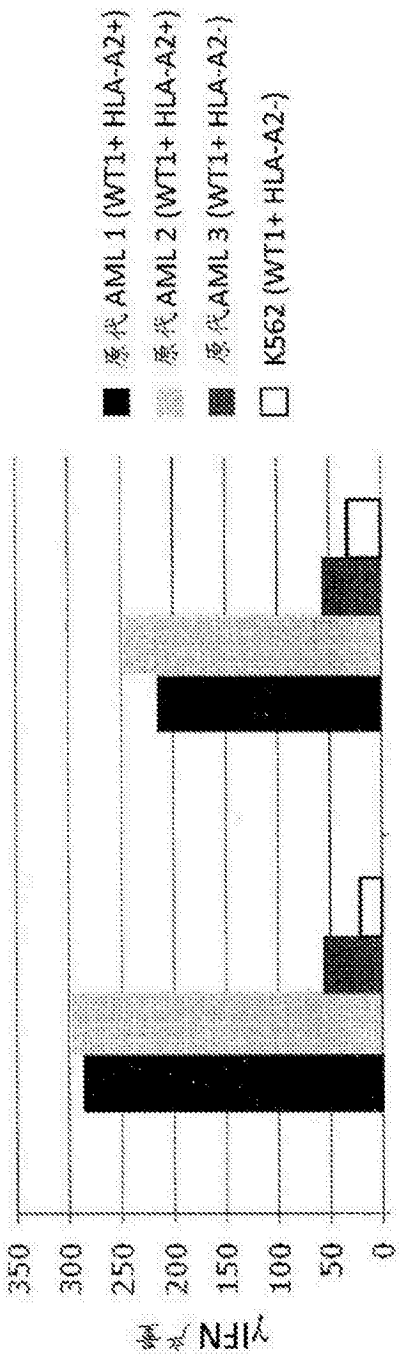

原代AML 1 (WT1+ HLA-A2+)
原代AML 2 (WT1+ HLA-A2+)
原代AML 3 (WT1+ HLA-A2-)
K562 (WT1+ HLA-A2-)
350
300
250
200
150
100
50
0

图 2A

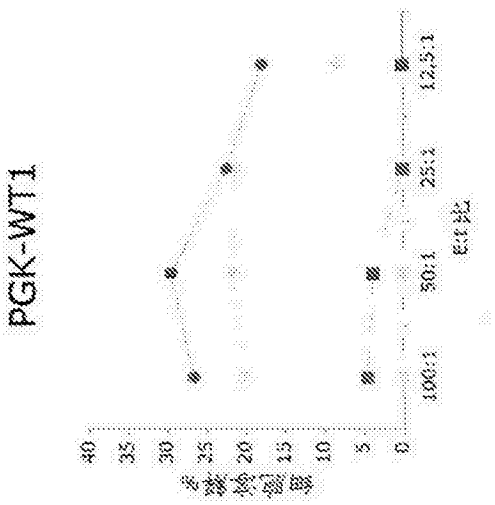

PGK-WT1
40
35
30
25
20
15
10
5
0
100:1
50:1
25:1
12.5:1

图 2B

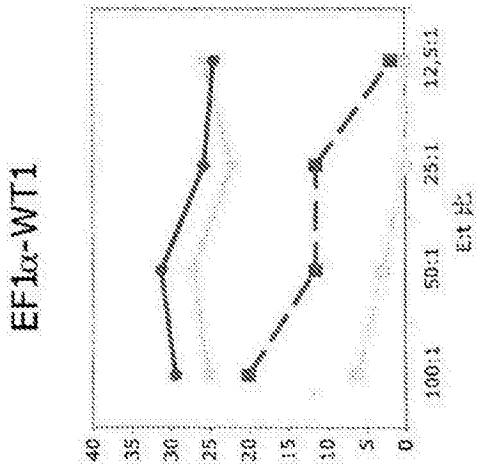
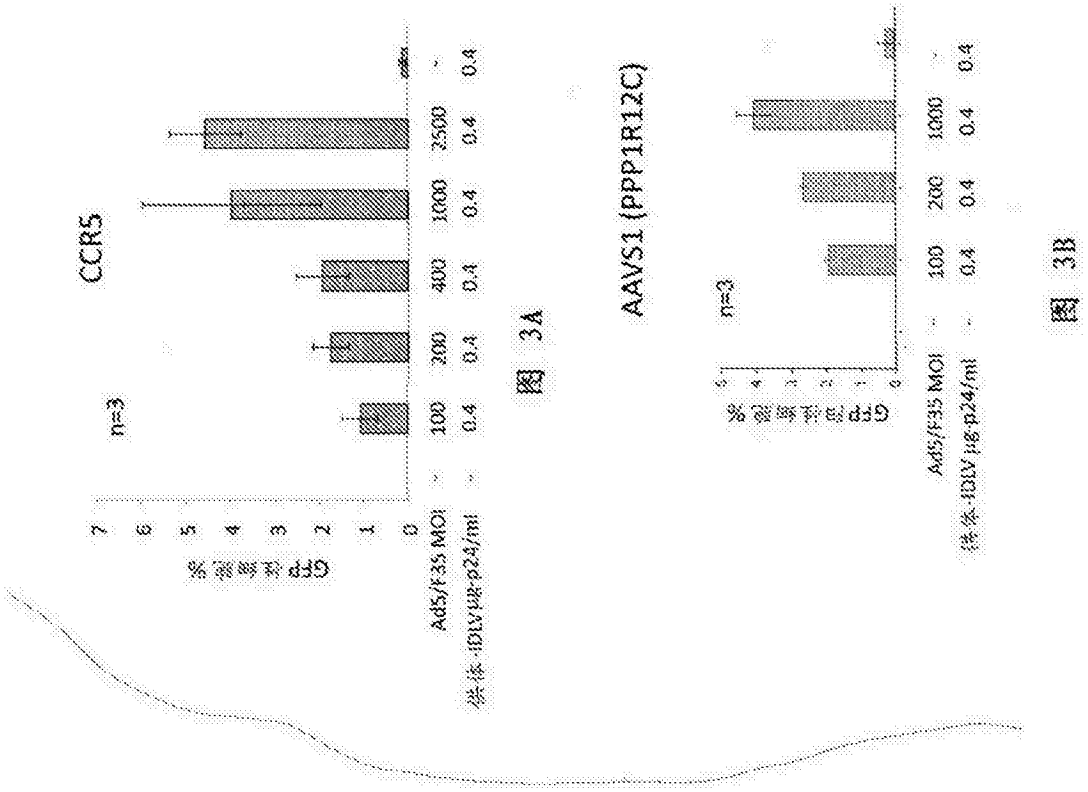

EF1α-WT1
40
35
30
25
20
15
10
5
0
100:1
50:1
25:1
12.5:1
E:T 比

图 2C


CCR5
n=3
GFP 阳性细胞 %
7
6
5
4
3
2
1
0
Ad5/F35 MOI - 100 200 400 1000 2500 -
慢体-IDLV μg-p24/ml - 0.4 0.4 0.4 0.4 0.4 0.4
图 3A
AAVS1 (PPP1R12C)
n=3
GFP 阳性细胞 %
5
4
3
2
1
0
Ad5/F35 MOI - 100 200 1000 -
慢体-IDLV μg-p24/ml - 0.4 0.4 0.4 0.4
图 3B

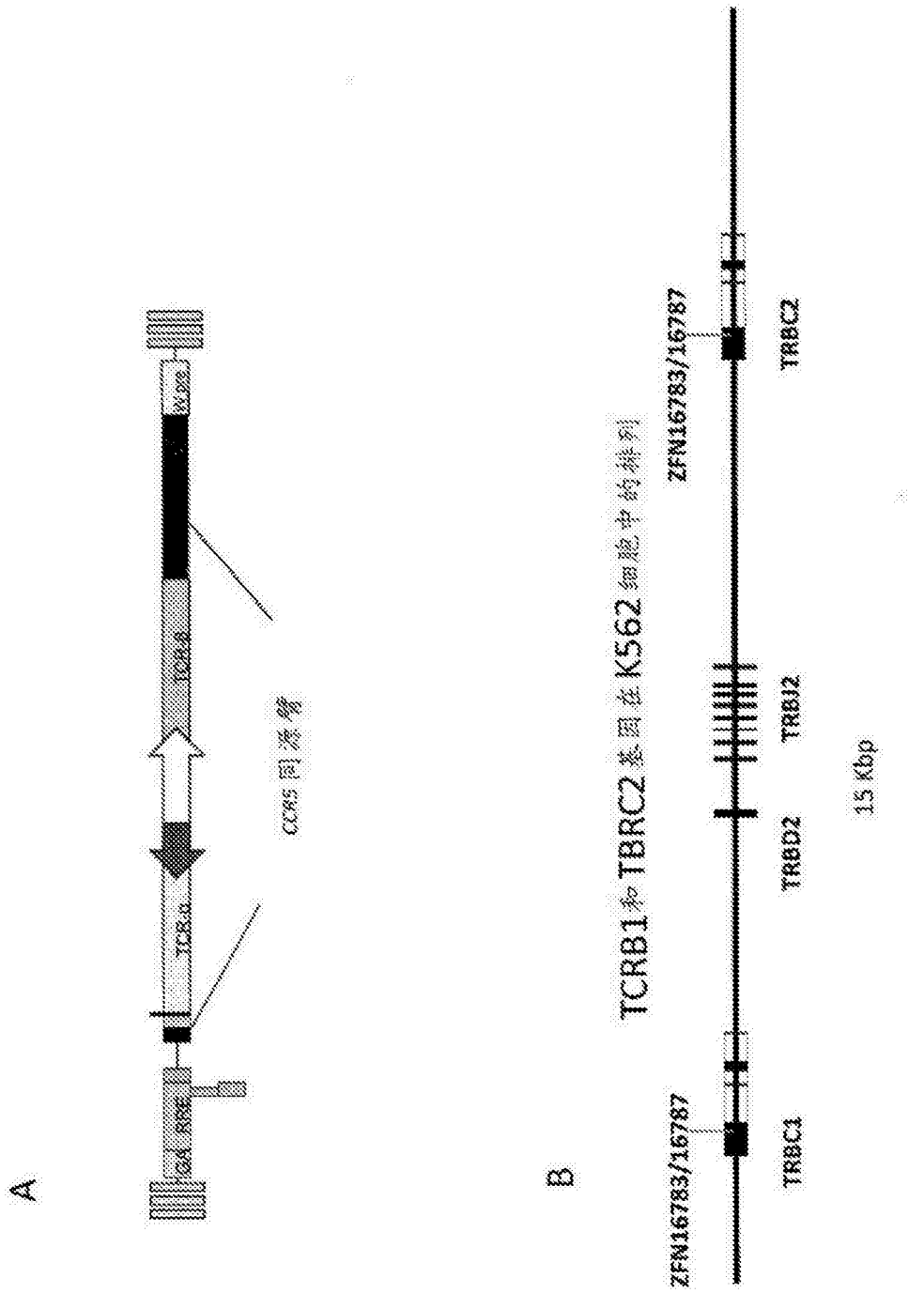

A
CCR5 同源臂
TCR α
TCR β
B
TCRB1 和 TBRC2 基因在 K562 细胞中的排列
ZFN16783/16787
TRBC2
TRBJ2
TRBD2
TRBC1
ZFN16783/16787
15 Kbp

图 4

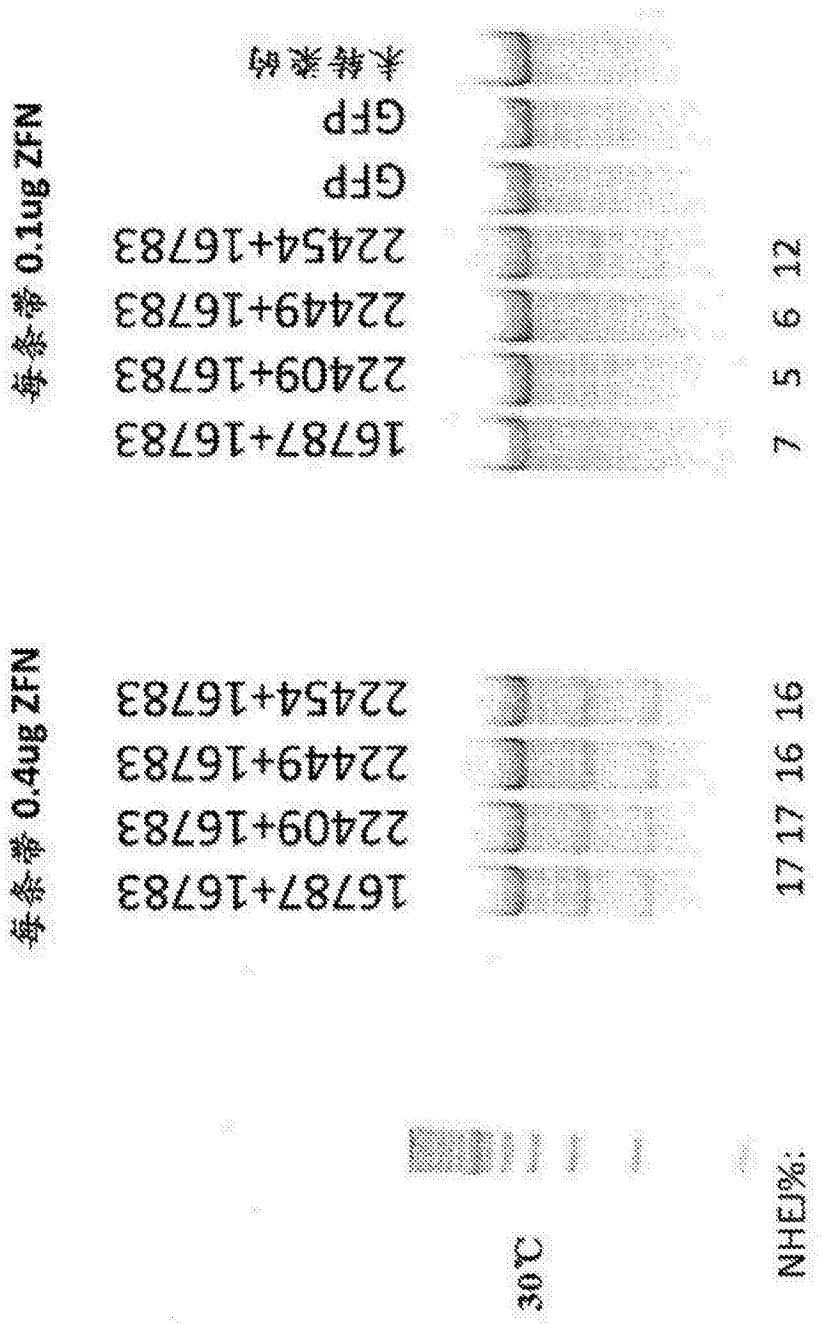


图 5 :K562 细胞中的 TCR-β 特异性 ZFN

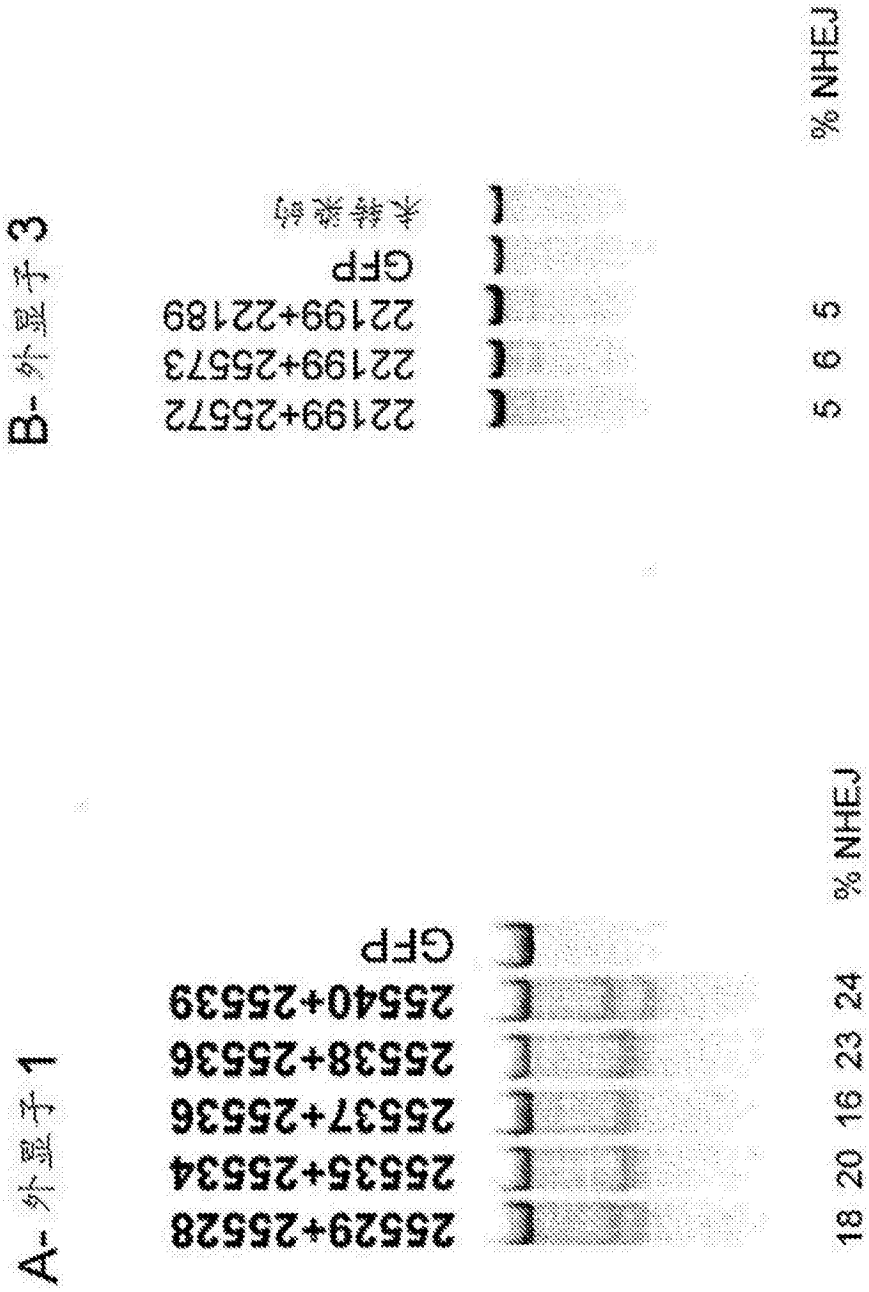


图 6-TCRα 特异性 ZFN

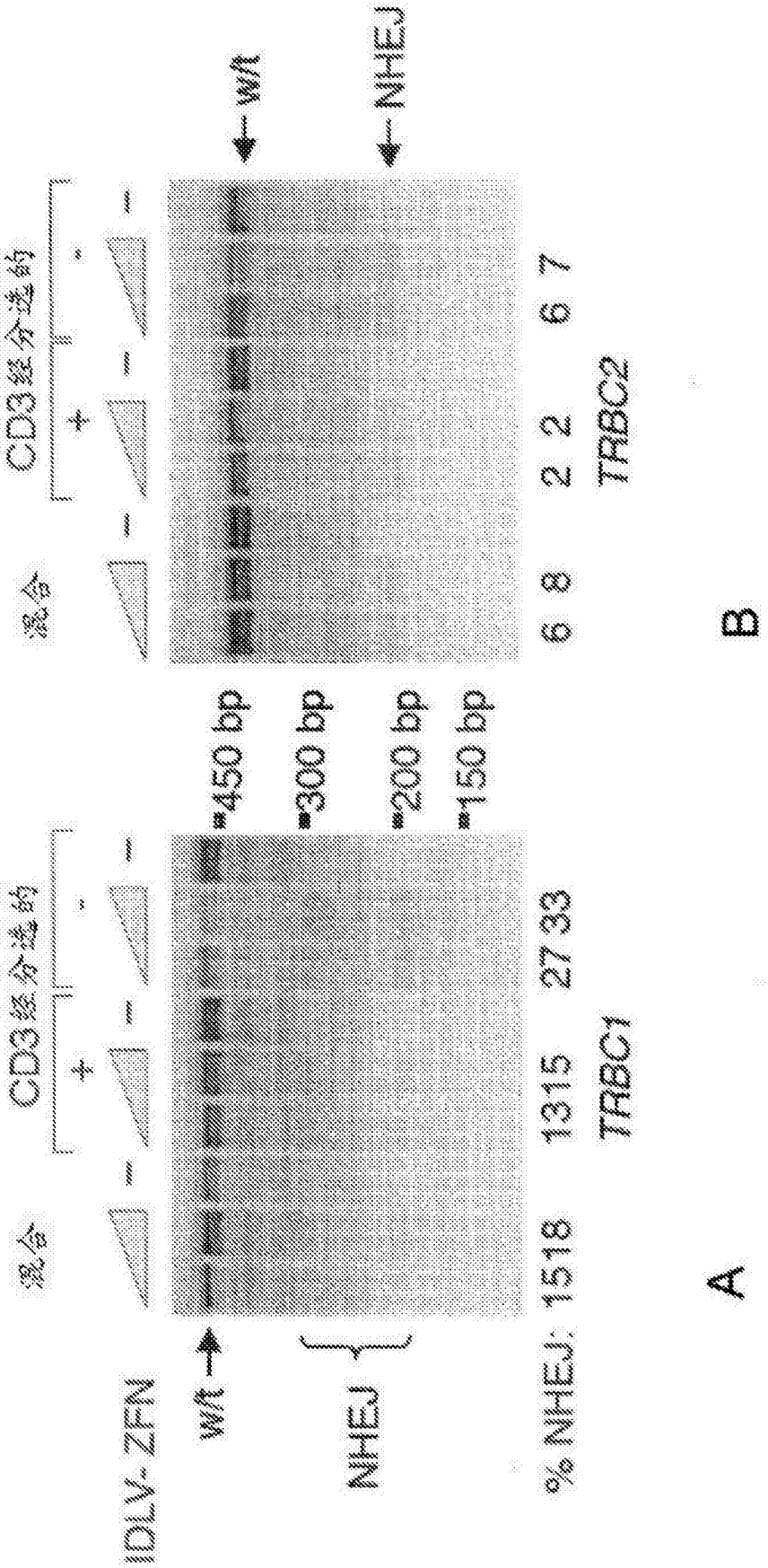

IDLV-ZFN
混合
CD3经分选的
w/t
NHEJ
% NHEJ: 15 18 13 15 27 33
TRBC1
A
450 bp
300 bp
200 bp
150 bp
混合
CD3经分选的
w/t
NHEJ
6 8 2 2 6 7
TRBC2
B

图 7

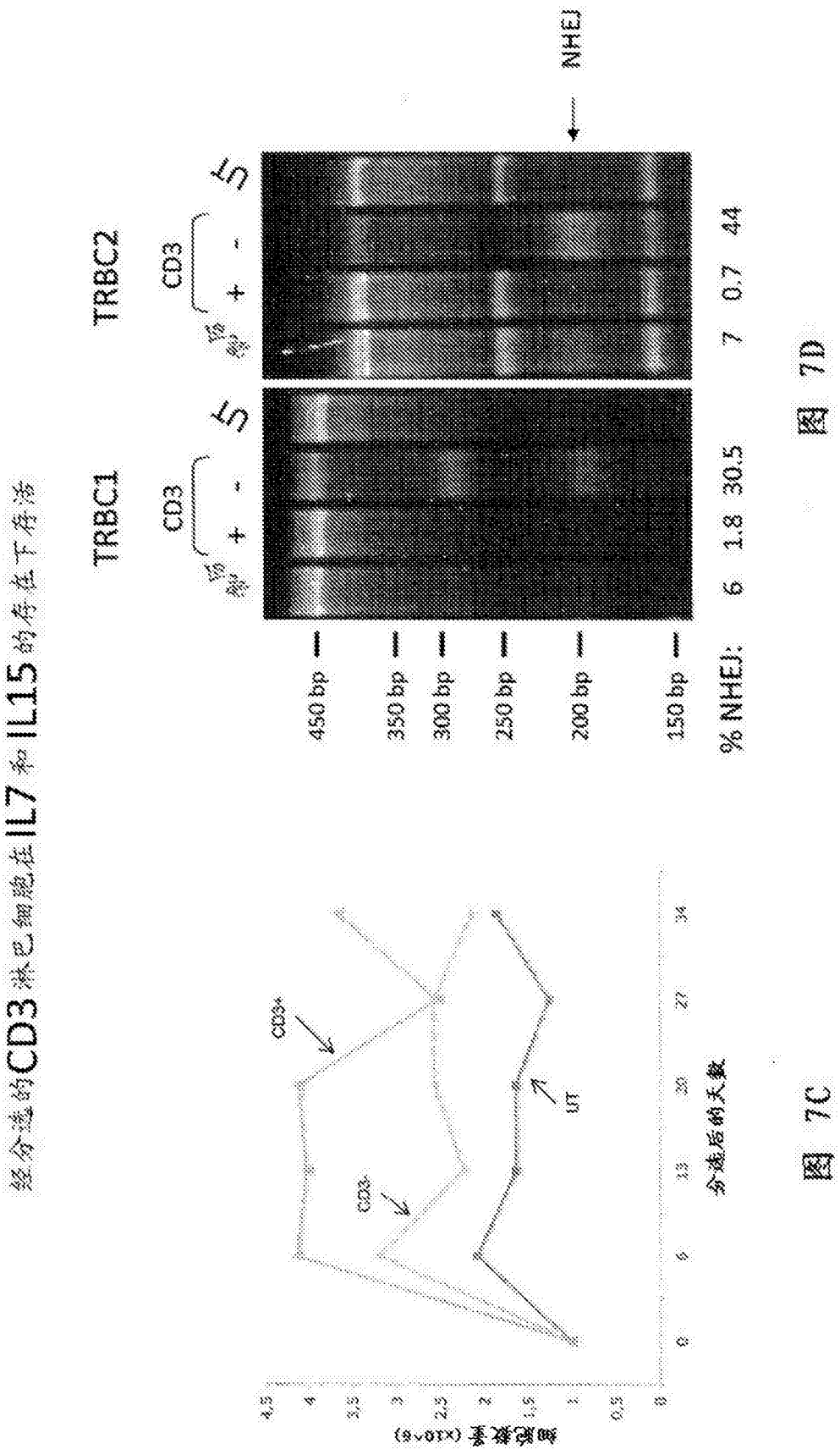
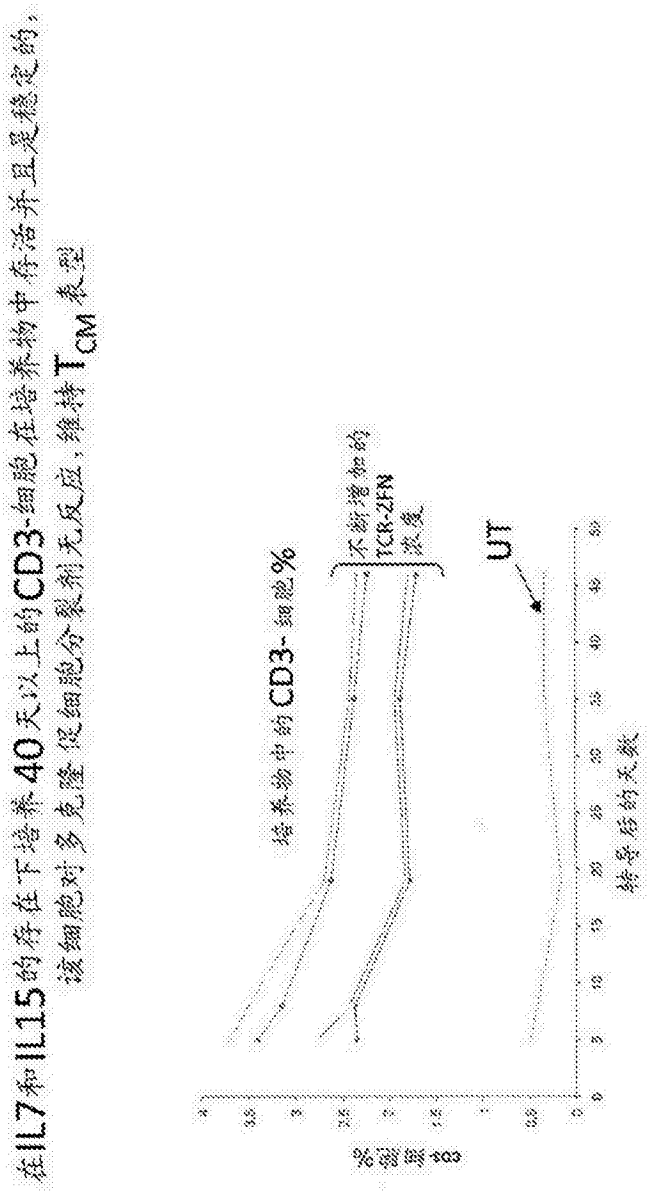
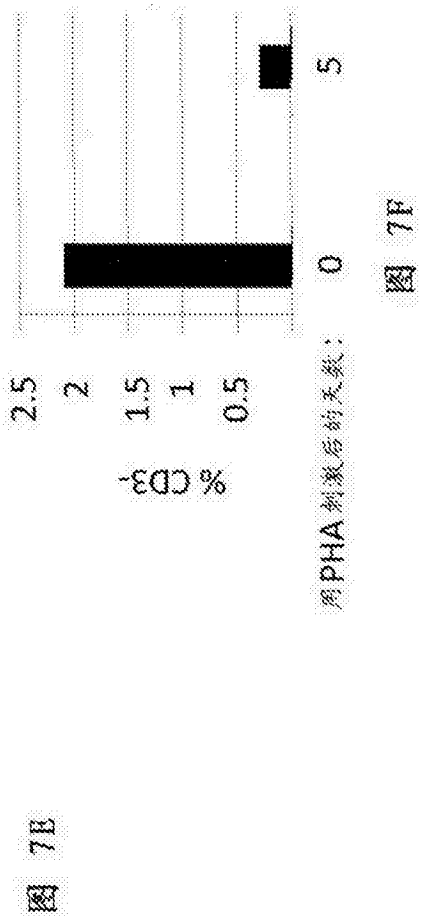

经分选的CD3淋巴细胞在IL7和IL15的存在下存活
细胞数量 (x10^6)
分选后的天数
CD3+
CD3-
UT
TRBC1
TRBC2
CD3
UT
NHEJ
450 bp
350 bp
300 bp
250 bp
200 bp
150 bp
% NHEJ: 6 1.8 30.5 7 0.7 44

图 7C

图 7D


在IL7和IL15的存在下培养40天以上的CD3-细胞在培养物中存活并且是稳定的，
该细胞对多克隆促细胞分裂剂无反应，维持T_{CM}表型
培养物中的CD3-细胞%
不断增加的
TCR-ZFN
浓度
UT
转导后的天数
图 7E


CD3-%
用PHA刺激后的天数：
图 7F

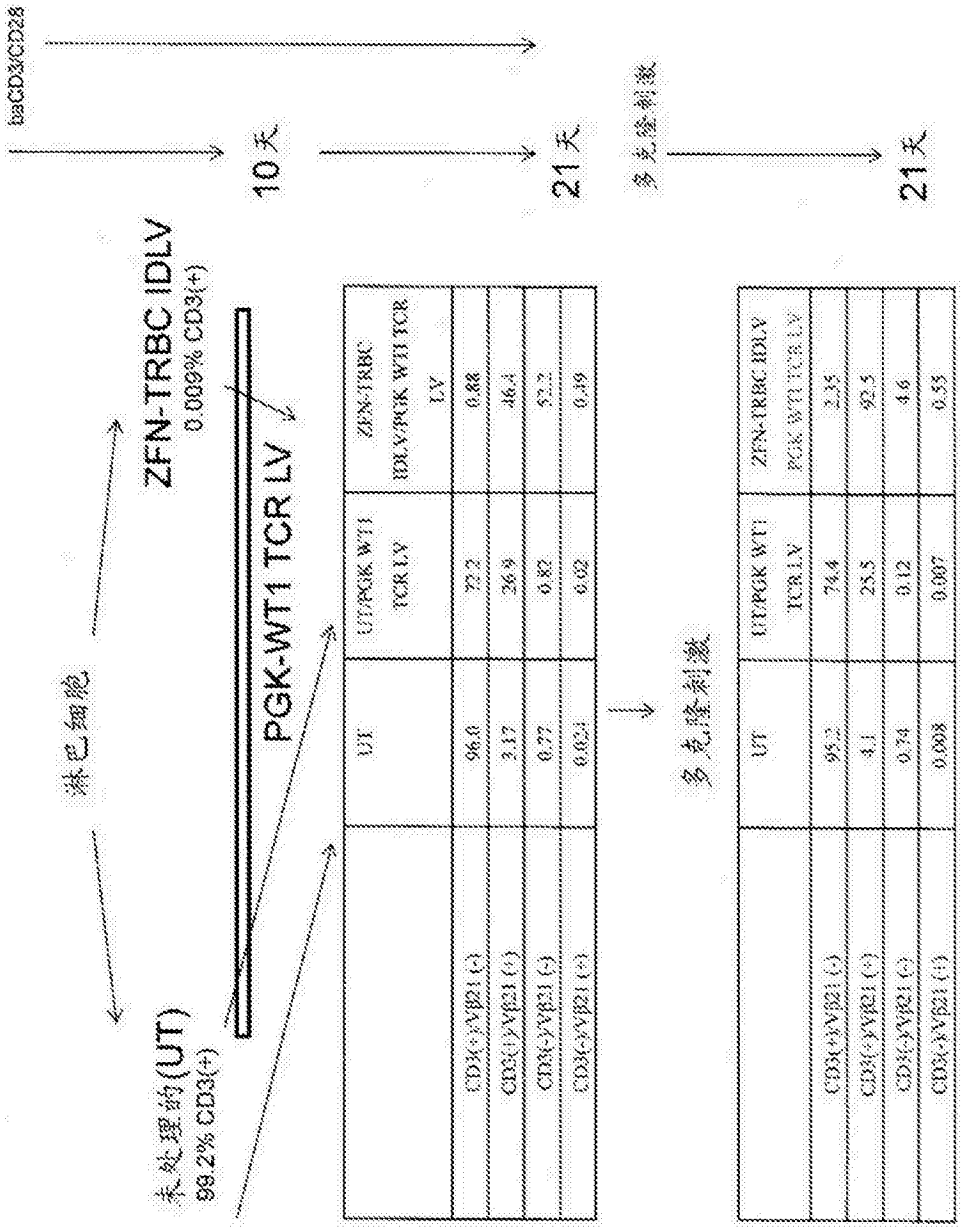

taaCD3/CD28
10天
21天
多克隆刺激
21天
ZFN-TRBC IDLV
0.009% CD3(+)
PGK-WT1 TCR LV
淋巴细胞
未处理的(UT)
99.2% CD3(+)
UT
UT/PGK WT1 TCR LV
ZFN-TRBC IDLV/PGK WT1 TCR LV
CD3(+)Vβ21(-)
CD3(+)Vβ21(+)
CD3(-)Vβ21(-)
CD3(-)Vβ21(+)
96.0
3.17
0.77
0.023
72.2
26.9
0.82
0.02
0.88
46.4
52.2
0.39
多克隆刺激
UT
UT/PGK WT1 TCR LV
ZFN-TRBC IDLV PGK WT1 TCR LV
95.3
4.1
0.34
0.008
74.4
25.5
0.12
0.007
2.35
92.5
4.6
0.55

图 8

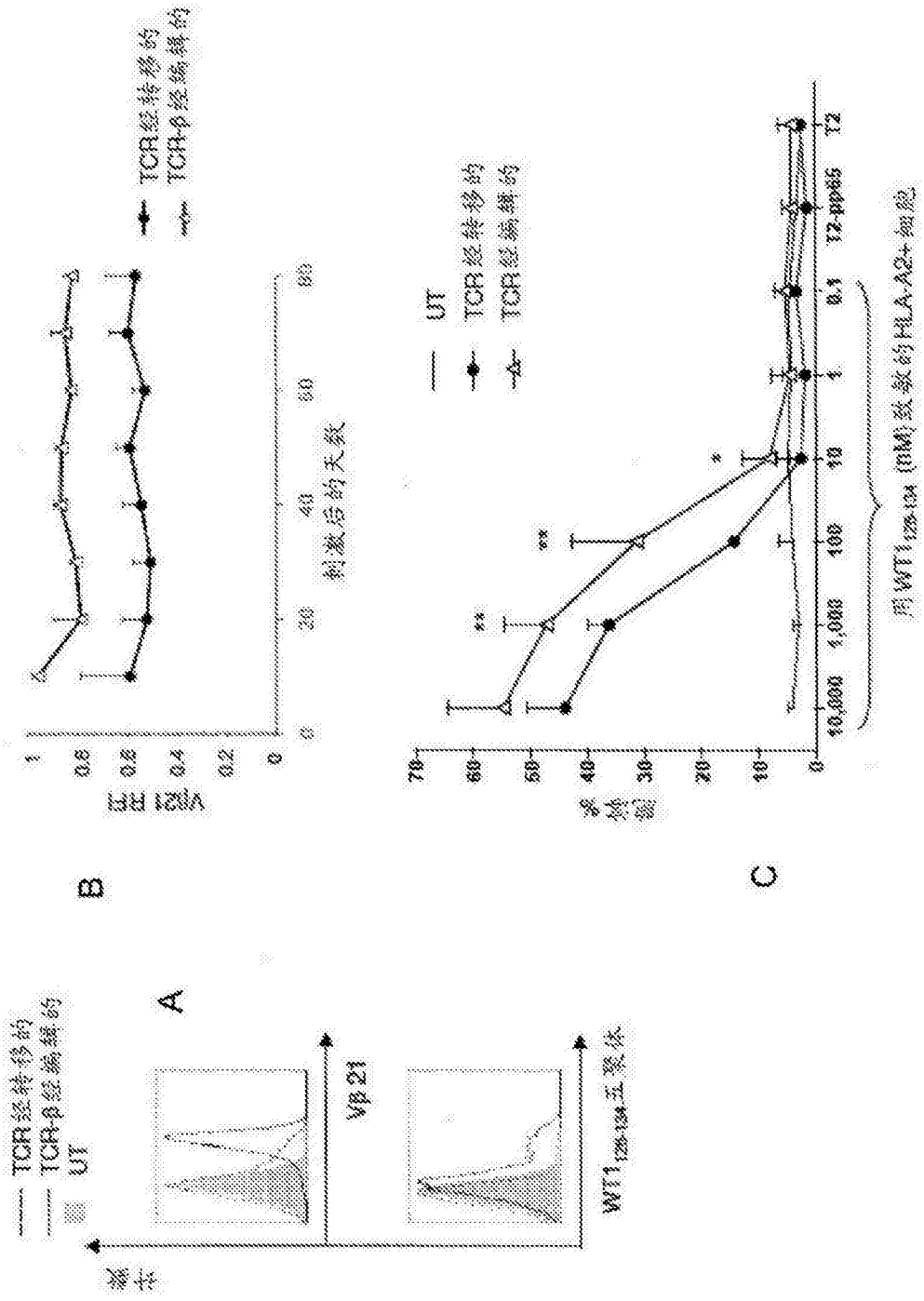

TCR经转移的
TCR-β经编辑的
UT
Vβ21
WT1126-134五聚体
A
B
C
刺激后的天数
%特异
用WT1126-134 (nM)致敏的HLA-A2+靶细胞
T2
T2-pp65

图 9

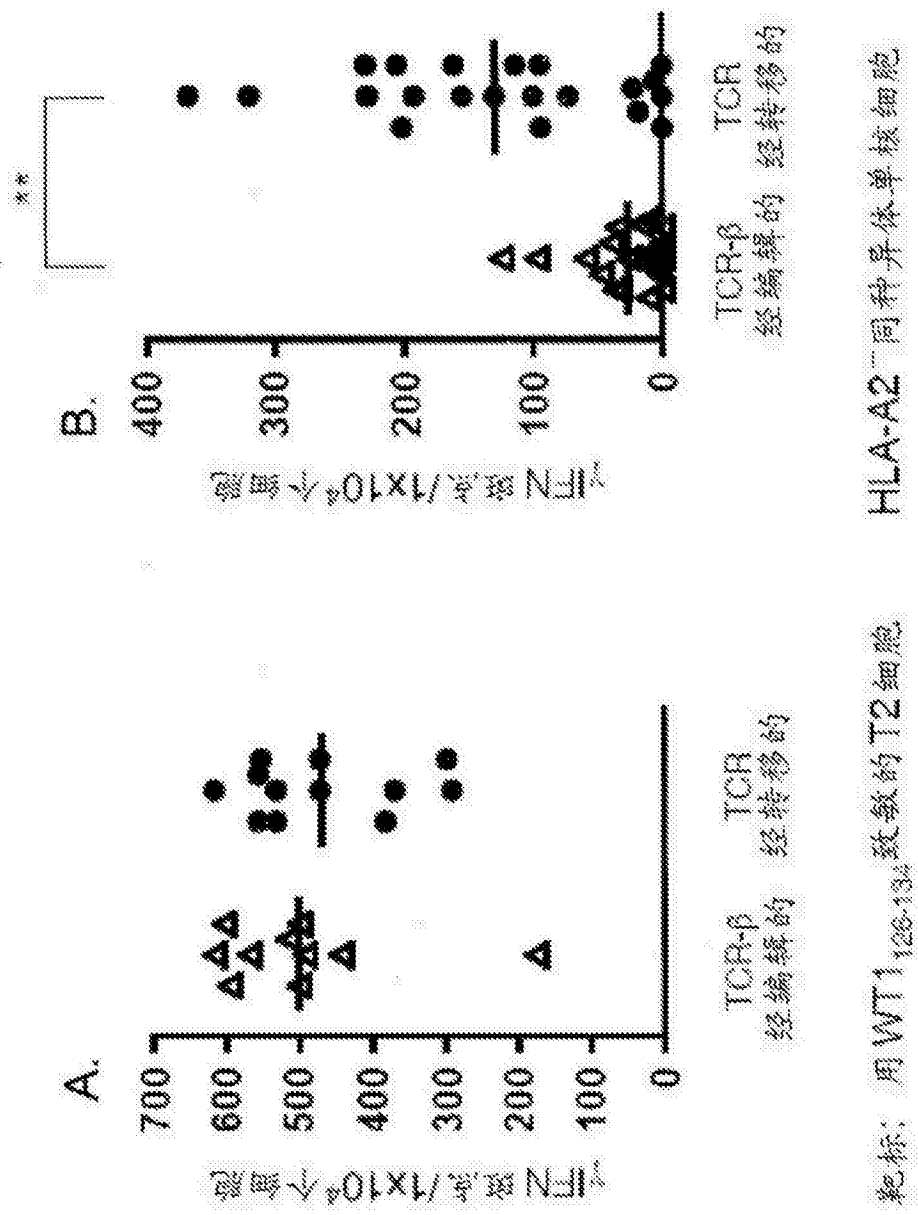

B.
A.
**
γIFN 斑点数/1X10⁶个细胞
400
300
200
100
0
700
600
500
TCR-β 经编辑的
TCR 经转移的
HLA-A2⁻同种异体单核细胞
靶标：用 WT1126-134 致敏的 T2 细胞

图 10

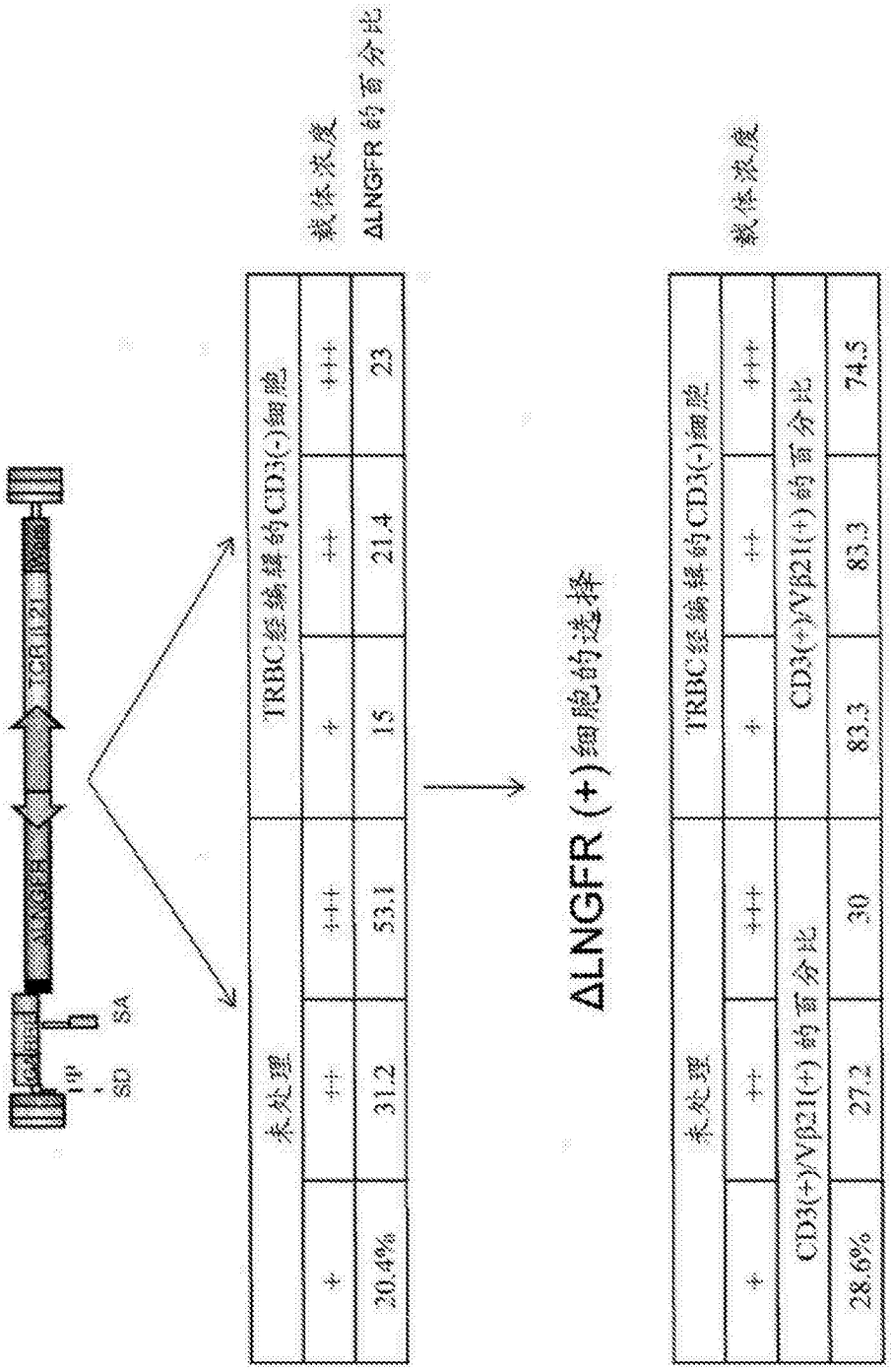

载体浓度
ΔLNGFR 的百分比
TRBC 经编辑的 CD3(-)细胞
未处理
+
++
+++
+
++
+++
20.4%
31.2
53.1
15
21.4
23
ΔLNGFR (+)细胞的选择
载体浓度
TRBC 经编辑的 CD3(-)细胞
未处理
+
++
+++
+
++
+++
CD3(+)/V821(+) 的百分比
CD3(+)/V821(+) 的百分比
28.6%
27.2
30
83.3
83.3
74.5
SD
SA

图 11

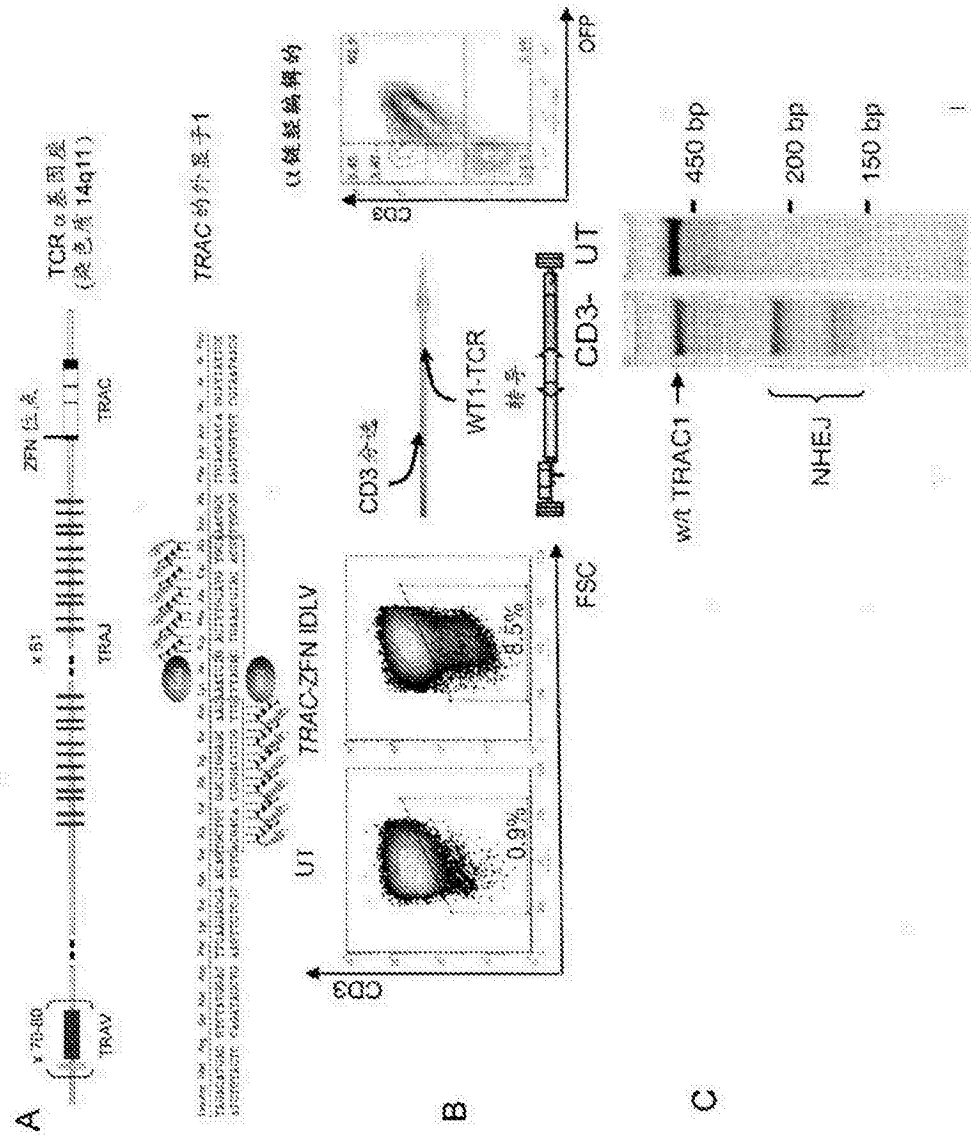

A
B
C
TRAV
TRAJ
TRAC
TCR α 基因座
(染色体 14q11)
ZFN 位点
TRAC 的外显子 1
TRAC-ZFN IDLV
UT
CD3
FSC
8.5%
0.9%
CD3 分选
WT1-TCR
转导
CD3- UT
OFP
wt TRAC1
NHEJ
450 bp
200 bp
150 bp

图 12

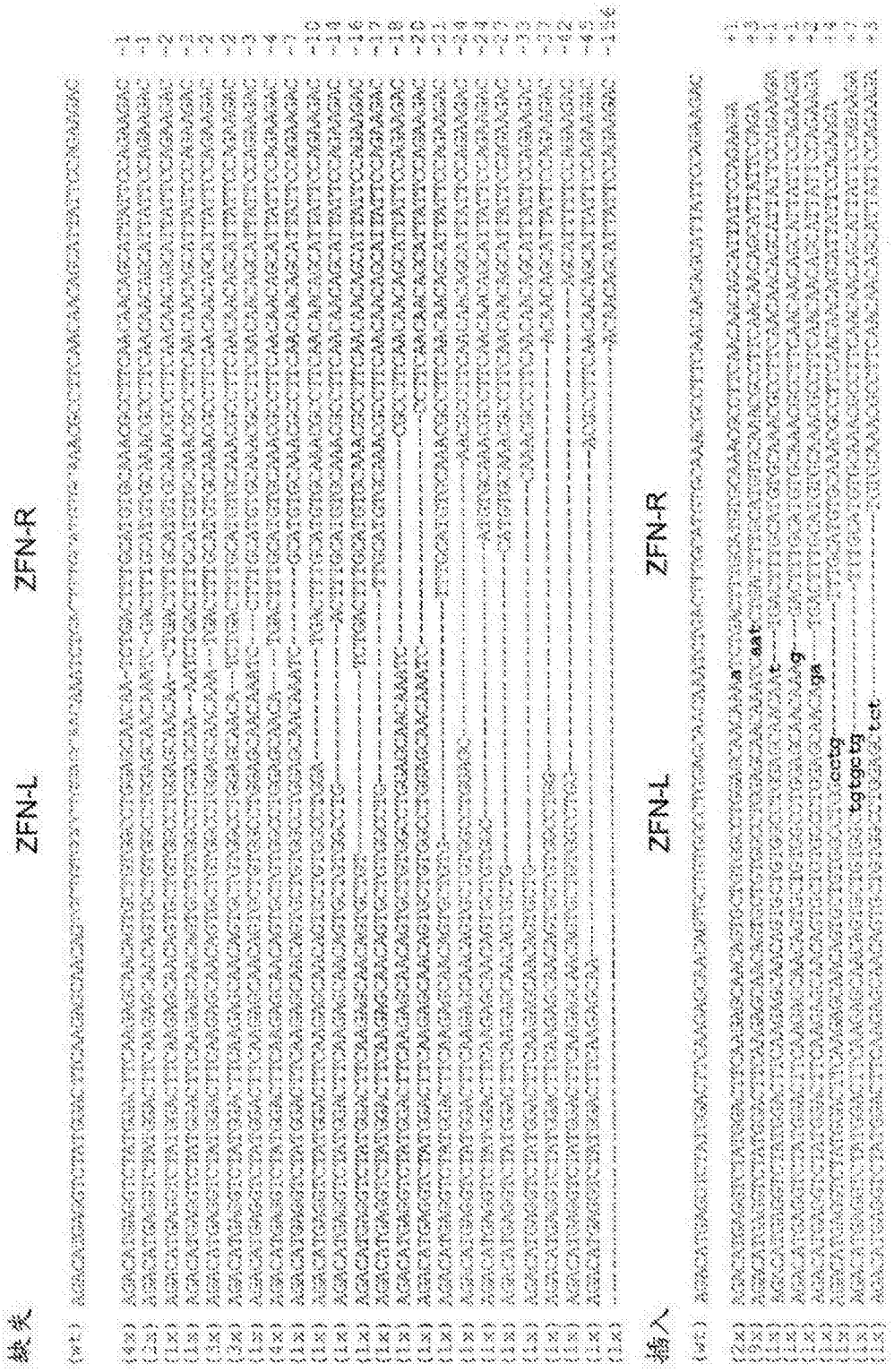


图 13

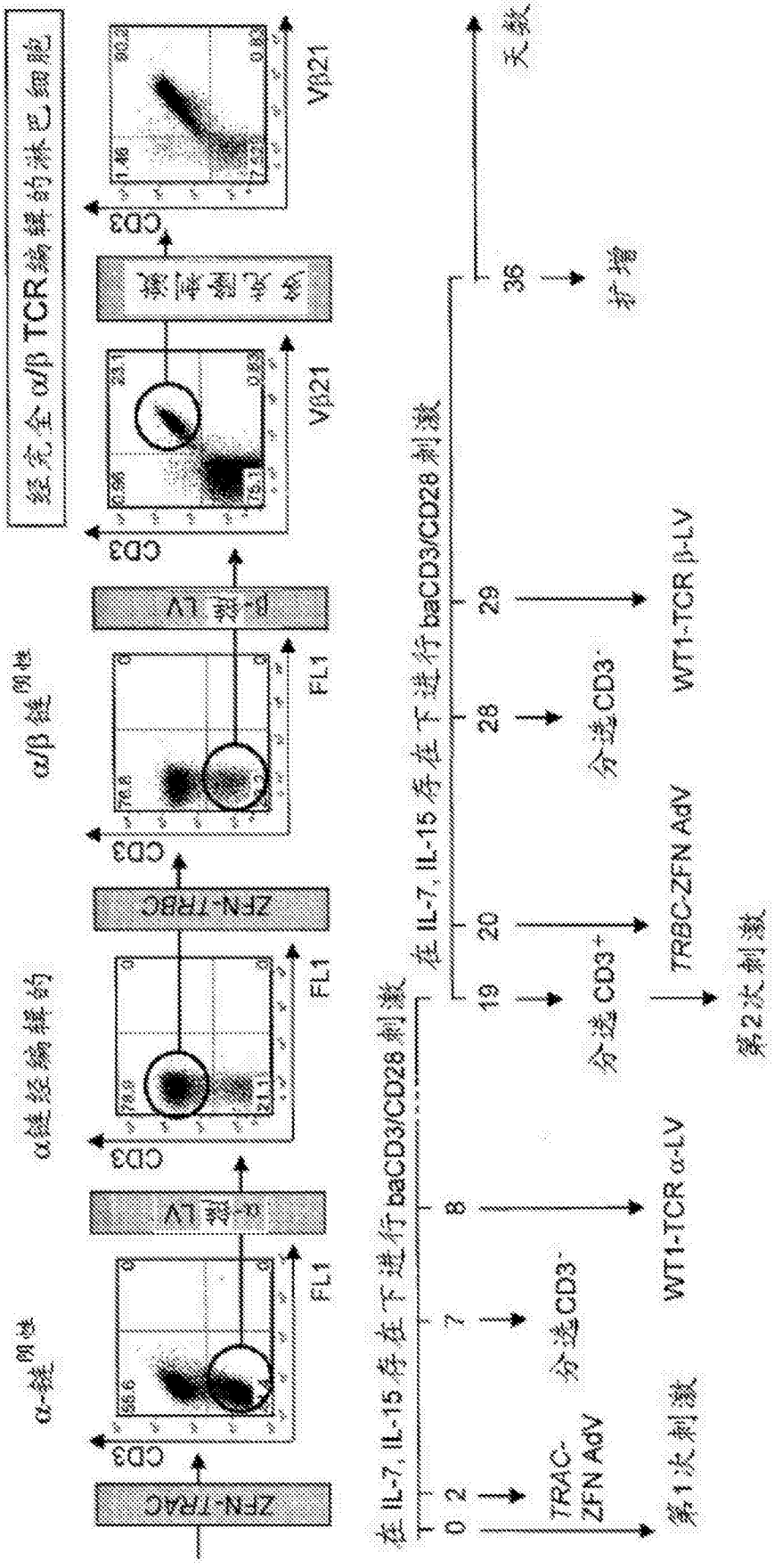

经完全αβ TCR编辑的淋巴细胞
α-链阴性
α链经编辑的
αβ链阴性
ZFN-TRAC
ZFN-TRBC
CD3
FL1
Vβ21
0 2
TRAC-ZFN AdV
分选CD3-
第1次刺激
7
8
WT1-TCR α-LV
在 IL-7, IL-15 存在下进行 baCD3/CD28 刺激
分选 CD3+
TRBC-ZFN AdV
第2次刺激
19
20
28
29
WT1-TCR β-LV
36
天数
扩增

图 14

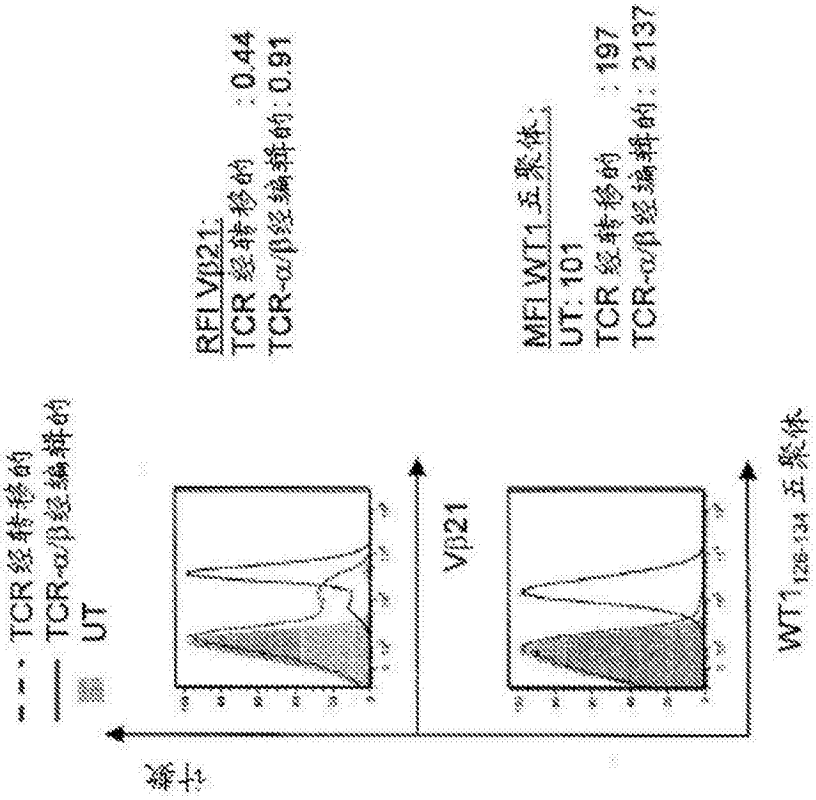

TCR经转移的
TCR-αβ经编辑的
UT
计数
Vβ21
WT1126-134五聚体
REL Vβ21:
TCR经转移的 : 0.44
TCR-αβ经编辑的: 0.91
MFI WT1五聚体:
UT: 101
TCR经转移的 : 197
TCR-αβ经编辑的: 2137

图 15

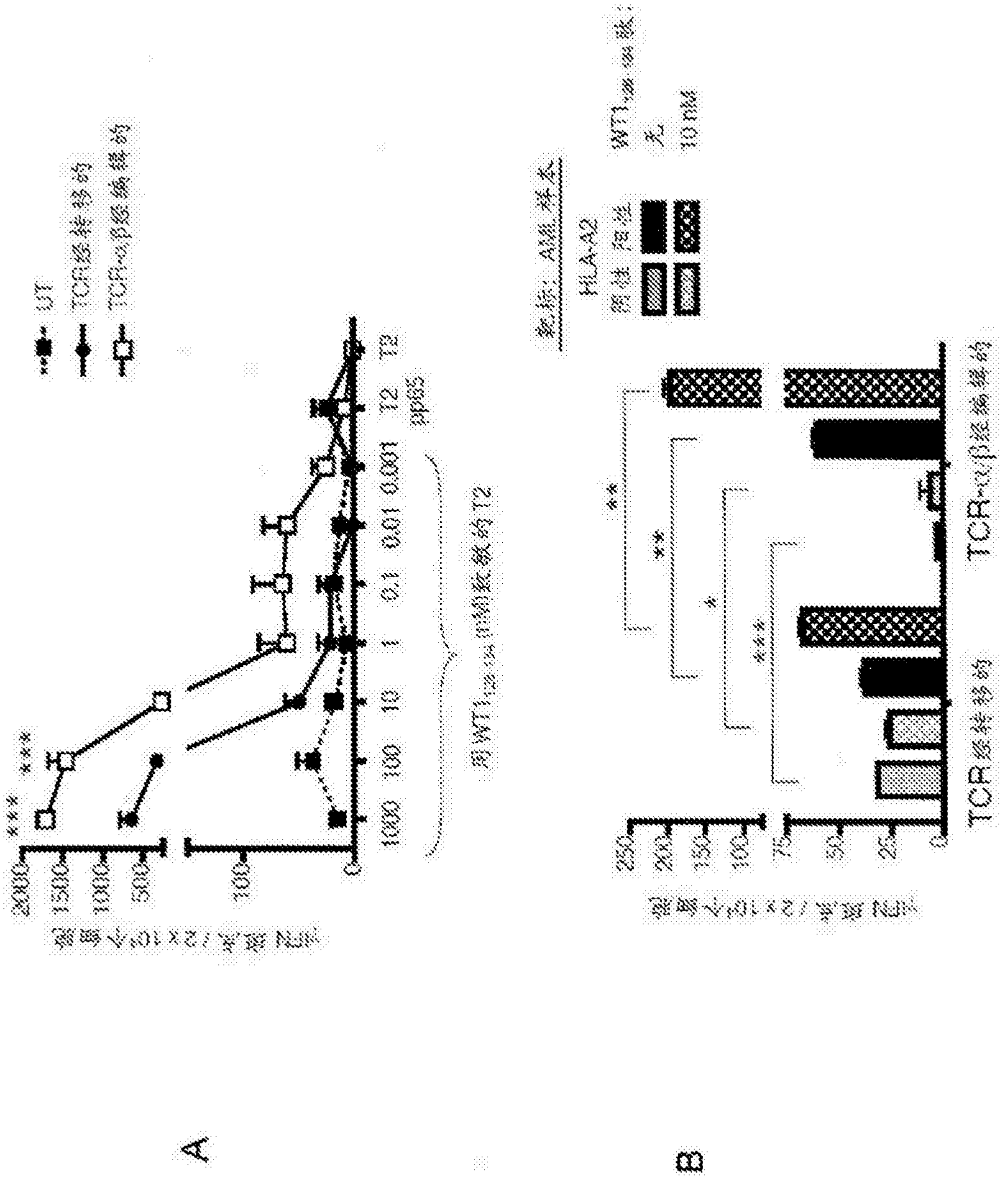


图 16

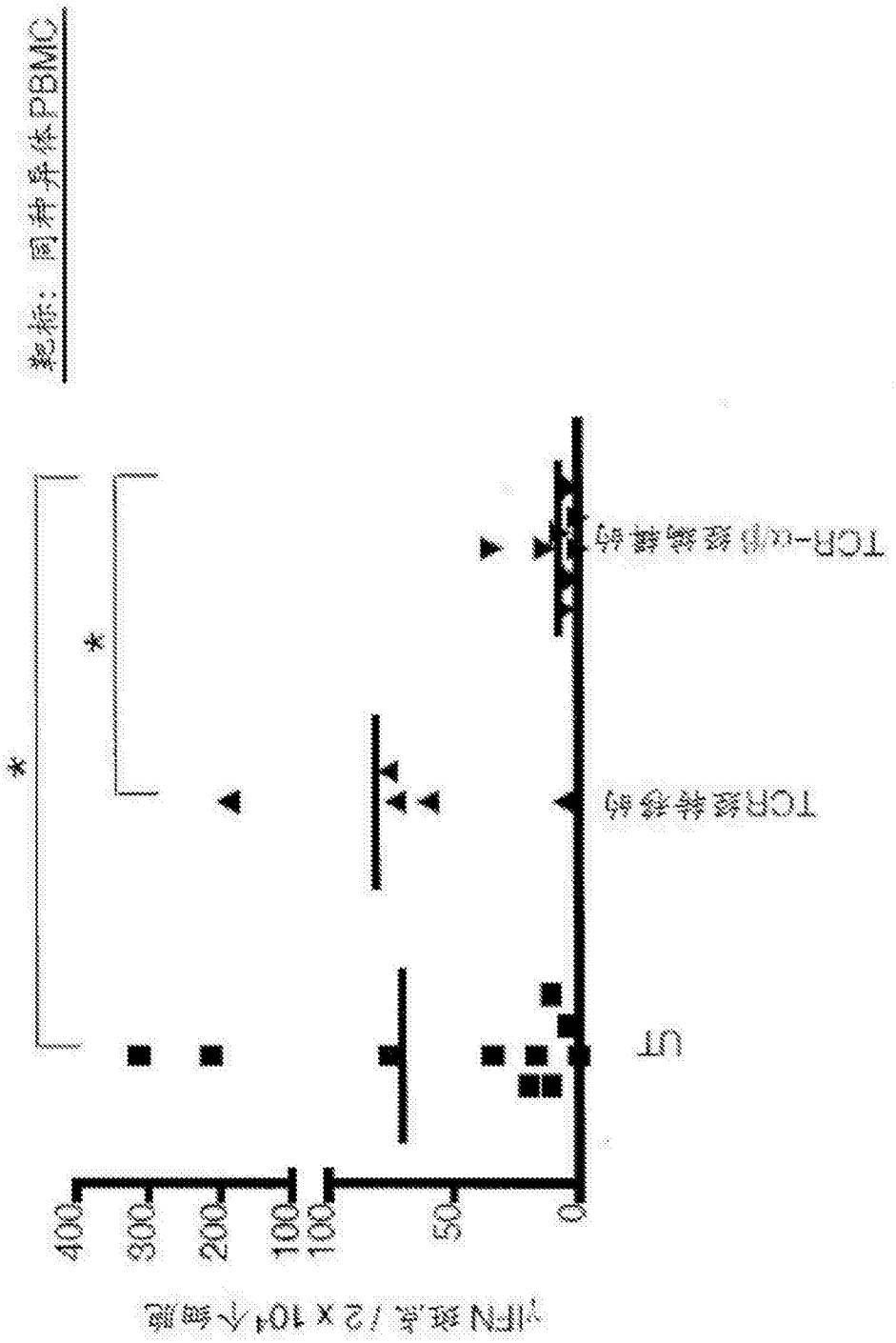


图 16C

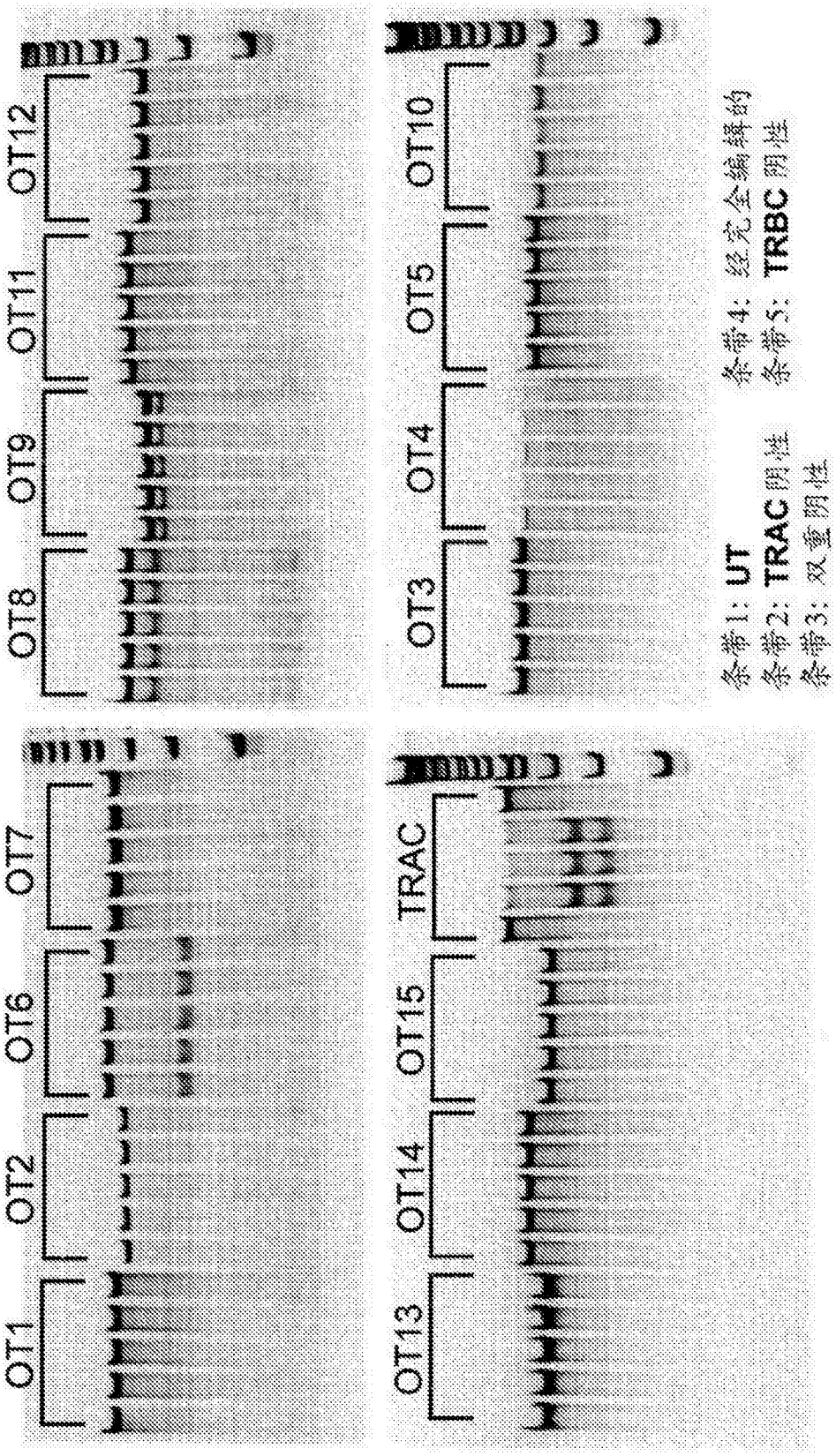


图 17 :TRAC 脱靶分析

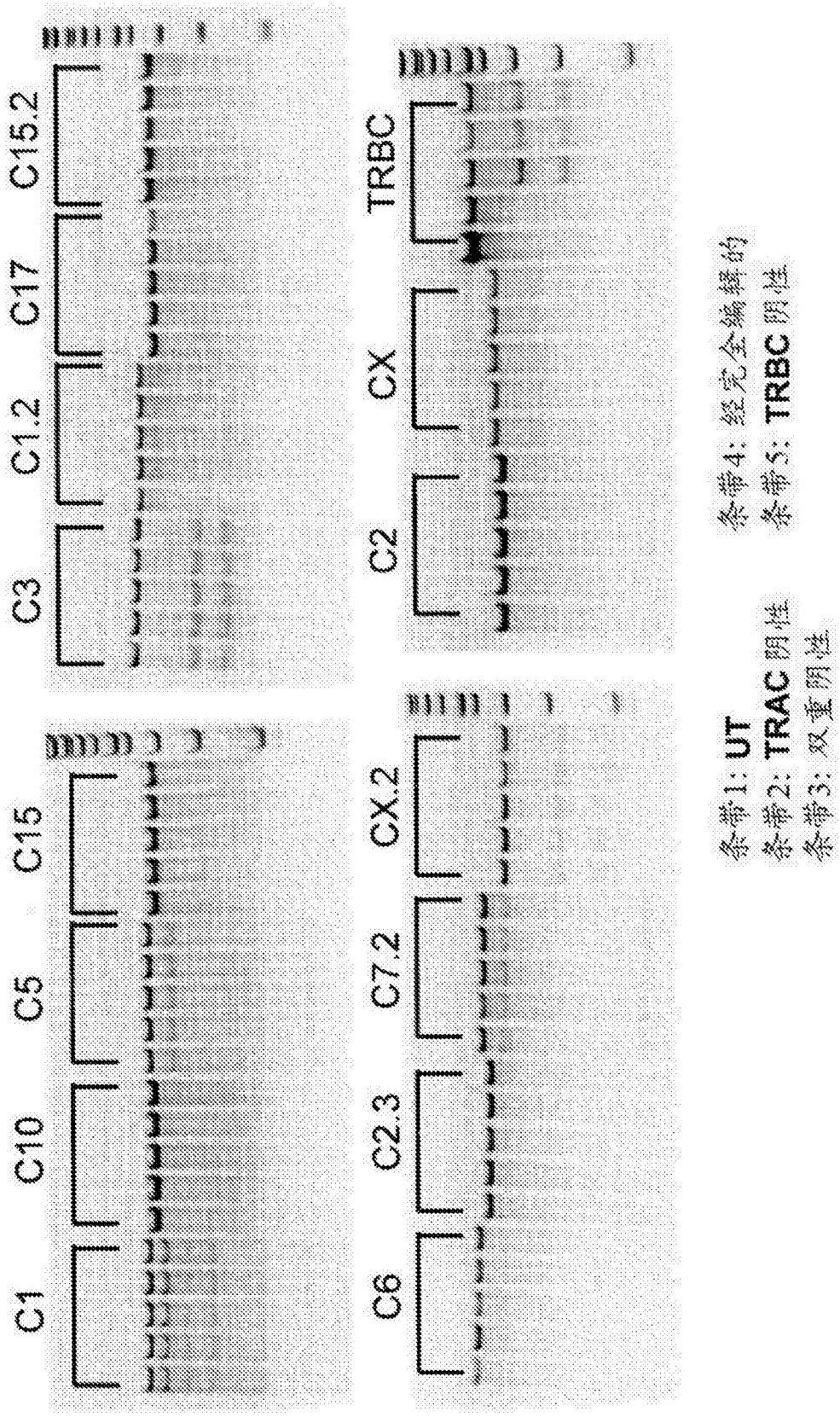


图 18 :TRBC 脱靶分析

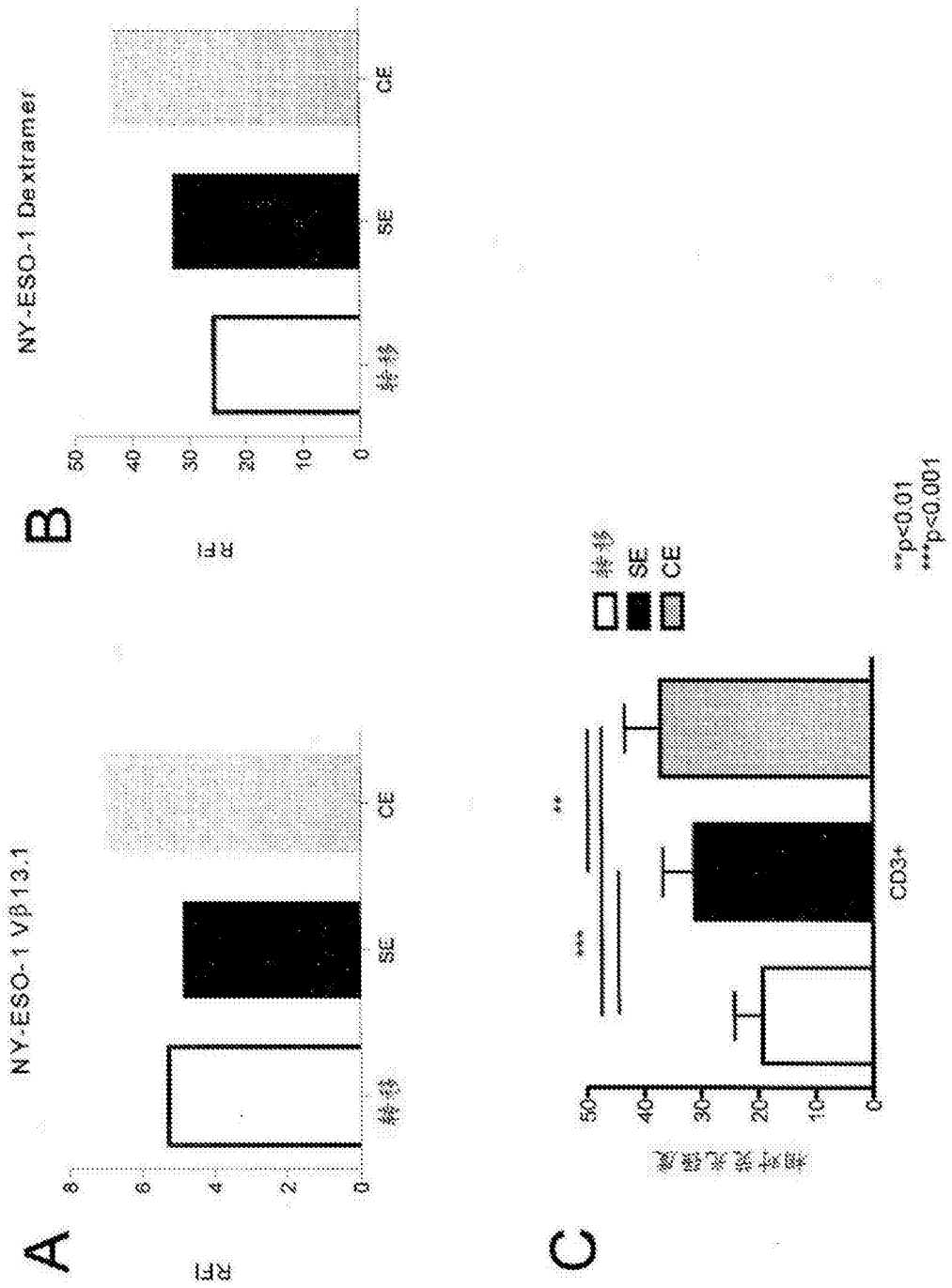

A
NY-ESO-1 Vβ13.1
RFI
0
2
4
6
8
转移
SE
CE
B
NY-ESO-1 Dextramer
RFI
0
10
20
30
40
50
转移
SE
CE
C
转移
SE
CE
**
***
CD3+
**p<0.01
***p<0.001

图 19

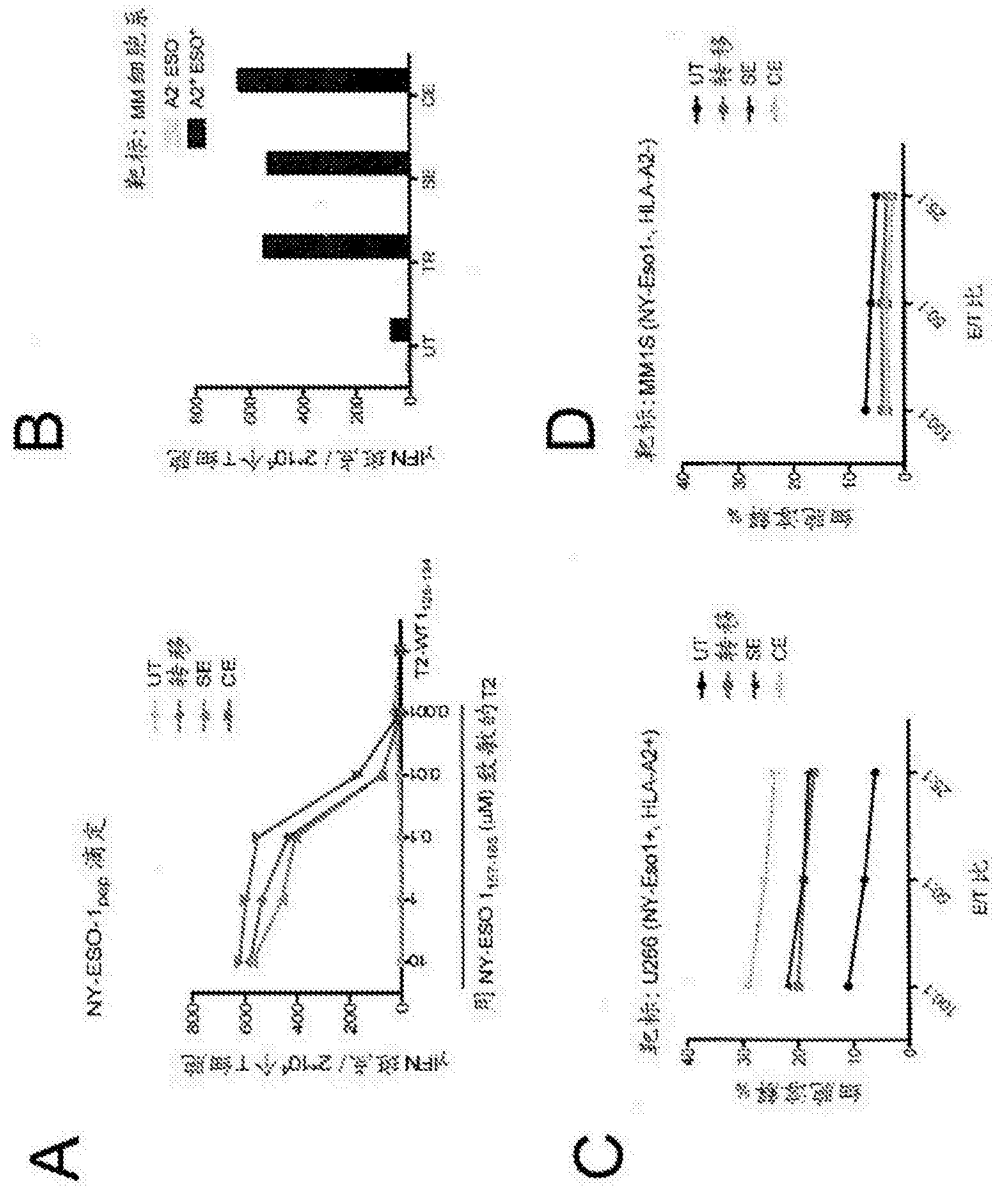
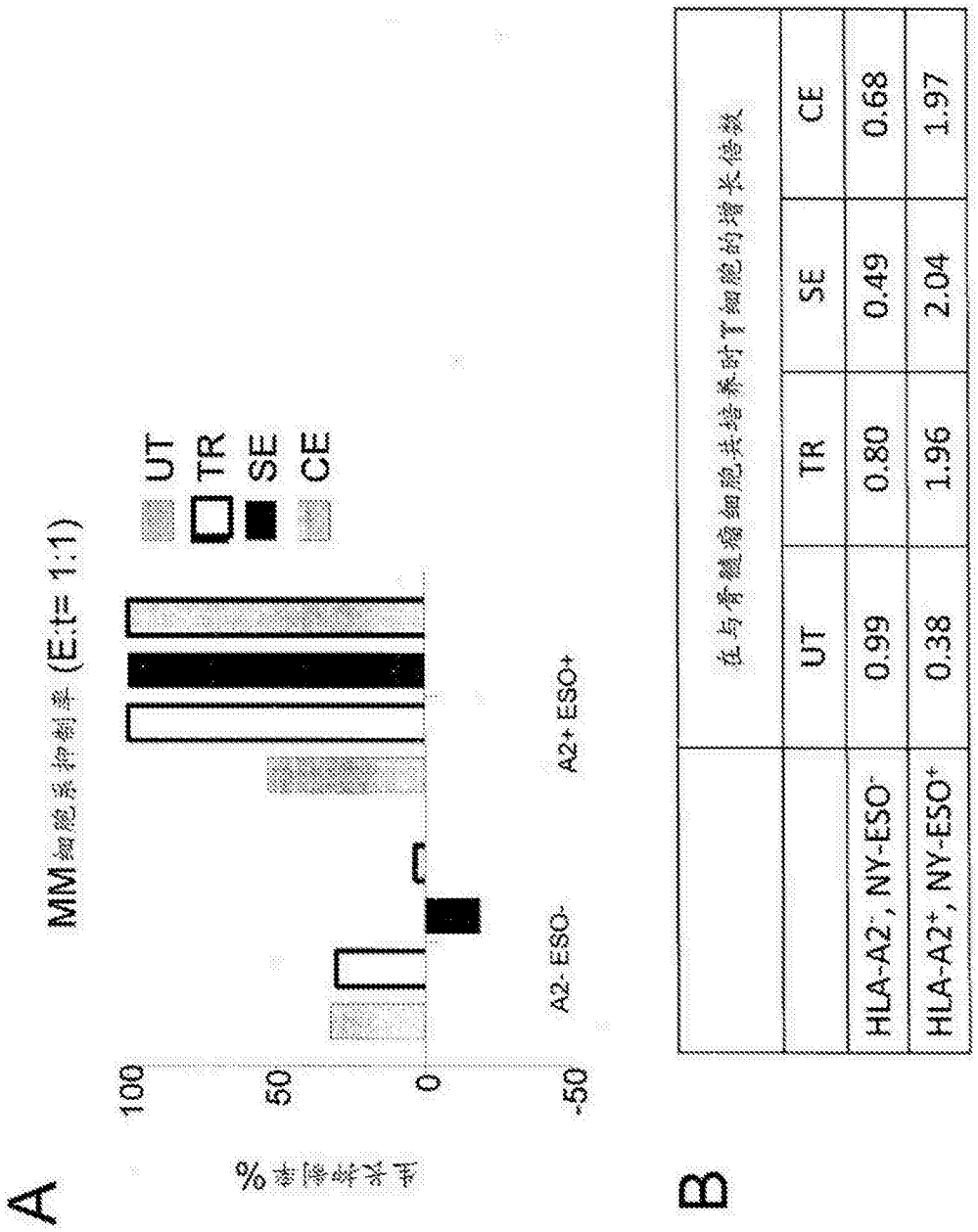

A
B
C
D
UT
SE
CE
E/T 比
细胞溶解 %
靶标：U266 (NY-Eso1+, HLA-A2+)
靶标：MM1S (NY-Eso1-, HLA-A2-)
靶标：MM 细胞系

图 20



	在与骨髓瘤细胞共培养时T细胞的增长倍数			
	UT	TR	SE	CE
HLA-A2$^-$, NY-ESO$^-$	0.99	0.80	0.49	0.68
HLA-A2$^+$, NY-ESO$^+$	0.38	1.96	2.04	1.97

图 21

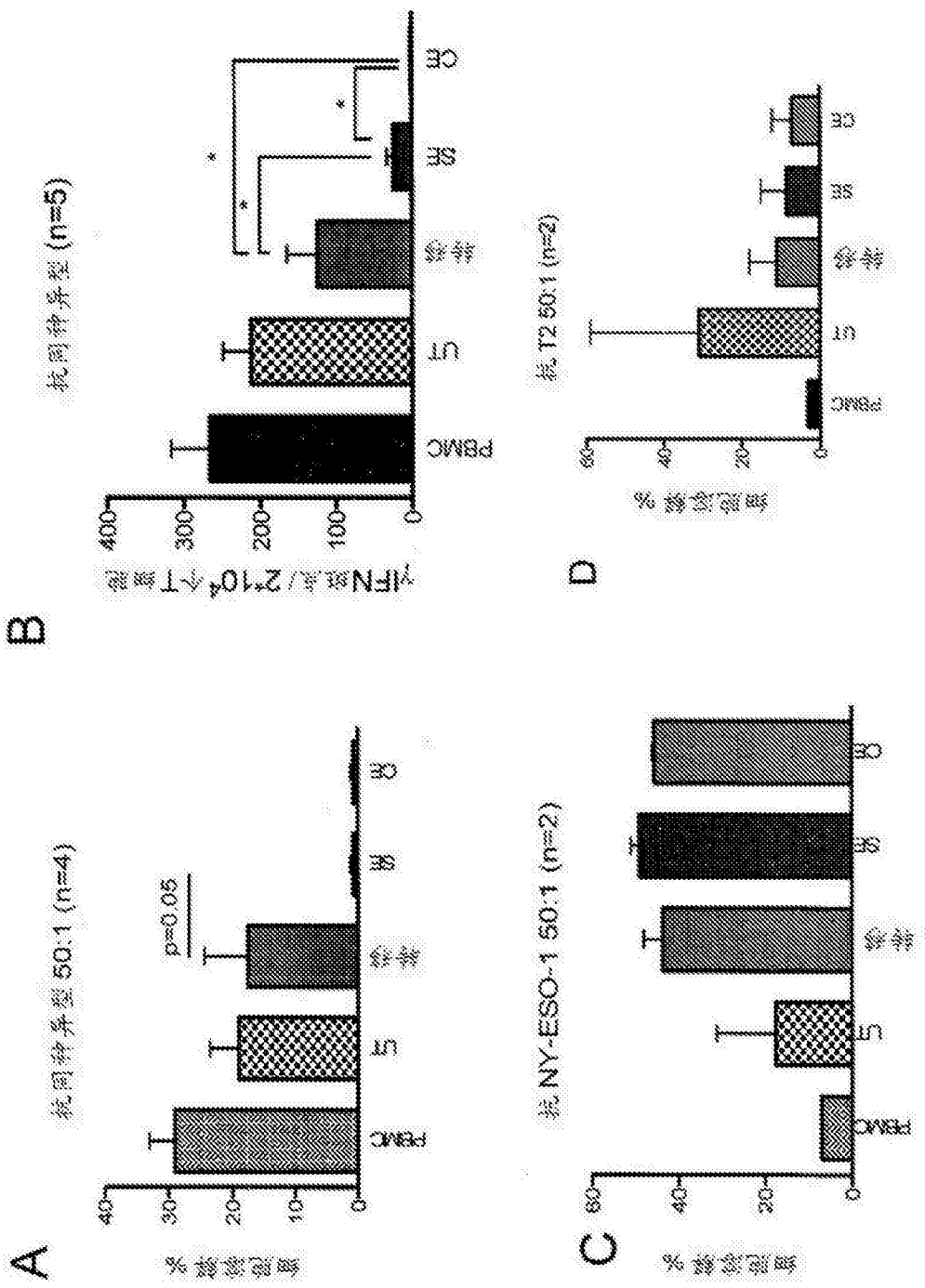

A
抗同种异型 50:1 (n=4)
p=0.05
特异裂解 %
PBMC
UT
转导
SE
CE
B
抗同种异型 (n=5)
IFNγ斑点/2*10^4个T细胞
C
抗 NY-ESO-1 50:1 (n=2)
D
抗 T2 50:1 (n=2)

图 22

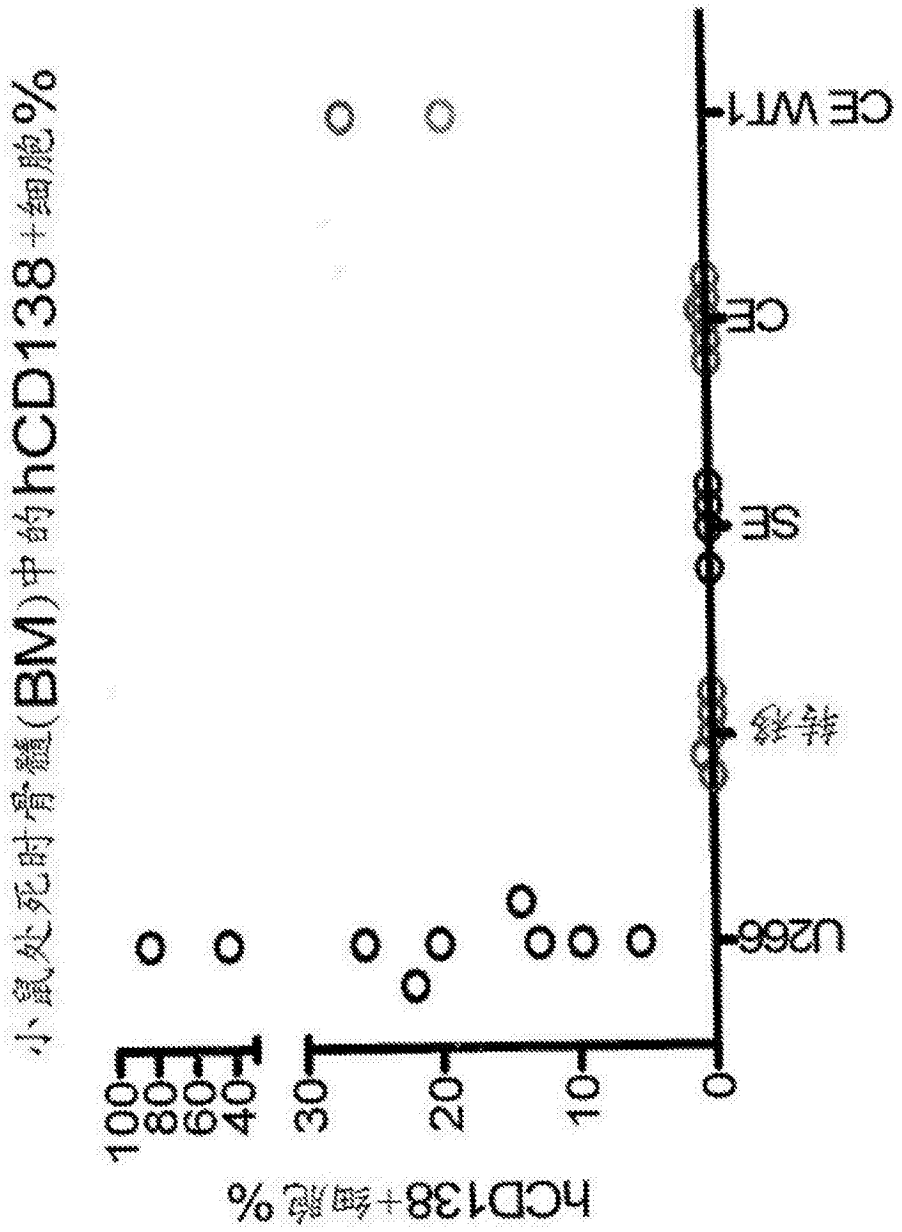

小鼠处死时骨髓(BM)中的hCD138+细胞%
hCD138+细胞%
100
80
60
40
30
20
10
0
U266
转移
SE
CE
CE WT1

图 23A

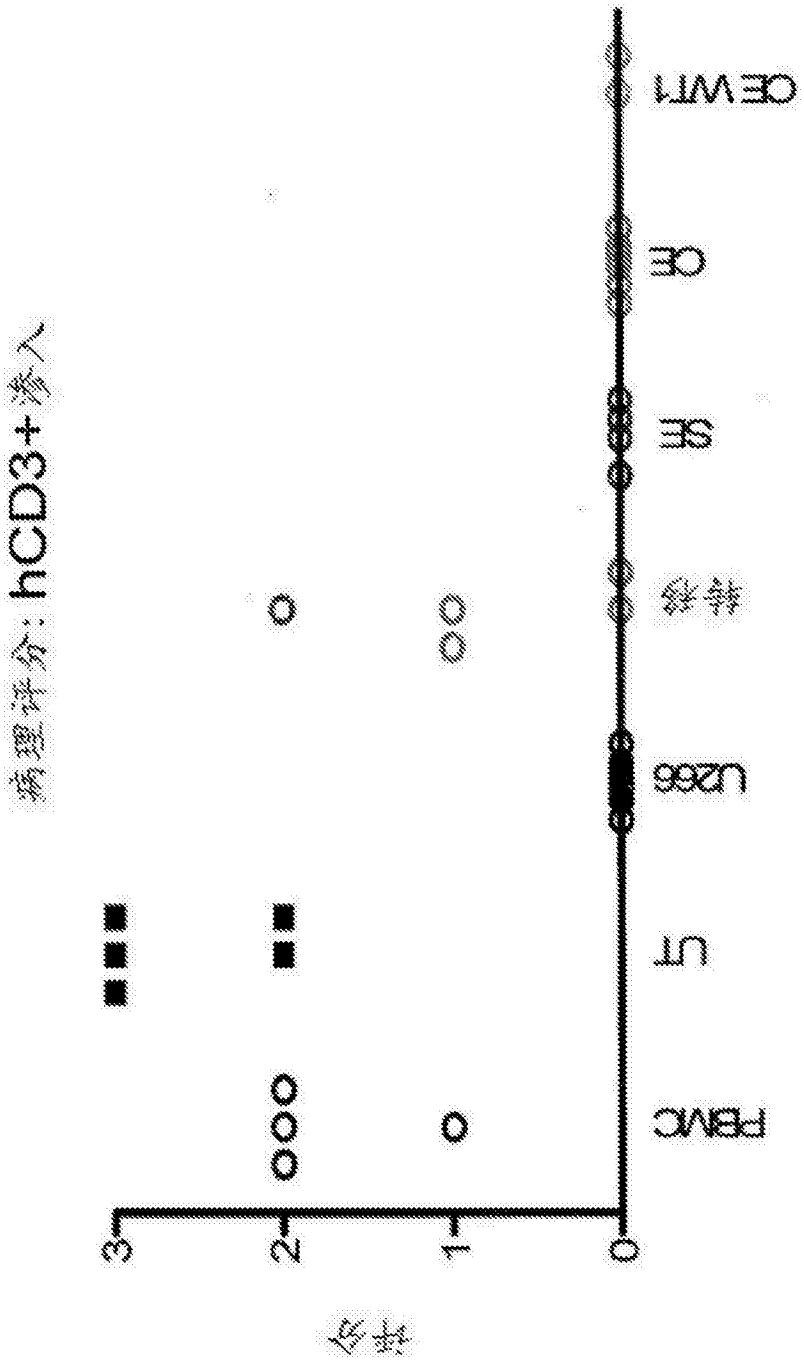


图 23B

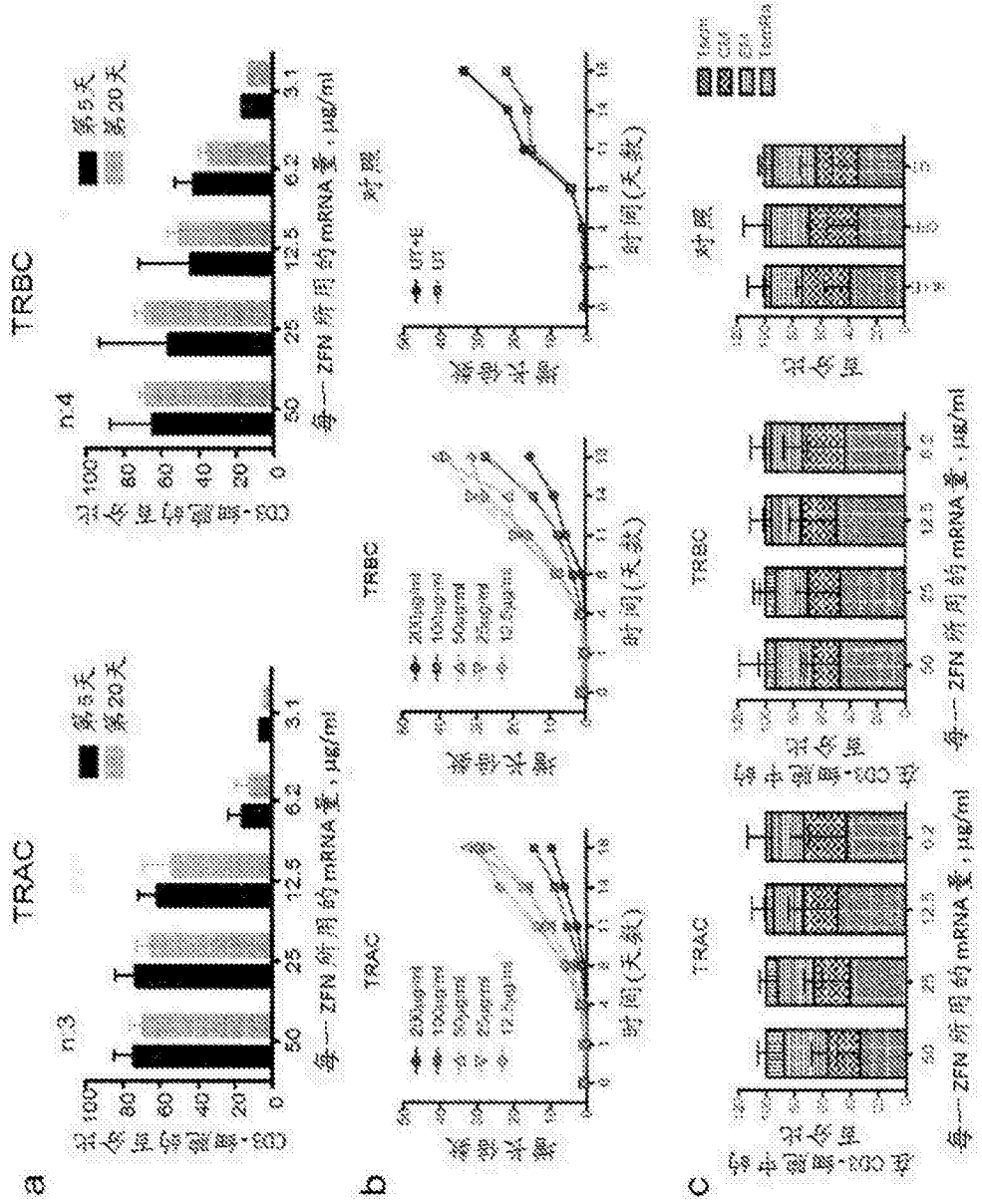

a
TRAC
TRBC
第5天
第20天
n:3
n:4
CD3-细胞的百分比
每一ZFN所用的mRNA量，μg/ml
b
TRAC
TRBC
对照
293ug/ml
100ug/ml
50ug/ml
25ug/ml
12.5ug/ml
时间(天数)
c
TRAC
TRBC
对照
在CD3-细胞中的百分比
每一ZFN所用的mRNA量，μg/ml

图 24

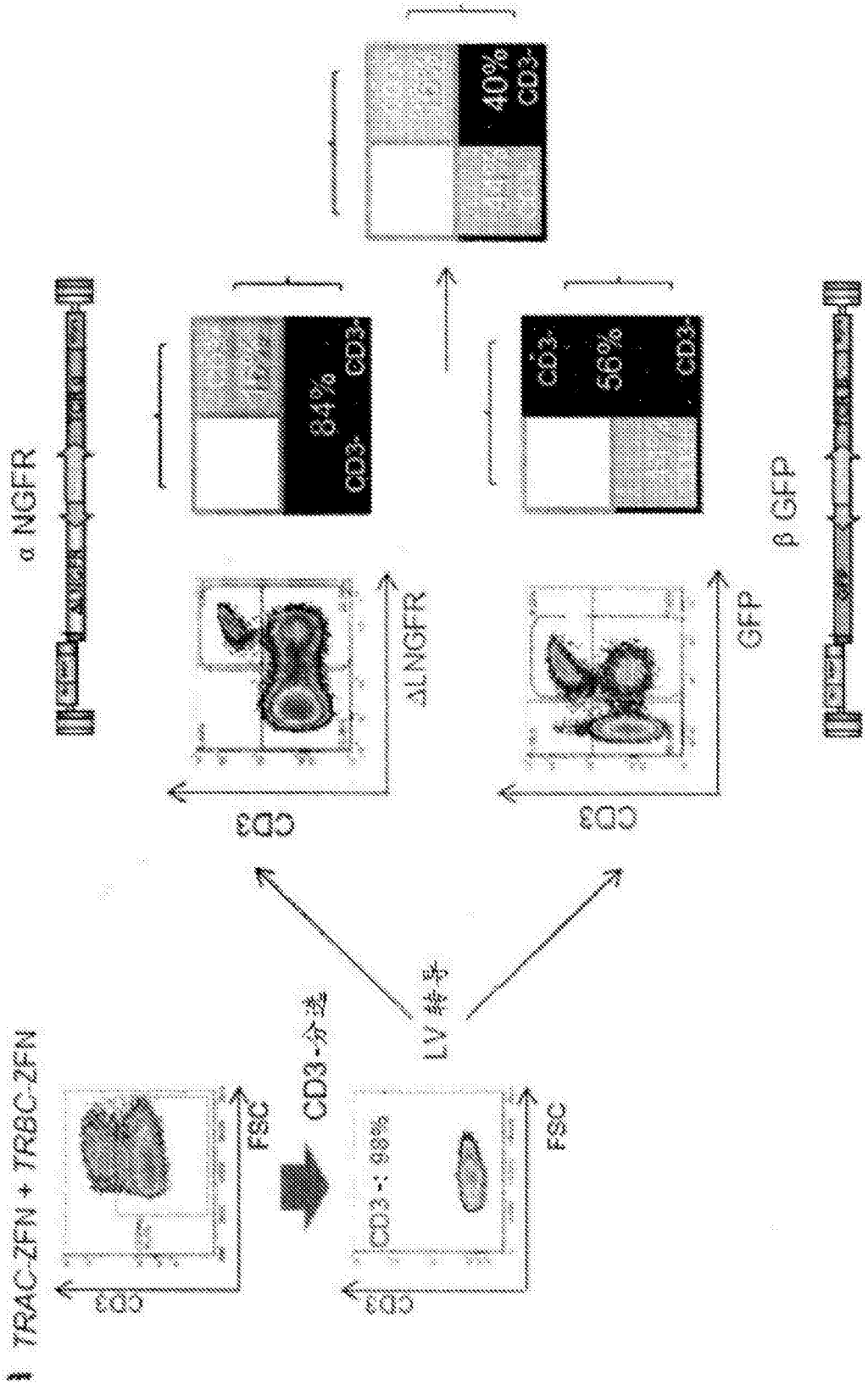

TRAC-ZFN + TRBC-ZFN
FSC
CD3
CD3-分选
CD3-: 98%
LV 转导
α NGFR
ΔLNGFR
CD3+
94%
CD3-
40%
CD3-
CD3+
56%
CD3-
β GFP
GFP

图 25A

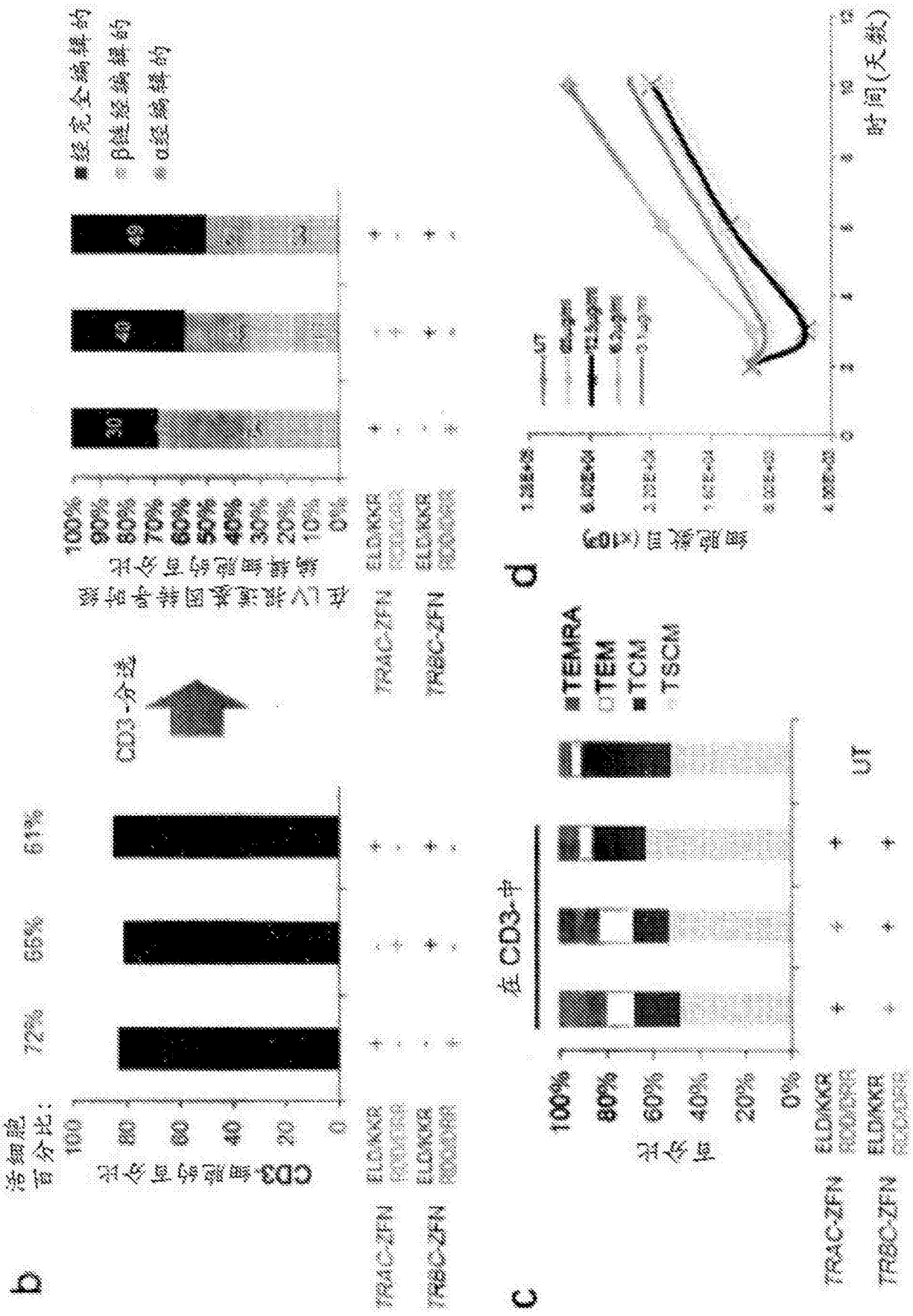

b
CD3-分选
CD3+细胞的百分比:
72%
65%
61%
CD3+细胞的百分比
在CD3+中
TRAC-ZFN
TRBC-ZFN
ELD/KKR
c
TEMRA
TEM
TCM
TSCM
UT
百分比
d
在小鼠淋巴瘤异种移植
存活细胞的百分比
时间(天数)
细胞数目(x10^9)

图 25B 图 25