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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
9 October 2014 (09.10.2014)

WIPO | PCT

(10) International Publication Number
WO 2014/165177 A1

(51) International Patent Classification:
C12N 5/00 (2006.01) *C12N 5/0783* (2010.01)
C12N 5/02 (2006.01)

(21) International Application Number:
PCT/US2014/024660

(22) International Filing Date:
12 March 2014 (12.03.2014)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/777,627 12 March 2013 (12.03.2013) US

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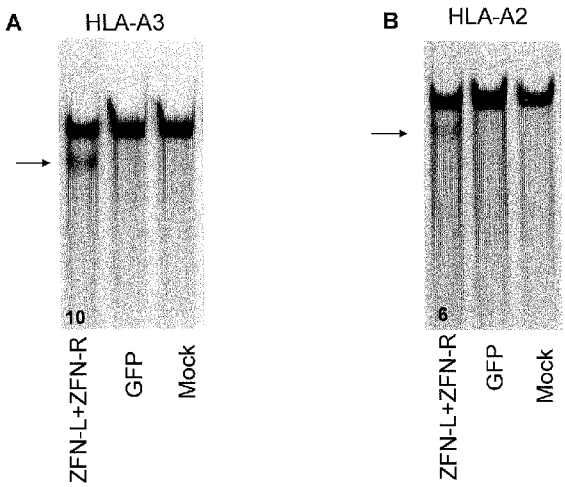
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JP, 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).

[Continued on next page]

(54) Title: METHODS AND COMPOSITIONS FOR MODIFICATION OF HLA

Figure 1



(57) Abstract: Disclosed herein are methods and compositions for modulating the expression of a HLA locus, including cells that lack expression of one or more classic HLA genes but are not targeted by Natural Killer (NK) cells for lysis.

WO 2014/165177 A1

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))*

METHODS AND COMPOSITIONS FOR MODIFICATION OF HLA

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims the benefit of U.S. Provisional Application No. 61/777,627, filed March 12, 2013, the disclosure of which is hereby incorporated by reference in its entirety.

STATEMENT OF RIGHTS TO INVENTIONS MADE UNDER FEDERALLY SPONSORED RESEARCH

[0002] Not applicable.

TECHNICAL FIELD

[0003] The present disclosure is in the fields of gene expression, genome engineering and gene therapy.

BACKGROUND

[0004] MHC antigens were first characterized as proteins that played a major role in transplantation reactions. Rejection is mediated by T cells reacting to the histocompatibility antigens on the surface of implanted tissues, and the largest group of these antigens is the major histocompatibility antigens (MHC). These proteins are expressed on the surface of all higher vertebrates and are called H-2 antigens in mice (for histocompatibility-2 antigens) and HLA antigens (for human leukocyte antigens) in human cells.

[0005] The MHC proteins serve a vital role in T cell stimulation. Antigen presenting cells (often dendritic cells) display peptides that are the degradation products of foreign proteins on the cell surface on the MHC. In the presence of a co-stimulatory signal, the T cell becomes activated, and will act on a target cell that also displays that same peptide/MHC complex. For example, a stimulated T helper cell will target a macrophage displaying an antigen in conjunction with its MHC, or a cytotoxic T cell (CTL) will act on a virally infected cell displaying foreign viral peptides.

[0006] MHC proteins are of two classes, I and II. The class I MHC proteins are heterodimers of two proteins, the α chain, which is a transmembrane protein

encoded by the MHC1 class I genes, and the β2 microblogulin chain, which is a small extracellular protein that is encoded by a gene that does not lie within the MHC gene cluster. The α chain folds into three globular domains and when the β2 microglobulin chain is associated, the globular structure complex is similar to an antibody complex. The foreign peptides are presented on the two most N-terminal domains which are also the most variable. Class II MHC proteins are also heterodimers, but the heterodimers comprise two transmembrane proteins encoded by genes within the MHC complex. The class I MHC:antigen complex interacts with cytotoxic T cells while the class II MHC presents antigens to helper T cells. In addition, class I MHC proteins tend to be expressed in nearly all nucleated cells and platelets (and red blood cells in mice) while class II MHC protein are more selectively expressed. Typically, class II MHC proteins are expressed on B cells, some macrophage and monocytes, Langerhans cells, and dendritic cells.

[0007] The class I HLA gene cluster in humans comprises three major loci, B, C and A, as well as several minor loci. The class II HLA cluster also comprises three major loci, DP, DQ and DR, and both the class I and class II gene clusters are polymorphic, in that there are several different alleles of both the class I and II genes within the population. There are also several accessory proteins that play a role in HLA functioning as well. The Tap1 and Tap2 subunits are parts of the TAP transporter complex that is essential in loading peptide antigens on to the class I HLA complexes, and the LMP2 and LMP7 proteosome subunits play roles in the proteolytic degradation of antigens into peptides for display on the HLA. Reduction in LMP7 has been shown to reduce the amount of MHC class I at the cell surface, perhaps through a lack of stabilization (see Fehling *et al* (1999) *Science* 265:1234-1237). In addition to TAP and LMP, there is the tapasin gene, whose product forms a bridge between the TAP complex and the HLA class I chains and enhances peptide loading. Reduction in tapasin results in cells with impaired MHC class I assembly, reduced cell surface expression of the MHC class I and impaired immune responses (see Grandea *et al* (2000) *Immunity* 13:213-222 and Garbi *et al* (2000) *Nat Immunol* 1:234-238).

[0008] Regulation of class I expression is generally at the transcriptional level, and several stimuli such as viral infection etc. can cause a change in transcription. The class I genes are down-regulated in some specific tissues, and the source of this down-regulation seems to be within the promoter and 3' intergenic sequences (see

Cohen *et al* (2009) *PLos ONE* 4(8): e6748). There is also evidence that microRNAs are capable of regulating some class I MHC genes (see Zhu *et al*, (2010) *Am. J. Obstet Gynecol* 202(6):592).

[0009] Regulation of class II MHC expression is dependent upon the activity of the MHCII enhanceosome complex. The enhanceosome components (one of the most highly studied components of the enhanceosome complex is the RFX5 gene product (see Villard *et al* (2000) *MCB* 20(10): 3364-3376)) are nearly universally expressed and expression of these components does not seem to control the tissue specific expression of MHC class II genes or their IFN-γ induced up-regulation. Instead, it appears that a protein known as CIITA (class II transactivator) which is a non-DNA binding protein, serves as a master control factor for MCHII expression. In contrast to the other enhanceosome members, CIITA does exhibit tissue specific expression, is up-regulated by IFN-γ, and has been shown to be inhibited by several bacteria and viruses which can cause a down regulation of MHC class II expression (thought to be part of a bacterial attempt to evade immune surveillance (see LeibundGut-Landmann *et al* (2004) *Eur. J. Immunol* 34:1513-1525)).

[0010] Regulation of the class I or II genes can be disrupted in the presence of some tumors and such disruption can have consequences on the prognosis of the patients. For example, in some melanomas, an observed reduction in Tap 1, Tap 2 and HLA class I antigens was found to be more common in metastatic melanomas ($P<0.05$) than in primary tumors (see, Kagashita *et al* (1999) *Am Jour of Pathol* 154(3):745-754).

[0011] In humans, susceptibility to several diseases is suspected to be tied to HLA haplotype. These diseases include Addison's disease, ankylosing spondylitis, Behçet's disease, Buerger's disease, celiac disease, chronic active hepatitis, Graves' disease, juvenile rheumatoid arthritis, psoriasis, psoriatic arthritis, rheumatoid arthritis, Sjögren syndrome, and lupus erythematosus, among others.

[0012] HLA also plays a major role in transplant rejection. The acute phase of transplant rejection can occur within about 1-3 weeks and usually involves the action of host T lymphocytes on donor tissues due to sensitization of the host system to the donor class I and class II HLA molecules. In most cases, the triggering antigens are the class I HLAs. For best success, donors are typed for HLA and matched to the patient recipient as completely as possible. But donation even between family

members, which can share a high percentage of HLA identity, is still often not successful. Thus, in order to preserve the graft tissue within the recipient, the patient often must be subjected to profound immunosuppressive therapy to prevent rejection. Such therapy can lead to complications and significant morbidities due to opportunistic infections that the patient may have difficulty overcoming.

[0013] Cell therapy is a specialized type of transplant wherein cells of a certain type (*e.g.* T cells reactive to a tumor antigen or B cells) are given to a recipient. Cell therapy can be done with cells that are either autologous (derived from the recipient) or allogenic (derived from a donor) and the cells may be immature cells such as stem cells, or completely mature and functional cells such as T cells. In fact, in some diseases such certain cancers, T cells may be manipulated *ex vivo* to increase their avidity for certain tumor antigens, expanded and then introduced into the patient suffering from that cancer type in an attempt to eradicate the tumor. This is particularly useful when the endogenous T cell response is suppressed by the tumor itself. However, the same caveats apply for cell therapy as apply for more well-known solid organ transplants in regards to rejection. Donor T cells express class I HLA antigens and thus are capable of eliciting a rejection response from the recipient's endogenous immune system.

[0014] U.S. Patent Publication No. 2012/0060230 describes specific zinc finger protein regulators of classic HLA genes such as HLA-A, HLA-B, HLA-C. These regulators can be used to make cells (*e.g.,* stem cells) that do not express one or more classic HLA genes and, accordingly, can be used for autologous transplants. However, the loss of classic HLA expression may render the genetically modified cells targets for natural killed (NK)-cell mediated cytotoxicity based on loss of ligands for KIR. *See, e.g.,* Parham *et al.* (2005) *Nat Rev Immunol.* 5(3):201-214.

[0015] Thus, there remains a need for compositions and methods for developing cells that lack some or all classic HLA expression but which cells are not targeted by NK cells for lysis.

SUMMARY

[0016] Disclosed herein are methods and compositions for modifying HLA expression. In particular, provided herein are methods and compositions for modulating expression of an HLA gene so as to treat HLA-related disorders, for example human disorders related to HLA haplotype of the individual. Additionally,

provided herein are methods and compositions for deleting (inactivating) or repressing an HLA gene to produce an HLA null cell, cell fragment (*e.g.* platelet), tissue or whole organism, for example a cell that does not express one or more classic HLA genes. Additionally, these methods and compositions may be used to create a cell, cell fragment, tissue or organism that is null for just one classic HLA gene, or more than one classic HLA gene, or is completely null for all classic HLA genes. In certain embodiments, the classic HLA null cells or tissues are human cells or tissues that are advantageous for use in transplants.

[0017] Thus, in one aspect, described herein are cells in which one or more classic HLA genes are inactivated and in which one or more non-classic HLA proteins (*e.g.,* HLA-E, HLA-F, HLA-G) are present within the cell. The non-classical class I HLA molecules may be expressed (over-expressed) from endogenous genes, may be added to the cell and/or may be expressed by genetic modification of the cell (*e.g.,* stable or transient transfection of polynucleotides expressing the one or more non-classical HLA molecules). In certain embodiments, the non-classical HLA molecules comprise HLA-E and/or HLA-G.

[0018] The modified cells may be a lymphoid cell (*e.g.,* natural killer (NK) cell, a T-cell, a B-cell), a myeloid cell (*e.g.,* monocyte, neutrophil, dendritic cell, macrophage, basophil, mast cell); a stem cell(*e.g.,* an induced pluripotent stem cell (iPSC), an embryonic stem cell (*e.g.,* human ES), a mesenchymal stem cell (MSC), a hematopoietic stem cell (HSC) or a neuronal stem cell) or a fragment of a cell (*e.g.,* platelet). The stem cells may be totitpotent or pluripotent (*e.g.,* partially differentiated such as an HSC that is a pluripotent myeloid or lymphoid stem cell). In some embodiments, the modified cells in which expression of more than one classic HLA gene have been altered, expression of one or more non-classic HLA(s) is also altered. In other embodiments, the invention provides methods for producing stem cells that have a null phenotype for one or more or all classic HLA genes. Any of the modified stem cells described herein (modified at the HLA locus/loci) may then be differentiated to generate a differentiated (*in vivo* or *in vitro*) cell descended from a stem cell as described herein.

[0019] In other embodiments, described herein are methods of reducing natural killer (NK) cell lysis of a cell lacking one or more classic HLA genes (*e.g.,* via nuclease-mediated inactivation of the one or more genes), the method comprising providing a cell as described herein (*e.g.,* a cell in which classic HLA gene(s) is(are)

inactivated and in in which one or more non-classic HLA molecules are present), thereby reducing NK mediated cell lysis.

[0020] In another aspect, the compositions (modified cells) and methods described herein can be used, for example, in the treatment or prevention or amelioration of any HLA-related disorder (*i.e.,* related to HLA haplotype). The methods typically comprise (a) cleaving an endogenous HLA gene or HLA regulator gene in an isolated cell (*e.g.,* T-cell or lymphocyte) using a nuclease (*e.g.,* ZFN or TALEN) or nuclease system such as CRISPR/Cas with an engineered crRNA/tracr RNA such that the HLA or HLA regulator gene is inactivated; (b) introducing a non-classic HLA molecule into the cell; and (c) introducing the cell into the subject, thereby treating or preventing an HLA-related disorder. In certain embodiments, the HLA-related disorder is graft-versus-host disease (GVHD). The nuclease(s) can be introduced as mRNA, in protein form and/or as a DNA sequence encoding the nuclease(s). Likewise the non-classic HLA molecules (*e.g.,* HLA-E and/or HLA-G) may be introduced as mRNA, in protein form and/or as a DNA sequence encoding the molecules. In certain embodiments, the isolated cell introduced into the subject further comprises additional genomic modification, for example, an integrated exogenous sequence (into the cleaved HLA or HLA regulatory gene or a different gene, for example a safe harbor gene) and/or inactivation (*e.g.,* nuclease-mediated) of additional genes, for example one or more TCR genes. The exogenous sequence may be introduced via a vector (*e.g.* Ad, AAV, LV), or by using a technique such as electroporation. In some aspects, the composition may comprise isolated cell fragments and/or differentiated (partially or fully) cells.

[0021] Also provided are pharmaceutical compositions comprising the modified cells as described herein (*e.g.,* stem cells with inactivated classic HLA gene(s) and which express non-classic HLA gene(s)). In certain embodiments, the pharmaceutical compositions further comprise one or more pharmaceutically acceptable excipients. Such pharmaceutical compositions may be used prophylactically or therapeutically and may comprise iPSCs, hES, MSCs, HSCs or combinations and/or derivatives thereof. In other embodiments, cells, cell fragments (*e.g.,* platelets) or tissues derived from such modified stem cells are provided such that such tissues are modified in the HLA loci as desired. In some aspects, such cells are partially differentiated (*e.g.* hematopoietic stem cells) while in others fully differentiated cells are provided (*e.g.* lymphocytes or megakarocytes) while in still

others, fragments of differentiated cells are provided. In other embodiments, stem cells, and/or their differentiated progeny are provided that contain an altered HLA or HLA regulator gene or genes, and they also can contain an additional genetic modification including a deletion, alteration or insertion of a donor DNA at another locus of interest.

[0022] In some embodiments, cells as described herein may be mature cells such as CD4+ T cells or NK cells. In some aspects, the mature cells may be used for cell therapy, for example, for a T cell transplant. In other embodiments, the cells for use in T cell transplant contain another gene modification of interest. In one aspect, the T cells contain an inserted chimeric antigen receptor (CAR) specific for a cancer marker. In a further aspect, the inserted CAR is specific for the CD19 marker characteristic of B cell malignancies. Such cells would be useful in a therapeutic composition for treating patients without having to match HLA, and so would be able to be used as an "off-the-shelf" therapeutic for any patient in need thereof. In some aspects, cells in which genes encoding the T-cell receptors (TCR) genes (*e.g.,* TCRα and/or TCRβ chains) have been manipulated or in which genes encoding TCR chains with desired specificity and affinity have been introduced are provided. In other embodiments, HLA modified platelets are provided for therapeutic use in treatment of disorders such as thromobytopenia or other bleeding disorders.

[0023] 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 stem cells, T-cells or NK cells prior to use for treating a subject in need thereof.

[0024] These and other aspects will be readily apparent to the skilled artisan in light of disclosure as a whole.

BRIEF DESCRIPTION OF THE DRAWINGS

[0025] **Figure 1, panels A and B,** shows levels of HLA-A3 (Figure 1A) and HLA-A2 (Figure 1B) genetic disruption assessed by the Surveyor™ nuclease assay. The lower (fast-moving) bands (arrows) are digestion products indicating ZFN-mediated gene modification. The numbers at the bottom of the lanes indicate the percentage of modified HLA-A alleles based on densitometry. DNA from mock transfected cells and cells transfected with a GFP expression vector was used for negative controls.

[0026] **Figure 2, panels A and B,** show isolation of HLA-A^{neg} HEK293. Figure 2A shows loss of HLA-A2 and HLA-A3 protein expression. Flow cytometry analysis of HLA-A2 and HLA-A3 expression on parental HEK293 cells and three derived genetically modified clones with loss of HLA-A (numbered 18.1, 8.18, 83). Dotted lines represent isotype (HLA-A2) or SA-PE (HLA-A3) controls, solid line represents HLA-A expression without IFN-γ and TNF-α, and filled lines represent HLA-A expression after culturing with 600 IU/mL of IFN-γ and 10 ng/mL of TNF-α for 48 hours. Dashed lines in the parental column represent HLA-A2 or HLA-A3 expression on EBV-LCL. Figure 2B shows resistance of the HLA modified clones to CTL-mediated lysis. Parental HEK293 and derived HLA-A^{neg} clones were cultured with IFN-γ and TNF-α for 48 hours and pulsed with serial dilutions of the cognate HLA-A3 peptide RVWDLPGVLK (SEQ ID NO:1, see also NP_001103685.1), derived from PANE1 (alternatively Centromere protein M isoform c) and recognized by CTL clone 7A7) or the HLA-A2 peptide CIPPDSLLFPA (SEQ ID NO:2, also alternative open reading frame of NM_199250.1) derived from C19ORF48/A2 and recognized by CTL clone GAS2B3-5) and evaluated for recognition by CTL clones in a 4-hour ^{51}Cr release assay at an effector to target ratio of 20:1.HLA-A2^{+} LCL (hatched bar) that expresses PANE1 mHAg (not peptide-loaded) were used as a positive control.

[0027] **Figure 3, panels A and B**, show loss of HLA-A expression on primary OKT3-propagated T cells after genetic editing with ZFNs. Figure 3A (top panel) shows loss of cell surface expression of HLA-A2 after electro-transfer of mRNA species encoding ZFN-L and ZFN-R targeting HLA-A2 (SBS#18889 and SBS#18881, respectively, see U.S. Patent Publication No. 20120060230). Coexpression of HLA-A2, CD4, and CD8 were analyzed 4 days after electro-transfer of graded doses of the mRNA species encoding ZFN-L and ZFN-R. Flow cytometry data were gated on the propidium iodide-negative, live cell population. Numbers in the lower right quadrant indicate the percentage of CD4 and CD8+ T cells that are HLA-A^{neg}. Figure 3A (bottom panel) shows improved disruption of HLA-A expression by "cold shock." Data were collected 4 days after electro-transfer of graded doses of the mRNA species encoding ZFN-L and ZFN-R. Cells were cultured at 30°C from days 1 to 3 after electro-transfer of ZFNs, returned to 37°C and cultured for one additional day before analysis. Figure 3B shows improved efficiency of HLA-

A disruption by ZFN-L and ZFN-R fused to the heterodimeric Fok I domain variants. mRNA species encoding the ZFN-L and ZFN-R heterodimeric Fok I mutants EL:KK targeting HLA-A were electro-transferred into primary T cells. HLA-A2 expression was analyzed after culturing the cells for 4 days at 37°C or 3 days at 30°C followed by 37°C for 1 day. X-axis represents CD4 and CD8 expression and y-axis represents HLA-A2 expression.

[0028] **Figure 4, panels A to C,** show that expression of non-classical HLA molecules protects against NK-mediated cell lysis. Figure 4A shows the immunophenotype of NK cells isolated from two individual PBMCs from healthy donor (each donor designated as NK-1 and NK-2). Flow cytometry data shown are gated for PI^{neg} population. The numbers represent percentage of each upper quadrant. Figure 4B shows genetic modification of HLA class I^{low}721.221 cells to express HLA-E and/or HLA-G. The SB transposon/transposase system was used to homogenously express HLA-E and/or HLA-G in three clones of 721.221 cells. Each number represents percentage expression of HLA-G, HLA-E, or both HLA-G and HLA-E as detected by flow cytometry. Figure 4 C shows specific lysis by NK cells targeting 721.221 cells. The relative ability of NK cells to kill parental (HLA class I^{low}), HLA-E^+, HLA-G^+, and both HLA-E^+HLA-G^+ 721.221 cells. Each column represents the mean ± standard deviation (SD) * $.01 < P < 0.05$, **$P < .01$; and ***$P < .001$

[0029] **Figure 5, panels A to C,** shows enrichment of HLA-A^{neg} primary T cells after genetic editing with ZFNs. Figure 5A shows generation of an HLA-$A2^{neg}$ T-cell population. HLA-$A2^{neg}$ T cells were enriched by magnetic bead-based selection. Input dose of mRNA coding for ZFN and 3-day culture conditions (37°C versus 30°C) after electro-transfer of mRNA are indicated. The numbers represent HLA-A2 negative population within CD4 and CD8 positive population. Figure 5B shows Surveyor™ nuclease assay of the HLA-$A2^{neg}$ T cells. Analysis of T cells enriched for loss of HLA-A2 expression demonstrates disruption in the HLA-A2 locus by the appearance of fast-moving band (arrow). Figure 5C shows results of sequencing of the HLA^{neg} T cells (SEQ ID NOs:39 to 53). PCR products using HLA-A2-specific primers from enriched cell (2.5 μg ZFNs, EL:KK *Fok* I domain, 30°C treatment) were cloned into a TOPO vector (Invitrogen) and plasmid products were sequenced. The wild type sequence is listed at the top with the expected ZFN binding sites underlined. Shown below are the sequences obtained from the ZFN-treated and

enriched cells. Deletions are indicated by hyphens and sequence changes are highlighted in bold. All 18 sequence changes result in frame shifts predicted to prevent protein translation.

[0030] **Figure 6, panels A to C,** show loss of HLA-A expression on primary CD19-specific CAR+ T cells genetically edited with ZFNs. Figure 6A shows disruption of HLA-A2 in CAR+ T cells by electro-transfer of mRNA encoding ZFNs. T cells from a HLA-A2+ donor were electroporated and propagated to express CD19-specific CAR (CD19RCD28). These T cells were re-electroporated with 2.5 μg of each mRNA encoding the heterodimeric *Fok* I domain variants of the HLA-A-specific ZFNs (ZFN-L-EL and ZFN-R-KK). HLA-A2 expression was analyzed after culturing at 30°C for 3 days followed by 37°C for 1 day. Enrichment of the HLA-$A2^{neg}$ population was performed by paramagnetic selection. Figure 6B shows HLA-A^{neg} CAR+ T cells evade lysis by HLA-A2 restricted CTL. Pools of the indicated CAR+ T cells were pulsed with serial dilutions of cognate peptide before being used as targets in a CRA. CTL clone GAS2B3-5, which is specific for C19ORF48/A2, was added at an effector-to-target ratio of 20:1. Figure 6C shows ZFN-modified HLA^{neg} CAR+ T cells maintain desired antigen-specific cytotoxicity. Redirected specificity for CD19 by HLA-A^{neg} T cells expressing CD19RCD28 CAR was demonstrated using the mouse T-cell line EL4 genetically modified to expresses a truncated variant of human CD19. Expression of introduced human CD19 on EL4 was 100%.

[0031] **Figure 7** shows ZFN-mediated elimination of HLA-A expression on human ESC. The HLAA2+ HLA-24+ hES parental cell line WIBR3 was modified by ZFN and donor plasmid coding for antibiotic resistance. Clones (5230, 5255, 5258) were chosen with loss of HLA-A expression and differentiated into fibroblasts. Expression of HLA-A2 and HLA-A24 on derived fibroblasts was assessed by flow cytometry after culturing with 600 IU/mL of IFN-γ and 10 ng/mL of TNF-α for 48 hours. Dashed line in parental panel represents isotype control.

DETAILED DESCRIPTION

[0032] Disclosed herein are compositions and methods for generating cells in which one or more classic HLA genes are inactivated but which express one or more non-classic HLA genes. Cells modified targeted in this manner can be used as therapeutics, for example, transplants, as the presence of the non-classic HLA gene(s)

reduces or eliminates NK-mediated lysis of HLA null cells. Additionally, other genes of interest may be inserted into cells in which the HLA genes have been manipulated.

[0033] Thus, the methods and compositions described herein provide methods for treatment of HLA related disorders, and these methods and compositions can comprise zinc finger transcription factors capable of modulating target genes as well as engineered zinc finger nucleases.

General

[0034] 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

[0035] 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.

[0036] 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 corresponding naturally-occurring amino acids.

[0037] "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.

[0038] 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.

[0039] 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.

[0040] 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. *See, e.g.,* U.S. Patent No. 8,586,526, incorporated by reference herein in its entirety.

[0041] Zinc finger and TALE DNA-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 protein or by engineering of the amino acids involved in DNA binding (the repeat variable diresidue or RVD region). Therefore, engineered zinc finger proteins or TALE proteins are proteins that are non-naturally occurring. Non-limiting

examples of methods for engineering zinc finger proteins and TALEs are design and selection. A designed 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 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.

[0042] 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.*, US 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 and WO 02/099084.

[0043] "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 via homology-directed repair mechanisms. 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.

[0044] In the methods of the disclosure, one or more targeted nucleases as described herein create a double-stranded break in the target sequence (*e.g.*, cellular chromatin) at a predetermined site, and a "donor" polynucleotide, having homology to the nucleotide sequence in the region of the break, can be introduced into the cell.

The presence of the double-stranded break has been shown to facilitate integration of the donor sequence. The donor sequence may be physically integrated or, alternatively, the donor polynucleotide is used as a template for repair of the break via homologous recombination, resulting in the introduction of all or part of the nucleotide sequence as in the donor into the cellular chromatin. Thus, a first sequence in cellular chromatin can be altered and, in certain embodiments, can be converted into a sequence present in a donor polynucleotide. Thus, the use of the terms "replace" or "replacement" can be understood to represent replacement of one nucleotide sequence by another, (*i.e.*, replacement of a sequence in the informational sense), and does not necessarily require physical or chemical replacement of one polynucleotide by another.

[0045] In any of the methods described herein, additional pairs of zinc-finger proteins can be used for additional double-stranded cleavage of additional target sites within the cell.

[0046] In certain embodiments of methods for targeted recombination and/or replacement and/or alteration of a sequence in a region of interest in cellular chromatin, a chromosomal sequence is altered by homologous recombination with an exogenous "donor" nucleotide sequence. Such homologous recombination is stimulated by the presence of a double-stranded break in cellular chromatin, if sequences homologous to the region of the break are present.

[0047] In any of the methods described herein, the first nucleotide sequence (the "donor sequence") can contain sequences that are homologous, but not identical, to genomic sequences in the region of interest, thereby stimulating homologous recombination to insert a non-identical sequence in the region of interest. Thus, in certain embodiments, portions of the donor sequence that are homologous to sequences in the region of interest exhibit between about 80 to 99% (or any integer therebetween) sequence identity to the genomic sequence that is replaced. In other embodiments, the homology between the donor and genomic sequence is higher than 99%, for example if only 1 nucleotide differs as between donor and genomic sequences of over 100 contiguous base pairs. In certain cases, a non-homologous portion of the donor sequence can contain sequences not present in the region of interest, such that new sequences are introduced into the region of interest. In these instances, the non-homologous sequence is generally flanked by sequences of 50-1,000 base pairs (or any integral value therebetween) or any number of base pairs

greater than 1,000, that are homologous or identical to sequences in the region of interest. In other embodiments, the donor sequence is non-homologous to the first sequence, and is inserted into the genome by non-homologous recombination mechanisms.

[0048] Any of the methods described herein can be used for partial or complete inactivation of one or more target sequences in a cell by targeted integration of donor sequence that disrupts expression of the gene(s) of interest. Cell lines with partially or completely inactivated genes are also provided.

[0049] Furthermore, the methods of targeted integration as described herein can also be used to integrate one or more exogenous sequences. The exogenous nucleic acid sequence can comprise, for example, one or more genes or cDNA molecules, or any type of coding or noncoding sequence, as well as one or more control elements (*e.g.,* promoters). In addition, the exogenous nucleic acid sequence may produce one or more RNA molecules (*e.g.*, small hairpin RNAs (shRNAs), inhibitory RNAs (RNAis), microRNAs (miRNAs), *etc.*).

[0050] "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.

[0051] 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.

[0052] 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.

[0053] 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.

[0054] "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.

[0055] 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.

[0056] 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.

[0057] 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.

[0058] 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.

[0059] 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.

[0060] 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. An exogenous molecule can also be the same type of molecule as an endogenous molecule but derived from a different species than the cell is derived from. For example, a human nucleic acid sequence may be introduced into a cell line originally derived from a mouse or hamster.

[0061] 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.

[0062] 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 one or more activation domains) 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.

[0063] 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.

[0064] 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.

[0065] "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 an 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.

[0066] "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. Genome editing (*e.g.,* cleavage, alteration, inactivation, random mutation) can be used to modulate expression. Gene inactivation refers to any reduction in gene expression as compared to a cell that does not include a ZFP as described herein. Thus, gene inactivation may be partial or complete.

[0067] 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.

[0068] "Eukaryotic" 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).

[0069] 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.

[0070] 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 (*e.g.,* ZFP, TALE) is fused to an activation domain, the DNA-binding domain and the activation 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 up-regulate gene expression. When a fusion polypeptide in which a DNA-binding domain is fused to a cleavage domain, 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 domain is able to cleave DNA in the vicinity of the target site. 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.

[0071] 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 or 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.

[0072] 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.

[0073] A "reporter gene" or "reporter sequence" refers to any sequence that produces a protein product that is easily measured, preferably although not necessarily in a routine assay. Suitable reporter genes include, but are not limited to, sequences encoding proteins that mediate antibiotic resistance (*e.g.*, ampicillin resistance, neomycin resistance, G418 resistance, puromycin resistance), sequences encoding colored or fluorescent or luminescent proteins (*e.g.*, green fluorescent protein, enhanced green fluorescent protein, red fluorescent protein, luciferase), and proteins which mediate enhanced cell growth and/or gene amplification (*e.g.*, dihydrofolate reductase). Epitope tags include, for example, one or more copies of FLAG, His, myc, Tap, HA or any detectable amino acid sequence. "Expression tags" include sequences that encode reporters that may be operably linked to a desired gene sequence in order to monitor expression of the gene of interest.

DNA-binding domains

[0074] Described herein are compositions comprising a DNA-binding domain that specifically binds to a target site in any gene comprising a HLA gene or a HLA regulator. Any DNA-binding domain can be used in the compositions and methods disclosed herein.

[0075] In certain embodiments, the DNA binding domain comprises a zinc finger protein. Preferably, the zinc finger protein is non-naturally occurring in that it is engineered to bind to a target site 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; U.S. Patent Nos. 6,453,242; 6,534,261; 6,599,692; 6,503,717; 6,689,558; 7,030,215; 6,794,136; 7,067,317; 7,262,054; 7,070,934; 7,361,635; 7,253,273; and U.S. Patent Publication Nos. 2005/0064474; 2007/0218528; 2005/0267061, all incorporated

herein by reference in their entireties. In certain embodiments, the DNA-binding domain comprises a zinc finger protein disclosed in U.S. Patent Publication No. 2012/0060230 (*e.g.*, Table 1), incorporated by reference in its entirety herein.

[0076] 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.

[0077] 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.

[0078] 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. In addition, enhancement of binding specificity for zinc finger binding domains has been described, for example, in U.S. Patent No. 6,794,136.

[0079] Selection of target sites; ZFPs 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. 6,140,0815; 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.

[0080] 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.

[0081] In certain embodiments, the DNA binding domain is an engineered zinc finger protein that binds (in a sequence-specific manner) to a target site in a HLA gene or HLA regulatory gene and modulates expression of HLA. The ZFPs can bind selectively to a specific haplotype of interest. For a discussion of HLA haplotypes identified in the United States population and their frequency according to different races, see Maiers *et al* (2007) *Human Immunology* 68: 779- 788, incorporated by reference herein.

[0082] Additionally, ZFPs are provided that bind to functional HLA regulator genes including, but not limited to, Tap1, Tap2, Tapascin, CTFIIA, and RFX5. HLA target sites typically include 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, which domains can be transcriptional activation or repression domains.

[0083] Specific examples of ZFPs are disclosed in Table 1 of U.S. Patent Publication No. 20120060230.

[0084] In some embodiments, 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; Paques *et al.* (2007) *Current Gene Therapy* **7:**49-66; U.S. Patent Publication No. 20070117128.

[0085] In other embodiments, the DNA binding domain comprises an engineered domain from a TAL effector similar to those derived from the plant pathogens *Xanthomonas* (see Boch *et al*, (2009) *Science* 326: 1509-1512 and Moscou and Bogdanove, (2009) *Science*326: 1501) and Ralstonia (see Heuer *et al* (2007) *Applied and Environmental Microbiology* 73(13): 4379-4384); U.S. Patent Application Nos. 20110301073 and 20110145940. 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). 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*.

[0086] 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.

Fusion proteins

[0087] Fusion proteins comprising DNA-binding proteins (*e.g.,* ZFPs or TALEs) as described herein and a heterologous regulatory (functional) domain (or functional fragment thereof) are also provided. Common domains include, *e.g.,* transcription factor domains (activators, repressors, co-activators, co-repressors), silencers, oncogenes (*e.g.*, myc, jun, fos, myb, max, mad, rel, ets, bcl, myb, mos family members etc.); DNA repair enzymes and their associated factors and modifiers; DNA rearrangement enzymes and their associated factors and modifiers; chromatin associated proteins and their modifiers (*e.g.* kinases, acetylases and deacetylases); and DNA modifying enzymes (*e.g.*, methyltransferases, topoisomerases, helicases, ligases, kinases, phosphatases, polymerases, endonucleases) and their associated factors and modifiers. U.S. Patent Application Publication Nos. 20050064474; 20060188987 and 2007/0218528 for details regarding fusions of DNA-binding domains and nuclease cleavage domains, incorporated by reference in their entireties herein.

[0088] Suitable domains for achieving activation include the HSV VP16 activation domain (*see, e.g.*, Hagmann *et al.*, *J. Virol.* 71, 5952-5962 (1997)) nuclear hormone receptors (*see, e.g.*, Torchia *et al.*, *Curr. Opin. Cell. Biol.* 10:373-383 (1998)); the p65 subunit of nuclear factor kappa B (Bitko & Barik, *J. Virol.* 72:5610-5618 (1998) and Doyle & Hunt, *Neuroreport* 8:2937-2942 (1997)); Liu et al., *Cancer Gene Ther.* 5:3-28 (1998)), or artificial chimeric functional domains such as VP64 (Beerli *et al.*, (1998) *Proc. Natl. Acad. Sci. USA* 95:14623-33), and degron (Molinari *et al.*, (1999) *EMBO J.* 18, 6439-6447). Additional exemplary activation domains include, Oct 1, Oct-2A, Sp1, AP-2, and CTF1 (Seipel *et al., EMBO J.* 11, 4961-4968

(1992) as well as p300, CBP, PCAF, SRC1 PvALF, AtHD2A and ERF-2. *See*, for example, Robyr *et al.* (2000) *Mol. Endocrinol.* 14:329-347; Collingwood *et al.* (1999) *J. Mol. Endocrinol.* 23:255-275; Leo *et al.* (2000) *Gene* 245:1-11; Manteuffel-Cymborowska (1999) *Acta Biochim. Pol.* 46:77-89; McKenna *et al.* (1999) *J. Steroid Biochem. Mol. Biol.* 69:3-12; Malik *et al.* (2000) *Trends Biochem. Sci.* 25:277-283; and Lemon *et al.* (1999) *Curr. Opin. Genet. Dev.* 9:499-504. Additional exemplary activation domains include, but are not limited to, OsGAI, HALF-1, C1, AP1, ARF-5,-6,-7, and -8, CPRF1, CPRF4, MYC-RP/GP, and TRAB1. *See*, for example, Ogawa *et al.* (2000) *Gene* 245:21-29; Okanami *et al.* (1996) *Genes Cells* 1:87-99; Goff *et al.* (1991) *Genes Dev.* 5:298-309; Cho *et al.* (1999) *Plant Mol. Biol.* 40:419-429; Ulmason *et al.* (1999) *Proc. Natl. Acad. Sci. USA* 96:5844-5849; Sprenger-Haussels *et al.* (2000) *Plant J.* 22:1-8; Gong *et al.* (1999) *Plant Mol. Biol.* 41:33-44; and Hobo *et al.* (1999) *Proc. Natl. Acad. Sci. USA* 96:15,348-15,353.

[0089] It will be clear to those of skill in the art that, in the formation of a fusion protein (or a nucleic acid encoding same) between a DNA-binding domain and a functional domain, either an activation domain or a molecule that interacts with an activation domain is suitable as a functional domain. Essentially any molecule capable of recruiting an activating complex and/or activating activity (such as, for example, histone acetylation) to the target gene is useful as an activating domain of a fusion protein. Insulator domains, localization domains, and chromatin remodeling proteins such as ISWI-containing domains and/or methyl binding domain proteins suitable for use as functional domains in fusion molecules are described, for example, in U.S. Patent Applications 2002/0115215 and 2003/0082552 and in WO 02/44376.

[0090] Exemplary repression domains include, but are not limited to, KRAB A/B, KOX, TGF-beta-inducible early gene (TIEG), v-erbA, SID, MBD2, MBD3, members of the DNMT family (*e.g.*, DNMT1, DNMT3A, DNMT3B), Rb, and MeCP2. *See*, for example, Bird *et al.* (1999) *Cell* 99:451-454; Tyler *et al.* (1999) *Cell* 99:443-446; Knoepfler *et al.* (1999) *Cell* 99:447-450; and Robertson *et al.* (2000) *Nature Genet.* 25:338-342. Additional exemplary repression domains include, but are not limited to, ROM2 and AtHD2A. *See*, for example, Chem *et al.* (1996) *Plant Cell* 8:305-321; and Wu *et al.* (2000) *Plant J.* 22:19-27.

[0091] Fusion molecules are constructed by methods of cloning and biochemical conjugation that are well known to those of skill in the art. Fusion molecules comprise a DNA-binding domain and a functional domain (*e.g.*, a

transcriptional activation or repression domain). Fusion molecules also optionally comprise nuclear localization signals (such as, for example, that from the SV40 medium T-antigen) and epitope tags (such as, for example, FLAG and hemagglutinin). Fusion proteins (and nucleic acids encoding them) are designed such that the translational reading frame is preserved among the components of the fusion.

[0092] Fusions between a polypeptide component of a functional domain (or a functional fragment thereof) on the one hand, and a non-protein DNA-binding domain (*e.g.,* antibiotic, intercalator, minor groove binder, nucleic acid) on the other, are constructed by methods of biochemical conjugation known to those of skill in the art. *See*, for example, the Pierce Chemical Company (Rockford, IL) Catalogue. Methods and compositions for making fusions between a minor groove binder and a polypeptide have been described. Mapp *et al.* (2000) *Proc. Natl. Acad. Sci. USA* 97:3930-3935.

[0093] In certain embodiments, the target site bound by the zinc finger protein is present in an accessible region of cellular chromatin. Accessible regions can be determined as described, for example, in U.S. Patent Nos. 7,217,509 and 7,923,542. If the target site is not present in an accessible region of cellular chromatin, one or more accessible regions can be generated as described in U.S. Patent Nos. 7,785,792 and 8,071,370. In additional embodiments, the DNA-binding domain of a fusion molecule is capable of binding to cellular chromatin regardless of whether its target site is in an accessible region or not. For example, such DNA-binding domains are capable of binding to linker DNA and/or nucleosomal DNA. Examples of this type of "pioneer" DNA binding domain are found in certain steroid receptor and in hepatocyte nuclear factor 3 (HNF3). Cordingley *et al.* (1987) *Cell* 48:261-270; Pina *et al.* (1990) *Cell* 60:719-731; and Cirillo *et al.* (1998) *EMBO J.* 17:244-254.

[0094] The fusion molecule may be formulated with a pharmaceutically acceptable carrier, as is known to those of skill in the art. *See*, for example, Remington's Pharmaceutical Sciences, 17th ed., 1985; and U.S. Patent Nos. 6,453,242 and 6,534,261.

[0095] The functional component/domain of a fusion molecule can be selected from any of a variety of different components capable of influencing transcription of a gene once the fusion molecule binds to a target sequence via its DNA binding domain. Hence, the functional component can include, but is not limited to, various

transcription factor domains, such as activators, repressors, co-activators, co-repressors, and silencers.

[0096] Additional exemplary functional domains are disclosed, for example, in U.S. Patent Nos. 6,534,261 and 6,933,113.

[0097] Functional domains that are regulated by exogenous small molecules or ligands may also be selected. For example, RheoSwitch® technology may be employed wherein a functional domain only assumes its active conformation in the presence of the external RheoChem™ ligand (*see* for example US 20090136465). Thus, the ZFP may be operably linked to the regulatable functional domain wherein the resultant activity of the ZFP-TF is controlled by the external ligand.

Nucleases

[0098] In certain embodiments, the fusion protein comprises a DNA-binding binding domain and cleavage (nuclease) domain. As such, gene modification can be achieved using a nuclease, for example an engineered nuclease. Engineered nuclease technology is based on the engineering of naturally occurring DNA-binding proteins. For example, engineering of homing endonucleases with tailored DNA-binding specificities has been described. Chames *et al.* (2005) *Nucleic Acids Res* 33(20):e178; Arnould *et al.* (2006) *J. Mol. Biol.* 355:443-458. In addition, engineering of ZFPs has also been described. See, *e.g.*, U.S. Patent Nos. 6,534,261; 6,607,882; 6,824,978; 6,979,539; 6,933,113; 7,163,824; and 7,013,219.

[0099] In addition, ZFPs and/or TALEs have been fused to nuclease domains to create ZFNs and TALENs – a functional entity that is able to recognize its intended nucleic acid target through its engineered (ZFP or TALE) DNA binding domain and cause the DNA to be cut near the DNA binding site via the nuclease activity. See, *e.g.*, Kim *et al.* (1996) *Proc Nat'l Acad Sci USA* 93(3):1156-1160. More recently, such nucleases have been used for genome modification in a variety of organisms. *See*, for example, United States Patent Publications 20030232410; 20050208489; 20050026157; 20050064474; 20060188987; 20060063231; and International Publication WO 07/014275.

[0100] Thus, the methods and compositions described herein are broadly applicable and may involve any nuclease of interest. Non-limiting examples of nucleases include meganucleases, TALENs and zinc finger nucleases. The nuclease may comprise heterologous DNA-binding and cleavage domains (*e.g.*, zinc finger

nucleases; 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).

[0101] In any of the nucleases described herein, the nuclease can comprise 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) or other DNA cleavage enzymes.

[0103] In certain embodiments, the nuclease comprises a meganuclease (homing endonuclease) or a portion thereof that exhibits cleavage activity. 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.

[0104] 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; 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; U.S. Patent Publication Nos. 20070117128; 20060206949; 20060153826; 20060078552; and 20040002092). In addition, naturally-occurring or engineered DNA-binding domains from meganucleases can be operably linked with a cleavage domain from a heterologous nuclease (*e.g., Fok*I) and/or cleavage domains from meganucleases can be operably linked with a heterologous DNA-binding domain (*e.g.,* ZFP or TALE).

[0105] In other embodiments, the nuclease is a zinc finger nuclease (ZFN) or TALE DNA binding domain-nuclease fusion (TALEN). ZFNs and TALENs comprise a DNA binding domain (zinc finger protein or TALE DNA binding domain) that has been engineered to bind to a target site in a gene of choice and cleavage domain or a cleavage half-domain (*e.g.,* from a restriction and/or meganuclease as described herein).

[0106] As described in detail above, zinc finger binding domains and TALE DNA 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 or TALE protein can have a novel binding specificity, compared to a naturally-occurring 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 or TALE amino acid sequences, in which each triplet or quadruplet nucleotide sequence is associated with one or more amino acid sequences of zinc fingers or TALE repeat units 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.

[0107] Selection of target sites; 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.

[0108] In addition, as disclosed in these and other references, zinc finger domains, TALEs 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. (*e.g.*, TGEKP (SEQ ID NO:3), TGGQRP (SEQ ID NO:4), TGQKP (SEQ ID NO:5), and/or TGSQKP (SEQ ID NO:6)). *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. *See, also,* U.S. Provisional Patent Application No. 61/343,729.

[0109] Thus, nucleases such as ZFNs, TALENs and/or meganucleases can comprise any DNA-binding domain and any nuclease (cleavage) domain (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 TAL-effector 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.

[0110] 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.

[0111] 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, US 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.

[0112] 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.

[0113] 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.

[0114] Exemplary Type IIS restriction enzymes are described in International Publication WO 07/014275, 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.

[0115] 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. 7,914,796; 8,034,598 and 8,623,618; and U.S. Patent Publication No. 20110201055, 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.

[0116] 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.

[0117] Thus, in one embodiment, a 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 Publication No. 2008/0131962, the disclosure of which is incorporated by reference in its entirety for all purposes. 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 FokI), 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). ((See US Patent Publication No. 20110201055).

[0118] 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. 7,914,796; 8,034,598 and 8,623,618; and U.S. Patent Publication No. 20110201055.

[0119] 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.

[0120] Nucleases (*e.g.*, ZFNs and/or TALENs) can be screened for activity prior to use, for example in a yeast-based chromosomal system as described in WO 2009/042163 and 20090068164. Nuclease expression constructs can be readily designed using methods known in the art. See, *e.g.*, United States Patent Publications 20030232410; 20050208489; 20050026157; 20050064474; 20060188987; 20060063231; and International Publication WO 07/014275. Expression of the nuclease may be under the control of a constitutive promoter or 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.

[0121] In certain embodiments, the nuclease comprises a CRISPR/Cas system. The CRISPR (clustered regularly interspaced short palindromic repeats) locus, which encodes RNA components of the system, and the cas (CRISPR-associated) locus, which encodes proteins (Jansen *et al.*, 2002. *Mol. Microbiol.* 43: 1565-1575; Makarova *et al.*, 2002. *Nucleic Acids Res.* 30: 482-496; Makarova *et al.*, 2006. *Biol. Direct* 1: 7; Haft *et al.*, 2005. *PLoS Comput. Biol.* 1: e60) make up the gene sequences of the CRISPR/Cas nuclease system. CRISPR loci in microbial hosts contain a combination of CRISPR-associated (Cas) genes as well as non-coding RNA elements capable of programming the specificity of the CRISPR-mediated nucleic acid cleavage.

[0122] The Type II CRISPR is one of the most well characterized systems and carries out targeted DNA double-strand break in four sequential steps. First, two non-coding RNA, the pre-crRNA array and tracrRNA, are transcribed from the CRISPR locus. Second, tracrRNA hybridizes to the repeat regions of the pre-crRNA and mediates the processing of pre-crRNA into mature crRNAs containing individual spacer sequences. Third, the mature crRNA:tracrRNA complex directs Cas9 to the target DNA via Watson-Crick base-pairing between the spacer on the crRNA and the protospacer on the target DNA next to the protospacer adjacent motif (PAM), an additional requirement for target recognition. Finally, Cas9 mediates cleavage of target DNA to create a double-stranded break within the protospacer. Activity of the CRISPR/Cas system comprises of three steps: (i) insertion of alien DNA sequences

into the CRISPR array to prevent future attacks, in a process called 'adaptation', (ii) expression of the relevant proteins, as well as expression and processing of the array, followed by (iii) RNA-mediated interference with the alien nucleic acid. Thus, in the bacterial cell, several of the so-called 'Cas' proteins are involved with the natural function of the CRISPR/Cas system and serve roles in functions such as insertion of the alien DNA etc.

[0123] In certain embodiments, Cas protein may be a "functional derivative" of a naturally occurring Cas protein. A "functional derivative" of a native sequence polypeptide is a compound having a qualitative biological property in common with a native sequence polypeptide. "Functional derivatives" include, but are not limited to, fragments of a native sequence and derivatives of a native sequence polypeptide and its fragments, provided that they have a biological activity in common with a corresponding native sequence polypeptide. A biological activity contemplated herein is the ability of the functional derivative to hydrolyze a DNA substrate into fragments. The term "derivative" encompasses both amino acid sequence variants of polypeptide, covalent modifications, and fusions thereof. Suitable derivatives of a Cas polypeptide or a fragment thereof include but are not limited to mutants, fusions, covalent modifications of Cas protein or a fragment thereof. Cas protein, which includes Cas protein or a fragment thereof, as well as derivatives of Cas protein or a fragment thereof, may be obtainable from a cell or synthesized chemically or by a combination of these two procedures. The cell may be a cell that naturally produces Cas protein, or a cell that naturally produces Cas protein and is genetically engineered to produce the endogenous Cas protein at a higher expression level or to produce a Cas protein from an exogenously introduced nucleic acid, which nucleic acid encodes a Cas that is same or different from the endogenous Cas. In some case, the cell does not naturally produce Cas protein and is genetically engineered to produce a Cas protein.

[0124] Exemplary CRISPR/Cas nuclease systems targeted to HLA and other genes are disclosed for example, in U.S. Provisional Application No. 61/823,689.

Delivery

[0125] The proteins (*e.g.*, nucleases and non-classic HLA molecules), polynucleotides encoding same and compositions comprising the proteins and/or polynucleotides described herein may be delivered to a target cell by any suitable means, including, for example, by injection of the protein and/or mRNA.

[0126] 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 T-cells, 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. Suitable cells also include stem cells such as, by way of example, embryonic stem cells, induced pluripotent stem cells (iPS cells), hematopoietic stem cells, neuronal stem cells and mesenchymal stem cells.

[0127] Methods of delivering proteins comprising DNA-binding domains as described herein 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.

[0128] DNA binding domains and fusion proteins comprising these DNA binding domains as described herein may also be delivered using vectors containing sequences encoding one or more of the DNA-binding protein(s). Additionally, additional nucleic acids (*e.g.*, donors and/or sequences encoding non-classic HLA proteins) also may be delivered via these vectors. 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 DNA-binding protein-encoding sequences and/or additional nucleic acids as appropriate. Thus, when one or more DNA-binding proteins as described herein are introduced into the cell, and additional DNAs as appropriate, they 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 DNA-binding proteins and additional nucleic acids as desired.

[0129] Conventional viral and non-viral based gene transfer methods can be used to introduce nucleic acids encoding engineered DNA-binding proteins in cells

(*e.g.*, mammalian cells) and target tissues and to co-introduce additional nucleotide sequences as desired. Such methods can also be used to administer nucleic acids (*e.g.*, encoding DNA-binding proteins, donors and/or non-classic HLA proteins) to cells *in vitro*. In certain embodiments, nucleic acids 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).

[0130] 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 U.S. Patent Nos. 7,074,596 and 8,153,773, incorporated by reference herein.

[0131] 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).

[0132] 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).

[0133] 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* vol 27(7) p. 643).

[0134] The use of RNA or DNA viral based systems for the delivery of nucleic acids encoding engineered DNA-binding proteins, non-classic HLA-molecules and/or other donors as desired takes 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 nucleic acids 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.

[0135] 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).

[0136] 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 are 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).

[0137] 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.

[0138] 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).

[0139] Recombinant adeno-associated virus vectors (rAAV) are a promising alternative gene delivery systems based on the defective and nonpathogenic parvovirus adeno-associated type 2 virus. All 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, AAV8, AAV8.2, AAV9 and AAVrh10 and pseudotyped AAV such as AAV2/8, AAV2/5 and AAV2/6 can also be used in accordance with the present invention.

[0140] Replication-deficient recombinant adenoviral vectors (Ad) can be produced at high titer and 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 human 293 cells that supply deleted gene function in *trans*. Ad vectors can transduce multiple types of tissues *in vivo*, including nondividing, 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.* 7:1083-9 (1998)). Additional examples of the use of adenovirus vectors for gene transfer in clinical trials include Rosenecker *et al., Infection* 24:1 5-10 (1996); Sterman *et al., Hum. Gene Ther.* 9:7 1083-1089 (1998); 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); Sterman *et al., Hum. Gene Ther.* 7:1083-1089 (1998).

[0141] Packaging cells are used to form virus particles that are capable of infecting a host cell. Such cells include 293 cells, which package adenovirus, 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.

[0142] 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.

[0143] 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 re-implantation of the cells into a patient, usually after selection for cells which have incorporated the vector.

[0144] *Ex vivo* cell transfection for diagnostics, research, transplant 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 DNA-binding proteins 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).

[0145] 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)).

[0146] 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)).

[0147] Stem cells that have been modified may also be used in some embodiments. For example, neuronal stem cells that have been made resistant to apoptosis may be used as therapeutic compositions where the stem cells also contain the ZFP TFs of the invention. Resistance to apoptosis may come about, for example, by knocking out BAX and/or BAK using BAX- or BAK-specific ZFNs (see, US patent application no. 12/456,043) in the stem cells, or those that are disrupted in a caspase, again using caspase-6 specific ZFNs for example. These cells can be transfected with the ZFP TFs that are known to regulate HLA.

[0148] Vectors (*e.g.*, retroviruses, adenoviruses, liposomes, etc.) containing therapeutic DNA-binding proteins (or nucleic acids encoding these proteins) can also

be administered directly to an organism for transduction of cells *in vivo*. Alternatively, naked DNA 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.

[0149] 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.

[0150] 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.

[0151] 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).

[0152] As noted above, the disclosed methods and compositions can be used in any type of cell including, but not limited to, prokaryotic cells, fungal cells, Archaeal cells, plant cells, insect cells, animal cells, vertebrate cells, mammalian cells and human cells, including T-cells and stem cells of any type. Suitable cell lines for protein expression are known to those of skill in the art and include, but are not limited to COS, CHO (*e.g.*, CHO-S, CHO-K1, CHO-DG44, CHO-DUXB11), VERO, MDCK, WI38, V79, B14AF28-G3, BHK, HaK, NS0, SP2/0-Ag14, HeLa, HEK293 (*e.g.*, HEK293-F, HEK293-H, HEK293-T), perC6, insect cells such as *Spodoptera fugiperda* (Sf), and fungal cells such as *Saccharomyces*, *Pichia* and *Schizosaccharomyces*. Progeny, variants and derivatives of these cell lines can also be used.

Applications

[0153] The disclosed compositions and methods can be used for any application in which it is desired to modulate HLA expression and/or functionality, including but not limited to, therapeutic and research applications in which HLA modulation is desirable.

[0154] Diseases and conditions which are tied to HLA include Addison's disease, ankylosing spondylitis, Behçet's disease, Buerger's disease, celiac disease, chronic active hepatitis, Graves' disease, juvenile rheumatoid arthritis, psoriasis, psoriatic arthritis, rheumatoid arthritis, Sjögren syndrome, and lupus erythematosus, among others. In addition, modification of a HLA gene may be useful in conjunction with other genetic modifications of a cell of interest. For example, modification of a target cell such as a CTL with a chimeric antigen receptor to change the CTL's specificity may be combined with HLA modification *ex vivo* as described herein in order to develop a cell therapeutic that may be used in most any patient in need thereof.

[0155] In addition, the materials and methods of the invention can be used in the treatment, prevention or amelioration of graft-versus-host-disease. Graft-versus-host disease (GVHD) is a common complication when allogenic T-cells (*e.g.,* bone marrow and/or blood transfusion) are administered to a patient. The functional immune cells in the infused material recognize the recipient as "foreign" and mount an immunologic attack. By modulating HLA and/or TCR expression in allogenic T cells, "off the shelf" T cells (*e.g.,* CD19-specific T-cells) can be administered on demand as "drugs" because the risk of GVHD is reduced or eliminated and, in addition, provision of non-classic HLA molecules reduces or eliminates NK-mediated lysis of the modified cells.

[0156] Methods and compositions also include stem cell compositions wherein one or more classic HLA genes within the stem cells has been inactivated and one or more non-classic HLA molecules activated. For example, HLA-modified hematopoietic stem cells can be introduced into a patient following bone marrow ablation. These altered HSC would allow the re-colonization of the patient without loss of the graft due to rejection and/or NK-mediated cell lysis. The introduced cells may also have other alterations to help during subsequent therapy (*e.g.*, chemotherapy resistance) to treat the underlying disease.

[0157] The methods and compositions of the invention are also useful for the development of HLA modified platelets, for example for use as therapeutics. Thus, HLA modified platelets may be used to treat thrombocytopenic disorders such as idiopathic thrombocytopenic purpura, thrombotic thrombocytopenic purpura and drug-induced thrombocytopenic purpura (*e.g.* heparin-induced thrombocytopenia). Other platelet disorders that may be treated with the HLA modified platelets of the invention include Gaucher's disease, aplastic anemia, Onyalai, fetomaternal alloimmune thrombocytopenia, HELLP syndrome, cancer and side effects from some chemotherapeutic agents. The HLA modified platelets also have use in as an "off the shelf" therapy in emergency room situations with trauma patients.

[0158] The methods and compositions of the invention can be used in xenotransplantation. Specifically, by way of example only, pig organs can be used for transplantation into humans wherein the porcine MHC genes have been deleted and/or replaced with human HLA genes. Strains of pigs can be developed (from pig embryos that have had HLA targeting ZFNs encoded by mRNAs injected into them such that the endogenous MHC genes are disrupted, or from somatic cell nuclear transfer into pig embryos using nuclei of cells that have been successfully had their HLA genes targeted) that contain these useful genetic mutations, and these animals may be grown for eventual organ harvest. This will prevent rejection of these organs in humans and increase the chances for successful transplantation.

[0159] The methods and compositions of the invention are also useful for the design and implementation of *in vitro* and *in vivo* models, for example, animal models of HLA or other disorders, which allows for the study of these disorders.

EXAMPLES

Example 1: Materials and Methods

ZFNs

[0160] HLA-A-binding ZFNs containing 5 or 6 fingers were designed and assembled using an established archive of pre-validated 2-finger and 1-finger modules as described in U.S. Patent Publication No. 20120060230. Exemplary ZFNs that may be used are shown below in Table 1. The first column in this table is an internal reference name (number) for a ZFP and corresponds to the same name in column 1 of Table 2. "F" refers to the finger and the number following "F" refers which zinc finger (*e.g.*, "F1" refers to finger 1).

Table 1: Zinc finger proteins

Target	SBS #	Design					
Class I		F1	F2	F3	F4	F5	F6
HLA A2	18889	QSSHLTR (SEQ ID NO:11)	RSDHLTT (SEQ ID NO:12)	RSDTLSQ (SEQ ID NO:13)	RSADLSR (SEQ ID NO:14)	QSSDLSR (SEQ ID NO:15)	RSDALTQ (SEQ ID NO:16)
HLA A2	18881	QKTHLAK (SEQ ID NO:17)	RSDTLSN (SEQ ID NO:18)	RKDVRIT (SEQ ID NO:19)	RSDHLST (SEQ ID NO:20)	DSSARKK (SEQ ID NO:21)	NA
HLA A2	24859	QNAHRKT (SEQ ID NO:22)	RSDSLLR (SEQ ID NO:23)	RNDDRKK (SEQ ID NO:24)	RSDHLST (SEQ ID NO:20)	DSSARKK (SEQ ID NO:21)	NA
HLA A3	25191	DRSHLSR (SEQ ID NO:25)	RSDDLTR (SEQ ID NO:26)	DRSDLSR (SEQ ID NO:27)	QSGHLSR (SEQ ID NO:28)	NA	NA
HLA A3	25190	DRSALSR (SEQ ID NO:29)	QSSDLRR (SEQ ID NO:30)	DRSALSR (SEQ ID NO:29)	DRSHLAR (SEQ ID NO:31)	RSDDLSK (SEQ ID NO:32)	DRSHLAR (SEQ ID NO:31)

[0161] The sequence for the target sites of exemplary HLA-A binding proteins are disclosed in Table 2. Table 2 shows target sequences for the indicated zinc finger proteins. 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 2: HLA-A Zinc finger target sites

Target	SBS #	Target site
Class I		
HLA A2	18889	gtATGGCTGCGACGTGGGGTcggacggg_(SEQ ID NO:34)
HLA A2	18881	ttATCTGGATGGTGTGAgaacctggccc_(SEQ ID NO:35)
HLA A2	24859	tcCTCTGGACGGTGTGAgaacctggccc_(SEQ ID NO:36)
HLA A3	25191	atGGAGCCGCGGGCgccgtggatagagc_(SEQ ID NO:37)
HLA A3	25190	ctGGCTCGcGGCGTCGCTGTCgaaccgc_(SEQ ID NO:38)

Cell Culture

[0162] HEK293 cells were maintained in Dulbecco's modified Eagle's medium (Lonza, Basel, Switzerland) supplemented with 10% heat-inactivated fetal bovine serum (FBS: Lonza) and 2 mmol/L L-glutamine (Glutamax-1: Invitrogen, Carlsbad, CA). EBV-LCL, 721.221 and EL-4 cell lines were maintained in RPMI 1640 (Lonza) supplemented with 10% heat-inactivated FBS and 2 mmol/L L-glutamine. Identity of these cell lines was confirmed by STR DNA fingerprinting. CD8+ CTL clones specific for mHAgs were: clone 7A7 (Brickner *et al.* (2006) *Blood* 107(9):3779-3786) recognizing peptide RVWDLPGVLK (SEQ ID NO:1) encoded by PANE1 transcripts in the context of HLA-A*0301 and clone GAS2B3-5 (Tykodi *et al.* (2008) *Clin Cancer Res.* 14(16):5260-5269) recognizing HLA-A*0201-restricted CIPPDSLLFPA (SEQ ID NO:2, alternative open reading frame of NM_199250.1) peptide from ORF +2/48 in C19ORF48. CTL clones were thawed one day before the ^{51}Chromium release assay, and maintained in RPMI 1640 supplemented with 10% human albumin serum, 2 mmol/L L-glutamine, 20 ng/mL of IL-15 (PeproTech, Rocky Hill, NJ), and 20 IU/mL of IL-2 (Chiron, Emeryville, CA).

Activation of primary T cells by OKT3-loaded artificial antigen presenting cells (aAPC)

[0163] T cells (CAR^{neg}) were activated for sustained proliferation by cross-linking CD3 *in vitro* by stimulating PBMC with OKT3 (eBioscience, San Diego, CA) pre-loaded onto aAPC (clone #4: K562 cells genetically modified to stably co-express CD19, CD64, CD86, CD137L, and a membrane-bound mutein of interleukin IL-15 synchronously expressed with EGFP17 (see, O'Connor *et al.* (2012) *Sci Rep* 2:249; Manuri *et al.* (2010) *Hum Gene Ther* 21(4):427-437)) at a ratio of 1:1 (T cells : γ-irradiated (100Gy) aAPC) in RPMI 1640 supplemented with 2 mmol/L L-glutamine and 10% FBS with 50 IU/mL of IL-2 (added every other day, beginning the day after addition of aAPC). OKT3-loaded aAPC were re-added every 14 days to sustain T-cell proliferation.

Generation of genetically modified CD19-specific CAR+ T cells and propagation on CD19+ aAPC

[0164] Our approach to manufacture clinical-grade CAR+ T cells was adapted to generate CD19-specific T cells. (See, *e.g.*, Singh *et al.* (2008) *Cancer Res.* 68(8):2961-2971). DNA plasmids coding for SB transposon CD19RCD28 and SB hyperactive transposase SB11 were simultaneously electro-transferred (Human T-Cell Nucleofector solution, program U-014) using a Nucleofector II device (Lonza) into T cells derived from PBMC. A population of CAR+ T cells was selectively numerically expanded by adding on the day of electroporation, and re-adding every 14 days (at 1 : 2 T cell : aAPC ratio) γ-irradiated (100 Gy) aAPC (clone #4 without OKT3 loading) in the presence of 50 IU/mL of IL-2 (added every other day, beginning the day after addition of aAPC).

***In vitro* transcription of messenger RNA**

[0165] *In vitro*-transcribed mRNA species were prepared as previously described in Torikai *et al.* (2012) *Blood* 119(24):5697-5705. In brief, the DNA template plasmids coding for ZFN-L and ZFN-R were linearized with XhoI. After *in vitro* transcription (RiboMAX™ Large Scale RNA Production System-T7, Promega, Madison, WI) and capping (ARCA cap analog, Ambion, Austin, TX) according to manufacturers' instructions, poly-adenines were added using the poly A tailing kit (Ambion). The integrity of the mRNA species was validated on a denaturing 1%

agarose gel with 3-(N-morpholino) propanesulphonic acid (MOPS) buffer and concentration was determined by spectrophotometer (BioRad, Hercules, CA) at OD260.The mRNA was vialed and stored at -80°C for one-time use.

Electro-transfer of DNA plasmids and mRNA species coding for ZFNs

[0166] For the modification of HEK293 cells, expression vectors encoding HLA-A targeting ZFNs were introduced by nucleofection (Lonza) using the manufacturer's protocol. T cells were harvested 6 days after initial stimulation or 2 to 3 days after re-stimulation with γ-irradiated aAPC. Five million T cells were pre-mixed with 2.5 to 10 μg of each ZFN-L and ZFN-R mRNA species in 100 μL of Human T Cell Nucleofector solution (Lonza) and electroporated in a cuvette using a Nucleofector II device with program T-20. Following electroporation, cells were immediately placed in pre-warmed RPMI 1640 supplemented with 2 mmol/L L-glutamine and 10% FBS, and cultured at 37°C and 5% CO2 for 4-6 hours, at which point 50 IU/mL of IL-2 was added for further culture. In "cold shock" experiments, after overnight culture in a 37°C-5% CO2 incubator, T cells were transferred to 30°C, 5% CO2 incubator and cultured for 3 days, and then returned to a 37°C, 5% CO2 incubator prior to analysis.

Enrichment of cells lacking expression of HLA-A

[0167] After washing cells with phosphate buffered saline (PBS) supplemented with 2% FBS and 2mM EDTA, cells were labeled with PE-conjugated monoclonal antibody (mAb) specific anti-HLA-A2 (BD Biosciences, San Jose, CA) at 4°C for 15 minutes, washed, and labeled with anti-PE microbeads (Miltenyi Biotec, Auburn, CA) for 10 minutes. After washing, labeled cells were passed through an LD column (MiltenyiBiotec) and the flow-through fraction was collected and cultured. T cells were propagated on γ-irradiated OKT3-loaded aAPC and CAR+ T cells were propagated on CD19+ aAPC (not OKT3-loaded) in RPMI 1640 supplemented with 2 mmol/L L-glutamine and 10% FBS with 50 IU/mL of IL-2 (added every other day).

Flow cytometry

[0168] The following antibodies were used: phycoerythrin (PE) anti HLA-A2 (clone BB7.2), FITC anti-CD4 (clone RPA-T4), FITC anti-CD8 (clone HIT8a), PE and APC anti-CD3 (clone SK7), PE anti-CD56 (clone B159), PE anti HLA-DR (clone

G46-6), PE-mouse IgG2bγ, PE mouse IgG2aκ, FITC non-specific mouse IgG1, secondary reagent streptavidin-PE (all from BD Biosciences), biotin-conjugated anti-HLA-A3(clone 4i153), APC anti-HLA-G (clone MEMG19), PE anti HLA class I (clone W6/32, from Abcam, Cambridge, MA), and PE anti-HLA-E (clone 3D12, Biolegend, San Diego, CA). The Alexa 488-conjugated anti-CD19RCD28 CAR antibody (clone no. 136-20-1) was generated in our laboratory. We added propidium iodide (Sigma-Aldrich) to exclude dead cells from analysis. Data was acquired on a FACS Calibur (BD Biosciences) using CellQuest version 3.3 (BD Biosciences) and analyzed by FlowJo version 7.6.1 (Tree Star, Inc. Ashland, OR).

Surveyor™ nuclease assay

[0169] The level of modification of the HLA-A gene sequence in ZFN transfected cells was determined by the Surveyor™ nuclease assay as described in Guschin *et al.* (2010) *Methods Mol Biol* 649:247-256. In brief, genomic DNA from ZFN-modified cells underwent PCR with oligonucleotide primers designed to amplify the ZFN target regions within HLA-A2 and HLA-A3 genetic loci. After denaturing and re-annealing, Surveyor endonuclease (Cel-1) (Transgenomic, Omaha, NE) was used to cut heteroduplex DNA products to reveal a fast-moving band on polyacrylamide gel that was interpreted as evidence of a mutation event. Percent target modification was quantified by densitometry. The PCR primers used for the amplification of target loci were;

HLA-A3 Forward; 5'- GGGGCCGGAGTATTGGGACCA -3'; (SEQ ID NO:7)

HLA-A3 Reverse; 5'- CCGTCGTAGGCGTCCTGCCG -3' (SEQ ID NO:8)

HLA-A2 Forward; 5'- GGGTCCGGAGTATTGGGACGG-3' (SEQ ID NO:9)

HLA-A2 Reverse; 5'- TTGCCGTCGTAGGCGTACTGGTG -3' (SEQ ID NO:10)

HLA-A2 and HLA-A3 sequences were obtained from IMGT/HLA database, for example IMGT/HLA Accession no.; HLA-A2:HLA00005, HLA-A3: HLA00037.

^{51}Chromium release assay (CRA)

[0170] Target cells were labeled with 0.1 mCi of ^{51}Cr for 2 hours. After washing thrice with ice-cold RPMI 1640 supplemented with 10% FBS, labeled cells were diluted and distributed at 10^3 target cells in 100 μL per well in 96-well, v-bottomed plates. In the peptide titration assay, target cells were incubated with 10-fold serial dilutions of the peptides for 30 minutes at room temperature. CTL were added

at indicated effector to target ratios. After 4-hour incubation at 37°C, in 5% CO2, 50 μL of cell-free supernatants were collected and counted on a TopCount device (Perkin Elmer, Shelton, CT). All assays were performed in triplicate. In some assays, parental HEK293 cell lines and HLA-A modified HEK293 clones were treated with 600 IU/mL of interferon-γ (IFN-γ; R&D systems, Minneapolis, MN) and 10 ng/mL of tissue necrosis factor-α (TNF-α; R&D systems) for 48 hours before assay. The percent specific lysis was calculated as follows: ((experimental cpm - spontaneous cpm) / (maximum cpm - spontaneous cpm)) × 100.

NK-cell isolation and enforced expression of non-classical HLA on 721.221 cells

[0171] NK cells were isolated from PBMC by CD56 microbeads (Miltenyi Biotec) and LS columns (Miltenyi Biotec) according to the manufacture's instruction. DNA plasmids coding for SB transposons HLA-E (accession no. 005516) and/or HLA-G (accession no. NM_002127) were co-electroporated with SB11 transposase into parental HLA class I^{low} 721.221 cells by Amaxa Nucleofector II device (program: A-016). HLA-E+ and HLA G+ clones exhibiting stable and homogeneous expression of introduced HLA molecules were derived by limiting dilution after sorting HLA-E and/or HLA-G positive cells by fluorescence-conjugated mAbs [PE anti-HLA-E, APC anti-HLA-G, and PE anti-HLA-G (clone 87G, Biolegend)] and paramagnetic beads [anti PE microbeads and anti APC microbeads (cat #s 130-048-801, 130-090-855 (Miltenyi Biotec)]. NK cell killing of 721.221 clones was assessed by 4-hr CRA and statistical differences of the data were calculated by one-way ANOVA followed by Tukey's multiple comparison in GraphPad Prism software (version 5, GraphPad Software, La Jolla, CA).

Culture and differentiation of hESC

[0172] The hESC line WIBR3 (Whitehead Institute Center for Human Stem Cell Research, Cambridge, MA) 22 was maintained as described previously (Soldner *et al.* (2009) *Cell* 136(5):964-977 on mitomycin C inactivated mouse embryonic fibroblast (MEF) feeder layers in hESC medium [DMEM/F12 (Invitrogen) supplemented with 15% FBS, 5% KnockOut™ Serum Replacement (Invitrogen), 1 mM glutamine (Invitrogen), 1% nonessential amino acids (Invitrogen), 0.1 mM β-mercaptoethanol (Sigma, St. Louis, MO) and 4 ng/ml FGF2 (R&D systems)]. Targeted hESC were differentiated into fibroblast-like cells as described previously

(Hockemeyer *et al.* (2008) *Cell Stem Cell* 3(3):346-353. Briefly, differentiation was induced by embryoid body (EB) formation in non-adherent suspension culture dishes (Corning, Corning, NY) in DMEM medium supplemented with 15% fetal bovine serum for 5 days. EBs were subsequently plated onto adherent tissue culture dishes and passaged according to primary fibroblast protocols using trypsin for at least four passages before the start of experiments.

ZFN-mediated genome editing of hESC

[0173] HESC were cultured in Rho-associated protein kinase (ROCK)-inhibitor (Stemolecule; Stemgent, Cambridge, MA) 24 hours prior to electroporation. Cells were harvested using 0.05% trypsin/EDTA solution (Invitrogen) and resuspended in PBS. Ten million cells were electroporated (Gene Pulser Xcell System, Bio-Rad: 250 V, 500μ F, 0.4 cm cuvettes) with 35 μg of donor plasmid encoding puromycin resistant gene under control of phosphoglycerate kinase (PGK) promoter flanked by 5' and 3' arms homologous to the putative ZFN binding region of HLA-A24 and 7.5 μg of each ZFN-encoding plasmid, or 35 μg of donor plasmid and 10 μg of each ZFN encoding mRNA. Cells were subsequently plated on DR4 MEF feeder layers in hESC medium supplemented with ROCK inhibitor for the first 24 hours. Puromycin selection (0.5 μg/ml) was initiated 72 hours after electroporation. Individual puromycin-resistant colonies were picked and expanded 10 to 14 days after electroporation. Correct targeting and gene disruption was verified by Southern blot analysis and sequencing of the genomic locus.

Example 2: Design and Validation of Zinc Finger Nucleases Targeting Multiple endogenous HLA-A Genes

[0174] ZFNs were designed to cleave a pre-defined site within the genomic coding sequence of the endogenous human HLA-A genes (see, *e.g.*, Table 2 of U.S. Patent Publication 2012/0060230). Expression of these ZFNs in human cells should eliminate expression of HLA-A molecules via error-prone repair of introduced double-strand breaks leading to disruption of the reading frame of the targeted HLA loci. To evaluate the ability of these ZFNs to disrupt HLA-A expression we initially used the human embryonic kidney cell line HEK293, which co-expresses HLA-A*03:01 (HLA-A3) and HLA-A*02:01 (HLA-A2). After transfecting HEK293 cells with expression plasmids encoding the ZFNs as described in Example 1, we used

allele-specific PCR and the Surveyor nuclease assay to quantify the level of gene modification at the anticipated ZFN target sites.

[0175] As shown in Figure 1, approximately 10% modification of HLA-A3 locus and ~6% modification of HLA-A2 locus were modified HLA-A targeted ZFNs.

Example 3: Isolation and Functional Validation of HLA-A^{neg} HEK293 Cells

[0176] To assess the impact of disrupting HLA-A expression, we used limiting dilution to obtain single-cell clones from the ZFN-modified HEK293 cell pool. Sequencing revealed clones that carried small insertions or deletions within the expected ZFN-binding sites in HLA-A2, HLA-A3, or both alleles, which resulted in a frame shift leading to premature termination of translation. Since the steady state level of HLA-A expression in HEK293 cells is low compared with hematopoietic cells, such as an EBV transformed lymphoblastoid cell line (EBV-LCL), we exposed the HEK293 cells to pro-inflammatory cytokines known to augment HLA levels. See, *e.g.*, Johnson (2003) *J Immunol.* 170(4):1894-1902.

[0177] The addition of interferon-gamma (IFN-γ) and tissue-necrosis-factor-alpha (TNF-α) increased expression of both HLA-A2 and HLA-A3 in parental HEK293 cells (Fig. 2A, top panel). In contrast, ZFN-treated HEK293 clones carrying mutations in HLA-A2 and/or HLA-A3 did not express these proteins even after induction by IFN-γ and TNF-α (Fig. 2A, bottom 3 panels). Thus, flow cytometry using mAbs specific for HLA-A2 or HLA-A3 confirmed the allele-specific loss of HLA-A expression on the cell surface.

[0178] Next, we asked whether the loss of HLA-A expression on the ZFN-modified clones would preclude T-cell recognition and this was tested using HLA-A3 and HLA-A2-restricted cytotoxic T-lymphocyte (CTL) clones. As expected, an HLA-A3-restricted CD8+ CTL clone 7A7 demonstrated robust specific lysis of the HLA-A3+ parental HEK293 cells loaded with serial dilutions of cognate peptide (RVWDLPGVLK, SEQ ID NO:1, NP_001103685) with 50% maximal lysis observed with 1 ng/mL of the pulsed cognate peptide (Fig. 2B, top panel). HEK293 clone 8.18 that has lost expression of HLA-A2 allele, but is wild type at HLA-A3, was also lysed by this HLA-A3 restricted CTL clone. In contrast, when pulsed with the same peptide, the HEK293 clone 18.1 that had been edited to eliminate HLA-A3 expression, was not lysed by the HLA-A3 restricted CTL clone 7A7, and neither was the HLA-A2/A3 double-knock out HEK293 clone 83 (Fig.2B, top panel). We also evaluated the

cytolytic activity of an HLA-A2 restricted CTL clone GAS2B3-5 and observed robust killing activity when presented with the parental HEK293 cells or the HLA-A2 wild type clone 18.1, while the ZFN-modified HLA-A2^{neg} HEK293 clone 8.18 and the HLA-A2/A3 double-knock out clone 83 were spared from lysis (Fig. 2B, bottom panel).

[0179] These data demonstrate that treatment with ZFNs completely eliminates HLA-A expression, resulting in protection from HLA-A restricted CTL-mediated killing, even under pro-inflammatory conditions that up-regulate endogenous HLA-A expression.

Example 4: NK-cell mediated lysis against HLA^{null} cells can be prevented by enforced expression of HLA-E and/or HLA-G

[0180] We envision that the approach we outline here, using ZFN targeting HLA class I genes combined with antibody based cell sorting, could ultimately be used to eliminate expression of HLA-A, -B and -C expression. Cells without classical HLA expression, especially HLA-B or HLA-C which are known to be the main ligands for killer inhibitory receptors (KIRs), may be eradicated through the loss of interaction between KIR and its ligand. Parham *et al.* (2005) *Nat Rev Immunol.* 5(3):201-214. To test whether NK-cell mediated cytoxicity would be reduced, we introduced non-classical HLA-E or HLA-G molecules, which have been shown to reduce NK-cell mediated cytotoxicity (Borrego *et al.* (1998) *J Exp Med.* 187(5):813-818; Riteau *et al.* (2001) *Int Immunol.* 13(2):193-201; Rouas-Freiss *et al.* (1997) *Proc Natl Acad Sci U S A.* 94(10):5249-5254; Braud *et al.* (1998) *Nature* 391(6669):795-799) and are much less polymorphic than classical HLA molecules, into the HLA class Ilow cell line 721.221 (Fig. 3A) and evaluated their susceptibility to be killed by NK cells.

[0181] The flow cytometry analysis of NK cells directly isolated from PBMC showed over 94% purity (CD56^{pos}CD3^{neg} population) (Fig. 4A) and HLA-E and/or HLA-G expression in genetically modified 721.221 clones at over 90% (Fig. 4B). We demonstrated that enforced expression of HLA-E and/or HLA-G on 721.221 significantly prevented these target cells from NK-cell mediated lysis (Fig. 4C).

[0182] This provides a solution to forestall elimination of administered HLA^{neg} allogeneic cells by recipient NK cells, thus rendering the complete HLA class

I knock out feasible for human application by avoiding the introduction of immunogenic transgenes.

Example 5: Disruption of HLA-A Genes in Primary T cells using a "Hit-and-Run" Strategy

[0183] To extend our results to clinically relevant primary cells, we evaluated the activity of the HLA-A-specific ZFNs in human T cells. Since ZFNs require only temporary expression to achieve stable disruption of desired target genes, we transiently expressed ZFNs from an in vitro transcribed mRNA. Electro-transfer of mRNA encoding the ZFNs into PBMC from an HLA-A2 homozygous donor (HLA-A2 being the most common HLA-A allele in Caucasians, see, e.g., Mori et al. (1997) Transplantation 64(7):1017-1027) rendered ~19% of these T cells HLA-A2 negative (Fig. 5A, top panel). We have previously demonstrated that transiently lowering the incubation temperature after transfection can increase ZFN activity. See, U.S. Patent Publication No. 2011/0129898.

[0184] Subjecting electroporated primary T cells to a transient hypothermia elevated the proportion of HLA-A2 negative cells by up to 57% in an mRNA dose dependent manner (Fig. 5A, bottom panel).

Example 6: Achieving a Clinically Relevant Level of HLA-A Disruption in Primary T Cells

[0185] With a view to the clinical application of the HLA-targeted ZFNs, we evaluated the use of the "high fidelity" obligate heterodimeric Fok I domains EL/KK, which are designed to decrease potential off-target cleavage events by preventing homodimerization33. Use of mRNA encoding the EL/KK ZFN variants of ZFN-L and ZFN-R resulted in an marked increase in HLA-A^{neg} T cells, eliminating HLA-A expression in up to 52% of T-cell population, despite limiting doses of mRNA (2.5 μg each ZFN) (Fig. 5B).

[0186] A single round of HLA-A positive T cell depletion with antibody-coated paramagnetic beads readily increased the HLA-$A2^{neg}$ T-cell fraction to over 95% of the population without impacting CD4 or CD8 expression (Fig. 6A). Analysis of this HLA-$A2^{neg}$ population by the Surveyor nuclease assay (Fig. 6B) and direct DNA sequencing (Fig. 6C) revealed nearly 100% editing of the HLA-A2 alleles precisely within the region targeted by the ZFNs.

[0187] Together these data demonstrate that ZFN-driven genome editing can rapidly generate an HLA-negative primary T cell population.

Example 7: Disruption of HLA-A Genes in T cells Genetically Modified to Redirect Specificity

[0188] To demonstrate the potential utility of HLA editing, we next focused on a specific class of cells that could be broadly used in allogeneic settings after elimination of HLA expression; namely cytotoxic T cells genetically modified to express a 'universal' chimeric antigen receptor (CAR) to redirect specificity towards tumor associated antigens independent of HLA recognition 34. Indeed, we and others are currently infusing patient-specific CAR+ T cells for the investigational treatment of CD19+ malignancies. (*See, e.g.,* Kalos *et al.* (2011) *Sci Transl Med* 3(95):95ra73; Porter *et al.* (2011) *N Engl J Med* 365(8):725-733). Recently, we have published that CAR+ T cells retain redirected specificity for CD19 when ZFNs are used to eliminate endogenous αβTCR expression (Torikai *et al.* (2012) *Blood* 119(24):5697-5705 and Provasi *et al*, (2012) *Nat Med* 18(5):807-15). Indeed, such TCR-edited T cells demonstrate both improved potency and safety (GVHD) *in vivo*.

[0189] To further our ability to generate "off-the-shelf" T cell therapies, we investigated whether ZFNs could eliminate HLA-A expression from primary T cells previously engineered to express a CD19-specific CAR. PBMC genetically modified by synchronous electro-transfer of DNA plasmids derived from the Sleeping Beauty (SB) transposon/transposase system followed by selective propagation on CD19+ aAPC, clone #439 resulted in expression of the CD19-specific CAR (designated CD19RCD28) in over 90% of the T cells. These SB and aAPC platforms have been adapted by us for human application in four clinical trials (INDs #14193, 14577, 14739, and 15180).

[0190] CAR+ T cells were subsequently electroporated with *in vitro*-transcribed mRNA encoding the obligate heterodimeric variants of the HLA-A ZFNs. ZFN treatment successfully disrupted HLA-A2 expression in ~22% of CAR+ T cells without selection, and this population was readily enriched to ~99% HLA-A2^{neg} cells by negative selection for HLA-positive cells (Fig. 6A). These cells were shown to maintain their new phenotype since after 50 days of continuous co-culture on CD19+ aAPC ~94% of the CAR+ T cells remained HLA-A2^{neg}. Importantly, these HLA-A2^{neg} T cells evaded attack by HLA-A2 restricted CTLs (Fig. 6B), and maintained

their anti-tumor activity as evidenced by CAR-dependent lysis of CD19+ tumor targets (Fig. 6C).

[0191] In aggregate, these data demonstrate that CAR redirected tumor specific T cells can be genetically modified by ZFNs to eliminate HLA-A expression. Such HLA-A^{neg} cells have the potential to enable "off the shelf" tumor-specific T cells that can be pre-prepared from one donor and infused on demand into multiple recipients.

Example 8: Disruption of the HLA-A Gene in hESC

[0192] To broaden the application of allogeneic cells for therapeutic applications, including tissue regeneration, we sought to generate hESC capable of evading T-cell recognition. By definition, all hESC are allogeneic with respect to potential recipients and upon differentiation will upregulate expression of HLAs40. To test the use of ZFNs for the generation of HLA-A^{neg} hESC, we genetically modified the HLA-A2+/A24+ hESC line WIBR3 with either mRNA or DNA plasmids encoding ZFNs targeting HLA-A loci. To facilitate generation of HLA-A^{neg} hESC we co-delivered a donor DNA plasmid encoding the puromycin resistance gene flanked by regions of homology surrounding the ZFN target site to mediate targeted integration by homology-directed repair. Puromycin-resistant clones were screened for modification of the HLA-A alleles by PCR sequencing of the ZFN target region and by Southern Blot analysis of the targeted region using probes located outside of the donor homology arms. There clones were containing mutations in the ZFN target region in both HLA-A alleles, and differentiated into fibroblast-like cells along with the unmodified parental hESC line. HLA expression was induced by treatment with IFN-γ and TNF-α and analyzed by flow cytometry with antibodies recognizing HLA-A2 and HLA-A24, respectively.

[0193] While the parental cell line exhibited strong expression of both HLA alleles, all 3 knockout lines lacked cell surface expression of both HLA-A alleles (Fig. 7). These data demonstrate the portability of the HLA-A knockout approach to hESCs - which may be a necessary step for cell persistence post-transplantation.

[0194] All patents, patent applications and publications mentioned herein are hereby incorporated by reference in their entirety.

[0195] 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 Natural Killer (NK) cell comprising one or more non-classic class I human leukocyte antigen (HLA) proteins and further wherein at least one classic endogenous HLA gene within the cell is inactivated by a zinc finger nuclease.

2. The NK cell of claim 1, wherein the non-classic class I HLA proteins are selected from the group consisting of HLA-E, HLA-F, HLA-G and combinations thereof.

3. The NK cell of claim 1 or claim 2, wherein the non-classical class I HLA proteins are expressed from endogenous genes.

4. The NK cell of any of claims 1 to 3, wherein the non-classic class I HLA proteins are expressed from exogenous sequences.

5. The NK cell of any of claims 1 to 4, wherein the zinc finger nuclease comprises a zinc finger protein comprising the recognition helix regions as shown in a single row of Table 1.

6. The NK cell of any of claims 1 to 5, wherein the cell comprises one or more additional genomic modifications.

7. A cell descended from the cell of claim 5 or claim 6.

8. A fragment of a cell according to any of claims 1 to 7.

9. A pharmaceutical composition comprising the NK cell of any of claims 1 to 8.

10. A method of reducing natural killer (NK) cell lysis of a cell, the method comprising providing a cell according to any of claims 1 to 9, wherein NK mediated cell lysis of the cell is reduced.

11. A method of treating an HLA-related disorder in a subject in need thereof, the method comprising administering a NK cell according to any of claims 1 to 9 to the subject.

12. The method of claim 11, wherein the HLA-related disorder is graft-versus-host disease (GVHD).

Figure 1

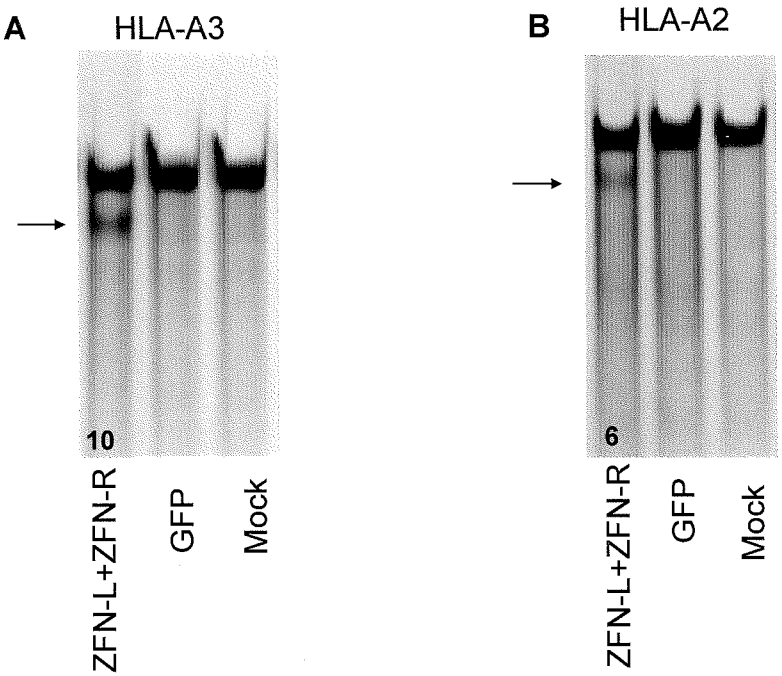


Figure 2A

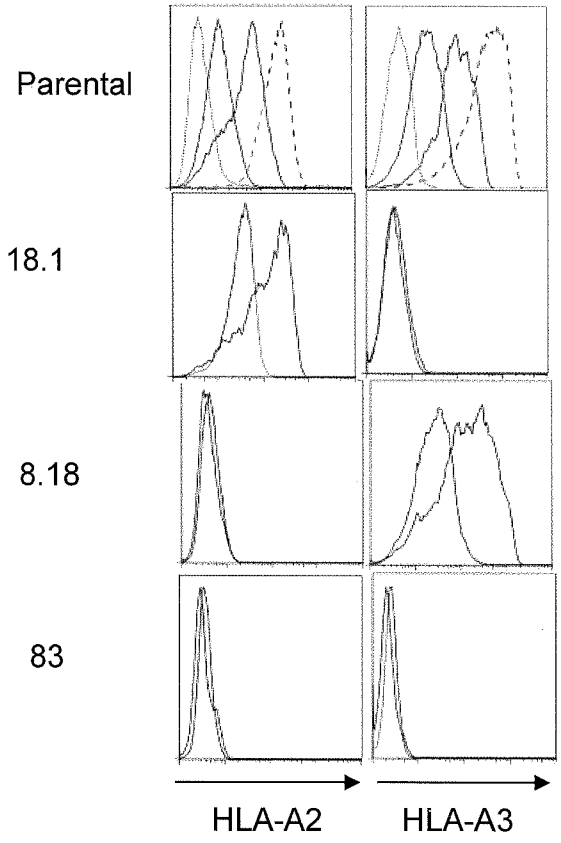
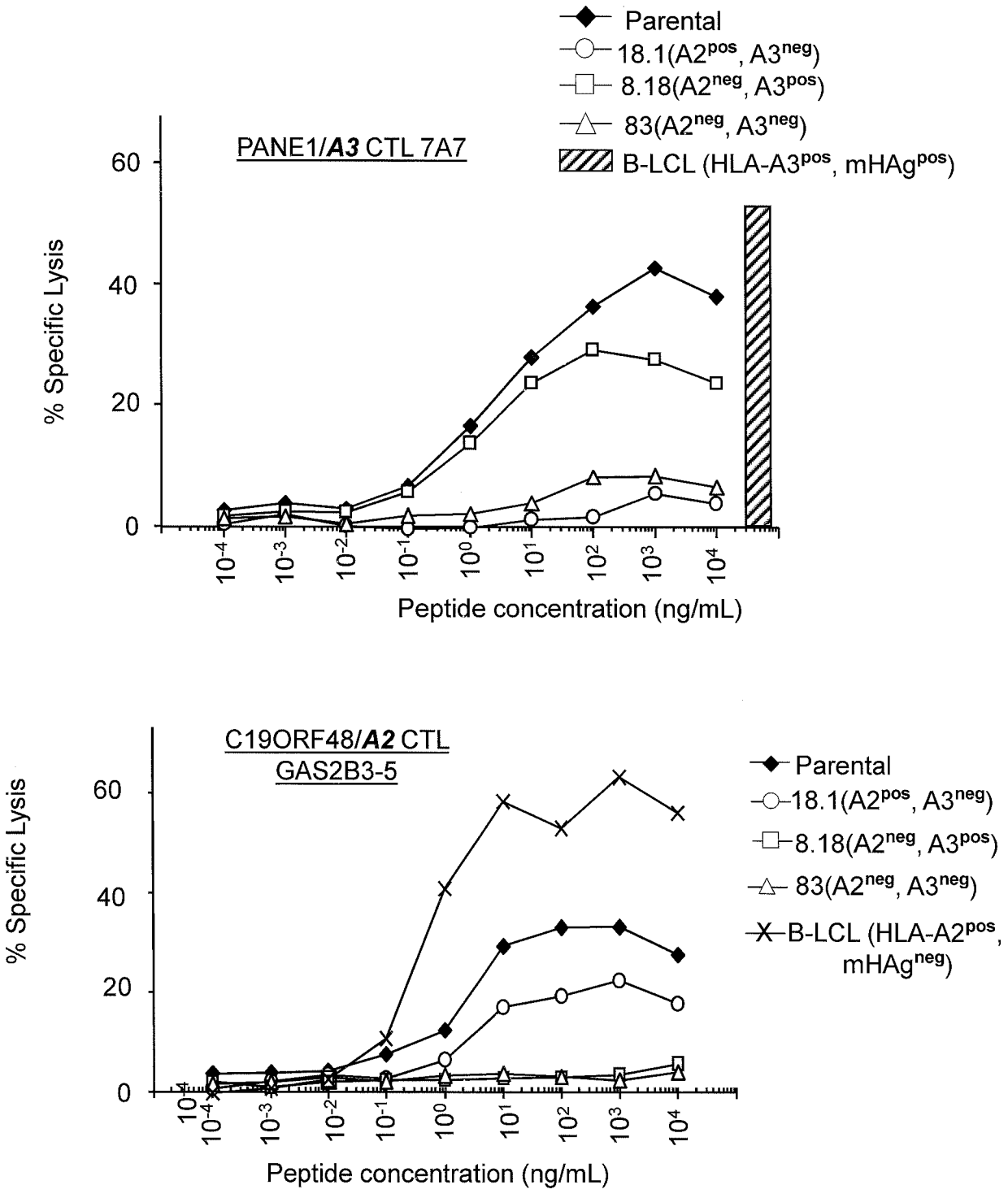


Figure 2B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83(A2neg, A3neg)
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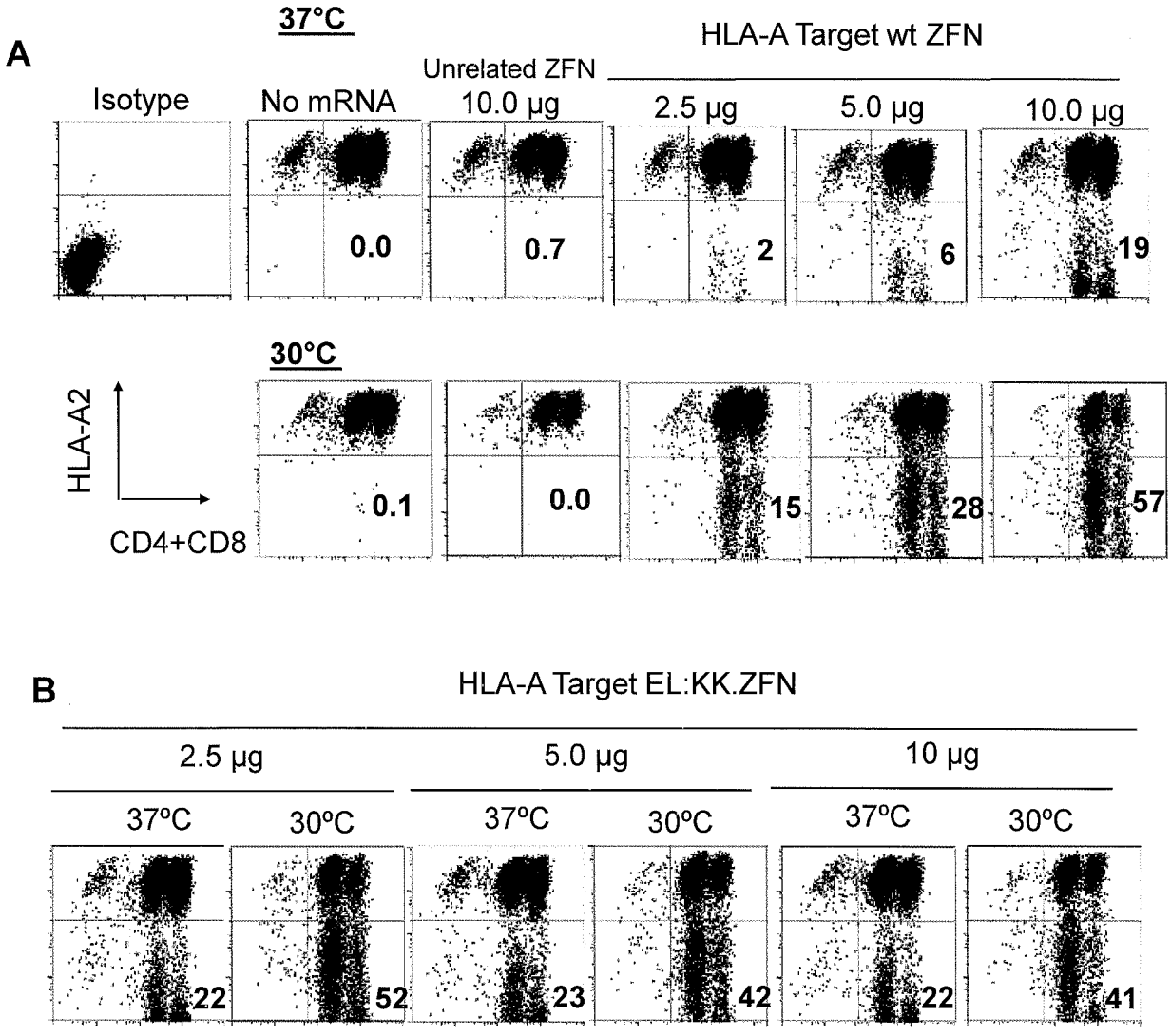
PANE1/A3 CTL 7A7
% Specific Lysis
Peptide concentration (ng/mL)
C19ORF48/A2 CTL
GAS2B3-5
Parental
18.1(A2pos, A3neg)
8.18(A2neg, A3pos)
83(A2neg, A3neg)
B-LCL (HLA-A2pos, mHAgneg)
% Specific Lysis
Peptide concentration (ng/mL)

Figure 3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Unrelated ZFN
Isotype
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2.5 µg
5.0 µg
10.0 µg
0.0
0.7
2
6
19
30°C
HLA-A2
CD4+CD8
0.1
0.0
15
28
57
B
HLA-A Target EL:KK.ZFN
2.5 µg
5.0 µg
10 µg
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30°C
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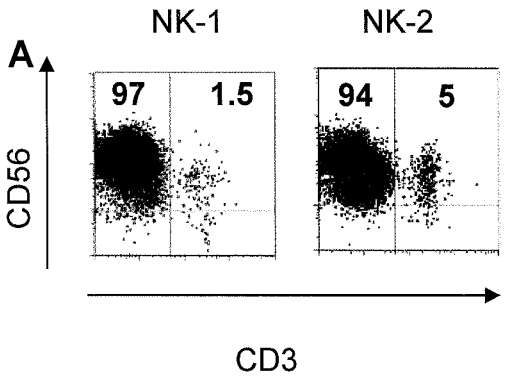
37°C
30°C
22
52
23
42
22
41

Figure 4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NK-2
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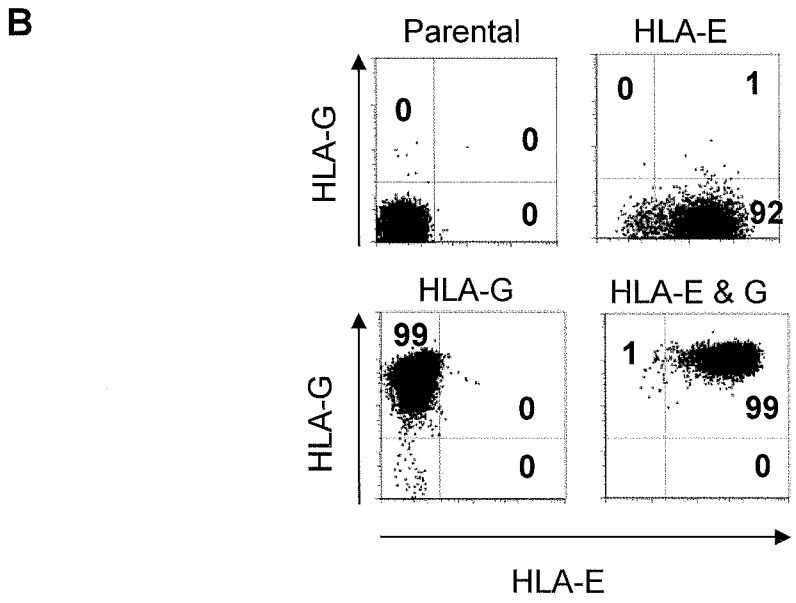
CD56
CD3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Parental
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HLA-G
HLA-E & G
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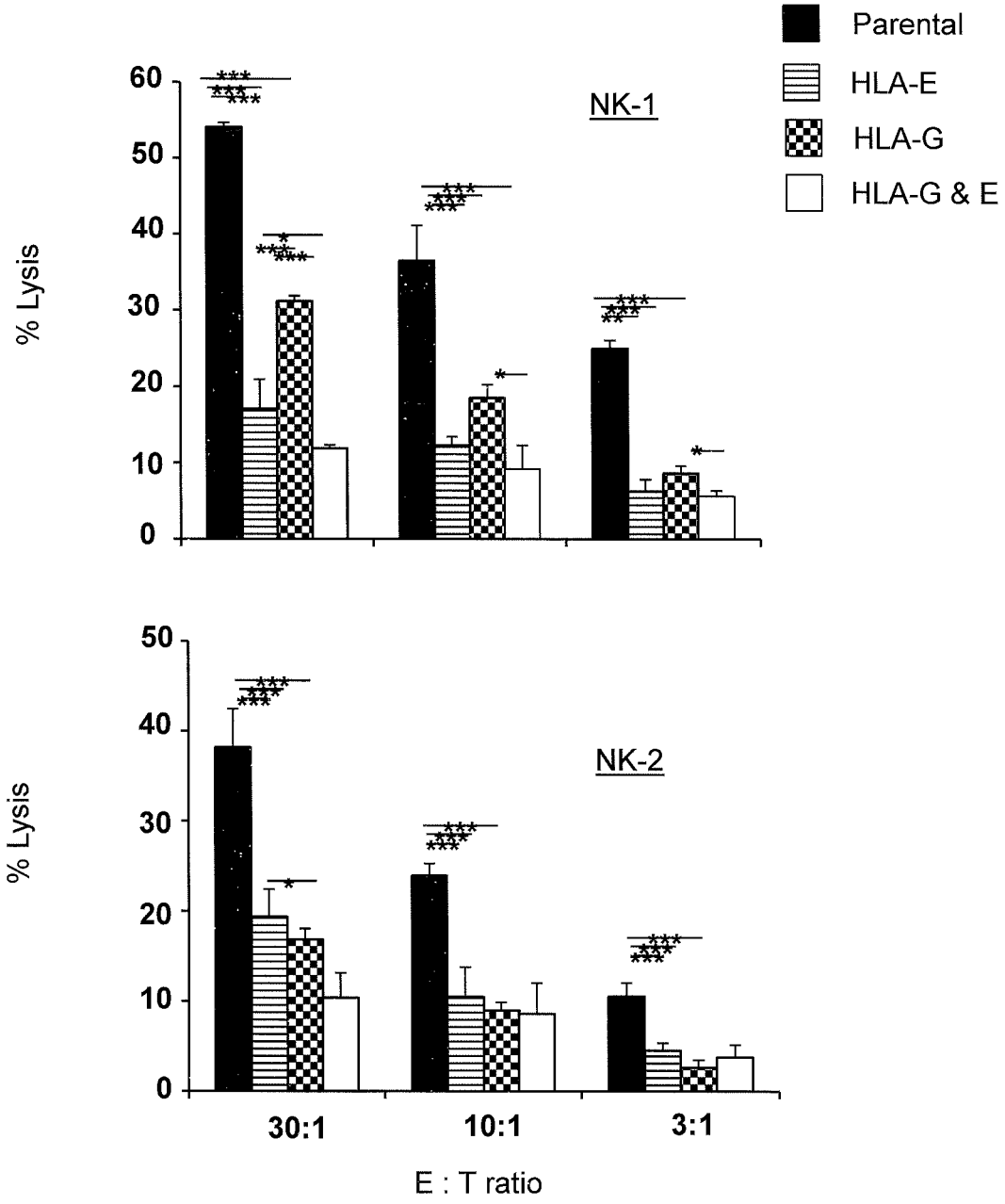
1
99
0
HLA-G
HLA-E

Figure 4C


Parental
HLA-E
HLA-G
HLA-G & E
NK-1
NK-2
% Lysis
% Lysis
60
50
40
30
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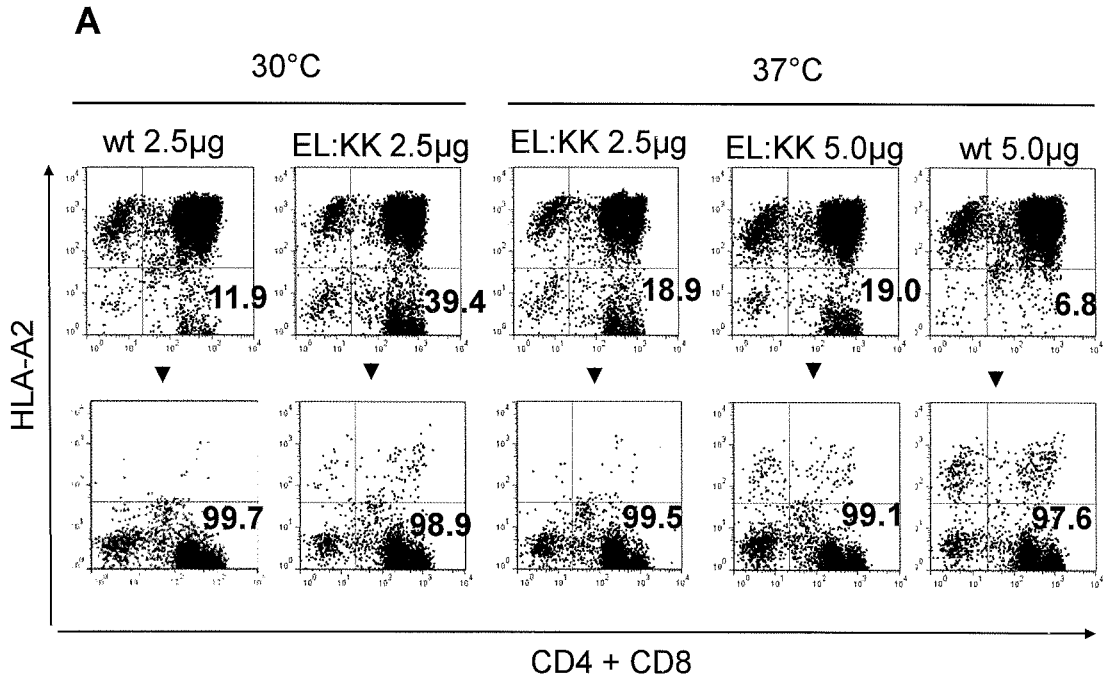
10
0
50
40
30
20
10
0
30:1
10:1
3:1
E : T ratio

Figure 5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37°C
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EL:KK 2.5µg
EL:KK 2.5µg
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wt 5.0µg
11.9
39.4
18.9
19.0
6.8
99.7
98.9
99.5
99.1
97.6
HLA-A2
CD4 + CD8

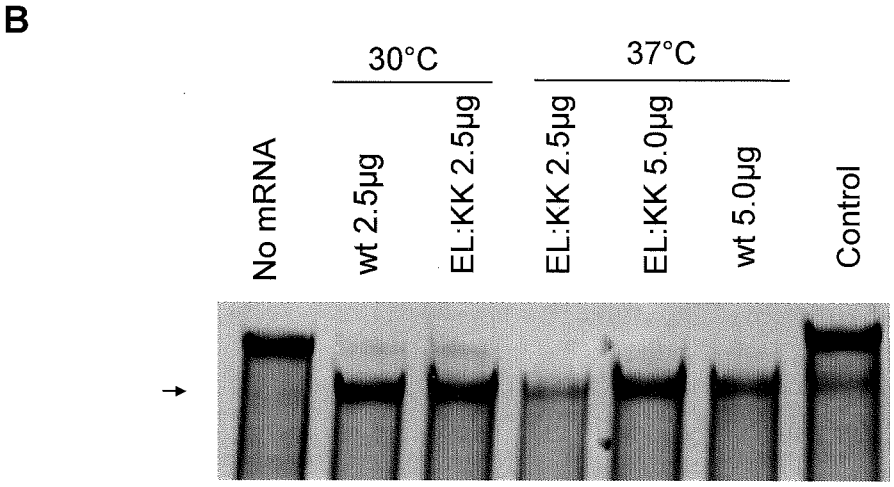

B
30°C
37°C
No mRNA
wt 2.5µg
EL:KK 2.5µg
EL:KK 2.5µg
EL:KK 5.0µg
wt 5.0µg
Control

Figure 5C

HLA-A2

GACCGCGGGGTCCGGGCCAGGTTCTCACACCGTCCAGAGGATGTATGGCTGCGACGTGGGGTCGGACTGGCGC

Sequences from sorted cells

GACCGCGGGGTCCGGGCCAGGTTCTCACACCGTCCAGAG-ATGTATGGCTGCGACGTGGGGTCGGACTGGCGC
GACCGCGGGGTCCGGGCCAGGTTCTCACACCGTCCAGAG-ATGTATGGCTGCGACGTGGGGTCGGACTGGCGC
GACCGCGGGGTCCGGGCCAGGTTCTCACACCGTCCAGAGGATGT--GGCTGCGACGTGGGGTCGGACTGGCGC
GACCGCGGGGTCCGGGCCAGGTTCTCACACCGTCCAGAGGA---ATGGCTGCGACGTGGGGTCGGACTGGCGC
GACCGCGGGGTCCGGGCCAGGTTCTCACACCGTCCAGAG----***C***ATGGCTGCGACGTGGGGTCGGACTGGCGC
GACCGCGGGGTCCGGGCCAGGTTCTCACACCGTCCAGAG----TATGGCTGCGACGTGGGGTCGGACTGGCGC
GACCGCGGGGTCCGGGCCAGGTTCTCACACCGTCCAGAG-----ATGGCTGCGACGTGGGGTCGGACTGGCGC
GACCGCGGGGTCCGGGCCAGGTTCTCACACCGTCCAGAG-----ATGGCTGCGACGTGGGGTCGGACTGGCGC
GACCGCGGGGTCCGGGCCAGGTTCTCACACCGTCCAGAG-----ATGGCTGCGACGTGGGGTCGGACTGGCGC
GACCGCGGGGTCCGGGCCAGGTTCTCACACCGTCCAGAG-----ATGGCTGCGACGTGGGGTCGGACTGGCGC
GACCGCGGGGTCCGGGCCAGGTTCTCACACCGTCCA---------TGGCTGCGACGTGGGGTCGGACTGGCGC
GACCGCGGGGTCCGGGCCAGGTTCTCACACCGT----------ATGGCTGCGACGTGGGGTCGGACTGGCGC
GACCGCGGGGTCCGGGCCAGGTTCTCACACCGTCCAGAG----------ACGTGGGGTCGGACTGGCGC
GACCGCGGGGTCCGGGCCAGGTTCTCACACC----------***A***TGGGGTCGGACTGGCGC
GACCGCGGGGTCCGGGCCAGGTTCTCACAC----------GTGGGGTCGGACTGGCGC
G----------CGACGTGGGGTCGGACTGGCGC
GACCGCGGGGTCCGGGCCAGGTTCTCACACCGTCCAGAGG***G***ATGTATGGCTGCGACGTGGGGTCGGACTGGCG
GACCGCGGGGTCCGGGCCAGGTTCTCACACCGTCCAGAG***GATGT***GATGTATGGCTGCGACGTGGGGTCGGACT

Figure 6

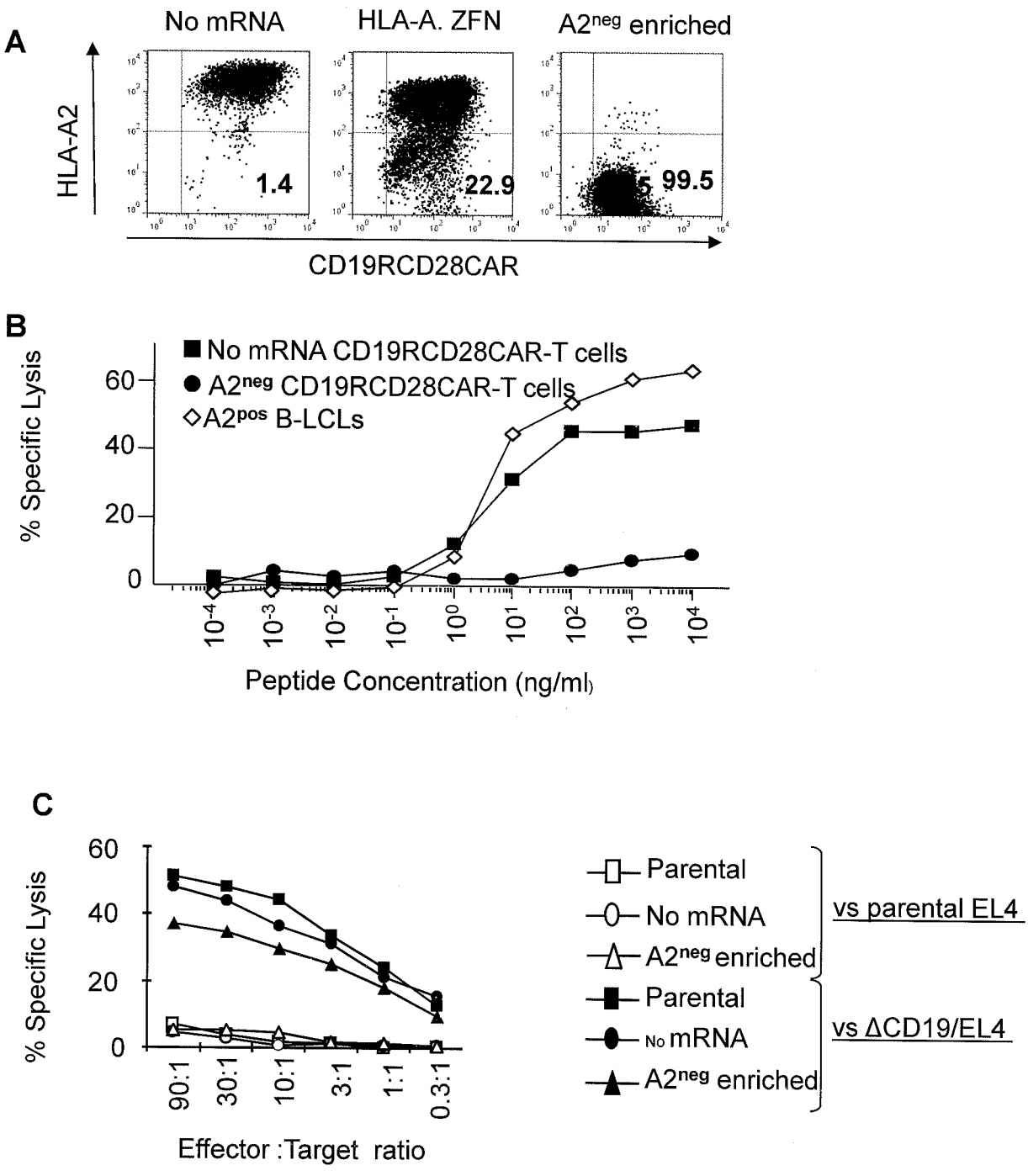
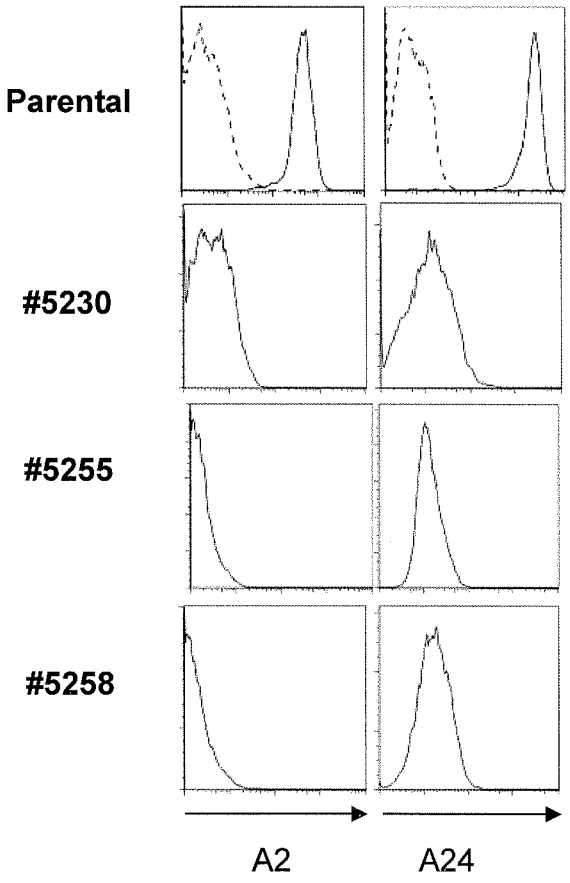

A
No mRNA
HLA-A. ZFN
A2neg enriched
HLA-A2
1.4
22.9
99.5
CD19RCD28CAR
B
No mRNA CD19RCD28CAR-T cells
A2neg CD19RCD28CAR-T cells
A2pos B-LCLs
% Specific Lysis
60
40
20
0
10-4
10-3
10-2
10-1
100
101
102
103
104
Peptide Concentration (ng/ml)
C
% Specific Lysis
60
40
20
0
90:1
30:1
10:1
3:1
1:1
0.3:1
Effector :Target ratio
Parental
No mRNA
A2neg enriched
Parental
No mRNA
A2neg enriched
vs parental EL4
vs ΔCD19/EL4

Figure 7



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US14/24660

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C12N 5/00 , 5/02, 5/0783 (2014.01)
USPC - 435/325, 363, 366
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): C12N 5/00 , 5/02, 5/0783 (2014.01)
USPC: 435/325, 363, 366

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MicroPatent (US-G, US-A, EP-A, EP-B, WO, JP-bib, DE-C,B, DE-A, DE-T, DE-U, GB-A, FR-A); Google Scholar; Pubmed; ScienceDirect; 'HLA,' 'non-classical,' 'HLA-E,' 'HLA-F,' 'HLA-G,' 'zinc finger,' 'NK cell,' 'natural killer'

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	TORIKAI, H et al. HLA And TCR Knockout By Zinc Finger Nucleases: Toward 'Off-The-Shelf' Allogenic T-Cell Therapy For CD19+ Malignancies. 53rd ASH Annual Meeting and Exposition. 10 December 2011; abstract 3766. Retrieved online on July 14, 2014 from <https://ash.confex.com/ash/2010/webprogram/Paper32101.html>.	1, 2, 3/1, 3/2
Y	BUKUR, J et al. The Role Of Classical And Non-Classical HLA Class I Antigens In Human Tumors. Semin Cancer Biol. August 2012; Vol. 22, No. 4; pages 350-358; abstract.	1, 2, 3/1, 3/2
A	US 2010/0291048 A1 (HOLMES, MC et al.) November 18, 2010; abstract; paragraphs [0007]-[0009]	1, 2, 3/1, 3/2

☐ 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
15 July 2014 (15.07.2014)	01 AUG 2014

Name and mailing address of the ISA/US	Authorized officer:
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US14/24660

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.: 4-12
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 105283539 A
(43)申请公布日 2016.01.27

(21)申请号 201480025999.3
(22)申请日 2014.03.12
(30)优先权数据
61/777,627 2013.03.12 US
(85)PCT国际申请进入国家阶段日
2015.11.06
(86)PCT国际申请的申请数据
PCT/US2014/024660 2014.03.12
(87)PCT国际申请的公布数据
WO2014/165177 EN 2014.10.09
(71)申请人 桑格摩生物科学股份有限公司
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申请人 得克萨斯州大学系统董事会
(72)发明人 P·D·格雷戈里 L·J·N·库珀 H·托里开
(74)专利代理机构 上海专利商标事务所有限公司 31100
代理人 杨昀 陶家蓉
(51)Int.Cl.
C12N 5/00(2006.01)
C12N 5/02(2006.01)
C12N 5/0783(2006.01)

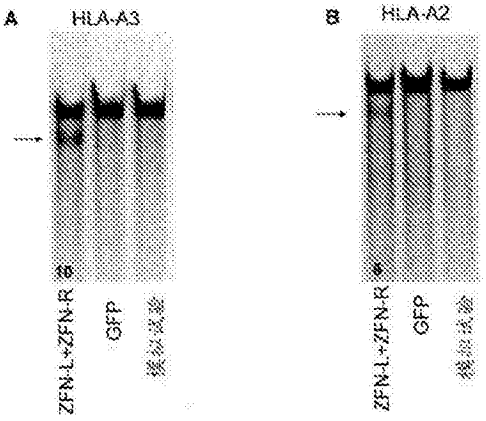
权利要求书1页 说明书33页 附图10页

(54)发明名称

用于HLA的修饰的方法和组合物

(57)摘要

本文中公开了用于调节HLA基因座的表达的方法和组合物，包括缺乏一种或多种经典HLA基因的表达但不被天然杀伤(NK)细胞靶向而被裂解的细胞。



CN 105283539 A

1. 一种分离的天然杀伤 (NK) 细胞，其包含一种或多种非经典 I 类人白细胞抗原 (HLA) 蛋白并且进一步地其中所述细胞内的至少一种经典内源 HLA 基因被锌指核酸酶灭活。

2. 根据权利要求 1 所述的 NK 细胞，其中所述非经典 I 类 HLA 蛋白选自由以下组成的组 :HLA-E、HLA-F、HLA-G 及其组合。

3. 根据权利要求 1 或权利要求 2 所述的 NK 细胞，其中所述非经典 I 类 HLA 蛋白由内源基因表达。

4. 根据权利要求 1-3 中任一项所述的 NK 细胞，其中所述非经典 I 类 HLA 蛋白由外源基因表达。

5. 根据权利要求 1-4 中任一项所述的 NK 细胞，其中所述锌指核酸酶包含含有如表 1 的单行中显示的识别螺旋区的锌指蛋白。

6. 根据权利要求 1-5 中任一项所述的 NK 细胞，其中所述细胞包含一个或多个另外的基因组修饰。

7. 一种细胞，其为权利要求 5 或权利要求 6 的细胞的后代。

8. 根据权利要求 1 至 7 中任一项所述的细胞的碎片。

9. 一种药物组合物，其包含权利要求 1 至 8 中任一项的 NK 细胞。

10. 一种减少细胞的天然杀伤 (NK) 细胞裂解的方法，所述方法包括提供根据权利要求 1 至 9 中任一项的细胞，其中所述细胞的 NK 介导的细胞裂解减少。

11. 一种治疗有需要的受试者的 HLA 相关病症的方法，所述方法包括向所述受试者施用权利要求 1 至 9 中任一项的 NK 细胞。

12. 根据权利要求 11 所述的方法，其中所述 HLA 相关病症是移植物抗宿主病 (GVHD)。

用于 HLA 的修饰的方法和组合物

[0001] 相关申请的交叉引用

[0002] 本申请要求 2013 年 3 月 12 日提交的美国临时申请第 61/777,627 号的权益，其内容据此通过引用整体并入。

[0003] 对在联邦资助的研究下进行的本发明的权利的声明

[0004] 不适用。

技术领域

[0005] 本公开属于基因表达、基因组工程和基因疗法的领域。

[0006] 背景

[0007] MHC 抗原最先被表征为在移植反应中起主要作用的蛋白。排斥通过 T 细胞对植入组织表面上的组织相容性抗原的反应的介导，并且这些抗原的最大的组是主要组织相容性抗原 (MHC)。这些蛋白在所有高等脊椎动物的表面上上表达，并且在小鼠中称为 H-2 抗原（对于组织相容性 -2 抗原而言）和在人细胞中称为 HLA 抗原（对于人白细胞抗原而言）。

[0008] MHC 蛋白在 T 细胞刺激中起着至关重要的作用。抗原呈递细胞（通常树突细胞）在细胞表面上展示在 MHC 上的作为外来蛋白质的降解产物的肽。在共刺激信号存在的情况下，T 细胞被激活，并将作用于也展示相同肽 /MHC 复合物的靶细胞。例如被刺激的 T 辅助细胞将靶向展示与其 MHC 结合的抗原的巨噬细胞，或细胞毒性 T 细胞 (CTL) 将作用于展示外灭病毒肽的病毒感染的细胞。

[0009] MHC 蛋白具有两种类型 I 类和 II 类。I 类 MHC 蛋白是两种蛋白（为由 MHC1I 类基因编码的跨膜蛋白的 α 链和为由不存在于 MHC 基因簇内的基因编码的小的细胞外蛋白的 β 2 微球蛋白链）的异二聚体。所述 α 链折叠成 3 个球状结构域，并且当 β 2 微球蛋白链被结合时，球状蛋白结构复合物与抗体复合物相似。外来肽被呈现在也是最可变的两个最 N 末端结构域上。II 类 MHC 蛋白也是异二聚体，但该异二聚体包含由 MHC 复合物内的基因编码的两个跨膜蛋白。I 类 MHC: 抗原复合物与细胞毒性 T 细胞相互作用，而 II 类 MHC 将抗原呈递至辅助 T 细胞。另外，I 类 MHC 蛋白倾向于在几乎所有有核细胞和血小板（以及在小鼠中红细胞）中表达，而 II 类 MHC 蛋白被更加选择性地表达。通常地，II 类 MHC 蛋白在 B 细胞、某些巨噬细胞和单核细胞、郎格尔汉斯细胞和树突细胞上表达。

[0010] 人中的 I 类 HLA 基因簇包含 3 个大基因座 B、C 和 A 以及几个小基因座。II 类 HLA 簇也包含 3 个大基因座 DP、DQ 和 DR，并且 I 类和 II 类基因簇是多态性的，因为在群体中存在 I 类和 II 类基因的几个不同等位基因。还存在也在 HLA 功能中起作用的几种辅助蛋白。Tap1 和 Tap2 亚单位是在将肽抗原加载至 I 类 HLA 复合物所必需的 TAP 转运蛋白复合物的部分，并且 LMP2 和 LMP7 蛋白体亚单位在将抗原蛋白水解成肽以在 HLA 上展示中起作用。已显示 LMP7 的减少使细胞表面上的 MHC I 类的量减少，可能通过稳定化的不存在来实现（见 Fehling 等 (1999)Science 265:1234-1237)。除了 TAP 和 LMP 以外，还存在甲巯蛋白基因，其表达产物形成 TAP 复合物与 HLA I 类链之间的桥并且增加肽加载。甲巯蛋白的减少导致具有受损的 MHC I 类装配的细胞，减少 MHC I 类的细胞表面表达和削弱免疫反应

(见Grandea等(2000)Immunity 13:213-222和Garbi等(2000)Nat Immunol 1:234-238)。

[0011] I类表达的调控通常是在转录水平上的,并且几种刺激物诸如病毒感染等可引起转录的变化。I类基因在一些特定组织中被下调,并且该下调的来源似乎在启动子和3'基因间序列内(见Cohen等(2009)PLos ONE 4(8):e6748)。还有证据表明微RNA能够调控一些I类MHC基因(见Zhu等,(2010)Am.J.Obstet Gynecol 202(6):592)。

[0012] II类MHC表达的调控取决于MHCII增强体复合物的活性。增强体组分(得到最高度研究的增强体复合物的组分之一是RFX5基因产物(见Villard等(2000)MCB 20(10):3364-3376))几乎被普遍地表达,并且这些组分的表达似乎不控制MHC II类基因的组织特异性表达或它们的IFN-γ诱导的上调。相反地,作为非DNA结合蛋白的称为CIITA的蛋白(II类反式激活因子)似乎用作MCHII表达的主控制因子。与其它增强体成员相反,CIITA确实展示组织特异性表达,被IFN-γ上调,并且已显示其被几种细菌和病毒抑制,所述细菌和病毒可引起MHC II类表达的下调(被认为是试图逃避免疫监督的细菌的部分(见LeibundGut-Landmann等(2004)Eur.J.Immunol 34:1513-1525))。

[0013] I或II类基因的调控可在一些肿瘤存在的情况下被破坏,并且此类破坏可对患者的预后具有后果。例如,在一些实施方案中,发现观察到的Tap 1、Tap 2和HLA I类抗原的减少在转移性黑素瘤中比在原发性肿瘤中更常见($P<0.05$)(见,Kagashita等(1999)Am Jour of Pathol 154(3):745-754)。

[0014] 在人中,对几种疾病的易感性被怀疑与HLA单体型密切相关。这些疾病包括阿狄森氏病、强直性脊柱炎、白塞氏病、伯格氏病、乳糜泻、慢性活动性肝炎、格雷夫斯病、幼年型类风湿关节炎、银屑病、银屑病性关节炎、类风湿性关节炎、斯耶格伦综合征和红斑狼疮等。

[0015] HLA还在移植排斥中起主要作用。移植排斥的急性期可在约1-3周内发生并且通常包括因宿主系统对供体I类和II类HLA分子的敏化而产生的宿主T淋巴细胞对供体组织的作用。在大多数情况下,触发抗原是I类HLA。为了获得最佳的成功,针对HLA对供体进行分型并尽可能完全地将其与患者受者匹配。但即使在家庭成员(其可共享高百分比的HLA同一性)之间捐赠,通常仍然是不成功的。因此,为了保护受者内的移植组织,患者常常必须经受深度的免疫抑制治疗,以防止排斥。这种治疗可导致因患者可能难以克服的机会致病菌感染而引起的并发症和显著的病态。

[0016] 细胞疗法是其中将特定类型的细胞(例如,对肿瘤抗原具有反应性的T细胞或B细胞)施予受者的特化类型的移植。细胞疗法可利用为自体的(来源于受者的)或同种异体的(来源于供体的)细胞来进行,并且所述细胞可以是是未成熟细胞诸如干细胞,或完全成熟的功能性细胞诸如T细胞。事实上,在某些疾病如某些癌症中,可离体操作T细胞来增强它们对某些肿瘤抗原的亲合力,扩增其,随后将其引入患有该癌症类型的患者以试图根除肿瘤。当内源性T细胞应答被肿瘤本身抑制时,这是特别有用的。然而,正如适用于关于排斥的更加众所周知的实体器官移植一样,同样的警告适用于细胞疗法。供体T细胞表达I类HLA抗原,并因此而能够引发来自受者的内源免疫系统的排斥应答。

[0017] 美国专利公布第2012/0060230号描述了经典HLA基因诸如HLA-A、HLA-B、HLA-C的特异性锌指蛋白调节剂。这些调节剂可用于产生不表达一种或多种经典HLA基因的细胞(例如,干细胞),并因此可用于自体移植物。然而,经典HLA表达的丢失可使经遗传修饰的细胞能够成为基于KIR的配体的丢失的天然杀伤(NK)细胞介导的细胞毒性的靶。参见,例

如，Parham 等 (2005)Nat Rev Immunol.5(3):201-214。

[0018] 因此，仍然需要对用于开发缺乏一些或所有经典 HLA 表达但细胞不被 NK 靶向而裂解的细胞的组合物和方法。

[0019] 概述

[0020] 本文中公开了用于修饰 HLA 表达的方法和组合物。具体地，本文中提供了用于调节 HLA 基因的表达以治疗 HLA 相关病症，例如与个体的 HLA 单体型相关的人病症的方法和组合物。另外，本文中提供了用于删除（灭活）或抑制 HLA 基因以产生 HLA 无效的细胞、细胞碎片（例如，血小板）、组织或完整生物，例如不表达一种或多种经典 HLA 基因的细胞的方法和组合物。另外，这些方法和组合物可用于产生仅对于正好一个经典 HLA 基因，或不止一个经典 HLA 基因无效的，或对于所有经典 HLA 基因完全无效的的癌细胞、细胞碎片、组织或生物体。在某些实施方案中，经典 HLA 无效细胞或组织是对于在移植中的使用是有利的人细胞或组织。

[0021] 因此，在一个方面，本文中描述了其中一种或多种经典 HLA 基因被灭活并且其中一种或多种非经典 HLA 蛋白（例如，HLA-E、HLA-F、HLA-G）存在于细胞内的细胞。非经典 I 类 HLA 分子可由内源基因表达（过表达），可被添加至细胞和 / 或可通过细胞铁遗传修饰来表达（例如，表达一种或多种非经典 HLA 分子的多核苷酸的稳定或瞬时转染）。在某些实施方案中，非经典 HLA 分子包含 HLA-E 和 / 或 HLA-G。

[0022] 经修饰的细胞何以是淋巴样细胞（例如，天然杀伤 (NK) 细胞、T 细胞、B 细胞）、髓样细胞（例如，单核细胞、嗜中性粒细胞、树突细胞、巨噬细胞、嗜碱粒细胞、肥大细胞）；干细胞（例如，诱导多能干细胞 (iPSC)、胚胎干细胞（例如，人 ES）、间充质干细胞 (MSC)、造血干细胞 (HSC) 或神经元干细胞）或其碎片（例如，血小板）。干细胞可以全能或多能的（例如，部分分化的，诸如为多能髓细胞样或淋巴样干细胞 HSC)。在一些实施方案中，其中不止一个经典 HLA 基因的表达已被改变的，一种或多种非经经典 HLA 的表达也被改变的经修饰的细胞。在其它实施方案中，本发明提供了用于产生具有一种或多种或全部经典 HLA 基因的无效表型的干细胞的方法。随后可将本文中描述的任何经修饰的干细胞（在 HLA 基因座上经修饰）分化以产生为本文中描述的干细胞后代的分化的（体内或体外）细胞。

[0023] 在其它实施方案中，本文中描述了减少缺乏一种或多种经典 HLA 基因（例如，通过一种或多种基因的核酸酶介导的灭活）的细胞的天然杀伤 (NK) 细胞裂解的方法，所述方法包括提供如本文所述的细胞（例如，其中经典 HLA 基因被灭活并且其中一种或多种非经典 HLA 分子存在的细胞），从而减少 NK 介导的细胞裂解。

[0024] 在另一个方面，本文中所述组合物（经修饰的细胞）和方法可用于例如治疗或预防或改善任何 HLA 相关病症（即，与 HLA 单体型相关的）。所述方法通常包括 (a) 使用核酸酶（例如，ZFN 或 TALEN) 或具有工程化 crRNA/tracr RNA 的核酸酶体系诸如 CRISPR/Cas 裂解分离的细胞（例如，T 细胞或淋巴细胞）中的内源 HLA 基因或 HLA 调控基因，以便 HLA 或 HLA 调控基因被灭活；(b) 将非经典 HLA 分子引入细胞；和 (c) 将所述细胞引入受试者，从而治疗或预防 HLA 相关病症。在某些实施方案中，所述 HLA 相关病症是移植物抗宿主病 (GVHD)。可将核酸酶作为 mRNA、以蛋白质形式和 / 或作为编码核酸酶的 DNA 序列引入。同样地，可将非经典 HLA 分子（例如，HLA-E 和 / 或 HLA-G) 作为 mRNA、以蛋白质形式和 / 或作为编码核酸酶的 DNA 序列引入。在某些实施方案中，被引入受试者的分离的细胞还包含另

外的基因组修饰，例如整合的外源序列（至裂解的 HLA 或 HLA 调控基因或不同的基因，例如安全港基因）和 / 或另外的基因诸如一种或多种 TCR 基因的灭活（例如，核酸酶介导的）。可通过载体（例如 Ad、AAV、LV）或通过使用技术诸如电穿孔引入外源序列。在一些方面，所述组合物可包含分离的细胞碎片和 / 或分化的（部分或完全地）细胞。

[0025] 还提供了包含如本文中所述的经修饰的细胞（例如，具有灭活的经典 HLA 基因并且表达非经典 HLA 基因的干细胞）的药物组合物。在某些实施方案中，所述药物组合物还包含一种或多种药学上可接受的赋形剂。此类药物组合物可被预防性或治疗性使用，并且可包含 iPSC、hES、MSC、HSC 或其组合和 / 或衍生物。在其它实施方案中，提供了细胞、细胞片段（例如，血小板）或来源于此类经修饰的干细胞的组织，以便必要时在 HLA 基因座中修饰此类组织。在一些方面，此类细胞被部分分化（例如造血干细胞）然而在其它方面提供了完全分化的细胞（例如淋巴细胞或巨核细胞），然而在其它方面，提供了分化的细胞的碎片。在其它实施方案中，提供了可含有改变的 HLA 或 HLA 调控基因的它们的分化的后代，并且它们还含有另外的遗传修饰，包括在另一个目标基因座上的供体 DNA 的缺失、改变或插入。

[0026] 在一些实施方案中，如本文中所述的细胞可以是成熟细胞诸如 CD4+T 细胞或 NK 细胞。在一些方面，所述成熟细胞可用于所有细胞疗法，例如用于 T 细胞移植。在其它实施方案中，用于 T 细胞移植的细胞含有另一个目标基因修饰。在一个方面，所述 T 细胞含有插入的对于癌症标志物是特异的嵌合抗原受体 (CAR)。在其它方面，所述插入的 CAR 对于 B 细胞恶性肿瘤的 CD19 标志物特征是特异的。此类细胞可用于用于治疗患者而不必匹配 HLA 的治疗性组合物，并且因此能够用作用于有此需要的任何患者的“下架”治疗剂。在一些方面，提供了其中编码 T 细胞受体 (TCR) 基因（例如，TCRα 和 / 或 TCRβ 链）的基因已被操作或其中编码具有所需的特异性和亲和力的 TCR 链的基因已被引入的细胞。在其它实施方案中，HLA 经修饰的血小板被提供来治疗性用于治疗病症诸如血小板减少症或其它出血障碍。

[0027] 可在体外、体内和 / 或活体外实践本文中所述的任何方法。在某些实施方案中，活体外实践所述方法例如以修饰干细胞、T 细胞或 NK 细胞，随后用于治疗有此需要的受试者。

[0028] 总体上根据公开内容，这些和其它方面将对技术人员显而易见。

[0029] 附图简述

[0030] 图 1，图框 A 和 B，显示通过 Surveyor™核酸酶测定评估的 HLA-A3（图 1A）和 HLA-A2（图 1B）的基因破坏的水平。较低（快速移动）的条带（箭头）是指示 ZFN 介导的基因修饰的消化产物。条带底部的数字表示基于光密度测定法的的经修饰的 HLA-A 等位基因的百分比。来自模拟转染的细胞和利用 GFP 表达载体转染的细胞的 DNA 用于阴性对照。

[0031] 图 2，图框 A 和 B，显示 HLA-A^{neg} HEK293 的分离。图 2A 显示 HLA-A2 和 HLA-A3 蛋白表达的丢失。亲代 HEK293 细胞和 3 个具有 HLA-A 丢失的衍生的经遗传修饰的克隆（编号为 18.1、8.18、83）上的 HLA-A2 和 HLA-A3 表达的流式细胞术分析。点线代表同种型 (HLA-A2) 或 SA-PE(HLA-A3) 对照，实线代表无 IFN-γ 和 TNF-α 的 HLA-A 表达，并且填充线代表在用 600IU/mL 的 IFN-γ 和 10ng/mL 的 TNF-α 培养 48 小时后 HLA-A 的表达。亲代栏中的虚线代表 EBV-LCL 上的 HLA-A2 或 HLA-A3 表达。图 2B 显示 HLA 经修饰的克隆对 CTL 介导的裂解的抗性。亲代 HEK293 和衍生的 HLA-A^{neg}克隆与 IFN-γ 和 TNF-α 一起培养 48 小时，随后用从 PANE1（可选地着丝粒蛋白 M 同种型 c）衍生的并且被 CTL 克隆 7A7 识别的同源 HLA-A3

肽 RVWDLPGVLK(SEQ ID NO:1,也见 NP_001103685.1) 或从 C19ORF48/A2 衍生的并且被 CTL 克隆 GAS2B3-5 识别的 HLA-A2 肽 CIPPDSLLFPA(SEQ ID NO:2,也是 NM_199250.1 的可选择的开放阅读框架) 脉冲,随后在 4 小时的 ^{51}Cr 释放测定中以 20:1 的效应子对靶的比率评价其被 CTL 克隆的识别。将表达 PANE1mHAg(未加载肽的) 的 HLA-A2^{+}LCL(有阴影线的条块) 用作阳性对照。

[0032] 图 3,图框 A 和 B,显示在用 ZFN 进行的遗传编辑后在原代 OKT3 繁殖的 T 细胞上的 HLA-A 表达的丢失。图 3A(顶图框) 显示在编码靶向 HLA-A2 的 ZFN-L 和 ZFN-R 的 mRNA 种类 (分别为 SBS#18889 和 SBS#18881,见美国专利公布第 20120060230 号) 电转移后 HLA-A2 的细胞表面表达的丢失。在分级剂量的编码 ZFN-L 和 ZFN-R 的 mRNA 种类的电转移后 4 天分析 HLA-A2、CD4 和 CD8 的共表达。对碘化丙啶阴性的活细胞群体的流式细胞术数据进行门控。右下象限中的数目表示为 HLA-A^{neg}的 CD4 和 CD8+T 的百分比。图 3A(底图框) 显示增加的“冷激”对 HLA-A 表达的破坏。在分级剂量的编码 ZFN-L 和 ZFN-R 的 mRNA 种类的电转移后 4 天收集数据。从 ZFN 的电转移后第 1 至 3 天在 30℃培养细胞,随后返回 37℃并再培养一天,随后进行分析。图 3B 显示提高的融合于异二聚体 Fok I 结构域变体的 ZFN-L 和 ZFN-R 对 HLA-A 的破坏效率。将编码 ZFN-L 和 ZFN-R 异二聚体 Fok I 突变体靶向 HLA-A 的 EL:KK 的 mRNA 种类电转移至原代 T 细胞中。在将细胞于 37℃培养 4 天,或在 30℃培养 3 天随后再在 37℃培养 1 天后分析 HLA-A2 表达。X 轴代表 CD4 和 CD8 表达,y- 轴代表 HLA-A2 表达。

[0033] 图 4,图框 A 至 C,显示非经典 HLA 分子的表达保护 NK 介导的细胞免于裂解。图 4A 显示从来自健康供体 (每一个供体被称为 NK-1 和 NK-2) 的两个单独 PBMC 分离的 NK 细胞的免疫表型。针对 PI^{neg}群体门控显示的流式细胞术数据。数字代表每一个上面的象限的非分比。图 4B 显示表达 HLA-E 和 / 或 HLA-G 的 HLA I 类 ^{low}721.221 细胞的遗传修饰。SB 转座子 / 转座酶用于在 721.221 细胞的 3 个克隆中同源表达 HLA-E 和 / 或 HLA-G。每一个数字代表 HLA-G、HLA-E 或 HLA-G 和 HLA-E 两者的百分比表达,如通过流式细胞术检测的。图 4C 显示由靶向 721.221 的 NK 细胞产生的特异性裂解。NK 细胞杀伤亲代 (HLA I 类 ^{low})、HLA-E^{+}、HLA-G^{+}和 HLA-E $^{+}$HLA-G^{+}721.221 细胞的相对能力。每一栏代表平均值 ± 标准差 (SD)*。01<P<0.05,**P<.01 ;和 ***P<.001。

[0034] 图 5,图框 A 至 C,显示在利用 ZFN 的遗传编辑后 HLA-A^{neg}原代 T 细胞的富集。图 5A 显示 HLA-A2^{neg} T- 细胞群体的产生。HLA-A2^{neg} T 细胞通过基于磁珠的选择来富集。显示编码 ZFN 的 mRNA 的输入剂量和 mRNA 的电转移后的 3 天培养条件 (37℃相对 30℃)。数字代表 CD4 和 CD8 阳性群体内的 HLA-A2 阴性群体。图 5B 显示 HLA-A2^{neg} T 细胞的 Surveyor™ 核酸酶测定。针对 HLA-A2 表达的丢失富集的 T 细胞的分析显示通过快速移动条带 (箭头) 的出现表示的 HLA-A2 基因座的破坏。图 5C 显示 HLA^{neg} T 细胞的测序的结果 (SEQ ID NO:39 至 53)。将使用 HLA-A2- 特异性引物从富集的细胞 (2.5μg ZFN,EL:KK Fok I 结构域,30℃处理) 产生的 PCR 产物克隆进 TOPO 载体 (Invitrogen),并且对质粒产物进行测序。野生型序列列于顶上,预期的 ZFN 结合位点加以下划线。下面显示的是获自 ZFN 处理的并且被富集的细胞的序列。缺失通过连字符来指示,序列变化以粗体突显。所有 18 个序列变化均导致经预测阻止蛋白质翻译的框内移码。

[0035] 图 6,图框 A 至 C,显示利用 ZFN 遗传编辑的原代 CD19- 特异性 CAR T 细胞上的 HLA-A

表达的丢失。图6A显示编码ZFN的mRNA的电转移对CAR_+T细胞中的HLA-A2的破坏。将来自HLA-A2+供体的T细胞电穿孔并繁殖以表达CD19-特异性CAR(CD19RCD28)。用2.5μg的每一种编码HLA-A-特异性ZFN的异二聚化Fok I结构域变体(ZFN-L-EL和ZFN-R-KK)的mRNA对这些T细胞进行重新电穿孔。在于30℃培养3天,随后在37℃培养1天后分析HLA-A2表达。通过顺磁性选择进行$HLA\text{-}A2^{neg}$群体的富集。图6B显示$HLA\text{-}A^{neg}$ CAR_+T逃避由HLA-A2限制的CTL产生的裂解。在于CRA中用作靶之前,用同源肽的系列稀释物脉冲指定的CAR_+T细胞的混合物。以20:1的效应子对靶的比率添加对于C190RF48/A2是特异的CTL克隆GAS2B3-5。图6C显示经ZFN修饰的HLA^{neg} CAR_+T细胞维持所需的抗原特异性细胞毒性。使用经遗传修饰以表达人CD19的截短的变体的小鼠T细胞系EL4证明表达CD19RCD28CAR的$HLA\text{-}A^{neg}$ T细胞对CD19的重定向特异性。EL4上引入的人CD19的表达是100%。

[0036] 图7显示人ESC上的HLA-A表达的ZFN介导的消除。利用ZFN和编码抗生素抗性的供体质粒修饰$HLAA2_+HLA\text{-}24_+hES$亲代细胞系WIBR3。选择具有HLA-A表达的丢失的克隆(5230、5255、5258),将其分化成成纤维细胞。在用600IU/mL的IFN-γ和10ng/mL的TNF-α培养48小时后通过流式细胞术评估衍生的成纤维细胞上的HLA-A2和HLA-A24的表达。亲代图中的虚线代表同种型对照。

[0037] 详述

[0038] 本文中公开了用于产生其中一种或多种经典HLA基因被灭活但表达一种或多种非经典HLA基因的细胞的组合物和方法。以该方式靶向修饰的细胞可用作治疗剂,例如,移植物,因为非经典HLA基因的存在减少或消除了HLA无效细胞的NK介导的裂解。另外,可将其它目标基因插入其中HLA基因被操作的细胞中。

[0039] 因此,本文所述方法和组合物提供了治疗HLA相关病症的方法,并且这些方法和组合物可包含能够调节靶基因的锌指转录因子以及工程化锌指核酸酶。

[0040] 概述

[0041] 除非另外指出,所述方法的实践以及本文公开的组合物的制备和使用采用分子生物学、生物化学、染色质结构和分析、计算化学、细胞培养、重组DNA和属于本领域技术范围内的相关领域的常规技术。在文献中全面解释了这些技术。见,例如,Sambrook等,MOLECULAR CLONING:A LABORATORY MANUAL,第2版,Cold Spring Harbor Laboratory Press,1989和第3版,2001;Ausubel等,CURRENT PROTOCOLS IN MOLECULAR BIOLOGY,John Wiley&Sons,New York,1987和定期更新;METHODS IN ENZYMOLOGY系列,Academic Press,San Diego;Wolffe,CHROMATIN STRUCTURE AND FUNCTION,第3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;和METHODS IN MOLECULAR BIOLOGY,第119卷,"Chromatin Protocols"(P.B.Becker编辑)Humana Press,Totowa,1999。

[0042] 定义

[0043] 术语"核酸"、"多核苷酸"和"寡核苷酸"可交换使用并且指呈线性或环状构象并且呈单链或双链形式的脱氧核糖核苷酸或核糖核苷酸聚合物。为了本公开的目的,这些术语不得视为关于聚合物长度的限制。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(硫代磷酸骨架)中经修饰的核苷酸。一般而言,特定核苷酸的类似物具

有相同碱基配对特异性；即，A 的类似物将与 T 碱基配对。

[0044] 术语“多肽”、“肽”和“蛋白质”可交换使用并且指氨基酸残基的聚合物。术语还适用于其中一种或多种氨基酸为相应天然存在的氨基酸的化学类似物或经修饰衍生物的氨基酸聚合物。

[0045] “结合”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。并非结合相互作用的所有组成部分均需要有序列特异性（例如，与 DNA 骨架中的磷酸残基接触），只要总体上相互作用有序列特异性。此类相互作用通常特征在于解离常数（K_d）为 $10^{-6}M^{-1}$ 或更低。“亲和力”指结合强度：结合亲和力增加与较低的 K_d 相关联。

[0046] “结合蛋白”是能够与另一分子非共价结合的蛋白质。例如，结合蛋白可与 DNA 分子（DNA 结合蛋白）、RNA 分子（RNA 结合蛋白）和 / 或蛋白质分子（蛋白质结合蛋白）结合。在为蛋白质结合蛋白的情况下，其可与自身结合（形成同型二聚体、同型三聚体等）和 / 或其可与不同蛋白质的一个或多个分子结合。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 结合和蛋白质结合活性。

[0047] “锌指 DNA 结合蛋白”（或结合结构域）是以序列特异性方式，通过一个或多个锌指结合 DNA 的蛋白质或较大蛋白质中的结构域，锌指是其结构通过锌离子配位稳定的结合结构域中的氨基酸序列区域。术语锌指 DNA 结合蛋白常常缩写为锌指蛋白或 ZFP。

[0048] “TALE DNA 结合结构域”或“TALE”是包含一个或多个 TALE 重复结构域 / 单元的多肽。重复结构域牵涉于 TALE 与其同源靶 DNA 序列的结合中。单个“重复单元”（也称为“重复”）通常长度为 33-35 个氨基酸并且与天然存在的 TALE 蛋白中的其它 TALE 重复序列至少表现出一定的序列同源性。参见，例如，美国专利号 8,586,526，通过引用整体并入本文。

[0049] 例如，可通过工程化天然存在的锌指蛋白的识别螺旋区（改变一种或多种氨基酸）或通过工程化参与 DNA 结合的氨基酸（重复可变的二残基或 RVD 区）将锌指和 TALE DNA- 结合结构域“工程化”为与预定核苷酸序列结合。因此，工程化锌指蛋白或 TALE 蛋白是非天然存在的蛋白质。工程化锌指蛋白和 TALE 的方法的非限制性实例为设计和选择。设计的蛋白是在自然界中不存在的蛋白质，其设计 / 组成主要由合理标准产生。设计的合理标准包括应用取代规则和计算机算法处理保存现有 ZFP 或 TALE 设计和结合数据信息的数据库中的信息。见，例如，美国专利第 8,586,526、6,140,081、6,453,242 和 6,534,261 号；还见 WO 98/53058、WO 98/53059、WO 98/53060、WO 02/016536 和 WO 03/016496。

[0050] “选定的”锌指蛋白或 TALE 是在自然界中未发现的蛋白质，其生成主要由经验过程引起，例如噬菌体展示、相互作用陷阱或杂交选择。见例如 US 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 和 WO 02/099084。

[0051] “重组”指两个多核苷酸之间交换遗传信息的过程。为了本公开的目的，“同源重组（HR）”指例如在细胞中通过同源介导修复机制修复双链断裂期间发生的此类交换的特化形式。这个过程需要核苷酸序列同源性，将“供体”分子用于“靶”分子（即，经历双链断裂的分子）的模板修复，并且因为其导致遗传信息从供体转移到靶标，所以不同地称为“非交换型基因转化（non-crossover gene conversion）”或“短序列基因转化（short tract gene conversion）”。不希望受任何特定理论约束，此类转移可涉及在断裂靶标和供体之间形成

的异源双链体DNA的错配校正和/或其中供体用于重新合成将成为靶标的一部分的遗传信息的“合成依赖式链退火”和/或相关过程。此类特化HR常常导致靶分子的序列改变，以致供体多核苷酸的部分或全部序列并入靶多核苷酸中。

[0052] 在本公开的方法中，如本文所述的一种或多种靶向核酸酶在靶序列（例如，细胞染色质）的预定位点产生双链断裂，并且可将与断裂区内的核苷酸序列具有同源性的“供体”多核苷酸引入细胞中。已经证实双链断裂的存在利于供体序列的整合。供体序列可物理整合，或可选地，将供体多核苷酸用作通过同源重组修复断裂的模板，导致与供体中一样，所有或部分核苷酸序列引入细胞染色质中。因此，细胞染色质中的第一序列可改变并且，在某些实施方案中，可转化为供体多核苷酸中存在的序列。因此，术语“置换(replace)”或“置换(replacement)”的使用可理解为表示一个核苷酸序列经另一个置换，(即，在信息意义上置换序列)，并不一定需要一个多核苷酸经另一个物理或化学置换。

[0053] 在本文所述的任何方法中，附加对的锌指蛋白可用于细胞中附加靶位点的附加双链裂解。

[0054] 在靶向重组和/或置换和/或改变细胞染色质中目标区域内的序列的方法的某些实施方案中，通过与外源“供体”核苷酸序列的同源重组改变染色体序列。如果存在与断裂区域同源的序列，则细胞染色质中双链断裂的存在刺激此类同源重组。

[0055] 在本文所述任何方法中，第一核苷酸序列(“供体序列”)可含有与目标区域内的基因组序列同源，但是不相同的序列，从而刺激同源重组以将不同序列插入目标区域。因此，在某些实施方案中，与目标区域内的序列同源的供体序列的部分表现出与被置换基因组序列约80-99％（或之间的任何整数）序列同一性。在其它实施方案中，例如如果在100个以上连续碱基对的供体和基因组序列之间仅1个核苷酸不同，则供体和基因组序列之间的同源性高于99％。在某些情况下，供体序列的非同源部分可含有在目标区域内不存在的序列，以致将新序列引入目标区域。在这些情况下，非同源序列两侧通常为与目标区域内的序列同源或相同的50-1,000个碱基对（或之间的任何整数值）或大于1,000的任何数量的碱基对的序列。在其它实施方案中，供体序列与第一序列非同源，并且通过非同源重组机制插入基因组中。

[0056] 本文所述任何方法均可用于通过靶向整合破坏目标基因表达的供体序列，使细胞中的一个或多个靶序列部分或完全失活。还提供了具有部分或完全失活基因的细胞系。

[0057] 此外，如本文所述靶向整合的方法也可用于整合一个或多个外源序列。例如，外源性核酸序列可包含一个或多个基因或cDNA分子或任何类型的编码或非编码序列以及一个或多个控制元件（例如，启动子）。另外，外源性核酸序列可产生一种或多种RNA分子（例如，小发夹RNA(shRNA)、抑制RNA(RNAi)、微RNA(miRNA)等）。

[0058] “裂解”指DNA分子的共价骨架的断裂。可通过多种方法，包括但不限于酶或化学水解磷酸二酯键引发裂解。单链裂解和双链裂解均有可能，并且双链裂解可由于两个不同单链裂解事件而发生。DNA裂解可导致产生平末端或交错末端。在某些实施方案中，融合多肽用于靶向双链DNA裂解。

[0059] “裂解半结构域”是连同第二多肽（相同或不同）一起形成具有裂解活性（优选为双链裂解活性）的复合物的多肽序列。术语“第一和第二裂解半结构域”、“+和-裂解半结构域”和“右侧和左侧裂解半结构域”可交换用于指二聚化的成对裂解半结构域。

[0060] “工程化裂解半结构域”是已经修饰以便与另一裂解半结构域（例如，另一工程化裂解半结构域）一起形成专性杂二聚体的裂解半结构域。同样见通过引用整体并入本文的美国专利公布第7,888,121、7,914,796、8,034,598、8,623,618号和美国专利公布2011/0201055号。

[0061] 术语“序列”指可为DNA或RNA，可为直链、环状或支链并且可为单链或双链的任何长度的核苷酸序列。术语“供体序列”指插入基因组中的核苷酸序列。供体序列可具有任何长度，例如长度介于2和10,000个核苷酸之间（或之间或以上的任何整数值），优选长度介于约100和1,000个核苷酸之间（或之间的任何整数值），更优选长度介于约200和500个核苷酸之间。

[0062] “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（主要为DNA）和蛋白质，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呈核小体形式存在，其中核小体核包含与包含组蛋白H2A、H2B、H3和H4各两个的八聚物缔合的DNA的约150个碱基对；并且接头DNA（根据生物体而言长度可变）在核小体核之间延伸。组蛋白H1的分子通常与接头DNA缔合。为了本公开的目的，术语“染色质”意在涵盖所有类型的原核和真核细胞核蛋白。细胞染色质包括染色体和附加体染色质。

[0063] “染色体”是包含细胞的全部或部分基因组的染色质复合物。细胞基因组常常特征在于其核型，这是包含细胞基因组的所有染色体的集合。细胞基因组可包含一条或多条染色体。

[0064] “附加体”为复制核酸、核蛋白复合物或包含并非细胞染色体核型的一部分的核酸的其它结构。附加体的实例包括质粒和某些病毒基因组。

[0065] “靶位点”或“靶序列”是限定结合分子将与之结合的核酸部分的核酸序列，条件是存在供结合的足够条件。例如，序列5’GAATTC 3’是Eco RI重组内切核酸酶的靶位点。

[0066] “外源”分子是细胞中通常不存在，但是可通过一种或多种遗传、生物化学或其它方法引入细胞中的分子。相对于细胞的特定发育阶段和环境条件测定“细胞中正常存在”。因此，例如，仅在肌肉胚胎发育期存在的分子相对于成人肌肉细胞为外源分子。类似地，由热休克诱导的分子相对于非热休克细胞为外源分子。例如，外源分子可包含功能形式的功能障碍内源分子或功能障碍形式的正常功能内源分子。

[0067] 其中，外源分子可为小分子，例如通过组合化学过程生成的小分子，或大分子例如蛋白质、核酸、碳水化合物、脂质、糖蛋白、脂蛋白、多糖、以上分子的任何经修饰衍生物或包含以上一种或多种分子的任何复合物。核酸包括DNA和RNA，可为单链或双链；可为直链、支链或环状；并且可具有任何长度。核酸包括能够形成双链体的核酸以及形成三链体的核酸。见，例如，美国专利第5,176,996和5,422,251号。蛋白质包括但不限于DNA结合蛋白、转录因子、染色质重构因子、甲基化DNA结合蛋白、聚合酶、甲基化酶、脱甲基酶、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、促旋酶和解螺旋酶。

[0068] 外源分子可为与内源分子相同类型的分子，例如外源蛋白质或核酸。例如，外源性核酸可包括感染病毒基因组、引入细胞的质粒或附加体或细胞中通常不存在的染色体。将外源分子引入细胞的方法为本领域的技术人员已知并且包括但不限于脂质介导的转移（即，脂质体，包括中性和阳离子性脂质）、电穿孔、直接注入、细胞融合、粒子轰击、磷酸钙共沉淀法、DEAE-葡聚糖介导的转移和病毒载体介导的转移。外源分子也可为与内源分子

相同类型的分子，但是源自与所述细胞来源不同的物种。例如，人核酸序列可引入最初源自小鼠或仓鼠的细胞系中。

[0069] 相反，“内源”分子是特定细胞中，在特定环境条件下的特定发育阶段通常存在的分子。例如，内源性核酸可包括染色体、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。另外的内源分子可包括蛋白质，例如转录因子和酶。

[0070] “融合”分子是其中两个或更多个亚基分子优选共价连接的分子。亚基分子可为相同化学类型的分子，或可为不同化学类型的分子。第一种类型的融合分子的实例包括但不限于融合蛋白（例如，ZFP 或 TALE DNA 结合结构域和一个或多个激活结构域之间的融合）和融合核酸（例如，编码如上所述融合蛋白的核酸）。第二种类型的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和小沟结合物与核酸之间的融合。

[0071] 细胞中融合蛋白的表达可由向细胞递送融合蛋白引起或通过向细胞递送编码融合蛋白的多核苷酸引起，其中转录多核苷酸并且翻译转录物以生成融合蛋白。在细胞中蛋白质的表达中还可牵涉反式剪接、多肽裂解和多肽连接。在本公开其它地方提出了向细胞递送多核苷酸和多肽的方法。

[0072] 为了本公开的目的，“基因”包括编码基因产物的 DNA 区域（见下文）以及调控基因产物的生成的所有 DNA 区域，不论此类调控序列是否与编码和 / 或转录序列相邻。相应地，基因包括但不一定限于启动子序列、终止子、翻译调控序列（例如核糖体结合位点和内部核糖体进入位点）、增强子、沉默子、绝缘子、边界元件、复制起点、基质附着位点和基因座控制区。

[0073] “基因表达”指将基因内所含信息转化为基因产物。基因产物可为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）或通过翻译 mRNA 生成的蛋白质。基因产物还包括通过例如加帽、聚腺苷酸化、甲基化和编辑的过程修饰的 RNA 和（例如）通过甲基化、乙酰化、磷酸化、泛素化、ADP- 核糖基化、豆蔻酰化和糖基化修饰的蛋白质。

[0074] 基因表达的“调节”指基因活性的变化。表达的调节可包括但不限于基因激活和基因阻遏。基因组编辑（例如，裂解、改变、失活、随机突变）可用于调节表达。基因失活指与不包括如本文所述的 ZFP 的细胞相比，基因表达的任何减少。因此，基因失活可为部分或全部。

[0075] “目标区域”为细胞染色质的任何区域，例如需要结合外源分子的基因或基因内或相邻的非编码序列。结合可能是为了靶向 DNA 裂解和 / 或靶向重组的目的。例如，目标区域可存在于染色体、附加体、细胞器基因组（例如，线粒体、叶绿体）或感染病毒基因组。目标区域可在基因的编码区内、经转录的非编码区例如前导序列、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。目标区域长度可小到单个核苷酸对或多达 2,000 个核苷酸对或任何整数值的核苷酸对。

[0076] “真核”细胞包括但不限于真菌细胞（例如酵母）、植物细胞、动物细胞、哺乳动物细胞和人细胞（例如，T 细胞）。

[0077] 术语“操作性连接”和“操作性地连接”（或“可操作地连接”）对于两个或更多个组成部分（例如序列元件）的并置可交换使用，其中组成部分排列成以致两个组成部分正常作用并且允许至少一个组成部分可介导对其它组成部分中的至少一个发挥的作用的可

能性。举例而言，如果转录调控序列控制编码序列响应于一种或多种转录调控因子的存在或缺乏而转录的水平，则转录调控序列例如启动子与编码序列操作性地连接。转录调控序列通常与编码序列顺式操作性地连接，但不需要与之直接相邻。例如，增强子是与编码序列操作性地连接的转录调控序列，即使它们不连续。

[0078] 对于融合多肽，术语“操作性地连接”可指每个组成部分在与其它组成部分的连接中，执行与如果不这样连接时相同的功能。例如，对于其中 DNA 结合结构域（例如，ZFP、TALE）与激活结构域融合的融合多肽，如果在融合多肽中，DNA 结合结构域部分能够结合其靶位点和 / 或其结合位点，而激活结构域能够上调基因表达，则 DNA 结合结构域和激活结构域处于操作性连接。对于 DNA 结合结构域与裂解结构域融合的融合多肽，如果在融合多肽中，DNA 结合结构域部分能够结合其靶位点和 / 或其结合位点，而裂解结构域能够在靶位点附近裂解 DNA，则 DNA 结合结构域和裂解结构域处于操作性连接。类似地，对于 DNA 结合结构域与激活或阻遏结构域融合的融合多肽，如果在融合多肽中，DNA 结合结构域部分能够结合其靶位点和 / 或其结合位点，而激活结构域能够上调基因表达或阻遏结构域能够下调基因表达，则 DNA 结合结构域和激活或阻遏结构域处于操作性连接。

[0079] 蛋白质、多肽或核酸的“功能片段”是其序列与全长蛋白质、多肽或核酸不同，但是保持了与全长蛋白质、多肽或核酸相同的功能的蛋白质、多肽或核酸。功能片段可具有比相应天然分子更多、更少或相同数量的残基，和 / 或可含有一种或多种氨基酸或核苷酸取代。测定核酸功能（例如，编码功能、与另一核酸杂交的能力）的方法在本领域中众所周知。类似地，测定蛋白质功能的方法众所周知。例如，可通过过滤结合、电泳迁移或免疫沉淀测定法测定多肽的 DNA 结合功能。可通过凝胶电泳测定 DNA 裂解。见 Ausubel 等，同上。例如，可通过遗传和生化免疫共沉淀、双杂交测定法或互补作用测定蛋白质与另一种蛋白质相互作用的能力。见，例如，Fields 等 (1989)Nature 340:245-246 ；美国专利第 5,585,245 号和 PCT WO 98/44350。

[0080] “载体”能够将基因序列转移到靶细胞中。通常，“载体构建体”、“表达载体”和“基因转移载体”意指能够指导目标基因的表达并且可将基因序列转移到靶细胞中的任何核酸构建体。因此，术语包括克隆和表达媒介物以及整合载体。

[0081] “报告基因”或“报告序列”指生成优选不一定在常规测定中易于测量的蛋白质产物的任何序列。适合的报告基因包括但不限于编码介导抗生素抗性（例如，氨苄青霉素 (ampicillin) 抗性、新霉素 (neomycin) 抗性、G418 抗性、嘌呤霉素 (puromycin) 抗性）的蛋白质的序列、编码有色或荧光或发光蛋白质（例如，绿色荧光蛋白、增强型绿色荧光蛋白、红色荧光蛋白、荧光素酶）和介导增强细胞生长和 / 或基因扩增的蛋白质（例如，二氢叶酸还原酶）的序列。表位标签包括（例如）一个或多个拷贝的 FLAG、His、myc、Tap、HA 或任何可检测的氨基酸序列。“表达标签”包括编码可能与所需基因序列可操作地连接以便监测目标基因的表达的报告基因的序列。

[0082] DNA 结合结构域

[0083] 本文描述了包含与包含 HLA 基因或 HLA 调节剂的任何基因中的靶位点特异性结合的 DNA 结合结构域的组合物。任何 DNA 结合结构域均可用于本文公开的组合物和方法中。

[0084] 在某些实施方案中，DNA 结合结构域包含锌指蛋白。优选地，锌指蛋白为非天然存在，因为其经工程化为与所选靶位点结合。见，例如，Beerli 等 (2002)Nature

Biotechnol.20:135-141;Pabo 等 (2001)Ann.Rev.Biochem.70:313-340;Isalan 等 (2001) Nature Biotechnol.19:656-660;Segal 等 (2001)Curr.Opin.Biotechnol.12:632-637; Choo 等 (2000)Curr.Opin.Struct.Biol.10:411-416;美国专利第 6,453,242、6,534,261、6,599,692、6,503,717、6,689,558、7,030,215、6,794,136、7,067,317、7,262,054、7,070,934、7,361,635、7,253,273 号;和美国专利公布第 2005/0064474、2007/0218528、2005/0267061 号,全部通过引用整体并入本文。在某些实施方案中,DNA 结合结构域包含美国专利公布第 2012/0060230 号(通过引用整体并入本文)中公开的锌指蛋白(例如,表 1)。

[0085] 与天然存在的锌指蛋白相比,工程化锌指结合结构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的设计和各种类型的选择。合理的设计包括(例如)使用包含三联体(或四联体)核苷酸序列和单独锌指氨基酸序列的数据库,其中每个三联体或四联体核苷酸序列与锌指的结合特定三联体或四联体序列的一个或多个氨基酸序列缔合。见,例如,美国专利 6,453,242 和 6,534,261,其通过引用整体并入本文。

[0086] 在美国专利 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 中公开了示例性选择方法,包括噬菌体展示和双杂交体系。另外,例如在美国专利号 6,794,136 中已描述了锌指结合结构域结合特异性的增强。

[0087] 另外,如这些和其它参考文献中所公开那样,锌指结构域和/或多指锌指蛋白可使用任何适合的接头序列,包括例如长度为 5 个或更多个氨基酸的接头连接在一起。对于长度为 6 个或更多个氨基酸的示例性接头,同样见美国专利第 6,479,626、6,903,185 和 7,153,949 号。本文所述蛋白质可包括在蛋白质的单独锌指之间适合接头的任何组合。另外,例如在美国专利号 6,794,136 中描述了对于锌指结合结构域的结合特异性的增强。

[0088] 靶位点的选择、ZFP 和设计和构建融合蛋白(和编码融合蛋白的多核苷酸)的方法为本领域的技术人员已知并且在美国专利第 6,140,0815、789,538、6,453,242、6,534,261、5,925,523、6,007,988、6,013,453、6,200,759 号、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 中有详细描述。

[0089] 另外,如这些和其它参考文献中所公开那样,锌指结构域和/或多指锌指蛋白可使用任何适合接头序列,包括例如长度为 5 个或更多个氨基酸的接头连接在一起。对于长度为 6 个或更多个氨基酸的示例性接头,同样见美国专利第 6,479,626、6,903,185 和 7,153,949 号。本文所述蛋白质可包括在蛋白质的单独锌指之间适合接头的任何组合。

[0090] 在某些实施方案中,DNA 结合结构域为结合(以序列特异性方式)结合 HLA 基因或 HLA 调控基因中的靶位点并且调节 HLA 的表达的工程化锌指蛋白。ZFP 可选择性结合特定的目标单体型。关于美国群体中鉴定的 HLA 单体型以及根据人种的其频率的论述,见 Maiers 等 (2007)Human Immunology 68:779-788(通过引用并入本文)。

[0091] 另外,提供了结合功能性 HLA 调控基因(包括但不限于 Tap1、Tap2、Tapascin、CTFIIA, 和 RFX5) 的 ZFP。HLA 靶位点通常包括至少一个锌指但可包括多个锌指(例如,2、3、4、5、6 或更多个指)。通常地,ZFP 包括至少 3 个指。某些 ZFP 包括 4、5 或 6 指。包括 3 指的 ZFP 通常识别包括 9 或 10 个核苷酸的靶位点;包括 4 指的 ZFP 通常识别包括 12 至 14

个核苷酸的靶位点；而具有 6 指的 ZFP 可识别包括 18 至 21 个核苷酸的靶位点。ZFP 还可以是融合蛋白，其包括一个或多个调控结构域，所述结构域可以是转录激活或阻遏结构域。

[0092] ZFP 的特定实例公开于美国专利公布第 20120060230 号中的表 1 中。

[0093] 在一些实施方案中，DNA 结合结构域可源自核酸酶。例如，已知归巢内切核酸酶和大范围核酸酶的识别序列，例如 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。还见美国专利第 5,420,032 号、美国专利第 6,833,252 号；Belfort 等 (1997)Nucleic Acids Res. 25:3379 - 3388；Dujon 等 (1989)Gene 82:115 - 118；Perler 等 (1994)Nucleic Acids Res. 22,1125 - 1127；Jasin(1996)Trends Genet. 12:224 - 228；Gimble 等 (1996)J. Mol. Biol. 263:163 - 180；Argast 等 (1998)J. Mol. Biol. 280:345 - 353 和 New England Biolabs 目录。另外，归巢内切核酸酶和大范围核酸酶的 DNA 结合特异性可经工程化为结合非天然靶位点。见，例如，Chevalier 等 (2002)Molec. Cell 10:895-905；Epinat 等 (2003)Nucleic Acids Res. 31:2952-2962；Ashworth 等 (2006)Nature 441:656-659；Paques 等 (2007) Current Gene Therapy 7:49-66；美国专利公布第 20070117128 号。

[0094] 在其它实施方案中，DNA 结合结构域包含来自与来源于植物病原菌黄单孢菌属 (Xanthomonas)（见 Boch 等，(2009)Science 326:1509-1512 以及 Moscou 和 Bogdanove,(2009)Science326:1501) 和罗尔斯通氏菌属 (Ralstonia)（见 Heuer 等 (2007)Applied and Environmental Microbiology 73(13):4379-4384)；美国专利申请第 20110301073 和 20110145940 号）的那些效应子相似的 TAL 效应子的工程化结构域。已知黄单孢菌属的植物病原菌在重要作物中引起许多疾病。黄单孢菌属的病原性取决于向植物细胞注入 25 种以上不同效应子蛋白的保守型 III 分泌 (T3S) 系统。其中注入的蛋白质为模拟植物转录激活因子并且操纵植物转录组的转录激活因子样效应子 (TALE)（见 Kay 等 (2007)Science 318:648-651)。这些蛋白质含有 DNA 结合结构域和转录激活结构域。表征最清楚的 TALE 之一为来自野油菜黄单胞菌疱病致病变种 (Xanthomonas campestgris pv. Vesicatoria) 的 AvrBs3（见 Bonas 等 (1989)Mol Gen Genet 218:127-136 和 WO2010079430)。TALE 含有串联重复的集中式结构域，每个重复含有对这些蛋白质的 DNA 结合特异性关键的大约 34 个氨基酸。另外，其含有核定位序列和酸性转录激活结构域（对于综述，见 Schornack S 等 (2006)J Plant Physiol 163(3):256-272)。另外，在植物病原细菌青枯病菌 (Ralstonia solanacearum) 中，已经发现在青枯病菌 (R. solanacearum) 生物变种 1 菌株 GMI1000 和生物变种 4 菌株 RS1000 中称为 brg11 和 hpx17 的两个基因与黄单孢菌属的 AvrBs3 家族同源（见 Heuer 等 (2007)Appl and Envir Micro 73(13):4379-4384)。这些基因在核苷酸序列上 98.9％彼此相同，但是不同之处在于 hpx17 的重复结构域中缺失 1,575bp。然而，两种基因产物与黄单孢菌属的 AvrBs3 家族蛋白具有低于 40％的序列同一性。

[0095] 另外，如这些和其它参考文献中公开的，可使用任何合适的接头序列，包括例如在长度上具有 5 或更多个氨基酸的的接头来将锌指结构域和 / 或多指化锌指蛋白或 TALE 连接在一起。关于例如长度为 6 或更多个氨基酸的示例性接头，也见，美国专利第 6,479,626、6,903,185 和 7,153,949 号。本文所述蛋白质可包括蛋白质的单独锌指之间的合适的接头的任意组合。另外，对于锌指结合结构域的结合特异性的增强已描述于例如美国专利第

6,794,136 号中。

[0096] 融合蛋白

[0097] 还提供了包含如本文所述的 DNA 结合蛋白（例如，ZFP 或 TALE）和异源调控（功能）结构域（或其功能片段）的融合蛋白。常见结构域包括（例如）转录因子结构域（激活因子、阻遏因子、辅助激活因子、辅助阻遏因子）、沉默子、致癌基因（例如，myc、jun、fos、myb、max、mad、rel、ets、bcl、myb、mos 家族成员等）；DNA 修复酶及其相关因子和修饰因子；DNA 重排酶及其相关因子和修饰因子；染色质相关蛋白及其修饰因子（例如，激酶、乙酰基转移酶和脱乙酰基酶）；和 DNA 修饰酶（例如，甲基转移酶、拓扑异构酶、解螺旋酶、连接酶、激酶、磷酸酶、聚合酶、内切核酸酶）及其相关因子和修饰因子。关于 DNA 结合结构域和核酸酶裂解结构域融合的详情，见美国专利申请公布第 20050064474、20060188987 和 2007/0218528 号，其通过引用整体并入本文。

[0098] 用于实现激活的适合结构域包括 HSV VP16 激活结构域（见，例如 Hagmann 等，J.Virol.71,5952-5962(1997)）核激素受体（见，例如 Torchia 等，Curr.Opin.Cell.Biol.10:373-383(1998)）；核因子 κB 的 p65 亚基（Bitko 和 Barik，J.Virol.72:5610-5618(1998) 及 Doyle 和 Hunt，Neuroreport 8:2937-2942(1997)）；Liu 等，Cancer Gene Ther.5:3-28(1998)）或人工嵌合功能结构域例如 VP64(Beerli 等，(1998)Proc.Natl.Acad.Sci.USA 95:14623-33) 和降解决定子 (Molinari 等，(1999)EMBO J.18,6439-6447)。另外的示例性激活结构域包括 Oct 1、Oct-2A、Sp1、AP-2 和 CTF1(Seipel 等，EMBO J.11,4961-4968(1992) 以及 p300、CBP、PCAF、SRC1PvALF、AtHD2A 和 ERF-2。见，例如，Robyr 等 (2000)Mol.Endocrinol.14:329-347；Collingwood 等 (1999)J.Mol.Endocrinol.23:255-275；Leo 等 (2000)Gene 245:1-11；Manteuffel-Cymborowska(1999) Acta Biochim.Pol.46:77-89；McKenna 等 (1999)J.Steroid Biochem.Mol.Biol.69:3-12；Malik 等 (2000)Trends Biochem.Sci.25:277-283；和 Lemon 等 (1999)Curr.Opin.Genet.Dev.9:499-504。另外的示例性激活结构域包括但不限于 OsGAI、HALF-1、C1、AP1、ARF-5、-6、-7、和 -8、CPRF1、CPRF4、MYC-RP/GP 和 TRAB1。见，例如，Ogawa 等 (2000)Gene 245:21-29；Okanami 等 (1996)Genes Cells 1:87-99；Goff 等 (1991)Genes Dev.5:298-309；Cho 等 (1999)Plant Mol.Biol.40:419-429；Ulmason 等 (1999)Proc.Natl.Acad.Sci.USA 96:5844-5849；Sprenger-Haussels 等 (2000)Plant J.22:1-8；Gong 等 (1999)Plant Mol.Biol.41:33-44；和 Hobo 等 (1999)Proc.Natl.Acad.Sci.USA 96:15,348-15,353。

[0099] 对于本领域的技术人员而言，明确的是在 DNA 结合结构域和功能结构域之间的融合蛋白（或编码融合蛋白的核酸）的形成中，激活结构域或与激活结构域相互作用的分子适合作为功能结构域。基本上能够为靶基因募集激活复合物和 / 或激活活性（例如，组蛋白乙酰化）的任何分子均可用作融合蛋白的激活结构域。例如在美国专利申请 2002/0115215 和 2003/0082552 及 WO 02/44376 中描述了适合用作融合分子中的功能结构域的绝缘子结构域、定位结构域和染色质重构蛋白例如含 ISWI 的结构域和 / 或甲基结合结构域蛋白。

[0100] 示例性阻遏结构域包括但不限于 KRAB A/B、KOX、TGF-β-诱导型早期基因 (TIEG)、v-erbA、SID、MBD2、MBD3、DNMT 家族成员（例如，DNMT1、DNMT3A、DNMT3B）、Rb 和 MeCP2。见，例如，Bird 等 (1999)Cell 99:451-454；Tyler 等 (1999)Cell 99:443-446；

Knoepfler等(1999)Cell 99:447-450;和Robertson等(2000)Nature Genet.25:338-342。另外的示例性阻遏结构域包括但不限于ROM2和AtHD2A。见,例如,Chem等(1996)Plant Cell 8:305-321;和Wu等(2000)Plant J.22:19-27。

[0101] 通过本领域技术人员众所周知的克隆和生物化学偶联方法构建融合分子。融合分子包含DNA结合结构域和功能结构域(例如,转录激活或阻遏结构域)。融合分子还任选包含核定位信号(例如,来自SV40基质T抗原)和表位标签(例如,FLAG和血细胞凝集素)。设计融合蛋白(和编码融合蛋白的核酸),以便将翻译阅读框保存在融合组成部分中。

[0102] 通过本领域技术人员众所周知的生物化学偶联方法构建一只手上功能结构域(或其功能片段)的多肽组成部分与另一只手上非蛋白DNA结合结构域(例如,抗生素、嵌入剂、小沟结合剂、核酸)之间的融合。见,例如,Pierce Chemical Company(Rockford,IL)目录。已经描述了用于在小沟结合剂与多肽之间产生融合的方法和组合物。Mapp等(2000)Proc.Natl.Acad.Sci.USA 97:3930-3935。

[0103] 在某些实施方案中,由锌指蛋白结合的靶位点存在于细胞染色质的可达区域。例如,可如美国专利第7,217,509和7,923,542号中所述测定可达区域。如果靶位点不存在于细胞染色质的可达区域,可如美国专利第7,785,792和8,071,370号中所述生成一个或多个可达区域。在另外的实施方案中,融合分子的DNA结合结构域能够与细胞染色质结合,不管其靶位点是否在可达区域内。例如,此类DNA结合结构域能够与接头DNA和/或核小体DNA结合。在某些类固醇受体和肝细胞核因子3(HNF3)中发现这类"先锋"DNA结合结构域的实例。Cordingley等(1987)Cell 48:261-270;Pina等(1990)Cell 60:719-731;和Cirillo等(1998)EMBO J.17:244-254。

[0104] 如本领域的技术人员所知,融合分子可与药学上可接受的载体配制。见,例如,Remington's Pharmaceutical Sciences,第17版,1985;和美国专利第6,453,242和6,534,261号。

[0105] 融合分子的功能组成部分/结构域可选自一旦融合分子经由其DNA结合结构域与靶序列结合,就能够影响基因转录的多种不同组成部分。因此,功能组成部分可包括但不限于各种转录因子结构域,例如激活因子、阻遏因子、辅助激活因子、辅助阻遏因子和沉默子。

[0106] 例如,在美国专利第6,534,261和6,933,113号中公开了另外的示例性功能结构域。

[0107] 也可选择受外源小分子或配体调控的功能结构域。例如,可采用RheoSwitch®技术,其中功能结构域只有在外部RheoChem™配体的存在下呈现其活性构象(见例如US 20090136465)。因此,ZFP可与可调控的功能结构域可操作地连接,其中所产生的ZFP-TF活性受外部配体控制。

[0108] 核酸酶

[0109] 在某些实施方案中,融合蛋白包含DNA结合结构域和裂解(核酸酶)结构域。同样,可使用核酸酶,例如工程化核酸酶实现基因修饰。工程化核酸酶技术基于天然存在的DNA结合蛋白的工程化。例如,已经描述了具有定制DNA结合特异性的归巢内勤核酸酶的工程化。(Chames等(2005)Nucleic Acids Res 33(20):e178;Arnould等(2006)J.Mol.Biol.355:443-458)。另外,还已经描述了ZFP的工程化。见,例如,美国专利第6,534,261、6,607,882、6,824,978、6,979,539、6,933,113、7,163,824和7,013,219号。

[0110] 另外，ZFP 和 / 或 TALE 已经与核酸酶结构域融合以产生能够通过其经工程化的 (ZFP 或 TALE)DNA 结合结构域识别其预期核酸靶标并经由核酸酶活性引起在 DNA 结合位点附近裂解 DNA 的 ZFN 和 TALEN- 功能实体。见，例如，Kim 等 (1996)Proc Nat'l Acad Sci USA 93(3):1156-1160。最近，此类核酸酶已经用于多种生物中的基因组修饰。见，例如，美国专利公布 20030232410、20050208489、20050026157、20050064474、20060188987、20060063231 和国际公布 WO 07/014275。

[0111] 因此，本文所述的方法和组合物广泛适用并且可能牵涉任何目标核酸酶。核酸酶的非限制性实例包括大范围核酸酶、TALEN 和锌指核酸酶。核酸酶可能包含异源 DNA 结合和裂解结构域 (例如，锌指核酸酶 ；大范围核酸酶 DNA 结合结构域与异源裂解结构域)，或可选地，天然存在的核酸酶的 DNA 结合结构域可改变为与所选靶位点结合 (例如，已经工程化为与不同于同源结合位点的位点结合的大范围核酸酶)。

[0112] 在本文所述的任何核酸酶中，所述核酸酶可包含工程化 TALE DNA 结合结构域和核酸酶结构域 (例如，内切核酸酶和 / 或大范围核酸酶结构域)，也称为 TALEN。用于工程化这些 TALEN 蛋白以与用户选择的靶序列发生强劲的位点特异性相互作用的组合物已被公布 (见，美国专利第 8,586,526 号)。在一些实施方案中，所述 TALEN 包含内切核酸酶 (例如，FokI) 裂解结构域或裂解半结构域。在其它实施方案中，所述 TALE 核酸酶是大范围 TAL。这些大范围 TAL 核酸酶是包含 TALE DNA 结构值勤玫大范围核酸酶裂解结构域的融合蛋白。大范围核酸酶裂解结构域作为单体是有活性的并且不需要二聚化俨产生活性。(见 Boissel 等，(2013)Nucl Acid Res:1-13,doi:10.1093/nar/gkt1224)。另外，核酸酶结构域还可展示 DNA 结合功能性。

[0113] 在另外的实施方案中，所述核酸酶包含紧凑型 TALEN(cTALEN)。这些核酸酶是将 TALE DNA 结合域连接于 TevI 核酸酶结构域的单链融合蛋白。所述融合蛋白可用作通过 TALE 区域定位的切口酶，或可产生双链断裂，这取决于其中 TALE DNA 结合结构域相对于 TevI 核酸酶结构域的定位 (见，Beurdeley 等 (2013)Nat Comm:1-8DOI:10.1038/ncomms2782)。可将任何 TALEN 与另外的 TALEN(例如，一种或多种具有一个或多个 mega-TAL 的 TALEN(cTALEN 或 FokI-TALEN)) 或其它 DNA 裂解酶组合使用。

[0114] 在某些实施方案中，核酸酶包含大范围核酸酶 (归巢内切核酸酶) 或展示裂解活性的其部分。天然存在的大范围核酸酶识别 15-40 个碱基对裂解位点并且常分为 4 个家族 ：LAGLIDADG 家族、GIY-YIG 家族、His-Cyst 盒家族和 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。其识别序列已知。同样见美国专利第 5,420,032 号 ；美国专利第 6,833,252 号 ；Belfort 等 (1997)Nucleic Acids Res.25:3379 - 3388 ；Dujon 等 (1989)Gene 82:115 - 118 ；Perler 等 (1994)Nucleic Acids Res.22,1125 - 1127 ；Jasin(1996)Trends Genet.12:224 - 228 ；Gimble 等 (1996)J.Mol.Biol.263:163 - 180 ；Argast 等 (1998)J.Mol.Biol.280:345 - 353 和 New England Biolabs 目录。

[0115] 来自主要来自 LAGLIDADG 家族的天然存在的大范围核酸酶的 DNA 结合结构域已经用于促进植物、酵母、果蝇属 (Drosophila)、哺乳动物细胞和小鼠中的位点特异性基因组修饰，但是这种方法已限于保存大范围核酸酶识别序列的同源基因的修饰 (Monet 等 (1999),Biochem.Biophysics.Res.Common.255:88-93) 或已经向其中引入了识别序列的预

工程化基因组 (Route 等 (1994), Mol. Cell. Biol. 14:8096-106 ;Chilton 等 (2003) Plant Physiology. 133:956-65 ;Puchta 等 (1996), Proc. Natl. Acad. Sci. USA 93:5055-60 ; Rong 等 (2002), Genes Dev. 16:1568-81 ;Gouble 等 (2006), J. Gene Med. 8(5):616-622)。相应地,已经尝试在医学或生物技术相关位点工程化大范围核酸酶以表现出新型结合特异性 (Porteus 等 (2005), Nat. Biotechnol. 23:967-73 ;Sussman 等 (2004), J. Mol. Biol. 342:31-41 ;Epinat 等 (2003), Nucleic Acids Res. 31:2952-62 ;Chevalier 等 (2002) Molec. Cell 10:895-905 ;Epinat 等 (2003) Nucleic Acids Res. 31:2952-2962 ;Ashworth 等 (2006) Nature 441:656-659 ;Paques 等 (2007) Current Gene Therapy 7:49-66 ;美国专利公布第 20070117128、20060206949、20060153826、20060078552 和 20040002092 号)。另外,来自大范围核酸酶的非天然存在或经工程化的 DNA 结合结构域也已经与来自异源核酸酶 (例如, FokI) 的裂解结构域可操作地连接和 / 或来自大范围核酸酶的裂解结构域可与异源 DNA 结合结构域 (例如, ZFP 或 TALE) 可操作地连接。

[0116] 在其它实施方案中,核酸酶为锌指核酸酶 (ZFN) 或 TALE DNA 结合结构域 - 核酸酶融合物 (TALEN)。ZFN 和 TALEN 包含已经工程化为与所选基因中的靶位点和裂解结构域或裂解半结构域结合 (例如,来自如本文所述的限制性和 / 或大范围核酸酶) 的 DNA 结合结构域 (锌指蛋白或 TALE DNA 结合结构域)。

[0117] 如以上所详细描述,锌指结合结构域和 TALE DNA 结合结构域可经工程化为与所选序列结合。见,例如, Beerli 等 (2002) Nature Biotechnol. 20:135-141 ;Pabo 等 (2001) Ann. Rev. Biochem. 70:313-340 ;Isalan 等 (2001) Nature Biotechnol. 19:656-660 ; Segal 等 (2001) Curr. Opin. Biotechnol. 12:632-637 ;Choo 等 (2000) Curr. Opin. Struct. Biol. 10:411-416。与天然存在的蛋白相比,工程化锌指结合结构域或 TALE 蛋白可具有新型结合特异性。工程化方法包括但不限于合理的设计和各种类型的选择。合理的设计包括 (例如) 使用包含三联体 (或四联体) 核苷酸序列和单独锌指或 TALE 氨基酸序列的数据库,其中每个三联体或四联体核苷酸序列与锌指或 TALE 重复单位的结合特定三联体或四联体序列的一个或多个氨基酸序列缔合。见,例如,美国专利 6,453,242 和 6,534,261,其通过引用整体并入本文。

[0118] 靶位点的选择 ;和用于融合蛋白 (和编码其的多核苷酸) 的设计和构建对于本领域普通技术人员来说是已知的并且描述于美国专利第 7,888,121 和 8,409,861 号 (通过引用整体并入本文) 中。

[0119] 另外,如这些和其它参考文献中所公开那样,锌指结构域、TALE 和 / 或多指锌指蛋白可使用任何适合的接头序列,包括例如长度为 5 个或更多个氨基酸的接头 (例如, TGEKP(SEQ ID NO:3)、TGGQRP(SEQ ID NO:4)、TGQKP(SEQ ID NO:5) 和 / 或 TGSQKP(SEQ ID NO:6)) 连接在一起。对于长度为 6 个或更多个氨基酸的示例性接头,见例如美国专利第 6,479,626、6,903,185 和 7,153,949 号。本文所述蛋白质可包括在蛋白质的单独锌指之间适合接头的任何组合。同样见,美国临时专利申请第 61/343,729 号。

[0120] 因此,核酸酶例如 ZFN、TALEN 和 / 或大范围核酸酶可包含任何 DNA 结合结构域和任何核酸酶 (裂解) 结构域 (裂解结构域、裂解半结构域)。如上所述,裂解结构域可能与 DNA 结合结构域异源,例如锌指或 TAL 效应子 DNA 结合结构域和来自核酸酶的裂解结构域,或大范围核酸酶 DNA 结合结构域和来自不同核酸酶的裂解结构域。异源裂解结构

域可从内切核酸酶或外切核酸酶获得。可从中得到裂解结构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。见，例如，2002-2003 目录，New England Biolabs,Beverly,MA；和 Belfort 等 (1997)Nucleic Acids Res.25:3379-3388。已知另外的裂解 DNA 的酶（例如，S1 核酸酶；绿豆核酸酶；胰腺 DNA 酶 I；微球菌核酸酶；酵母 HO 内切核酸酶；同样见 Linn 等（编辑）Nucleases,Cold Spring Harbor Laboratory Press,1993)。这些酶中的一种或多种（或其功能片段）可用作裂解结构域和裂解半结构域的来源。

[0121] 类似地，裂解半结构域可源自正如上面提出的对于裂解活性需要二聚化的任何核酸酶或其部分。一般而言，如果融合蛋白包含裂解半结构域，则裂解需要两个融合蛋白。可选地，可使用包含两个裂解半结构域的单一蛋白质。所述两个裂解半结构域可源自相同内切核酸酶（或其功能片段），或每个裂解半结构域可源自不同内切核酸酶（或其功能片段）。另外，两个融合蛋白的靶位点优选相对于彼此布置，以致两个融合蛋白与其各自靶位点的结合使裂解半结构域处于允许（例如）通过二聚化使裂解半结构域形成功能裂解结构域的相互空间定位。因此，在某些实施方案中，靶位点的近边间隔 5-8 个核苷酸或 15-18 个核苷酸。然而，任何整数数量的核苷酸或核苷酸对可插入两个靶位点之间（例如，2 至 50 个核苷酸对或更多）。一般而言，裂解位点位于靶位点之间。

[0122] 限制性内切核酸酶（限制酶）存在于许多物种中并且能够与 DNA 进行序列特异性结合（在识别位点），并在结合位点或附近裂解 DNA。某些限制酶（例如 IIS 型）在从识别位点移出的位点处裂解 DNA 并且具有可分离的结合和裂解结构域。例如，IIS 型酶 Fok I 在从一条链上的识别位点开始 9 个核苷酸处和从另一条链上的识别位点开始 13 个核苷酸处催化 DNA 的双链裂解。见，例如，美国专利 5,356,802、5,436,150 和 5,487,994；以及 Li 等 (1992)Proc.Natl.Acad.Sci.USA 89:4275-4279；Li 等 (1993)Proc.Natl.Acad.Sci.USA 90:2764-2768；Kim 等 (1994a)Proc.Natl.Acad.Sci.USA 91:883-887；Kim 等 (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 型限制酶的裂解结构域（或裂解半结构域）和可能或可能未经工程化的一种或多种锌指结合结构域。

[0123] 裂解结构域可与结合结构域分离的示例性 IIS 型限制酶为 Fok I。这种特殊的酶呈二聚体时有活性。Bitinaite 等 (1998)Proc.Natl.Acad.Sci.USA 95:10,570-10,575。相应地，为了本公开的目的，将 Fok I 酶用于公开的融合蛋白中的部分视为裂解半结构域。因此，为了使用锌指 -Fok I 融合进行靶向双链裂解和 / 或靶向置换细胞序列，各包含 Fok I 裂解半结构域的两种融合蛋白可用于重新组成有催化活性的裂解结构域。可选地，也可使用含有锌指结合结构域和两个 Fok I 裂解半结构域的单一多肽分子。在本公开的其它地方提供了使用锌指 -Fok I 融合进行靶向裂解和靶向序列改变的参数。

[0124] 裂解结构域或裂解半结构域可为保持裂解活性，或保持多聚化（例如，二聚化）以形成功能裂解结构域的能力的蛋白质的任何部分。

[0125] 在整体并入本文的国际公布 WO 07/014275 中描述了示例性 IIS 型限制酶。另外的限制酶还含有可分离的结合和裂解结构域，并且这些为本公开所涵盖。见，例如，Roberts 等 (2003)Nucleic Acids Res.31:418-420。

[0126] 在某些实施方案中，例如美国专利第 7,914,796、8,034,598 和 8,623,618 号；和美

国专利公布第 20110201055 号中所述，裂解结构域包含最小化或防止均二聚的一个或多个工程化裂解半结构域（也称为二聚化结构域突变体），其全部内容通过引用整体并入本文。Fok I 的位置 446、447、479、483、484、486、487、490、491、496、498、499、500、531、534、537 和 538 的氨基酸残基全部为影响 Fok I 裂解半结构域二聚化的靶标。

[0127] 形成专性杂二聚体的 Fok I 的示例性工程化裂解半结构域包括一对，其中第一裂解半结构域包括在 Fok I 的位置 490 和 538 的氨基酸残基处的突变而第二裂解半结构域包括在氨基酸残基 486 和 499 处的突变。

[0128] 因此，在一个实施方案中，490 处的突变以 Lys(K) 置换 Glu(E)；538 处的突变以 Lys(K) 置换 Iso(I)；486 处的突变以 Glu(E) 置换 Gln(Q)；并且位置 499 处的突变以 Lys(K) 置换 Iso(I)。具体地，通过使一个裂解半结构域中的位置 490(E → K) 和 538(I → K) 突变以生成命名为“E490K:I538K”的工程化裂解半结构域并且通过使另一裂解半结构域中的位置 486(Q → E) 和 499(I → L) 突变以生成命名为“Q486E:I499L”的工程化裂解半结构域制备本文所述的工程化裂解半结构域。本文所述的工程化裂解半结构域为其中异常裂解减到最少或消除的专性杂二聚体突变体。见，例如，美国专利公布第 2008/0131962 号，出于种种目的，其公开内容通过引用整体并入。在某些实施方案中，工程化裂解半结构域包含位置 486、499 和 496（相对于野生型 FokI 编号）处的突变，例如以 Glu(E) 残基置换位置 486 处的野生型 Gln(Q) 残基，以 Leu(L) 残基置换位置 499 处的野生型 Iso(I) 残基和以 Asp(D) 或 Glu(E) 残基置换位置 496 处的野生型 Asn(N) 残基的突变（也分别称为“ELD”和“ELE”结构域）。在其它实施方案中，工程化裂解半结构域包含位置 490、538 和 537（相对于野生型 FokI 编号）处的突变，例如以 Lys(K) 残基置换位置 490 处的野生型 Glu(E) 残基，以 Lys(K) 残基置换位置 538 处的野生型 Iso(I) 残基和以 Lys(K) 残基或 Arg(R) 残基置换位置 537 处的野生型 His(H) 残基的突变（也分别称为“KKK”和“KKR”结构域）。在其它实施方案中，工程化裂解半结构域包含位置 490 和 537（相对于野生型 FokI 编号）处的突变，例如以 Lys(K) 残基置换位置 490 处的野生型 Glu(E) 残基和以 Lys(K) 残基或 Arg(R) 残基置换位置 537 处的野生型 His(H) 残基的突变（也分别称为“KIK”和“KIR”结构域）。（见美国专利公布第 20110201055 号）。

[0129] 可使用任何适合方法，例如如美国专利第 7,914,796、8,034,598 和 8,623,618 号；和美国专利公布第 20110201055 号中所述，通过定点诱变野生型裂解半结构域 (Fok I) 制备本文所述的工程化裂解半结构域。

[0130] 可选地，可使用所称的“裂解酶”技术，在体内在核酸靶位点处组装核酸酶（见例如美国专利公布第 20090068164 号）。此类裂解酶的组成部分可在任一单独表达构建体上表达，或者可连接在一开放阅读框中，其中例如通过自我裂解 2A 肽或 IRES 序列将单独组成部分分隔开。组成部分可为单独锌指结合结构域或大范围核酸酶核酸结合结构域的结构域。

[0131] 使用之前，例如在如 WO 2009/042163 和 20090068164 中所述的基于酵母的染色体体系中，可筛选核酸酶（例如，ZFN 和 / 或 TALEN) 的活性。使用本领域已知的方法可容易地设计核酸酶表达构建体。见，例如，美国专利公布 20030232410、20050208489、20050026157、20050064474、20060188987、20060063231；和国际公布 WO 07/014275。核酸酶的表达受组成型启动子或诱导型启动子，例如在棉子糖和 / 或半乳糖的存在下激活（去阻遏）而在葡

葡糖的存在下阻遏的半乳糖激酶启动子的控制。

[0132] 在某些实施方案中，核酸酶包含 CRISPR/Cas 体系。CRISPR（成簇的规律性间隔的短回文重复序列）基因座（其编码所述体生活经验的 RNA 组分）和 cas(CRISPR- 结合的）基因座（其编码蛋白质 (Jansen 等，2002. Mol. Microbiol. 43:1565-1575 ;Makarova 等，2002. Nucleic Acids Res. 30:482-496 ;Makarova 等，2006. Biol. Direct 1:7 ;Haft 等，2005. PLoS Comput. Biol. 1:e60)）组成 CRISPR/Cas 核酸酶体系的基因序列。微生物宿主中的 CRISPR 基因座含有 CRISPR- 结合的 (Cas) 基因以及能够对 CRISPR 介导的核酸裂解的特异性编程的非编码 RNA 元件的组合。

[0133] II 型 CRISPR 是最充分表征的体系之一并且在 4 个连续步骤中进行靶向 DNA 双链断裂。首先，从 CRISPR 基因座转录两个非编码 RNA、前 -crRNA 阵列和 tracrRNA。第二，tracrRNA 与前 -crRNA 的重复区域杂交，并且介导前 -crRNA 至含有单个间隔子序列的成熟 crRNA 的加工。第三，所述成熟的 crRNA:tracrRNA 复合物通过 crRNA 上的间隔子与紧接原型间隔序列邻近基序 (PAM) 的靶 DNA 上的原型间隔序列之间的沃尔森 - 克里克碱基配（靶识别的额外要求）对将 Cas9 导向靶 DNA。最后，Cas9 介导靶 DNA 的裂解以在原型间隔序列内产生双链断裂。CRISPR/Cas 体系的活性由 3 个步骤组成 :(i) 外来 DNA 序列至 CRISPR 阵列的插入，以在称为‘适应’的过程中预防将来的攻击，(ii) 相关蛋白质的表达，以及阵列的表达和加工，随后 (iii)RNA 介导的干扰异形核酸。因此，在细菌细胞中，几种所谓的‘Cas’蛋白牵涉 CRISPR/Cas 体系的天然功能并且在功能诸如外来 DNA 的插入等中起作用。

[0134] 在某些实施方案中，Cas 蛋白可以是天然存在的 Cas 蛋白的 " 功能性衍生物 "。天然序列多肽的 " 功能性衍生物 " 是具有与天然序列多肽共同的定性生物学性质的化合物。" 功能性衍生物 " 包括，但不限于，天然序列的片段和天然序列多肽的衍生物及其片段，只要它们具有与相应天然序列多肽共同的生物活性。本文中涉及的生物活性是功能性衍生物将 DNA 底物水解成片段的能力。术语 " 衍生物 " 包括多肽的氨基酸序列变体、共价修饰和其融合物。Cas 多肽的合适的衍生物或其片段包括，但不限于 Cas 蛋白或其片段的突变体、融合物、共价修饰。包括 Cas 蛋白或其片段的 Cas 蛋白以及 Cas 蛋白的衍生物或其片段可从细胞获得或化学合成，或通过这两种方法的组合获得。所述细胞可以是天然产生 Cas 蛋白的细胞，或天然产生 Cas 蛋白和经遗传工程化以更高表达水平产生内源 Cas 蛋白或从外源引入的核酸产生 Cas 蛋白的细胞，所述核酸编码与内源 Cas 相同或不同的 Cas。在一些情况下，所述细胞不天然产生 Cas 蛋白并且经遗传工程化产生 Cas 蛋白。

[0135] 在例如美国临时申请第 61/823,689 号中公开了靶向 HLA 和其它基因的示例性 CRISPR/Cas 核酸酶体系。

[0136] 递送

[0137] 可通过任何适合方式，包括（例如）通过注射蛋白和 / 或 mRNA，向靶细胞递送蛋白质（例如，核酸酶和非经典 HLA 分子）、编码蛋白质的多核苷酸和包含本文所述的蛋白质和 / 或多核苷酸的组合物。

[0138] 适合的细胞包括但不限于真核和原核细胞和 / 或细胞系。此类细胞或由此类细胞产生的细胞系的非限制性实例包括 T 细胞、COS、CHO（例如，CHO-S、CHO-K1、CHO-DG44、CHO-DUXB11、CHO-DUKX、CHOK1SV）、VERO、MDCK、WI38、V79、B14AF28-G3、BHK、HaK、NSO、SP2/0-Ag14、HeLa、HEK293（例如，HEK293-F、HEK293-H、HEK293-T）和 perC6 细

胞以及昆虫细胞例如草地贪夜蛾 (Spodoptera fugiperda)(Sf) 或真菌细胞例如酵母属 (Saccharomyces)，毕赤酵母 (Pichia) 和裂殖酵母 (Schizosaccharomyces)。在某些实施方案中，细胞系为 CHO-K1、MDCK 或 HEK293 细胞系。适合的细胞还包括干细胞，举例而言，例如胚胎干细胞、诱导多能干细胞 (iPS 细胞)、造血干细胞、神经元干细胞和间充质干细胞。

[0139] 例如，在美国专利第 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 和 7,163,824 号中描述了递送包含如本文所述的 DNA 结合结构域的蛋白质的方法，所述专利全部的公开内容通过引用整体并入本文。

[0140] 还可使用含有编码一种或多种 DNA 结合蛋白的序列的载体来递送如本文所述的 DNA 结合结构域和包含这些 DNA 结合结构域的融合蛋白。另外，还可通过这些载体递送另外的核酸 (例如，供体和 / 或编码非经典 HLA 蛋白的序列)。可使用任何载体体系，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、疱疹病毒载体和腺相关病毒载体等。同样，见，美国专利第 6,534,261、6,607,882、6,824,978、6,933,113、6,979,539、7,013,219 和 7,163,824 号，其通过引用整体并入本文。此外，明显的是，任何这些载体可包含一个或多个编码 DNA 结合蛋白的序列和 / 或适当时另外的核酸。因此，当向细胞引入一种或多种如本文所述的 DNA 结合蛋白和适当时另外的 DNA 时，可在相同载体或不同载体上携带它们。当使用多个载体时，每个载体均可包含编码一个或多个 DNA 结合蛋白的序列和必需时另外的核酸。

[0141] 基于病毒和非病毒的常规基因转移方法可用于在细胞 (例如，哺乳动物细胞) 和靶组织中引入编码工程化 DNA 结合蛋白的核酸以及必要时共引入另外的核苷酸序列。此类方法也可用于在体外向细胞施用核酸 (例如，编码 DNA 结合蛋白、供体和 / 或非经典 HLA 蛋白)。在某些实施方案中，施用核酸用于体内或体外基因治疗用途。非病毒载体递送系统包括 DNA 质粒、裸露核酸和与递送媒介物例如脂质体或泊洛沙姆 (poloxamer) 复合的核酸。病毒载体递送系统包括在递送到细胞后具有附加体或整合基因组的 DNA 和 RNA 病毒。对于基因治疗过程的综述，见 Anderson, Science 256:808-813(1992) ;Nabel 和 Felgner, TIBTECH 11:211-217(1993) ;Mitani 和 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 和 Perricaudet, British Medical Bulletin 51(1):31-44(1995) ; Haddada 等，在 Current Topics in Microbiology and Immunology 中，Doerfler 和 Böhm (编辑)(1995) ;和 Yu 等，Gene Therapy 1:13-26(1994)。

[0142] 核酸的非病毒递送方法包括电穿孔、脂质转染、显微注射、基因枪法、病毒颗粒、脂质体、免疫脂质体、聚阳离子或脂质 : 核酸缀合物、裸 DNA、mRNA、人工病毒粒子和试剂增强的 DNA 摄取。使用 (例如)Sonitron 2000 系统 (Rich-Mar) 的声致穿孔法也可用于核酸的递送。在一个优选实施方案中，将一种或多种核酸作为 mRNA 递送。还优选使用加帽的 mRNA 以增加翻译效率和 / 或 mRNA 稳定性。尤其优选 ARCA(抗 - 反向帽类似物) 帽或其变体。见美国专利第 7,074,596 和 8,153,773 号，其通过引用并入本文。

[0143] 另外的示例性核酸递送系统包括 Amaxa Biosystems(Cologne, Germany)、Maxcyte, Inc.(Rockville, Maryland)、BTX Molecular Delivery Systems(Holliston, MA)

和 Copernicus Therapeutics Inc. 提供的核酸递送系统（见例如 US6008336）。例如 US 5,049,386、US 4,946,787 和 US 4,897,355 中描述了脂质转染并且脂质转染试剂在市场上有售（例如，Transfectam™、Lipofectin™和 Lipofectamine ™RNAiMAX）。适合多核苷酸的有效受体 - 识别脂质转染的阳离子和中性脂质包括 Felgner（WO 91/17424、WO 91/16024）的脂质。可向细胞（活体外施用）或靶组织（体内施用）递送。

[0144] 脂质：核酸复合物，包括靶向脂质体例如免疫脂质复合物的制备为本领域的技术人员众所周知（见，例如，Crystal, Science 270:404-410(1995)；Blaese 等，Cancer Gene Ther. 2:291-297(1995)；Behr 等，Bioconjugate Chem. 5:382-389(1994)；Remy 等，Bioconjugate Chem. 5:647-654(1994)；Gao 等，Gene Therapy 2:710-722(1995)；Ahmad 等，Cancer Res. 52:4817-4820(1992)；美国专利第 4,186,183、4,217,344、4,235,871、4,261,975、4,485,054、4,501,728、4,774,085、4,837,028 和 4,946,787 号）。

[0145] 另外的递送方法包括利用将待递送核酸包装到 EnGeneIC 递送媒介物 (EDV) 中。使用双特异性抗体将这些 EDV 特异性地递送到靶组织，其中所述抗体的一臂对靶组织具有特异性而另一臂对 EDV 具有特异性。抗体将 EDV 带到靶细胞表面，然后通过胞吞作用将 EDV 带入细胞。一旦进入细胞，就释放内含物（见 MacDiarmid 等 (2009) Nature Biotechnology 第 27 卷 (7) 第 643 页）。

[0146] 使用基于 RNA 或 DNA 病毒的系统递送编码工程化 DNA 结合蛋白、非经典 HLA 分子和 / 或所需的其它供体的核酸利用使病毒靶向体内的特定细胞并且将病毒有效载荷运输到核的高度进化过程。病毒载体可直接向患者（体内）施用或其可用于在体外处理细胞并且向患者（在活体外）施用经修饰的细胞。递送核酸的基于病毒的常规系统包括但不限于用于基因转移的逆转录病毒、慢病毒、腺病毒、腺相关、牛痘和单纯性疱疹病毒载体。用逆转录病毒、慢病毒和腺相关病毒基因转移方法可能整合在宿主基因组中，常常导致插入的转基因长期表达。另外，已经在许多不同细胞类型和靶组织中观察到高转导效率。

[0147] 可通过并入外源包膜蛋白，扩增靶细胞的潜在靶群体改变逆转录病毒的向性。慢病毒载体是能够转导或感染非分裂细胞并且通常产生高病毒滴度的逆转录病毒载体。逆转录病毒基因转移系统的选择取决于靶组织。逆转录病毒载体由包装容量高达 6-10kb 外源序列的顺式作用长末端重复组成。最小的顺式作用 LTR 足以复制和包装载体，然后用于将治疗性基因整合到靶细胞中以提供永久性转基因表达。广泛使用的逆转录病毒载体包括基于小鼠白血病病毒 (MuLV)、长臂猿白血病病毒 (GaLV)、猿免疫缺陷病毒 (SIV)、人免疫缺陷病毒 (HIV) 及其组合的那些（见，例如，Buchscher 等，J. Virol. 66:2731-2739(1992)；Johann 等，J. Virol. 66:1635-1640(1992)；Sommerfelt 等，Virol. 176:58-59(1990)；Wilson 等，J. Virol. 63:2374-2378(1989)；Miller 等，J. Virol. 65:2220-2224(1991)；PCT/US94/05700）。

[0148] 在其中优选瞬时表达的应用中，可使用基于腺病毒的系统。基于腺病毒的载体在许多细胞类型中能够具有非常高的转导效率并不需要细胞分裂。用此类载体，已经获得高滴度和高水平的表达。在相对简单的系统中可大量生成这种载体。腺相关病毒（“AAV”）载体也用于例如在核酸和肽的体外生成中用核酸转导细胞，并且用于体内和活体外基因治疗过程（见，例如，West 等，Virology 160:38-47(1987)；美国专利第 4,797,368 号；WO 93/24641；Kotin, Human Gene Therapy 5:793-801(1994)；Muzyczka, J. Clin.

Invest.94:1351(1994)。在许多出版物中描述了重组AAV载体的构建,包括美国专利第5,173,414 号;Tratschin 等,Mol.Cell.Biol.5:3251-3260(1985);Tratschin 等,Mol.Cell.Biol.4:2072-2081(1984);Hermonat 和 Muzyczka,PNAS 81:6466-6470(1984); 和 Samulski 等,J.Virol.63:03822-3828(1989)。

[0149] 至少6种病毒载体方法目前可用于临床试验中的基因转移,其利用牵涉由插入辅助细胞系中的基因互补缺陷型载体的方法以生成转导剂。

[0150] pLASN和MFG-S为已经用于临时试验的逆转录病毒载体的实例(Dunbar等,Blood 85:3048-305(1995);Kohn等,Nat.Med.1:1017-102(1995);Malech等,PNAS 94:22 12133-12138(1997))。PA317/pLASN为用于基因治疗试验的首例治疗性载体。(Blaese等,Science 270:475-480(1995))。对于MFG-S包装载体已经观察到50%或更高的转导效率。(Ellem等,Immunol Immunother.44(1):10-20(1997);Dranoff 等,Hum.Gene Ther.1:111-2(1997)。

[0151] 重组腺相关病毒载体(rAAV)是基于缺陷型和非病原性细小病毒腺相关2型病毒的有前景的可供选择的基因递送系统。所有载体源自仅保留了在转基因表达盒两侧的AAV 145bp反向末端重复的质粒。由于整合到转导细胞基因组中的有效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,Lancet 351:9117 1702-3(1998),Kearns等,Gene Ther.9:748-55(1996))。也可根据本发明使用其它AAV血清型,包括AAV1、AAV3、AAV4、AAV5、AAV6、AAV8、AAV8.2、AAV9和AAV rh10及假型AAV例如AAV2/8、AAV2/5和AAV2/6。

[0152] 复制缺陷型重组腺病毒载体(Ad)可在高滴度下生成并且容易感染许多不同细胞类型。大部分腺病毒载体经工程化,以致转基因置换Ad E1a、E1b和/或E3基因;随后使复制缺陷型载体在反式供给缺失基因功能的人293细胞中增殖。Ad载体可在体内转导多种类型的组织,包括非分裂、分化细胞例如存在于肝脏、肾脏和肌肉中的细胞。常规Ad载体承载容量大。临床试验中使用Ad载体的实例包括经肌肉内注射进行抗肿瘤免疫的多核苷酸疗法(Sterman等,Hum.Gene Ther.7:1083-9(1998))。在临床试验中使用腺病毒载体进行基因转移的另外实例包括Rosenecker等,Infection 24:1 5-10(1996);Sterman等,Hum.Gene Ther.9:7 1083-1089(1998);Welsh等,Hum.Gene Ther.2:205-18(1995);Alvarez等,Hum.Gene Ther.5:597-613(1997);Topf等,Gene Ther.5:507-513(1998);Sterman等,Hum.Gene Ther.7:1083-1089(1998)。

[0153] 包装细胞用于形成能够感染宿主细胞的病毒粒子。此类细胞包括包装腺病毒的293细胞和包装逆转录病毒的ψ2细胞或PA317细胞。通常由将核酸载体包装在病毒粒子中的生产细胞系生成用于基因治疗的病毒载体。所述载体通常含有包装和后续整合到宿主(若适用)所需的最小病毒序列,由编码待表达蛋白质的表达盒置换的其它病毒序列。由包装细胞系反式供给缺少的病毒功能。例如,用于基因治疗的AAV载体通常仅仅具有来自于AAV基因组的包装和整合到宿主基因组中所需的反向末端重复(ITR)序列。病毒DNA包装在含有编码其它AAV基因,即rep和cap的辅助质粒,但是缺乏ITR序列的细胞系中。细胞系还受作为辅助因子的腺病毒感染。辅助病毒促进AAV载体的复制和AAV基因由辅助质粒表达。由于缺乏ITR序列,辅助质粒未大量包装。例如,可通过腺病毒比AAV更敏感的热处理减轻受腺病毒的污染。

[0154] 在许多基因治疗应用中，以对特定组织类型的高度特异性，递送基因治疗载体是可取的。相应地，可通过表达配体作为与病毒外表面上的病毒外壳蛋白的融合蛋白，将病毒载体修饰为对指定细胞类型具有特异性。选择所述配体对已知存在于目标细胞类型上的受体具有亲和力。例如，Han 等，Proc. Natl. Acad. Sci. USA 92:9747-9751(1995) 报道可将莫洛尼鼠白血病病毒修饰为表达与 gp70 融合的人调蛋白 (heregulin)，并且重组病毒感染表达人表皮生长因子受体的某些人乳腺癌细胞。这种原理可延伸到其它病毒 - 靶细胞对，其中靶细胞表达受体而病毒表达包含细胞表面受体的配体的融合蛋白。例如，丝状噬菌体可经工程化为展示对几乎所选任何细胞受体具有特异性结合亲和力的抗体片段（例如，FAB 或 Fv）。虽然以上描述主要适用于病毒载体，但是可将相同原理应用于非病毒载体。此类载体可经工程化为含有利于被特定靶细胞摄取的特定摄取序列。

[0155] 可通过向患者个体施用，通常通过如下所述的全身施用（例如，静脉内、腹膜内、肌肉日、皮下或颅内输注）或局部应用，在体内递送基因治疗载体。可选地，可在活体外向细胞，例如从患者个体移植的细胞（例如淋巴细胞、骨髓穿刺液、组织活检）或万能供血者造血干细胞递送载体，接着通常在为已经并入载体的细胞选择后，将细胞再植入患者体内。

[0156] 用于诊断、研究、移植或基因治疗的活体外细胞转染（例如，通过将转染细胞再次输注到宿主生物中）为本领域的技术人员众所周知。在一个优选实施方案中，从受试生物分离细胞，用 DNA 结合蛋白的核酸（基因或 cDNA）转染，并且再次输注至受试生物（例如，患者）体内。适合活体外转染的各种细胞类型为本领域的技术人员众所周知（见，例如，Freshney 等，Culture of Animal Cells, A Manual of Basic Technique(1994 年第 3 版)）和本文引用以讨论如何从患者分离和培养细胞的参考文献）。

[0157] 在一个实施方案中，干细胞用于细胞转染和基因治疗的活体外过程。使用干细胞的优点是它们可在体外分化为其它细胞类型，或可引入哺乳动物（例如细胞供体）中，在这里它们将植入骨髓中。已知使用细胞因子例如 GM-CSF、IFN-γ 和 TNF-α 使 CD34+ 细胞在体外分化成临床上重要的免疫细胞类型的方法（见 Inaba 等，J. Exp. Med. 176:1693-1702(1992)）。

[0158] 使用已知方法分离干细胞进行转导和分化。例如，通过用结合不必要的细胞，例如 CD4+ 和 CD8+(T 细胞）、CD45+(panB 细胞）、GR-1（粒细胞）和 Iad（分化的抗原呈递细胞）的抗体，淘选骨髓细胞，而从骨髓细胞分离干细胞（见 Inaba 等，J. Exp. Med. 176:1693-1702(1992)）。

[0159] 在一些实施方案中也可使用已经修饰的干细胞。例如，已经使其抗细胞凋亡的神经元干细胞可用作治疗组合物，其中干细胞还含有本发明的 ZFP TF。例如，通过在干细胞中使用 BAX- 或 BAK- 特异性 ZFN（见，美国专利申请第 12/456,043 号）或在半胱天冬酶中破坏的干细胞中，例如再次使用半胱天冬酶 -6 特异性 ZFN 敲除 BAX 和 / 或 BAK，可能出现对细胞凋亡的抗性。可用已知调控 HLA 的 ZFP TF 转染这些细胞。

[0160] 也可向生物直接施用含有治疗性 DNA 结合蛋白（或编码这些蛋白的核酸）的载体（例如，逆转录病毒、腺病毒、脂质体等）以在体内进行细胞转导。可选地，可施用裸 DNA。通过一般用于将分子引入与血液或组织细胞的最终接触的任何途径施用，包括但不限于注射、输注、局部应用和电穿孔。施用此类核酸的适合方法可用并且为领域的技术人员众所周知，并且，虽然可使用一种以上的途径施用特定组合物，但是常常一种特定途径可以提供比

另一途径更直接且更有效的反应。

[0161] 例如，在美国专利第 5,928,638 号中公开了将 DNA 引入造血干细胞的方法。用于将转基因引入造血干细胞，例如 $CD34^+$ 细胞的载体包括 35 型腺病毒。

[0162] 适合将转基因引入免疫细胞（例如，T 细胞）的载体包括非整合慢病毒载体。见，例如，Ory 等 (1996)Proc.Natl.Acad.Sci.USA 93:11382-11388 ;Dull 等 (1998) J.Virol.72:8463-8471 ;Zuffery 等 (1998)J.Virol.72:9873-9880 ;Follenzi 等 (2000) Nature Genetics 25:217-222。

[0163] 部分由施用的特定组合物以及由用于施用组合物的特定方法决定药学上可接受的载体。相应地，如下所述，存在可用药物组合物的多种适合配方（见，例如，Remington's Pharmaceutical Sciences，第 17 版，1989）。

[0164] 如上所述，公开的方法和组合物可用于任何类型的细胞，包括但不限于原核细胞、真菌细胞、古细菌细胞、植物细胞、昆虫细胞、动物细胞、脊椎动物细胞、哺乳动物细胞和人细胞，包括任何类型的 T 细胞和干细胞。用于蛋白质表达的适合细胞系为本领域的技术人员已知并且包括但不限于 COS、CHO（例如，CHO-S、CHO-K1、CHO-DG44、CHO-DUXB11）、VERO、MDCK、WI38、V79、B14AF28-G3、BHK、HaK、NS0、SP2/0-Ag14、HeLa、HEK293（例如，HEK293-F、HEK293-H、HEK293-T）、perC6、昆虫细胞例如草地贪夜蛾 (Sf) 和真菌细胞例如酵母属，毕赤酵母和裂殖酵母。也可使用这些细胞系的子代、变体和衍生物。

[0165] 应用

[0166] 公开的组合物和方法可用于其中需要调节 HLA 表达和 / 或功能性的任何应用，包括但不限于其中 HLA 调节是所需的治疗和研究应用。

[0167] 与 HLA 密切相关的疾病和病况包括阿狄森氏病、强直性脊柱炎、白塞氏病、伯格氏病、乳糜泻、慢性活动性肝炎、格雷夫斯病、幼年型类风湿关节炎、银屑病、银屑病性关节炎、类风湿性关节炎、斯耶格伦综合征和红斑狼疮等。另外，HLA 基因的调节可与目标细胞的其它遗传修饰结合使用。例如，可将利用嵌合抗原受体对靶细胞诸如 CTL 的修饰（以改变 CTL 的特异性）与如本文所述的活体外 HLA 修饰组合，以开发可用于大多数有此需要的任何患者的细胞治疗。

[0168] 另外，本发明的材料和方法可用于治疗、预防或改善移植物抗宿主病。当向患者施用同种异体 T 细胞（例如，骨髓和 / 或输血）时，移植物抗宿主病 (GVHD) 是常见并发症。输注的材料中的功能性免疫细胞将受者识别为 " 外来物 " 并且激发免疫攻击。通过调节同种异体 T 细胞中的 HLA 和 / 或 TCR 表达，可将“下架”T 细胞（例如，CD19- 特异性 T 细胞）作为“药物”按需施用，因为 GVHD 的风险下降或被消除并且，另外地，非经典 HLA 分子的提供减少或消除经修饰的细胞的 NK 介导的裂解。

[0169] 方法和组合物还包括其中干细胞内的一种或多种经典 HLA 基因已被灭活并且一种或多种非经典 HLA 分子被激活的干细胞组合物。例如，已将 HLA 经修饰的造血干细胞引入骨髓去除后的患者。这些改变的 HSC 可允许患者的再定殖而无因排斥和 / 或 NK 介导的细胞裂解产生的丢失。引入的细胞还可具有其它改变来在随后的治疗（例如，化学疗法抗性）过程中帮助治疗基础疾病。

[0170] 本发明的方法和组合物也用于 HLA 经修饰的血小板（例如用作治疗剂）的开发。因此，HLA 经修饰的血小板可用于治疗血小板减少症诸如特发性血小板减少性紫癜、血栓性

血小板减少性紫癜和药物诱发的血小板减少性紫癜（例如，肝素诱发的血小板减少症）。可用本发明的 HLA 经修饰的血小板治疗的其它血小板病症包括戈谢病、再生障碍性贫血、奥尼赖病、胎儿母亲异源免疫性血小板减少症、HELLP 综合征、癌症以及来自一些化疗治疗剂的副作用。HLA 经修饰的血小板还作为“下架”疗法在急诊室情况下用于创伤患者。

[0171] 本发明的方法可用于异种移植术。具体地，仅通过举例，可将猪的器官用于移植进人中，其中猪类 MHC 基因已被删除和 / 或被人 HLA 基因置换。可开发（从已具有由被注射入它们的 mRNA 编码的靶向 HLA 的 ZFN 以便内源 MHC 基因被破坏的猪胚胎，或从使用它们的 HLA 基因已被成功地靶向的细胞的细胞核将体细胞的细胞核转移进猪胚胎）含有这些有用的基因突变的猪的品系，并将可培养这些动物直至最终器官收获。这将在人中阻止对这些器官的排拆和增加成功移植的机会。

[0172] 本发明的方法和组合物还用于设计和实现体外和体内模型，例如，允许研究这些病症的 HLA 或其它病症的动物模型。

实施例

[0173] 实施例 1：材料和方法

[0174] ZFN

[0175] 使用如美国专利第 20120060230 号中所述的已预先验证的 2- 指和 1- 指模块的的已建立的档案设计和组装含有 5 或 6 个指的 HLA-A 结合 ZFN。可使用的示例性 ZFN 示于下表 1 中。该表中的第 1 栏是 ZFP 的内部参照名称（编号）并且对应于表 2 的第 1 栏中的相同名称。“F”指的是指并且“F”后的数字是指哪个锌指（例如，“F1”指的是指 1）。

[0176] 表 1：锌指蛋白

[0177]

靶	SBS #	设计					
I 类		F1	F2	F3	F4	F5	F6
HLA A2	18889	QSSNLTR (SEQ ID NO:11)	RSDNLTT (SEQ ID NO:12)	RSDTLSQ (SEQ ID NO:13)	RSADLSR (SEQ ID NO:14)	QSSDLSR (SEQ ID NO:15)	RSDALTQ (SEQ ID NO:16)
HLA A2	18891	QRTHLAK (SEQ ID NO:17)	RSDTLSR (SEQ ID NO:18)	RSDVRIT (SEQ ID NO:19)	RSDHLST (SEQ ID NO:20)	DSSARKN (SEQ ID NO:21)	NA
HLA A2	24859	QNARKRT (SEQ ID NO:22)	RSDSLLR (SEQ ID NO:23)	RNDCRKR (SEQ ID NO:24)	RSDELST (SEQ ID NO:20)	DSSRRKR (SEQ ID NO:21)	NA
HLA A3	25191	DRSHLSR (SEQ ID NO:25)	RSDDLTR (SEQ ID NO:26)	DRSDLSR (SEQ ID NO:27)	QSGHLSR (SEQ ID NO:28)	NA	NA
HLA A3	25193	DRSALSR (SEQ ID NO:29)	QSGDLSR (SEQ ID NO:30)	DRSALSR (SEQ ID NO:29)	EPSHLAR (SEQ ID NO:31)	RSDDLSK (SEQ ID NO:32)	DRSHLAR (SEQ ID NO:31)

[0178] 表 2 中公开了示例性 HLA-A 结合蛋白的靶位点的序列。表 2 显示指定的锌指蛋白的靶序列。被 ZFP 识别螺旋接触的靶位点中的核苷酸以大写字母指示；非接触核苷酸以小写字母指示。

[0179] 表 2:HLA-A 锌指靶位点

[0180]

类	SEQ #	靶位点
I类		
HLA A2	19989	gtATGGCTGCCGACGTGGGGTcggacgggg (SEQ ID NO:34)
HLA A2	19991	ttASCTGGATGGTGTGAgaacctggcac (SEQ ID NO:35)
HLA A2	24059	tcCTCTGGACGGTGTGAgaacctggcac (SEQ ID NO:36)
HLA A3	25191	atGGAGCCGCGGGCgccgtggatagagc (SEQ ID NO:37)
HLA A3	25190	ctGGCTCGnGGCGTCGCTGTCgaaccgc (SEQ ID NO:38)

[0181] 细胞培养

[0182] 将HEK293细胞维持在补充有10％热灭活胎牛血清(FBS:Lonza)和2mmol/L L-谷氨酰胺(Glutamax-1:Invitrogen,Carlsbad,CA)的达尔伯克改良伊格尔培养基(Lonza,Basel,Switzerland)中。将EBV-LCL、721.221和EL-4细胞系维持在补充有10％热灭活的FBS和2mmol/L L-谷氨酰胺的RPMI 1640(Lonza)中。这些细胞系的鉴定通过STR DNA指纹分析来确认。对于mHAg是特异的CD8+CTL克隆是:在HLA-A*0301的背景中的识别由PANE1编码的肽RVWDLPGVLK(SEQ ID NO:1)的克隆7A7(Brickner等(2006)Blood 107(9):3779-3786)和识别来自C19ORF48中的ORF+2/48的HLA-A*0201-限制的CIPPDSLLFPA(SEQ ID NO:2,NM_199250.1的可选的开放阅读框架)肽的克隆GAS2B3-5(Tykodi等(2008)Clin Cancer Res.14(16):5260-5269)。在^{51}铬释放测定前一天解冻CTL克隆,将其维持在补充有10％人白蛋白血清、2mmol/L L-谷氨酰胺、20ng/mL的IL-15(PeproTech,Rocky Hill,NJ)和20IU/mL的IL-2(Chiron,Emeryville,CA)的RPMI 1640中。

[0183] 载有OKT3的人工抗原呈递细胞(aAPC)对原代T细胞的激活

[0184] 通过在补充有2mmol/L L-谷氨酰胺和10％FBS以及50IU/mL的IL-2的RPMI 1640(始于aAPC添加后第二天,每隔一天添加的)中以1:1(T细胞:γ-辐照的(100Gy)aAPC)的比率用加载至aAPC(克隆#4:通常经修饰稳定地共表达CD19、CD64、CD86、CD137L和与EGFP17同步表达的白细胞介素IL-15的膜结合的突变蛋白的K562细胞(见,O'Connor等(2012)Sci Rep 2:249;Manuri等(2010)Hum Gene Ther 21(4):427-437))上的OKT3(eBioscience,San Diego,CA)刺激PBMC,来通过体外交联CD3激活T细胞(CAR^{neg})以使其持续增殖。每14天再添加载有OKT3的aAPC一次以持续T细胞增殖。

[0185] 经遗传修饰的CD19-特异性CAR+T细胞的产生和在CD19+aAPC上的增殖

[0186] 我们制造临床级CAR+T细胞的方法经改造适合于产生CD19-特异性T细胞。(见,例如,Singh等(2008)Cancer Res.68(8):2961-2971)。使用Nucleofector II装置(Lonza)将编码SB转座子CD19RCD28和SB极度活跃的转座酶SB11的DNA质粒同时电转移(人T细胞细胞核转染溶液,程序U-014)进来源于PBMC的T细胞。通过在50IU/mL的IL-2(始于aAPC添加后第二天,每隔一天添加)存在的情况下,在电穿孔当天添加以及每隔14天再添加(以1:2的T细胞:aAPC比率)γ-辐照的(100Gy)aAPC(无OKT3加载的克隆#4)来选择性地在数目上扩增一组CAR+T细胞。

[0187] 信使RNA的体外转录

[0188] 如先前Torikai等(2012)Blood 119(24):5697-5705中所述制备体外转录的mRNA种类。简言之,用XhoI线性化编码ZFN-L和ZFN-R的DNA模板质粒。在按照制造商的说明书体外转录(RiboMAX™大规模RNA产生系统-T7,Promega,Madison,WI)和加帽(ARCA帽类

似物，Ambion, Austin, TX）后，使用 poly A 加尾试剂盒（Ambion）添加多聚腺嘌呤酸。在 1％变性琼脂糖凝胶上利用 3-(N-吗啉代）丙烷磺酸（MOPS）缓冲液验证 mRNA 种类的完整性，并通过分光光度计（BioRad, Hercules, CA）在 OD260 测定浓度。将 mRNA 装入小瓶并于 -80℃贮存以用于一次性使用。

[0189] 编码 ZFN 的 DNA 质粒和 mRNA 种类的电穿孔

[0190] 为了修饰 HEK293 细胞，使用制造商的方案通过核转染法（Lonza）引入编码靶向 HLA-A 的 ZFN 的表达载体。在初始刺激后 6 天或在用 γ-辐照的 aAPC 再刺激后 2 至 3 天收获 T 细胞。将 500 万个 T 细胞与 2.5 至 10 μg 的每一种 ZFN-L 和 ZFN-R mRNA 种类于 100 μL 的人 T 细胞细胞核转染溶液（Lonza）预混合，随后使用 Nucleofector II 设备利用程序 T-20 在电击杯中进行电穿孔。在电穿孔后，立即将细胞置于预先加温的补充有 2mmol/L L-谷氨酰胺和 10％ FBS 的 RPMI 1640 中，并在 37℃和 5％ CO2 下培养 4-6 小时，在该点上添加 50IU/mL 的 IL-2 以进一步培养。在“冷激”实验中，在于 37℃ -5％ CO2 培养箱中培养过夜后，将 T 细胞转移至 30℃、5％ CO2 培养箱中，并培养 3 天，随后在进行分析之前返回 37℃、5％ CO2 培养箱。

[0191] 缺少 HLA-A 的表达的细胞的富集

[0192] 在用补充有 2％ FBS 和 2mM EDTA 的磷酸盐缓冲盐水（PBS）洗涤细胞后，在 4℃用缀合有 PE 的单克隆抗体（mAb）特异性抗 -HLA-A2（BD Biosciences, San Jose, CA）标记细胞，进行 15 分钟，随后洗涤，然后并用抗 -PE 微珠（Miltenyi Biotec, Auburn, CA）标记 10 分钟。洗涤后，将标记的细胞通过 LD 柱（MiltenyiBiotec），收集流过级分，并进行培养。将 T 细胞在 γ-辐照的加载有 OKT3 的 aAPC 上繁殖，并且将 CAR+T 细胞在 CD19+aAPC（未加载 OKT3 的）上于补充有 2mmol/L L-谷氨酰胺和 10％ FBS 及 50IU/mL 的 IL-2 的 RPMI 1640（每隔一天添加）中繁殖。

[0193] 流式细胞术

[0194] 使用以下抗体：藻红蛋白（PE）抗 HLA-A2（克隆 BB7.2）、FITC 抗 -CD4（克隆 RPA-T4）、FITC 抗 -CD8（克隆 HIT8a）、PE 和 APC 抗 -CD3（克隆 SK7）、PE 抗 -CD56（克隆 B159）、PE 抗 HLA-DR（克隆 G46-6）、PE- 小鼠 IgG2bγ、PE 小鼠 IgG2aκ、FITC 非特异性小鼠 IgG1、第二试剂链霉抗生物素蛋白 -PE（全部来自 BD Biosciences）、缀合有生物素的抗 -HLA-A3（克隆 4i153）、APC 抗 -HLA-G（克隆 MEMG19）、PE 抗 HLA I 类（克隆 W6/32，来自 Abcam, Cambridge, MA）和 PE 抗 -HLA-E（克隆 3D12, Biolegend, San Diego, CA）。缀合有 Alexa 488 的抗 -CD19RCD28CAR 抗体（克隆号 136-20-1）产生于我们的实验室中。我们将添加碘化丙啶（Sigma-Aldrich）以将死亡细胞排除分析。使用 CellQuest 3.3 版（BD Biosciences）在 FACS Calibur（BD Biosciences）上获得数据，利用 FlowJo 7.6.1 版（Tree Star, Inc. Ashland, OR）分析所述数据。

[0195] Surveyor™核酸酶测定

[0196] 通过如 Guschin 等（2010）Methods Mol Biol 649:247-256 中所述的 Surveyor™核酸酶测定确定 ZFN 转染的细胞中的 HLA-A 基因序列的修饰水平。简言之，来自经 ZFN 修饰的细胞的基因组 DNA 经历利用被设计用来扩增 HLA-A2 和 HLA-A3 基因座内的 ZFN 靶区域的寡核苷酸引物的 PCR。在变性和重退火后，将 Surveyor 内切核酸酶（Cel-1）（Transgenomic, Omaha, NE）用于裂解异源双链体 DNA 产物以在聚丙烯酰胺凝胶上显现快速

移动的条带，所述条带被解释为突变事件的证据。通过光密度测定法定量百分比靶修饰。用于扩增靶基因座的 PCR 引物是：

[0197] HLA-A3 正向：5’-GGGGCCGGAGTATTGGGACCA-3’；(SEQ ID NO:7)

[0198] HLA-A3 反向：5’-CCGTCGTAGGCGTCCTGCCG-3’(SEQ ID NO:8)

[0199] HLA-A2 正向：5’-GGGTCCGGAGTATTGGGACGG-3’(SEQ ID NO:9)

[0200] HLA-A2 反向：5’-TTGCCGTCGTAGGCGTACTGGTG-3’(SEQ ID NO:10)

[0201] HLA-A2 和 HLA-A3 序列获自 IMGT/HLA 数据库，例如 IMGT/HLA 登录号：HLA-A2：HLA00005、HLA-A3：HLA00037。

[0202] ^{51}铬释放测定 (CRA)

[0203] 用 0.1mCi 的 ^{51}Cr 标记靶细胞 2 小时。在用冰冷的补充有 10％ FBS 的 RPMI 1640 洗涤 3 次后，稀释标记的细胞，随后以 10^3个靶细胞于每孔 100μL 中分配在 96 孔 v 型底板中。在肽滴定测定中，在室温下以肽的 10 倍系列稀释物孵育靶细胞，进行 30 分钟。以指定的效应子对靶的比率添加 CTL。在于 37℃、5％ CO2 中孵育 4 小时后，收集 50μL 的无细胞上清液，随后在 TopCount 仪 (Perkin Elmer,Shelton,CT) 上进行计数。以一式三份进行所有测定。在一些测定中，用 600IU/mL 的干扰素－γ (IFN-γ；R&D systems,Minneapolis,MN) 和 10ng/mL 的组织坏死因子－α (TNF-α；R&D systems) 处理亲代 HEK293 细胞系和以 HLA-A 修饰的 HEK293 克隆 48 小时，随后进行测定。如下计算百分比特异性裂解：((实验 cpm－自发 cpm)/(最大 cpm-自发 cpm))×100。

[0204] NK 细胞分离和非经典 HLA 在 721.221 细胞上的强制表达

[0205] 按照制造商的方法，利用 CD56 微珠 (Miltenyi Biotec) 和 LS 柱 (Miltenyi Biotec) 从 PBMC 分离 NK 细胞。利用 Amaxa Nucleofector II 装置（程序：A-016）将编码 SB 转座子 HLA-E（登录号 005516）和／或 HLA-G（登录号 NM_002127）的 DNA 质粒与 SB11 转座酶共电穿至亲代 HLA Ⅰ类^{low} 721.221 细胞中。在利用缀合有荧光的 mAb[PE 抗-HLA-E、APC 抗-HLA-G 和 PE 抗-HLA-G（克隆 87G,Biolegend)] 和顺磁性珠粒 [抗 PE 微珠和抗 APC 微珠（目录号 130-048-801、130-090-855(Miltenyi Biotec)] 分选 HLA-E 和／或 HLA-G 阳性细胞后，通过有限稀释衍生显示稳定且均一地表达引入的 HLA 分子的 HLA-E+ 和 HLA G+ 克隆。通过 4 小时的 CRA 评估 721.221 克隆的 NK 细胞杀伤，通过单因素 ANOVA，随后 GraphPad Prism 软件 (5 版，GraphPad Software,La Jolla,CA) 中的 Tukey 多重比较来计算数据的统计显著性。

[0206] hESC 的培养和分化

[0207] 如先前所述 (Soldner 等 (2009)Cell 136(5):964-977)，在丝裂霉素 C 灭活的小鼠胚胎成纤维细胞 (MEF) 滋养层上于 hESC 培养基 [补充有 15％ FBS、5％ KnockOut™血清替代品 (Invitrogen)、1mM 谷氨酰胺 (Invitrogen)、1％非必需氨基酸 (Invitrogen)、0.1mMβ－巯基乙醇 (Sigma,St.Louis,MO) 和 4ng/ml FGF2(R&D systems) 的 DMEM/F12(Invitrogen)] 中维持 hESC 品系 WIBR3(Whitehead Institute Center for Human Stem Cell Research,Cambridge,MA)22。如先前所述 (Hockemeyer 等 (2008)Cell Stem Cell 3(3):346-353)，将靶向 hESC 分化成成纤维细胞样细胞。简言之，通过在非贴壁悬浮培养皿 (Corning,Corning,NY) 中于补充有 15％胎牛血清的 DMEM 培养基中形成拟胚体 (EB)（进行 5 天）来诱导分化。随后将 EB 涂板在贴壁组织培养皿上，按照原代成纤维细胞方案，使用胰

蛋白酶处理至少 4 代，随后开始实验。

[0208] ZFN- 介导的 hESC 的基因组编辑

[0209] 在电穿孔之前，将 HESC 在 Rho 相关蛋白激酶 (ROCK)- 抑制剂 (Stemolecule ; Stemgent, Cambridge, MA) 中培养 24 小时。使用 0.05％胰蛋白酶 /EDTA 溶液 (Invitrogen) 收获细胞，随后重悬浮于 PBS 中。用 35μg 编码侧翼连接有与 HLA-A24 的假定的 ZFN 结合区同源的 5’和 3’臂的磷酸甘油酸激酶 (PGK) 启动子控制下的嘌令霉素抗性基因的供体质粒和 7.5μg 每一种编码 ZFN 的质粒，或 35μg 供体质粒和 10μg 的每一种编码 ZFN 的 mRNA 对 1000 万个细胞进行电穿孔 (Gene Pulser Xcell System, Bio-Rad:250V, 500μF, 0.4cm 电击杯)。随后将细胞涂板于在补充有 ROCK 抑制剂的 hESC 培养基中的 DR4MEF 滋养层上，进行前 24 小时。在电穿孔后 72 小时开始嘌呤霉素选择 (0.5μg/ml)。在电穿孔后 10 至 14 天挑拣单个抗嘌呤霉素的集落，并进行扩增。正确靶向和基因破坏通过基因组基因座的 Southern 印迹分析和测序来确认。

[0210] 实施例 2 :靶向多个内源 HLA-A 基因的锌指核酸酶的设计和验证

[0211] ZFN 被设计来裂解内源性人 HLA-A 基因的基因组编码序列内的预定位点 (见，例如，美国专利公布 2012/0060230 的表 2)。这些 ZFN 在人细胞中的表达应当通过引入的双链断裂的易错修复 (从而导致靶向 HLA 基因座的阅读框架的破坏) 来消除 HLA-A 分子的表达。为了评价这些 ZFN 破坏 HLA-A 表达的能力，我们最初使用共表达 HLA-A*03:01(HLA-A3) 和 HLA-A*02:01(HLA-A2) 的人胚胎肾细胞系 HEK293。在如实施例 1 中所述用编码的 ZFN 的表达质粒转染 HEK293 细胞后，我们使用等位基因特异性 PCR 和 Surveyor 核酸酶测定来定量预期的 ZFN 靶位点上的基因修饰的水平。

[0212] 如图 1 中所示，约 10％的 HLA-A3 基因座的和～ 6％的 HLA-A2 基因座的修饰是经修饰的 HLA-A 靶向 ZFN。

[0213] 实施例 3 :HLA-A^{neg} HEK293 细胞的分离和功能验证

[0214] 为了评估破坏 HLA-A 表达的影响，我们使用有限稀释来从经 ZFN 修饰的 HEK293 细胞库获得单细胞克隆。测序显示在 HLA-A2、HLA-A3 或这两个等位基因中的预期的 ZFN 结合位点内具有小的插入或缺失的克隆，所述插入或缺失导致移码，从而导致翻译的提前终止。由于 HEK293 细胞中的 HLA-A 表达的稳定状态水平低于造血细胞诸如 EBV 转化的类成淋巴细胞系 (EBV-LCL)，因此我们将 HEK293 细胞暴露于已知提高 HLA 水平的促炎细胞因子。见，例如，Johnson(2003)J Immunol. 170(4):1894-1902。

[0215] 干扰素 -γ (IFN-γ) 和组织坏死因子 -α (TNF-α) 的添加增加亲代 HEK293 细胞中的 HLA-A2 和 HLA-A3 表达 (图 2A，上图框)。相反地，具有 HLA-A2 和 / 或 HLA-A3 的突变的经 ZFN 处理的 HEK293 克隆即使在被 IFN-γ 和 TNF-α 诱导后也不表达这些蛋白 (图 2A，底下 3 个图框)。因此，使用对于 HLA-A2 或 HLA-A3 是特异的 mAb 的流式细胞术确定细胞表面上 HLA-A 表达的等位基因特异性丢失。

[0216] 随后，我们问经 ZFN 修饰的克隆上的 HLA-A 表达的丢失是否可避免 T 细胞识别，并使用 HLA-A3 和 HLA-A2 限制性细胞毒性 T 淋巴细胞 (CTL) 克隆来测试该设想。如所预期的，HLA-A3- 限制性 CD8+CTL 克隆 7A7 显示强劲的载有同源肽 (RVWDLPGVLK, SEQ ID NO:1, NP_001103685) 的系列稀释物的 HLA-A3+ 亲代 HEK293 细胞的特异性裂解，对于 1ng/mL 的脉冲同源肽观察到 50％的最大裂解 (图 2B，顶图框)。已失去 HLA-A2 等位基因的表

达但在 HLA-A3 上为野生型的 HEK293 克隆 8.18 也被该 HLA-A3 限制性 CTL 克隆裂解。相反地，当用相同肽脉冲时，已被编辑来消除 HLA-A3 表达的 HEK293 克隆 18.1 未被 HLA-A3 限制性 CTL 克隆 7A7 裂解，HLA-A2/A3 双敲除的 HEK293 克隆 83 也不被其裂解（图 2B，顶图框）。我们还评价 HLA-A2 限制性 CTL 克隆 GAS2B3-5 的催化活性，并且当利用亲代 HEK293 细胞或 HLA-A2 野生型克隆 18.1 速递时，观察到强劲的杀伤活性，然而经 ZFN 修饰的 HLA-$A2^{neg}$ HEK293 克隆 8.18 和 HLA-A2/A3 双敲除克隆 83 免于裂解（图 2B，底图框）。

[0217] 这些数据表明利用 ZFN 的处理完全消除 HLA-A 表达，从而导致免受 HLA-A 限制性 CTL 介导的杀伤，即使在上调内源 HLA-A 表达的促炎症条件下亦如此。

[0218] 实施例 4：针对 HLA^{null} 细胞的 NK 细胞介导的裂解可被 HLA-E 和 / 或 HLA-G 的强制表达阻止

[0219] 我们设想我们在此概述的与基于抗体的细胞分选组合的使用 ZFN 靶向 HLA I 类基因的方法，可最终用于消除 HLA-A、HLA-B 和 HLA-C 表达的表达。可通过 KIR 与其配体之间的相互作用的丢失来根除无经典 HLA（尤其是已知为杀伤抑制性受体 (KIP) 的主要配体的 HLA-B 或 HLA-C) 表达的细胞。Parham 等 (2005)Nat Rev Immunol.5(3):201-214。为了测试 NK 细胞介导的细胞毒性是否可降低，我们将非经典 HLA-E 或 HLA-G 分子（已显示所述分子降低 NK 细胞介导的细胞毒性 (Borrego 等 (1998)J Exp Med.187(5):813-818；Riteau 等 (2001)Int Immunol.13(2):193-201；Rouas-Freiss 等 (1997)Proc Natl Acad Sci U S A.94(10):5249-5254；Braud 等 (1998)Nature 391(6669):795-799) 并且多态性远少于经典 HLA 分子）引入 HLA I 类低细胞系 721.221（图 3A)，并评价它们对被 NK 细胞杀伤的易感性。

[0220] 从 PBMC 直接分离的 NK 细胞的流式细胞术分析显示超过 94％的纯化 ($CD56^{pos}CD3^{neg}$ 群体）（图 4A) 并且经遗传修饰的 721.221 克隆中的 HLA-E 和 / 或 HLA-G 表达高于 90％（图 4B)。我们证明 721.221 上的 HLA-E 和 / 或 HLA-G 的强制表达显著地使这些靶细胞免于 NK 细胞介导的裂解（图 4C)。

[0221] 这为预先阻止受者 NK 细胞对施用的 HLA^{neg} 异体移植细胞的消除，从而通过避免免疫原性转基因的引入而使得完全 HLA I 类敲除能够可行地用于人应用提供了解决务法。

[0222] 实施例 5：使用“肇事逃逸 (Hit-and-Run)”策略破坏原代 T 细胞中的 HLA-A 基因

[0223] 为了将我们的结果扩展至临床相关原代细胞，我们评价人 T 细胞中的 HLA-A 特异性 ZFN 的活性。由于 ZFN 只需要短暂表达来实现所需靶基因的稳定破坏，因此我们从体外转录的 mRNA 瞬时表达 ZFN。编码 ZFN 的 mRNA 至来自 HLA-A2 纯合供体的 PBMC(HLA-A2 是高加索人中最常见的 HLA-A 等位基因，见，例如，Mori 等 (1997)Transplantation 64(7):1017-1027) 中的电转移使～ 19％的这些 T 细胞成为 HLA-A2 阴性（图 5A，顶图框）。我们先前已证明在转染后瞬时降低孵育温度可增强 ZFN 活性。见，美国专利公布第 2011/0129898 号。

[0224] 将电穿孔的原代 T 细胞经历瞬间低温使 HLA-A2 阴性细胞的比例以 mRNA 剂量依赖性方式提高达到 57％（图 5A，底图框）。

[0225] 实施例 6：在原代 T 细胞中实现临床相关水平的 HLA-A 破坏

[0226] 为了靶向 HLA 的 ZFN 的临床应用，我们评价＂高保真＂强制异二聚化 Fok I 结构域 EL/KK（其被设计来通过阻止同二聚化 33 以减少潜在的脱靶裂解事件）的用途。编码

ZFN-L 和 ZFN-R 的 EL/KK ZFN 变体的 mRNA 的使用导致 HLA-A^{neg} T 细胞的显著增加，从而在达到 52％的 T 细胞群体中消除 HLA-A 表达，尽管使用有限剂量的 mRNA(2.5μg 每一种 ZFN)(图 5B)。

[0227] 单轮利用涂覆有抗体的顺磁性珠粒进行的 HLA-A 阳性 T 细胞耗竭容易地使 HLA-A2^{neg} T 细胞级分增加至超过 95％的群体而不影响 CD4 或 CD8 表达(图 6A)。通过 Surveyor 核酸酶测定(图 6B)和直接 DNA 测序(图 6C)分析该 HLA-A2^{neg}群体显示几乎 100％的精确地在被 ZFN 靶向的区域内的 HLA-A2 等位基因的编辑。

[0228] 这些数据一起显示 ZFN 驱动的基因组编辑可快速地产生 HLA- 阴性原代 T 细胞群。

[0229] 实施例 7:HLA-A 基因在经遗传修饰以重定向特异性的 T 细胞中的分配

[0230] 为了证明 HLA 编辑的潜在效用，我们随后聚焦于在 HLA 表达消除后被广泛用于异体移植背景中的特定类的细胞；即细胞毒性 T 细胞经遗传修饰以表达不依赖于 HLA 识别 34 将特异性重新导向肿瘤相关抗原的‘通用’嵌合抗原受体(CAR)。事实上，我们和其它研究人员目前输入患者特异性 CAR+T 细胞以研究性治疗 CD19+ 恶性肿瘤。(见，例如，Kalos 等(2011)Sci Transl Med 3(95):95ra73；Porter 等(2011)N Engl J Med 365(8):725-733)。最近，我们已公布：，当将 ZFN 用于消除内源 α β TCR 表达时，CAR+T 细胞保留对 CD19 的重定向特异性(Torikai 等(2012)Blood 119(24):5697-5705 和 Provasi 等，(2012)Nat Med 18(5):807-15)。事实上，此类 TCR 编辑的 T 细胞显示增强的体内效力和安全性(GVHD)。

[0231] 为了增强我们产生“下架”T 细胞疗法的能力，我们调查 ZFN 是否可从先前工程化以表达 CD19- 特异性 CAR 的原代 T 细胞消除 HLA-A 表达。通过来源于白花酢浆草(SB)转座子 / 转座酶体系的 DNA 质粒的同时电转移遗传修饰的，随后在 CD19+aAPC 上进行选择性繁殖的 PBMC 克隆 #439 导致 CD19- 特异性 CAR(命名为 CD19RCD28)在超过 90％的 T 细胞中表达。这些 SB 和 aAPC 平台已被我们改造来在 4 个临床试验(IND#14193、14577、14739 和 15180)中用于人应用。

[0232] 随后用编码 HLA-A ZFN 的强制异二聚变体的体外转录的 mRNA 对 CAR+T 细胞进行电穿孔。ZFN 治疗成功地破坏～ 22％的 CAR+T 细胞中的 HLA-A2 表达而无需选择，并且可通过 HLA- 阳性细胞的阴性选择容易地将该群体富集成～ 99％ HLA-A2^{neg}细胞(图 6A)。已显示自在 CD19+aAPC 上连续共培养 50 天后，这些细胞维持它们的新表型，～ 94％的 CAR+T 细胞保持 HLA-A2^{neg}。重要的是，这些 HLA-A2^{neg} T 细胞逃避了 HLA-A2 限制性 CTL 的攻击(图 6B)，并且维持它们的抗肿瘤活性，如通过 CD19+ 肿瘤靶的 CAR 依赖性裂解证明的(图 6C)。

[0233] 总的来说，这些数据表明 CAR 重定向的肿瘤特异性 T 细胞可通过 ZFN 遗传修饰来消除 HLA-A 表达。此类 HLA-A^{neg}细胞具有举能来使〞下架〞肿瘤特异性 T 细胞可从一个供体预先制备的并按需要输注至多个受者中。

[0234] 实施例 8：hESC 中的 HLA-A 基因的破坏

[0235] 为了拓宽异体移植物用于治疗应用(包括组织再生)的应用，我们寻求产生能够逃避 T 细胞识别的 hESC。按照定义，所有 hESC 对于潜在受者是异基因的，并且当分化时将上调 HLAs40 的表达。为了测试 ZFN 用于产生 HLA-A^{neg} hESC 的用途，我们利用编码 ZFN 靶向 HLA-A 基因座的 mRNA 或 DNA 质粒遗传修饰 HLA-A2+/A24+hESC 品系 WIBR3。为了促进 HLA-A^{neg} hESC 的产生，我们共递送编码侧翼连接有围绕 ZFN 靶位点的同源物的区域的嘌呤霉素抗性基因的供体 DNA 质粒以通过同源介导的修复来介导靶向整合。通过 ZFN 靶区域的

PCR 测序和通过使用位于供体同源臂外的探针的靶向区域的 Southern 印迹分析来筛选抗嘌呤霉素的克隆的 HLA-A 等位基因的修饰。这些克隆在两个 HLA-A 等位基因中的 ZFN 靶区域中都含有突变，并且与未经修饰的亲代 hESC 细胞一起分化成成纤维细胞样细胞。通过利用 IFN-γ 和 TNF-α 的处理来诱导 HLA 表达，并且通过分别用识别 HLA-A2 和 HLA-A24 的抗体进行流式细胞术来进行分析。

[0236] 虽然亲代细胞系显示两个 HLA 等位基因的强表达，但所有 3 个敲除品系不存在两个 HLA-A 等位基因的细胞表面表达（图 7）。这些数据表明针对 hESC 的 HLA-A 敲除法的可携带性－这可以是细胞移植后保持所必需的步骤。

[0237] 本文提到的所有专利、专利申请和出版物据此通过引用整体并入。

[0238] 虽然为了理解清楚的目的，已经通过举例说明和实施例的方式相当详细地提供了公开内容，但是对于本领域的技术人员显而易见的是，在不背离公开内容的精神或范围的前提下，可实践各种变化和修改。相应地，不得将前面的描述和实施例视为限制。

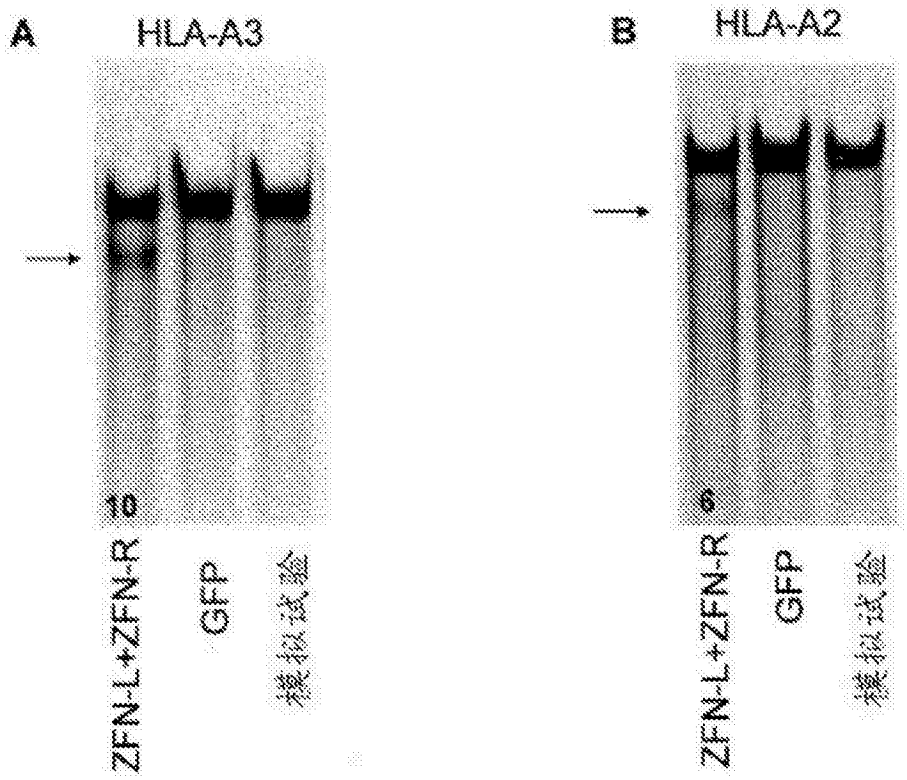


图 1

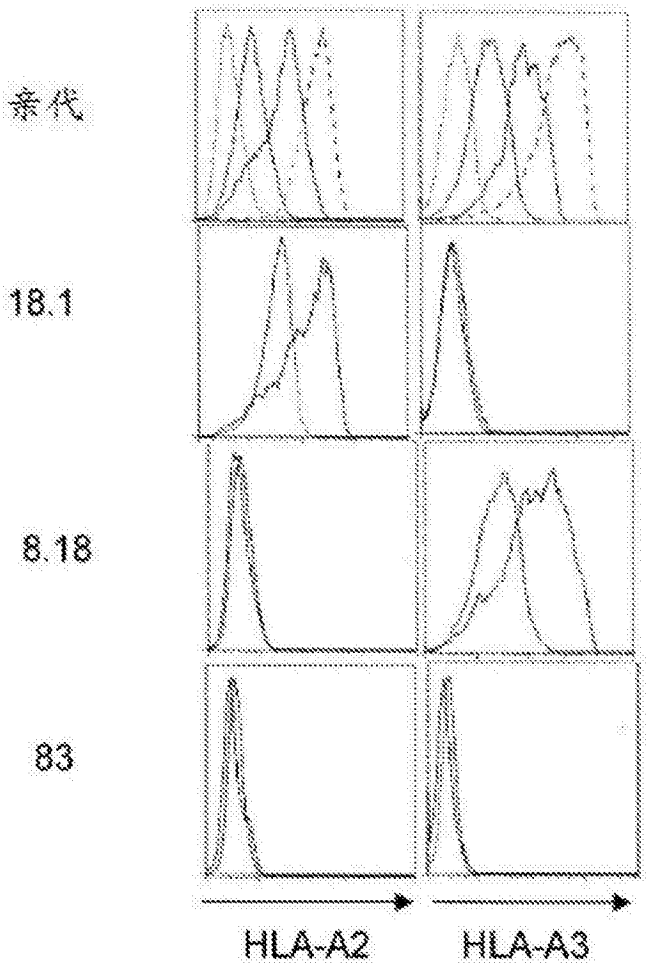


图 2A

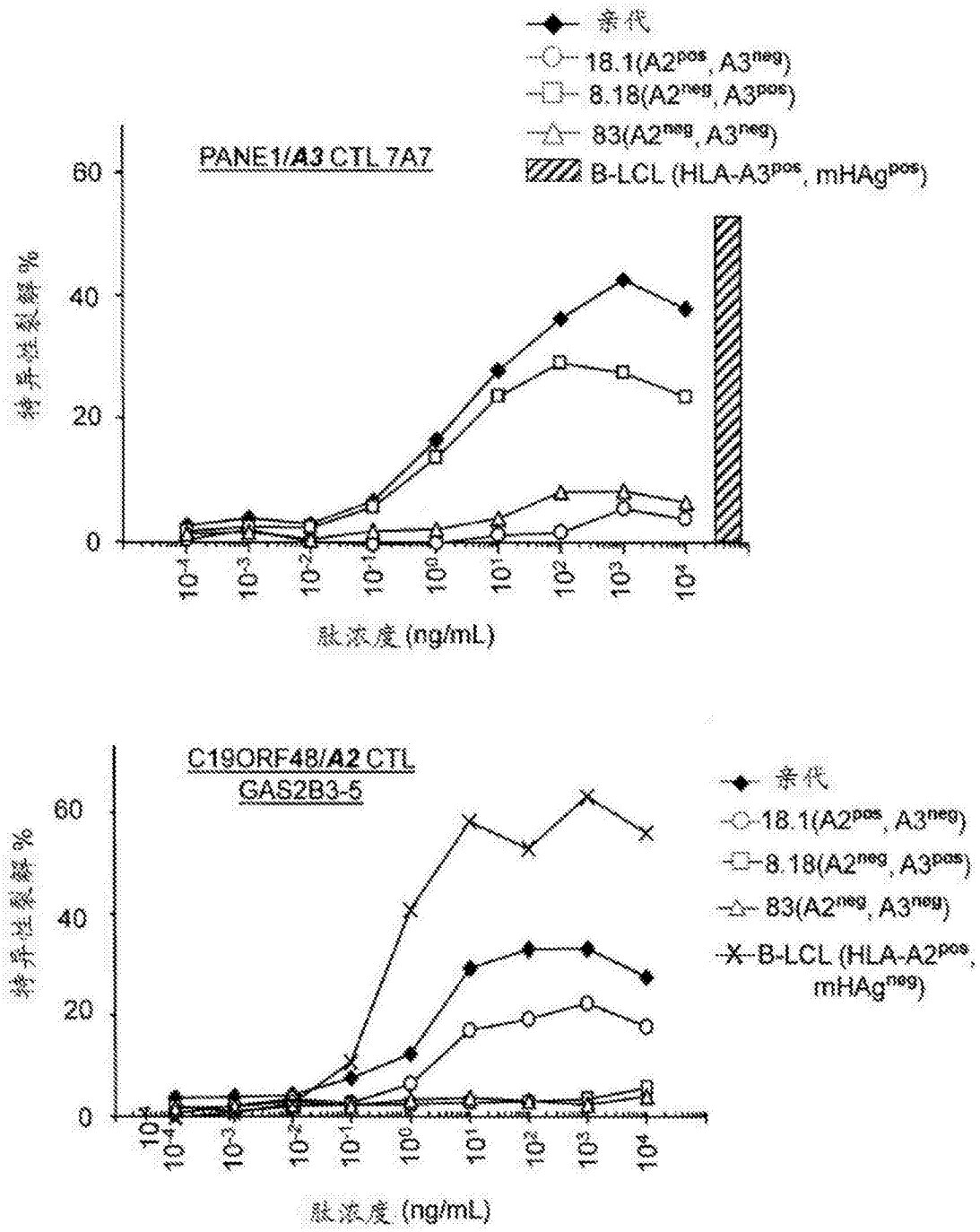

亲代
18.1(A2pos, A3neg)
8.18(A2neg, A3pos)
83(A2neg, A3neg)
B-LCL (HLA-A3pos, mHAgpos)
PANE1/A3 CTL 7A7
特异性裂解 %
肽浓度 (ng/mL)
C19ORF48/A2 CTL
GAS2B3-5
亲代
18.1(A2pos, A3neg)
8.18(A2neg, A3pos)
83(A2neg, A3neg)
B-LCL (HLA-A2pos, mHAgneg)
特异性裂解 %
肽浓度 (ng/mL)

图 2B

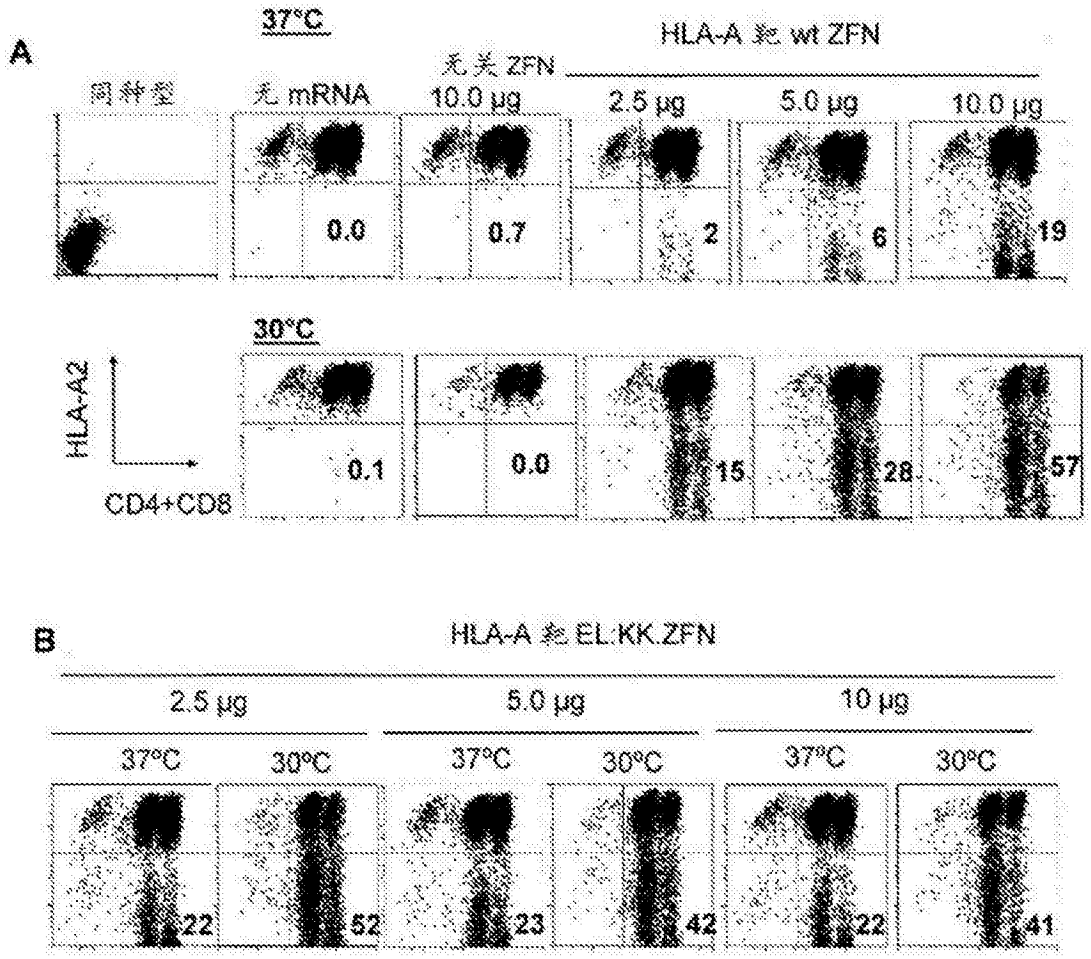

A
37°C
HLA-A 处 wt ZFN
无关 ZFN
同种型
无 mRNA
10.0 μg
2.5 μg
5.0 μg
10.0 μg
0.0
0.7
2
6
19
30°C
HLA-A2
CD4+CD8
0.1
0.0
15
28
57
B
HLA-A 处 EL:KK.ZFN
2.5 μg
5.0 μg
10 μg
37°C
30°C
37°C
30°C
37°C
30°C
22
52
23
42
22
41

图 3

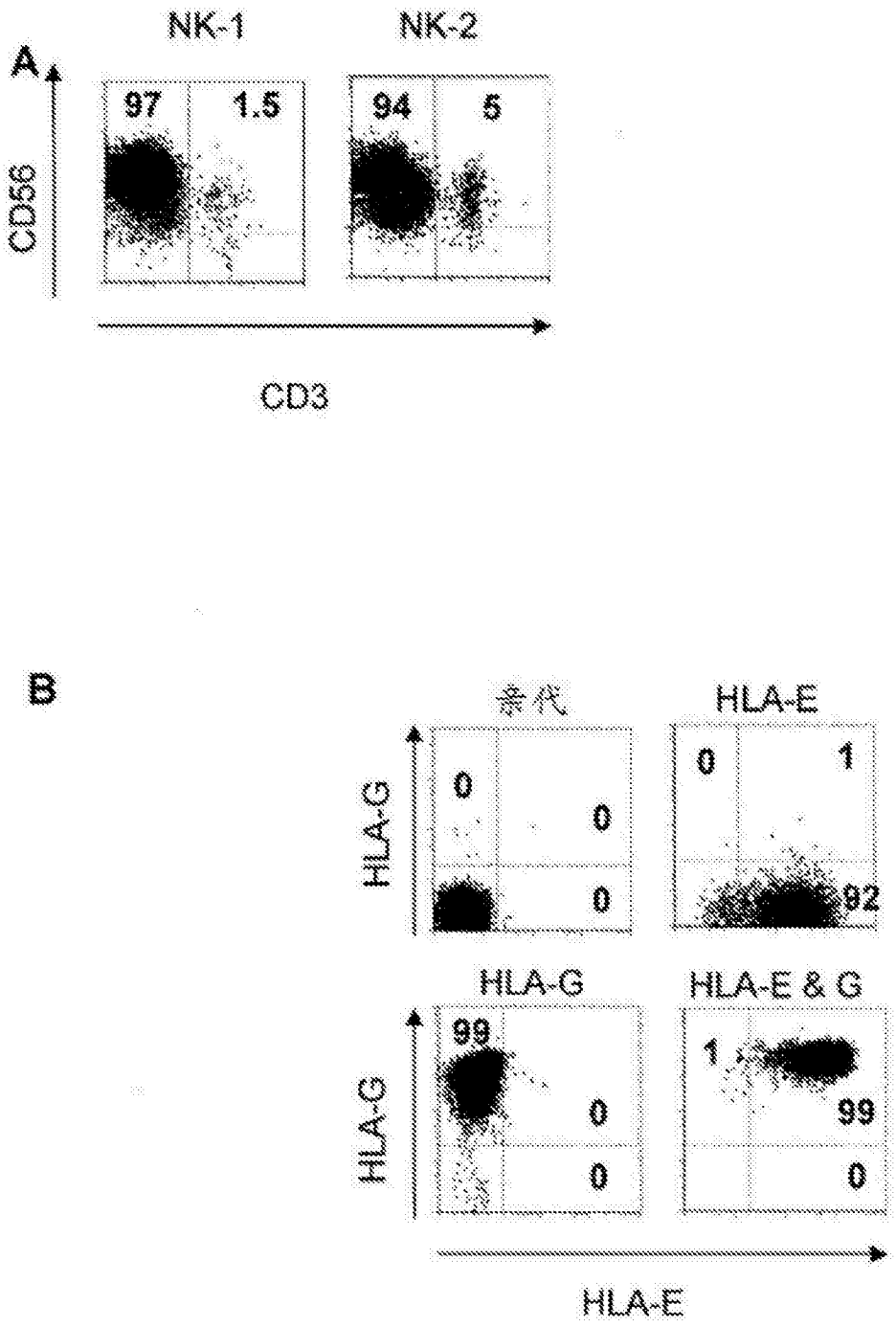

A
NK-1
NK-2
97
1.5
94
5
CD56
CD3
B
未转染
HLA-E
0
0
0
0
1
92
HLA-G
HLA-E & G
99
0
0
1
99
0
HLA-G
HLA-E

图 4

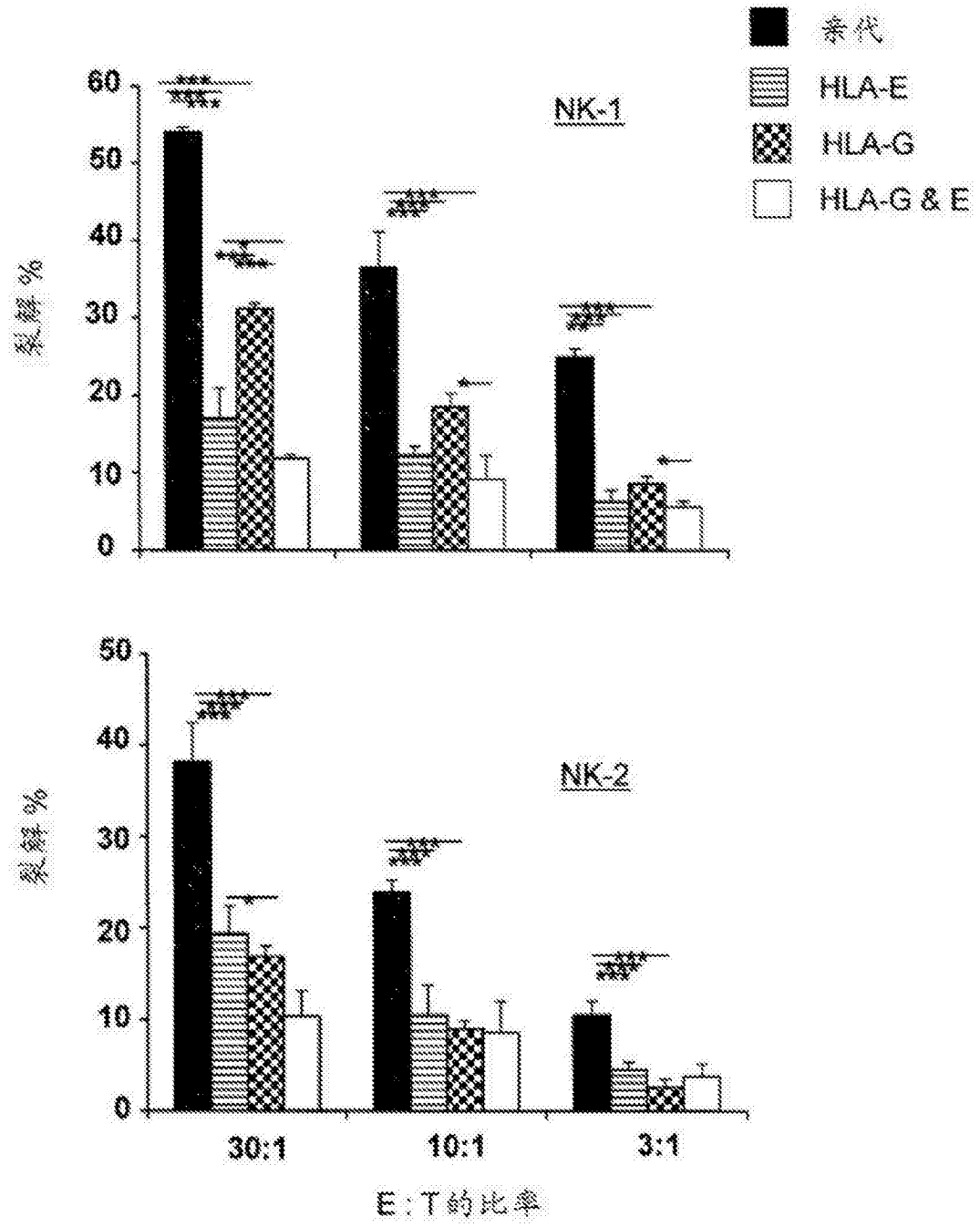


图 4C

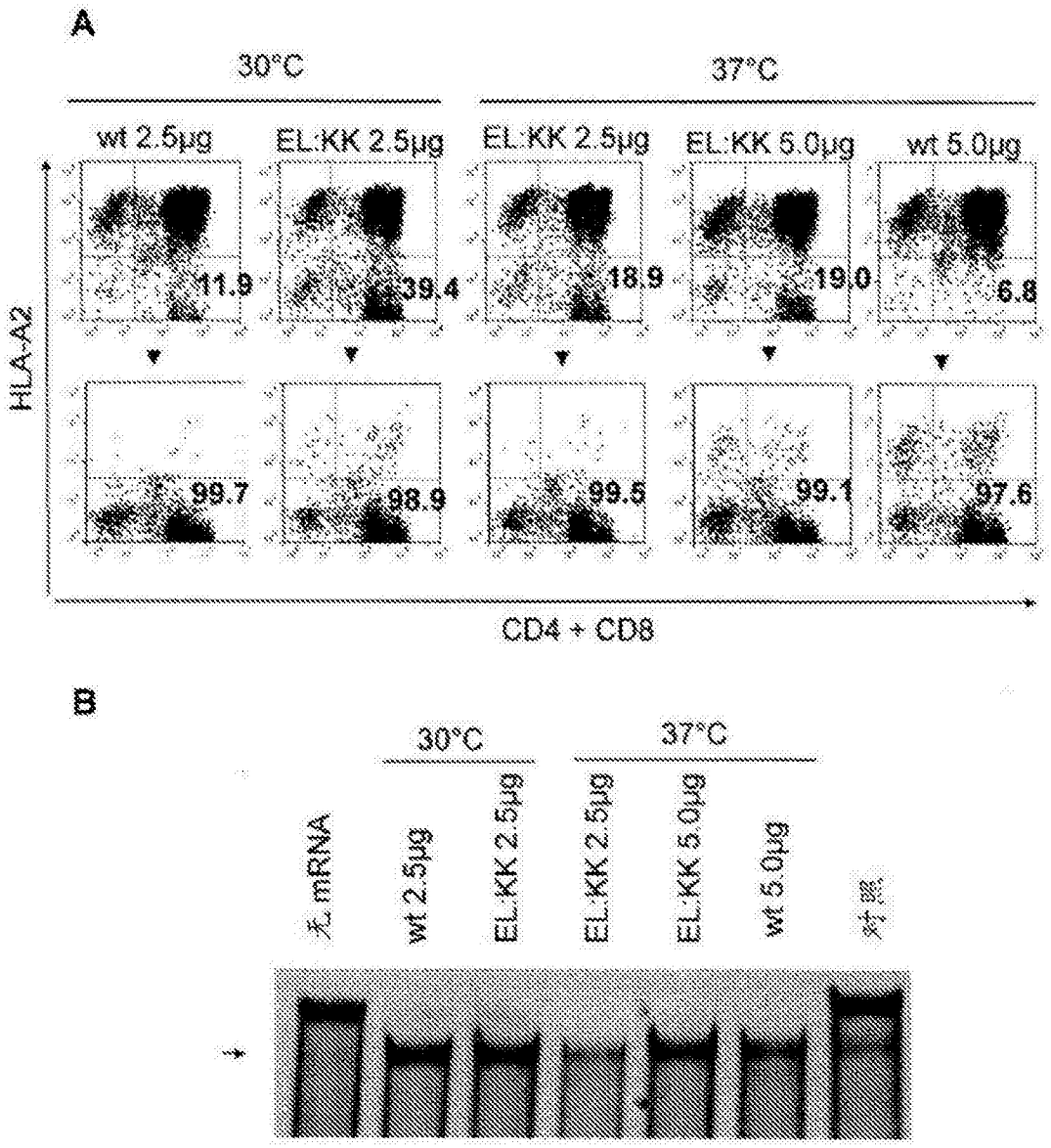

A
30°C
37°C
wt 2.5μg
EL:KK 2.5μg
EL:KK 2.5μg
EL:KK 5.0μg
wt 5.0μg
11.9
39.4
18.9
19.0
6.8
99.7
98.9
99.5
99.1
97.6
HLA-A2
CD4 + CD8
B
30°C
37°C
无 mRNA
wt 2.5μg
EL:KK 2.5μg
EL:KK 2.5μg
EL:KK 5.0μg
wt 5.0μg
对照

图 5

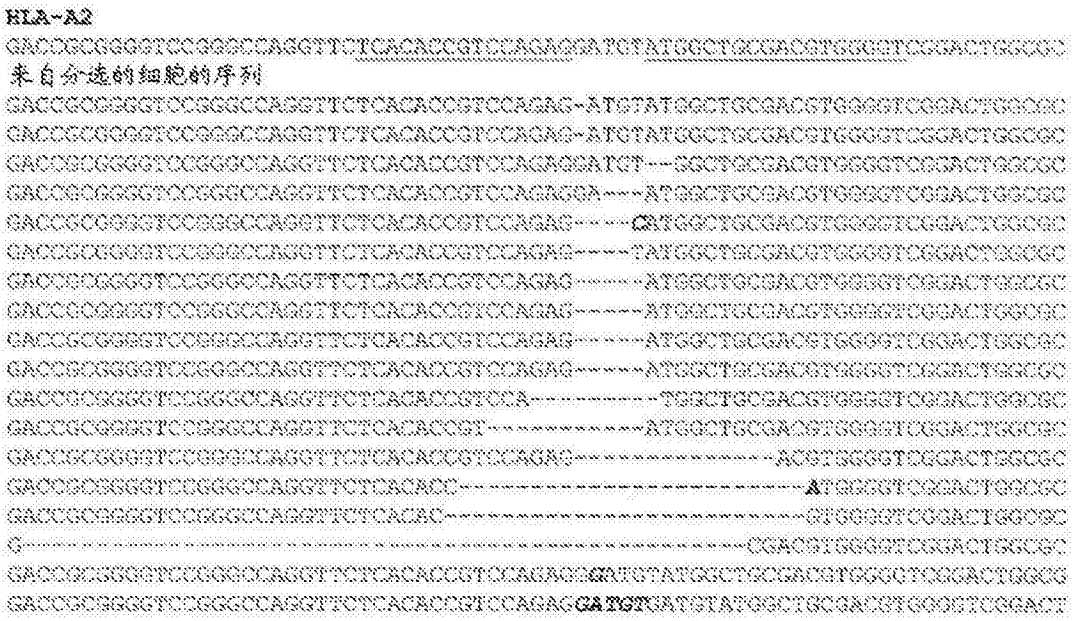


图 5C

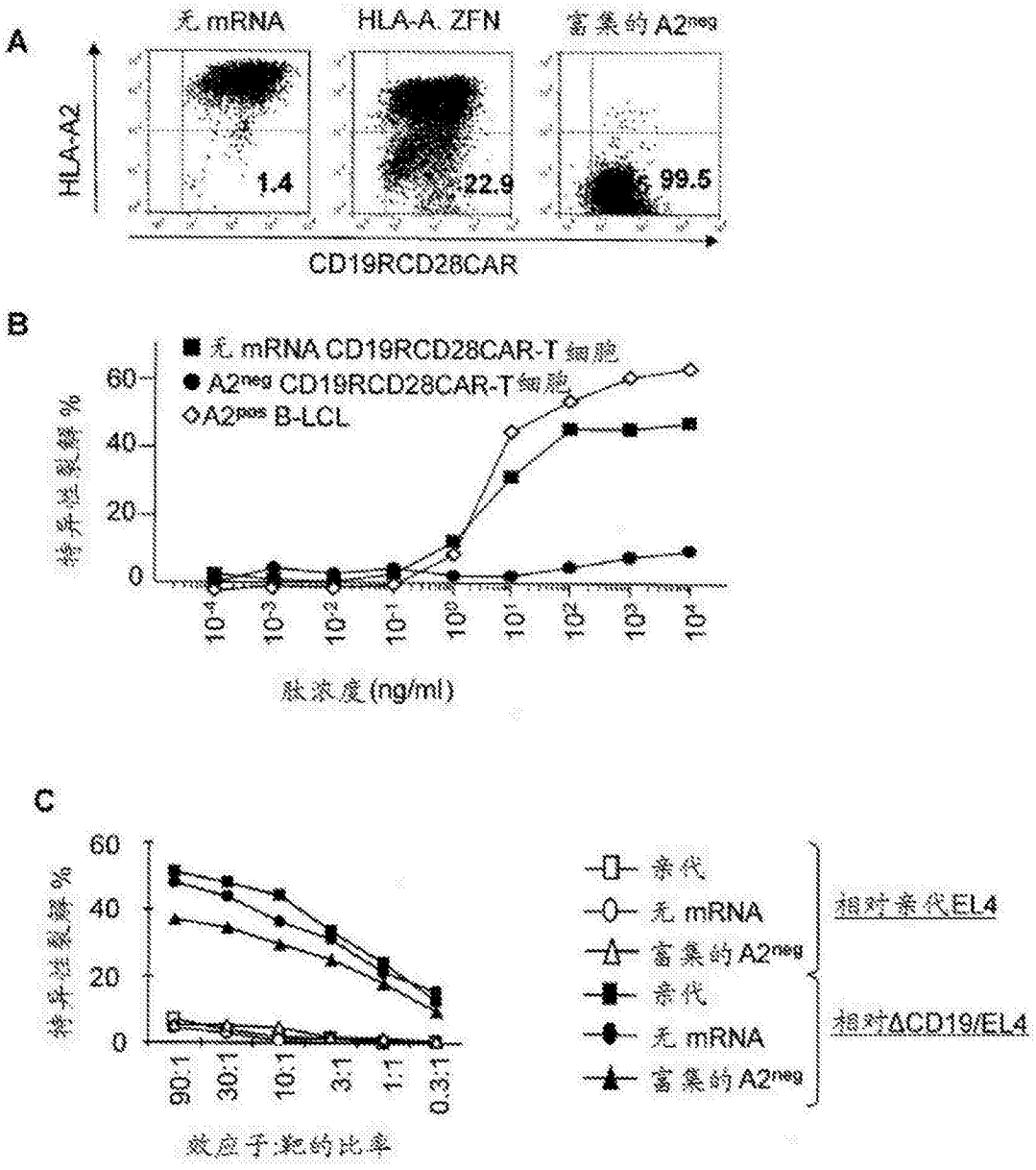

A
无 mRNA
HLA-A ZFN
富集的 A2neg
HLA-A2
1.4
22.9
99.5
CD19RCD28CAR
B
无 mRNA CD19RCD28CAR-T 细胞
A2neg CD19RCD28CAR-T 细胞
A2pos B-LCL
特异性裂解 %
60
40
20
0
10-4
10-3
10-2
10-1
100
101
102
103
104
肽浓度(ng/ml)
C
特异性裂解 %
60
40
20
0
90:1
30:1
10:1
3:1
1:1
0.3:1
效应子:靶的比率
亲代
无 mRNA
富集的 A2neg
亲代
无 mRNA
富集的 A2neg
相对亲代EL4
相对ΔCD19/EL4

图 6

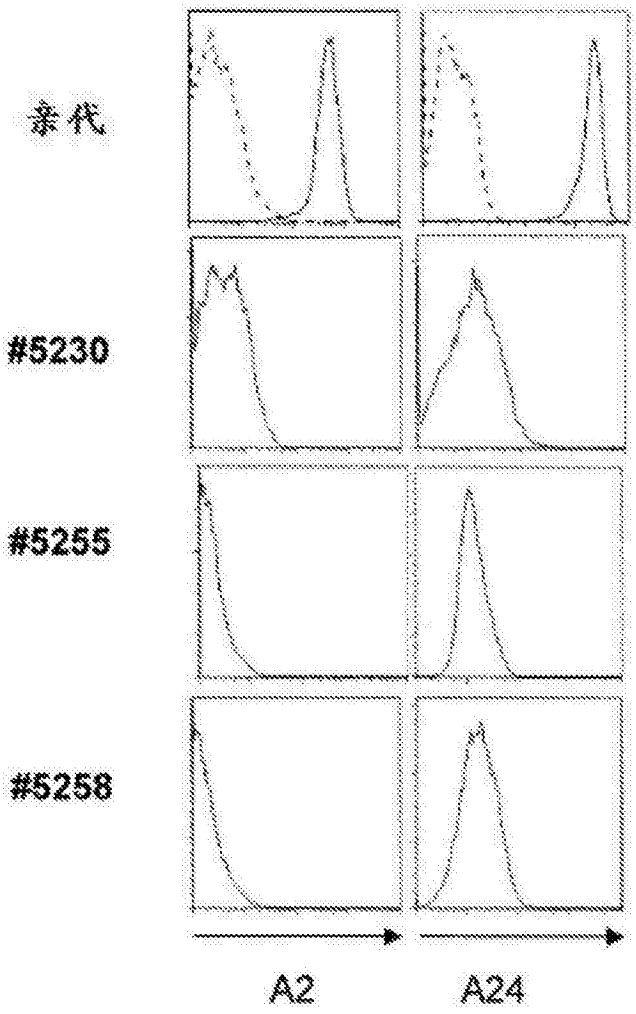


图 7