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(54) **Titre : METHODES D'INDUCTION DE LA CYTOTOXICITE CELLULAIRE DEPENDANT DES ANTICORPS (ADCC) A L'AIDE DE
CELLULES TUEUSES NATURELLES (NK) MODIFIEES**
(54) **Title: METHODS OF INDUCING ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY (ADCC) USING MODIFIED NATURAL
KILLER (NK) CELLS**

(57) **Abrégé/Abstract:**

The present disclosure is directed to the use of modified NK cells for immunotherapy in combination with an antibody, or antigen-binding fragment thereof, to induce an enhanced antibody-dependent cellular cytotoxicity (ADCC) effect.

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(54) Title: METHODS OF INDUCING ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY (ADCC) USING MODIFIED NATURAL KILLER (NK) CELLS

(57) Abstract: The present disclosure is directed to the use of modified NK cells for immunotherapy in combination with an antibody, or antigen-binding fragment thereof, to induce an enhanced antibody-dependent cellular cytotoxicity (ADCC) effect.

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**METHODS OF INDUCING ANTIBODY-DEPENDENT CELLULAR CYTOTOXICITY
(ADCC) USING MODIFIED NATURAL KILLER (NK) CELLS**

RELATED APPLICATIONS

[1] This application claims priority to U.S. Provisional Application No. 63/105,464, filed on October 26, 2020; U.S. Provisional Application No. 63/115,112, filed on November 18, 2020; and U.S. Provisional Application No. 63/165,786, filed on March 25, 2021, the entire contents of each of which are expressly incorporated herein by reference.

SEQUENCE LISTING

[2] The instant application contains a Sequence Listing which has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. Said ASCII copy, created on March 7, 2021, is named 126454-02620_SL.txt and is 415,455 bytes in size.

BACKGROUND

[3] NK cells are useful for immunotherapy approaches, for example, in the context of immunoncology. NK cells are a type of cytotoxic innate lymphocyte. NK cells play an important role in tumor immunity, and the cytotoxic activity of NK cells is tightly regulated by a network of activating and inhibitory pathways (see, *e.g.*, Bald, T., Krummel, M.F., Smyth, M.J. *et al.* (2020) *Nat Immunol* 21, 835–847; and Huntington, N.D., Cursons, J. & Rautela, J. (2020) *Nat Rev Cancer* 20, 437–454;; incorporated in their entireties herein by reference).

[4] The use of naturally occurring or modified NK cells in immunotherapy approaches, *e.g.*, via autologous or allogeneic NK cell transfer, has been reported, and while some success has been achieved, such approaches are typically characterized by a suboptimal NK cell response. In the context of immune-oncology, it is believed that this suboptimal response is, at least in part, to tumors harnessing NK cell inhibitory pathways to suppress cytotoxic NK cell activity, limit NK cell invasion, and/or inhibit NK cell proliferation and survival. Thus, application of NK cells in the therapy of solid tumors has seen limited success to date.

[5] Initial work has been performed in trying to focus NK cell response on specific cells, *e.g.*, by expressing a chimeric antigen receptor in NK cells that targets the NK cells to tumor cells, or by modulating activating or inhibitory NK cell pathways to achieve a stronger and/or more sustained NK cell response. See, *e.g.*, Liu *et al.* (2020) *New England J. Medicine* 382(6):545-553; incorporated in its entirety herein by reference.

[6] In pursuit of an off-the shelf allogeneic NK cell therapy, an induced pluripotent stem cell line has been developed in which cells express an enhanced version of CD16 (hnCD16), and NK cells

have been derived from this iPSC line. See, *e.g.*, Li et al., *Cell Stem Cell*. 2018 Aug 2;23(2):181-192.e5; incorporated in its entirety herein by reference.

[7] However, to date all of these approaches have seen limited success. Therefore, there remains a need for the development of better therapeutic approaches for immunotherapy.

SUMMARY

[8] The present disclosure provides modified NK cells (or other lymphocytes) that are useful in NK cell therapy, *e.g.*, in the context of immunotherapeutic approaches, particularly in combination with a therapeutic antibody, or antigen-binding portion thereof, to generate striking antibody-dependent cellular cytotoxicity (ADCC) effects, thereby surprisingly increasing the effectiveness of the modified NK cells in killing target cells, *e.g.* cancer cells. ADCC is a mechanism of cell-mediated immune defense, where an immune effector cell actively lyses a target cell after its membrane-surface antigens have been bound by specific antibodies. To participate in ADCC, the immune effector cells must express Fc-gamma receptors (FcγR) to be able to recognize the Fc region of the antibodies that bind to the target cells. Most immune effector cells have both activating and inhibitory FcγR. An advantage of using NK cells to target cancer cells via ADCC is that, unlike other effector cells, NK cells only have activating FcγRs (*e.g.*, FcγR IIIa, also known as CD16a, and FcγR IIc, also known as CD32c) and are believed to be the most important effectors of ADCC in humans. Thus, the use of the modified NK cells disclosed herein and antibodies targeting cancer cell-specific antigens to elicit ADCC provides novel and surprisingly effective immunotherapies.

[9] In some embodiments, the modified NK cells provided herein can serve as an off-the-shelf clinical solution for patients having, or having been diagnosed with, a hyperproliferative disease, such as, for example, a cancer. In some embodiments, the modified NK cells exhibit an enhanced survival, proliferation, NK cell response level, NK cell response duration, resistance against reduction of NK cell functional persistence, and/or target recognition as compared to non-modified NK cells. For example, the modified NK cells provided herein may comprise genomic edits that result in a loss-of-function in TGF beta receptor 2 (TGFbetaR2) and/or a loss-of-function of CISH. In some embodiments, the modified NK cells comprise genomic edits that result in a loss-of-function of TGFbetaR2. In some embodiments, the modified NK cells comprise genomic edits that result in a loss-of-function of CISH. In some embodiments, the modified NK cells comprise genomic edits that result in a loss-of-function of TGFbetaR2 and a loss-of-function of CISH. In some embodiments, the modified NK cells consist of genomic edits that result in a loss-of-function of TGFbetaR2. In some embodiments, the modified NK cells consist of genomic edits that result in a loss-of-function of CISH. In some embodiments, the modified NK cells consist of genomic edits that result in a loss-of-function of TGFbetaR2 and a loss-of-function of CISH. Other modified NK cells that may be useful in the methods described herein are described in WO2020/168300, published on 17 September 2020, the entire contents of which are expressly incorporated by reference herein.

[10] In some embodiments, the modified NK cells provided herein may comprise genomic edits that result in: expression of a chimeric antigen receptor (CAR) of interest, *e.g.*, a CAR targeting mesothelin, EGFR, HER2 and/or MICA/B; expression of a CD16 variant, *e.g.*, a non-naturally occurring CD16 variant such as, for example, hnCD16 (see, *e.g.*, Zhu *et al.*, Blood 2017, 130:4452, the contents of which are incorporated herein in their entirety by reference); expression of an IL15/IL15RA fusion; a loss-of-function in TGF beta receptor 2 (TGFbetaR2); and/or expression of a dominant-negative variant of TGFbetaR2; a loss-of-function of ADORA2A; a loss-of-function of B2M; expression of HLA-G; a loss-of-function of a CIITA ; a loss-of-function of a PD1 ; a loss-of-function of TIGIT; and/or a loss-of-function of CISH; or any combination of two or more thereof in the modified NK cell. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of TGFbetaR2 and a loss-of-function of CISH. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of TGFbetaR2 and a loss-of-function of TIGIT. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of TGFbetaR2 and a loss-of-function of ADORA2A. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of TGFbetaR2 and a loss-of-function of NKG2A. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of CISH and a loss-of-function of TIGIT. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of CISH and a loss-of-function of ADORA2A. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of CISH and a loss-of-function of NKG2A. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of TIGIT and a loss-of-function of ADORA2A. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of TIGIT and a loss-of-function of NKG2A. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of ADORA2A and a loss-of-function of NKG2A. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of TGFbetaR2, a loss-of-function of CISH, and a loss-of-function of TIGIT. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of TGFbetaR2, a loss-of-function of CISH, and a loss-of-function of ADORA2A. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of TGFbetaR2, a loss-of-function of CISH, and a loss-of-function of NKG2A. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of TGFbetaR2, a loss-of-function of TIGIT, and a loss-of-function of ADORA2A. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of TGFbetaR2, a loss-of-function of TIGIT, and a loss-of-function of NKG2A. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of TGFbetaR2, a loss-of-function of ADORA2A, and a loss-of-function of NKG2A. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of CISH, a loss-of-function of TIGIT, and a loss-of-function of ADORA2A. In one embodiment, the

modified NK cell comprises genomic edits that result in a loss-of-function of CISH, a loss-of-function of TIGIT, and a loss-of-function of NKG2A. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of CISH, a loss-of-function of ADORA2A, and a loss-of-function of NKG2A. In one embodiment, the modified NK cell comprises genomic edits that result in a loss-of-function of TIGIT, a loss-of-function of ADORA2A, and a loss-of-function of NKG2A.

[11] In some embodiments, the modified NK cells provided herein may comprise genomic edits that result in: expression of an exogenous a CD16 variant, *e.g.*, hnCD16, expression of an exogenous IL15/IL15RA fusion, expression of an exogenous HLA-G, expression of an exogenous DN-TGFBetaR2, a loss of function in TGFBetaR2, a loss of function in B2M, a loss of function of PD1, a loss of function of TIGIT, and/or a loss of function of ADORA2A.

[12] In some embodiments, the modified NK cells provided herein may comprise genomic edits that result in: expression of an exogenous a CD16 variant, *e.g.*, hnCD16, expression of an exogenous IL15/IL15RA fusion, expression of an exogenous HLA-G, expression of an exogenous DN-TGFBetaR2, expression of a soluble MICA and/or MICB, a loss of function in TGFBetaR2, a loss of function in B2M, a loss of function of PD1, a loss of function of TIGIT, and/or a loss of function of ADORA2A.

[13] In some embodiments, the modified NK cells provided herein may comprise genomic edits that result in: expression of an exogenous a CD16 variant, *e.g.*, hnCD16, expression of an exogenous IL15/IL15RA fusion, expression of an exogenous HLA-G, expression of an exogenous DN-TGFBetaR2, expression of a soluble MICA and/or MICB, expression of an exogenous IL-12, expression of an exogenous IL-18, a loss of function in TGFBetaR2, a loss of function in B2M, a loss of function of PD1, a loss of function of TIGIT, and/or a loss of function of ADORA2A.

[14] In some embodiments, the modified NK cells provided herein may comprise genomic edits that result in: expression of an exogenous a CD16 variant, *e.g.*, hnCD16, expression of an exogenous IL15/IL15RA fusion, expression of an exogenous HLA-G, expression of an exogenous DN-TGFBetaR2, expression of an exogenous IL-12, expression of an exogenous IL-18, a loss of function in TGFBetaR2, a loss of function in B2M, a loss of function of PD1, a loss of function of TIGIT, and/or a loss of function of ADORA2A.

[15] In some embodiments, the disclosure features a modified NK cell, wherein the modified NK cell does not express endogenous CD3, CD4, and/or CD8; and expresses at least one endogenous gene encoding: (i) CD56 (NCAM), CD49, and/or CD45; (ii) NK cell receptor (cluster of differentiation 16 (CD16)); (iii) natural killer group-2 member D (NKG2D); (iv) CD69; (v) a natural cytotoxicity receptor; or any combination of two or more thereof; wherein the modified NK cell further: (1) comprises at least one exogenous nucleic acid construct encoding: (i) a chimeric antigen receptor (CAR); (ii) a non-naturally occurring variant of immunoglobulin gamma Fc region receptor III (FcγRIII, CD16); (iii) interleukin 15 (IL-15); (iv) IL-15 receptor (IL-15R), or a variant thereof; (v) interleukin 12 (IL-12); (vi) interleukin-12 receptor (IL-12R), or a variant thereof; (vii) human

leukocyte antigen G (HLA-G); (viii) human leukocyte antigen E (HLA-E); (ix) a nucleic acid sequence encoding leukocyte surface antigen cluster of differentiation CD47 (CD47); or any combination of two or more thereof; and/or (2) exhibits a loss of function of at least one of: (i) transforming growth factor beta receptor 2 (TGF β R2); (ii) adenosine A2a receptor (ADORA2A); (iii) T cell immunoreceptor with Ig and ITIM domains (TIGIT); (iv) β -2 microglobulin (B2M); (v) programmed cell death protein 1 (PD-1); (vi) cytokine inducible SH2 containing protein (CISH); (vii) class II, major histocompatibility complex, transactivator (CIITA); (viii) natural killer cell receptor NKG2A (natural killer group 2A); (ix) two or more HLA class II histocompatibility antigen alpha chain genes, and/or two or more HLA class II histocompatibility antigen beta chain genes; (x) cluster of differentiation 32B (CD32B, FCGR2B); (xi) T cell receptor alpha constant (TRAC); or any combination of two or more thereof. In one embodiment, the modified NK cell exhibits a loss of function of TGF β R2 and a loss-of-function of CISH. In one embodiment, the modified NK cell exhibits a loss-of-function of TGFbetaR2 and a loss-of-function of TIGIT. In one embodiment, the modified NK cell exhibits a loss-of-function of TGFbetaR2 and a loss-of-function of ADORA2A. In one embodiment, the modified NK cell exhibits a loss-of-function of TGFbetaR2 and a loss-of-function of NKG2A. In one embodiment, the modified NK cell exhibits a loss-of-function of CISH and a loss-of-function of TIGIT. In one embodiment, the modified NK cell exhibits a loss-of-function of CISH and a loss-of-function of ADORA2A. In one embodiment, the modified NK cell exhibits a loss-of-function of CISH and a loss-of-function of NKG2A. In one embodiment, the modified NK cell exhibits a loss-of-function of TIGIT and a loss-of-function of ADORA2A. In one embodiment, the modified NK cell exhibits a loss-of-function of TIGIT and a loss-of-function of NKG2A. In one embodiment, the modified NK cell exhibits a loss-of-function of ADORA2A and a loss-of-function of NKG2A. In one embodiment, the modified NK cell exhibits a loss-of-function of TGFbetaR2, a loss-of-function of CISH, and a loss-of-function of TIGIT. In one embodiment, the modified NK cell exhibits a loss-of-function of TGFbetaR2, a loss-of-function of CISH, and a loss-of-function of ADORA2A. In one embodiment, the modified NK cell exhibits a loss-of-function of TGFbetaR2, a loss-of-function of CISH, and a loss-of-function of NKG2A. In one embodiment, the modified NK cell exhibits a loss-of-function of TGFbetaR2, a loss-of-function of TIGIT, and a loss-of-function of ADORA2A. In one embodiment, the modified NK cell exhibits a loss-of-function of TGFbetaR2, a loss-of-function of TIGIT, and a loss-of-function of NKG2A. In one embodiment, the modified NK cell exhibits a loss-of-function of TGFbetaR2, a loss-of-function of ADORA2A, and a loss-of-function of NKG2A. In one embodiment, the modified NK cell exhibits a loss-of-function of CISH, a loss-of-function of TIGIT, and a loss-of-function of ADORA2A. In one embodiment, the modified NK cell exhibits a loss-of-function of CISH, a loss-of-function of TIGIT, and a loss-of-function of NKG2A. In one embodiment, the modified NK cell exhibits a loss-of-function of CISH, a loss-of-function of ADORA2A, and a loss-of-function of NKG2A. In one embodiment, the modified NK cell

exhibits a loss-of-function of TIGIT, a loss-of-function of ADORA2A, and a loss-of-function of NKG2A.

[16] In one embodiment, the modified NK cell does not express endogenous CD3, CD4, and/or CD8; and expresses at least one endogenous gene encoding: (i) CD56 (NCAM), CD49, and/or CD45; (ii) NK cell receptor (cluster of differentiation 16 (CD16)); (iii) natural killer group-2 member D (NKG2D); (iv) CD69; (v) a natural cytotoxicity receptor; or any combination of two or more thereof; wherein the modified NK cell further: (1) comprises at least one exogenous nucleic acid construct encoding: (i) a chimeric antigen receptor (CAR); (ii) a non-naturally occurring variant of immunoglobulin gamma Fc region receptor III (FcγRIII, CD16); (iii) interleukin 15 (IL-15); (iv) IL-15 receptor (IL-15R), or a variant thereof; (v) interleukin 12 (IL-12); (vi) interleukin-12 receptor (IL-12R), or a variant thereof; (vii) human leukocyte antigen G (HLA-G); (viii) human leukocyte antigen E (HLA-E); (ix) a nucleic acid sequence encoding leukocyte surface antigen cluster of differentiation CD47 (CD47); or any combination of two or more thereof; and/or (2) exhibits a loss of function of transforming growth factor beta receptor 2 (TGFβR2), cytokine inducible SH2 containing protein (CISH), or a combination thereof.

[17] In some embodiments, the modified NK cells comprise genomic edits that result in: expression of a CD16 variant, *e.g.*, a non-naturally occurring CD16 variant such as, for example, hnCD16 (see, *e.g.*, Zhu *et al.*, Blood 2017, 130:4452, the contents of which are incorporated herein in their entirety by reference); expression of an IL15/IL15RA fusion; a loss-of-function in TGF beta receptor 2 (TGFβR2); and a loss-of-function of CISH.

[18] In another aspect, disclosed herein is a method of treating cancer in a subject, the method comprising administering to the subject a modified natural killer (NK) cell and a molecule comprising an Fc domain that binds cancer cells, *e.g.*, an antibody, or an antigen-binding portion thereof, wherein the modified NK cell exhibits a loss of function of transforming growth factor beta receptor 2 (TGFβR2) and cytokine inducible SH2 containing protein (CISH), wherein the administering induces ADCC of a cancer cell in the subject, thereby treating the cancer in the subject.

[19] In one aspect, disclosed herein is a method of inducing antibody-dependent cell-mediated cytotoxicity (ADCC) of a cancer cell, the method comprising contacting the cancer cell with a modified natural killer (NK) cell and a molecule comprising an Fc domain that binds cancer cells, *e.g.*, an antibody, or antigen-binding portion thereof, wherein the modified NK cell exhibits a loss of function of transforming growth factor beta receptor 2 (TGFβR2) and cytokine inducible SH2 containing protein (CISH), thereby inducing ADCC of the cancer cell. In one embodiment, the contact is *in vivo* in a subject.

[20] In one embodiment, the administration increases ADCC or enhances ADCC. In one embodiment, the administration increases ADCC by at least about 10%, at least about 15%, 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at

least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, at least about 100%, at least about 125%, at least about 150%, at least about 175%, at least about 2-fold, at least about 3-fold, at least about 4-fold, at least about 5-fold, at least about 6-fold, at least about 7-fold, at least about 8-fold, at least about 9-fold, or at least about 10-fold as compared to ADCC of a cancer cell using an unmodified NK cell and the antibody.

[21] In another embodiment, the administering decreases tumor volume in the subject by at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, or at least about 95% after administering. In one embodiment, the administration decreases tumor volume in the subject at the values listed above at least about 5 days, 7 days, 10 days, 14 days, 21 days, 30 days, 1 month, 40 days, two months, three months, four months, five months, six months, seven months, eight months, nine months, ten months, eleven months, one year after administering.

[22] In one embodiment, the administering increases the survival time of the subject. In one embodiment, the survival time of the subject is increased by at least about two-fold, about three-fold, about four-fold, or about five-fold as compared to a subject, *e.g.*, comparator subject, who has not been administered the modified NK cell and the antibody; by at least about two-fold, about three-fold, about four-fold, or about five-fold as compared to a subject, *e.g.*, comparator subject, who has been administered the antibody alone; and/or by at least about 50% about 75%, about 100%, about 150%, about two-fold, about three-fold, about four-fold, or about five-fold as compared to a subject, *e.g.*, comparator subject, who has been administered the modified NK cell alone. In one embodiment, the comparator subject is a subject with the same type of cancer cell as the subject. In one embodiment, the comparator subject is a subject with the same type of cancer cell as the subject and a comparable tumor burden as the subject. In one embodiment, the survival time of the comparator subject is an average survival time calculated from a population of subjects having the same type of cancer cell, and/or the same stage of cancer, and/or the same amount of tumor burden as the subject.

[23] In one embodiment, the contacting is *in vitro*. In one embodiment, the contacting is in a subject.

[24] In one embodiment, the administration increases a level of TNF α by at least about two fold, at least about three-fold, at least about four-fold, or at least about five-fold as compared to a control level expression of TNF α . In one embodiment, the control level of TNF α is a level of TNF α produced by an unmodified NK cell under the same conditions. In another embodiment, the control level of TNF α is a reference level of TNF α . In one embodiment, the modified NK cell comprises an increase in level of TNF α by at least about two fold as compared to a control level expression of TNF α , wherein the control level of TNF α is a level of TNF α produced by an unmodified NK cell under the same

conditions. In one embodiment, the modified NK cell comprises an increase in level of $\text{TNF}\alpha$ by at least about three fold as compared to the control level expression of $\text{TNF}\alpha$.

[25] In one embodiment, the administration increases a level of $\text{IFN}\gamma$ by at least about two fold, at least about three-fold, at least about four-fold, or at least about five-fold as compared to a control level expression of $\text{IFN}\gamma$. In one embodiment, the control level of $\text{IFN}\gamma$ is a level of $\text{IFN}\gamma$ produced by an unmodified NK cell under the same conditions. In another embodiment, the control level of $\text{IFN}\gamma$ is a reference level of $\text{IFN}\gamma$. In one embodiment, the modified NK cell comprises an increase in level of $\text{IFN}\gamma$ by at least about two fold as compared to a control level expression of $\text{IFN}\gamma$, wherein the control level of $\text{IFN}\gamma$ is a level of $\text{IFN}\gamma$ produced by an unmodified NK cell under the same conditions. In one embodiment, the modified NK cell comprises an increase in level of $\text{IFN}\gamma$ by at least about three fold as compared to the control level expression of $\text{IFN}\gamma$.

[26] In one embodiment, the administration decreases normalized total integrated red object intensity in a tumor spheroid assay by at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95% or 100% as compared to a control level of normalized total integrated red object intensity, wherein the control level of normalized total integrated red object intensity is a level of normalized total integrated red object intensity produced using an unmodified NK cell under the same conditions. In one embodiment, the modified NK cell comprises a decrease in normalized total integrated red object intensity in a tumor spheroid assay by at least about 20% as compared to a control level of normalized total integrated red object intensity. In one embodiment, the control level of normalized total integrated red object intensity is a level of normalized total integrated red object intensity produced using an unmodified NK cell under the same conditions. In one embodiment, the modified NK cell comprises a decrease in normalized total integrated red object intensity in the tumor spheroid assay by at least about 25%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 75%, or about 100% as compared to the control level of normalized total integrated red object intensity.

[27] In one embodiment, the administration increases a level of a cytolytic granule produced by the modified NK cell by at least about two fold, at least about three fold, at least about four fold, at least about five fold, at least about ten fold, or at least about twenty fold as compared to a control level expression of the cytolytic granule. In one embodiment, the control level of cytolytic granule is a level of cytolytic granule produced by an unmodified NK cell under the same conditions. In another embodiment, the control level of a cytolytic granule is a reference level of cytolytic granule. In one embodiment, the cytolytic granule is selected from the group consisting of GZMB, GZMA and GZMH. In one embodiment, the modified NK cell comprises an increase in level of a cytolytic granule by at least about two fold as compared to a control level expression of the cytolytic granule. In one embodiment, the cytolytic granule is selected from the group consisting of GZMB, GZMA and

GZMH. In one embodiment, the control level of cytolytic granule is a level of cytolytic granule produced by an unmodified NK cell under the same conditions. In one embodiment, the modified NK cell comprises an increase in level of the cytolytic granule by at least about three fold as compared to the control level expression of the cytolytic granule.

[28] In one embodiment, the administration increases a level of a cytolytic granule produced by the modified NK cell by at least about one hour, at least about two hours, at least about three hours, at least about four hours, or at least about five hours earlier as compared to a control level expression of the cytolytic granule. In one embodiment, the control level of cytolytic granule is a level of cytolytic granule produced by an unmodified NK cell under the same conditions. For example, the administration increases the level of the cytolytic granule produced by the modified NK cell by at least about one hour, at least about two hours, at least about three hours, at least about four hours, or at least about five hours earlier as compared to an observed increase in the level of the cytolytic granule produced by the unmodified NK cell under the same conditions. In another embodiment, the control level of a cytolytic granule is a reference level of cytolytic granule. In one embodiment, the cytolytic granule is selected from the group consisting of GZMB, GZMA and GZMH.

[29] In one embodiment, the administration increases a production rate of a cytolytic granule by the modified NK cell by at least about two fold, at least about three fold, at least about four fold, or at least about five fold as compared to a control production rate of the cytolytic granule. In one embodiment, the control production rate of cytolytic granule is a production rate of cytolytic granule by an unmodified NK cell under the same conditions. In another embodiment, the control production rate of a cytolytic granule is a reference production rate of cytolytic granule. In one embodiment, the cytolytic granule is selected from the group consisting of GZMB, GZMA and GZMH. In one embodiment, the modified NK cell comprises an increase in production rate of a cytolytic granule by at least about two fold as compared to a control production rate of the cytolytic granule. In one embodiment, the cytolytic granule is selected from the group consisting of GZMB, GZMA and GZMH. In one embodiment, the control production rate of cytolytic granule is a production rate of cytolytic granule by an unmodified NK cell under the same conditions. In one embodiment, the modified NK cell comprises an increase in production rate of the cytolytic granule by at least about three fold as compared to the control production rate of the cytolytic granule.

[30] In one embodiment, the administration increases a level of CD107a in the modified NK cells by at least about two fold, at least about three fold, at least about four fold, or at least about five fold as compared to a control level expression of CD107a. In one embodiment, the control level of CD107a is a level of CD107a in an unmodified NK cell under the same conditions. In another embodiment, the control level of CD107a is a reference level of CD107a. In one embodiment, the modified NK cell comprises an increase in level of CD107a by at least about two fold as compared to a control level expression of CD107a. In one embodiment, the control level of CD107a is a level of CD107a in an unmodified NK cell under the same conditions. In one embodiment, the modified NK

cell comprises an increase in level of CD107a by at least about three fold as compared to the control level expression of CD107a.

[31] In one embodiment, the cytotoxicity activity of the modified NK cell under a nutrient-depriving condition is at least about 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95% or 100% higher as compared to a control level of cytotoxicity activity, wherein the control level of cytotoxicity activity is a cytotoxicity level of an unmodified NK cell under the same conditions. In one embodiment, the modified NK cell comprises an increase in cytotoxicity activity under a nutrient-depriving condition by at least about 20% as compared to a control level of cytotoxicity activity. In one embodiment, the control level of cytotoxicity activity is a cytotoxicity level of an unmodified NK cell under the same conditions. In one embodiment, the modified NK cell comprises an increase in cytotoxicity activity under the nutrient-depriving condition by at least about 25%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 75% or about 100% as compared to the control level of cytotoxicity activity.

[32] In one embodiment, the spare respiratory capacity of the modified NK cell is at least 20%, at least about 25%, at least about 30%, at least about 35%, at least about 40%, at least about 45%, at least about 50%, at least about 55%, at least about 60%, at least about 65%, at least about 70%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95% or 100% higher as compared to a control level of spare respiratory capacity, wherein the control level of spare respiratory capacity is a level of spare respiratory capacity of an unmodified NK cell under the same conditions. In one embodiment, the modified NK cell comprises an increase in spare respiratory capacity by at least 20% as compared to a control level of spare respiratory capacity. In one embodiment, the control level of spare respiratory capacity is a level of spare respiratory capacity of an unmodified NK cell under the same conditions. In one embodiment, the modified NK cell comprises an increase in spare respiratory capacity by at least about 25%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 75% or about 100% as compared to the control level of spare respiratory capacity.

[33] In one embodiment, the molecule comprising an Fc domain that binds cancer cells, *e.g.*, antibody, or antigen-binding portion thereof, binds epidermal growth factor receptor (EGFR), HER2, or CD20. In one embodiment, the antibody is cetuximab, trastuzumab, or rituximab, or an antigen-binding portion thereof.

[34] In one embodiment, the modified NK cell is administered concurrently with the antibody. In one embodiment, the antibody is administered prior to the modified NK cell. In one embodiment, the antibody is administered 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, or 2 weeks prior to the modified NK cell. In one embodiment, the modified NK cell is administered prior to the antibody. In one embodiment, the modified NK cell is

administered 1 day, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 8 days, 9 days, 10 days, 11 days, 12 days, 13 days, or 2 weeks prior to the antibody. In another embodiment, the modified NK cell is administered once, and the antibody is administered at least two, three, four, or five times. In another embodiment, the modified NK cell is administered at least one, two, three, four or five times, and the antibody is administered at least one, two, three, four or five times, either concurrently or sequentially.

[35] In one embodiment, the cancer cell is a head and neck cancer cell, breast cancer cell, colorectal cancer cell, gastric cancer cell, renal cell carcinoma (RCC) cell, or non-small cell lung cancer (NSCLC) cell, solid tumor cell, bladder cancer cell, hepatocellular carcinoma cell, prostate cancer cell, ovarian/uterine cancer cell, pancreatic cancer cell, mesothelioma cell, melanoma cell, glioblastoma cell, cervical cancer cell, oral cavity cancer cell, cancer of the pharynx, thyroid cancer cell, gallbladder cancer cell, soft tissue sarcoma, or a hematological cancer cell. In one embodiment, the cancer cell is a head and neck cancer cell.

[36] In one embodiment, the modified NK cell has been modified using CRISPR prior to the administering. In one embodiment, the modified NK cell has been modified using a RNA guided nuclease and at least one guide RNA (gRNA). In one embodiment, the RNA guided nuclease comprises a sequence of SEQ ID NO:1142, SEQ ID NO:1143, SEQ ID NO:1144, SEQ ID NO:1145, SEQ ID NO:1146, SEQ ID NO:1147, SEQ ID NO:1148, SEQ ID NO:1149, or SEQ ID NO:1150. In one embodiment, the RNA guided nuclease comprises a sequence of SEQ ID NO:1146. In one embodiment, the gRNA targets a DNA sequence of any one of SEQ ID NOs:769-875 or 1174. In one embodiment the gRNA targets a DNA sequence of any one of SEQ ID NOs:540-768 or 1173. In one embodiment, the gRNA comprises a sequence of SEQ ID NO:1164 or SEQ ID NO:1170, and/or SEQ ID NO:1166 or SEQ ID NO:1172. In one embodiment, the modified NK cell was generated from a NK cell, *e.g.*, a mature NK, or a stem cell. In one embodiment, the stem cell is an induced pluripotent stem cell (iPS) cell, a hematopoietic stem cell (HSC), or an embryonic stem cell. In one embodiment, the NK cell is an iNK cell.

BRIEF DESCRIPTION OF THE DRAWINGS

[37] **Figs. 1A and 1B** depict that robust single and double-gene editing of TGFBR2 and CISH was achieved in NK cells. 72 hours after CRISPR-EngCas12a editing for each KO combination, editing at CISH and TGFBR2 were assessed by NGS in **Fig. 1A**, and viability was assessed by AO/PI staining in **Fig. 1B**. Data were obtained from three unique NK cell donors, representative of a minimum of five independent experiments.

[38] **Figs. 2A and 2B** depict that knockout (KO) of CISH and TGFBR2 by CRISPR-EngCas12a increased phosphorylation of STAT5 (pSTAT5) upon IL-15 stimulation and reduced phosphorylation of SMAD2/3 (pSMAD2/3) upon TGF- β stimulation. NK cells were cytokine-starved for 18 hours, 72 hours after CRISPR-EngCas12a editing, followed by re-stimulation for 120 min with IL-15 (**Fig. 2A**) or IL-15 and TGF- β (**Fig. 2B**), and analyzed by phosphoflow cytometry assay. Data are representative

of four unique NK cell donors in two independent experiments. Statistical difference is the result of 1-way ANOVA analysis (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$).

[39] **Figs. 3A, 3B, 3C, and 3D** depict that double KO (DKO) of CISH/TGFBR2 in NK cells by CRISPR-EngCas12a editing increased inflammatory cytokine production after co-culturing with spheroids of ovarian cancer cell line SK-OV-3 (**Figs. 3A and 3B**) and prostate cancer cell line PC-3 (**Figs. 3C and 3D**) in comparison to unedited control NK cells. Supernatants were harvested at the conclusion of the spheroid assay (120 hrs) and analyzed for TNF- α and IFN- γ by AlphaLISA (+TGF- β conditions). Statistical difference is the result of 2-way ANOVA analysis (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$).

[40] **Figs. 4A, 4B, 4C, and 4D** depict that CRISPR-EngCas12a editing enhanced anti-tumor activity of NK cells against SK-OV-3 ovarian tumor compared with unedited control NK cells in the *in vitro* spheroid assay at different effector cell to target cell (E:T) ratios. **Figs. 4A and 4B** depict the tumor spheroid analysis at 10:1 E:T ratio in the presence of 10 ng/ml TGF- β , without and with the addition of 10 μ g/mL trastuzumab, respectively, as analyzed across a minimum of 4 unique donors and 3 independent experiments. Red object intensity was measured every two hours for 5 days on an Incucyte imaging system. **Figs. 4C and 4D** depict the tumor spheroid analysis at 1.25:1, 2.5:1, 5:1 and 10:1 E:T ratios in the presence of 10 ng/ml TGF- β , without and with the addition of 10 μ g/mL trastuzumab, respectively, as analyzed across a minimum of 4 unique donors and 3 independent experiments. Red object intensity is shown at 100 hours following NK cell addition.

[41] **Fig. 5** depicts amplified tumor killing by NK cells through antibody-dependent cellular cytotoxicity *in vitro*. At low E:T ratio of 1.25:1, the addition of 10 μ g/mL trastuzumab significantly increased killing of SK-OV-3 tumor spheroids by both unedited and CISH/TGFBR2 DKO NK cells, as analyzed across a minimum of 4 unique donors and 3 independent experiments.

[42] **Figs. 6A, 6B, 6C, and 6D** depict that CRISPR-EngCas12a-edited NK cells reduced SK-OV-3 ovarian tumor burden more effectively than unedited control NK cells, leading to an increased median survival time in an *in vivo* mouse model. NSG mice (n=8 per group in two independent experiments) were inoculated via intraperitoneal (i.p.) with 0.5 million (**Figs. 6A and 6C**) or 1 million (**Figs. 6B and 6D**) luciferase-expressing SK-OV-3 cells. Seven days later, the mice were administered 10 million unedited NK cells or 10 million DKO NK cells by i.p. infusion. Tumor burden measured by bioluminescence signal from SK-OV-3 cells are shown in **Figs. 6A and 6B**, and overall survival of mice are shown in **Figs. 6C and 6D**. Data are representative of two independent experiments. Statistical difference is the result of 2-way ANOVA (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$) for bioluminescence and log rank test for overall survival.

[43] **Figs. 7A, 7B, 7C and 7D** depict that trastuzumab mediated antibody-dependent cellular toxicity in NK cell treatments of SK-OV-3 tumor bearing mice. NSG mice (n=8 per group) were inoculated via intraperitoneal (i.p.) with 0.5 million luciferase-expressing SK-OV-3 cells. On day 7, mice were treated with 2.5 mpk isotype, 2.5 mpk trastuzumab, 10 million unedited CD56+ NK cells,

10 million DKO CD56+ NK cells or the combination of DKO CD56+ NK cells with trastuzumab. The average tumor volumes are shown as mean $\pm$ SEM (**** $p < 0.0001$, ** $p < 0.01$, * $p < 0.05$, 2-way analysis of variance) (**Figs. 7A and 7B**). Kaplan-Meier survival curves shown for the treatment groups as indicated (* $p < 0.05$; ** $p < 0.01$; Gehan-Wilcoxon test) (**Figs. 7C and 7D**). **Figs. 7A and 7C** show that the DKO NK cells are effective at controlling tumor growth and increased mouse lifespan. **Figs. 7B and 7D** show that administration of trastuzumab further reduced SK-PV-3 ovarian tumor burden and extended lifespan of tumor-bearing mice in treatments with DKO NK cells.

[44] **Figs. 8A and 8B** depict that DKO NK cells demonstrate more robust serial killing of Raji tumor cells over a tested period of more than 7 days with multiple de novo additions of Raji tumor target cells relative to control NK cells, and that combination with rituximab improved killing by both control and DKO NK cells. **Fig. 8A** shows the experimental set up of the assay. 200 thousand NK cells were seeded in each well. 10 thousand Raji tumor cells were added to the NK cells at the beginning of the assay, and subsequently 5 thousand tumor cells and IL-15 were bolused into each well every 48 hours. Surviving tumor cells were quantified by normalized total red object area. **Fig. 8B** shows that DKO NK cells demonstrate increased killing of Raji tumor cells relative to control NK and that the addition of rituximab improved killing by both types of NK cells.

[45] **Fig. 9A** depicts upregulation of granzyme transcripts, *GZMB*, *GZMA* and *GZMH* in CISH^{-/-} NK cells as assessed by NanoString analysis.

[46] **Fig. 9B** depicts that *GZMB* transcripts were upregulated 22-fold in CISH/TGFBR2 DKO NK cells as quantified by RT-qPCR. TBP (TATA box binding protein) was used as a reference transcript.

[47] **Fig. 9C** depicts that CISH/TGFBR2 DKO NK cells demonstrated enhanced tumor cytotoxicity relative to unedited control NK cells. CISH/TGFBR2 DKO NK cells were co-cultured with SK-OV-3 tumor spheroids in the presence of 10 ng/mL TGF- β over a time period of 36 hours at a 5:1 effector tumor ratio. Error bars represent standard deviation.

[48] **Fig. 9D** shows representative Incucyte images of SK-OV3::GzmB cells co-cultured with CISH/TGFBR2 DKO NK cells or unedited NK control cells for 4 hours. **Fig. 9D** depicts that CISH/TGFBR2 DKO NK cells released more GzmB than unedited control NK cells when co-cultured with SK-OV-3 tumor cells.

[49] **Fig. 9E** depicts that CISH/TGFBR2 DKO NK cells demonstrated higher levels of GzmB granulation at earlier time points relative to unedited NK control cells.

[50] **Fig. 10A** depicts that CISH/TGFBR2 DKO NK cells had enhanced cytotoxicity when compared to unedited control NK cells in unfavorable metabolic conditions in isolation. CISH/TGFBR2 DKO NK cells were co-cultured with SK-OV-3 tumor spheroids without TGF- β at a 10:1 effector tumor ratio.

[51] **Fig. 10B** depicts that CISH/TGFBR2 DKO NK cells had enhanced cytotoxicity when compared to unedited control NK cells in multifactorially unfavorable metabolic conditions. The

CISH/TGFBR2 DKO NK cells or the unedited control cells were co-cultured with SK-OV-3 tumor spheroids in the presence of 10 ng/mL TGF- β at a 5:1 effector tumor ratio.

[52] **Fig. 10C** depicts that CISH/TGFBR2 DKO NK cells had enhanced cytotoxicity when compared to unedited control NK cells against tumor cells evolved to grow in unfavorable metabolic conditions. The CISH/TGFBR2 DKO NK cells or the unedited control cells were co-cultured with SK-OV-3 tumor spheroids that were selectively evolved to grow in unfavorable metabolic conditions in the presence of 10 ng/mL TGF- β at a 10:1 effector tumor ratio. EC50 was measured at 100 hours.

[53] **Fig. 10D** depicts that CISH/TGFBR2 DKO NK cells had a greater cytotoxicity potential in unfavorable metabolic conditions than in control media compared to unedited control NK cells. The CISH/TGFBR2 DKO NK cells or the unedited control cells were co-cultured with SK-OV-3 tumor spheroids that were selectively evolved to grow in unfavorable metabolic conditions in the presence of 10 ng/mL TGF- β at 100 hours at various effector target ratios as indicated.

[54] **Fig. 10E** depicts that CISH/TGFBR2 DKO NK cells exhibited significantly greater metabolic fitness (*i.e.*, greater spare respiratory capacity (SRC)) than unedited control NK cells after overnight IL-15 starvation. * $p < 0.05$.

[55] **Figs. 11A and 11B** depict that CISH/TGFBR2 DKO NK cells enhanced anti-tumor activity against Nalm6 cells in the presence of TGF- β , respectively, as analyzed across a minimum of 5 unique donors and 2 independent experiments. CISH/TGFBR2 DKO NK cells and unedited control NK cells were co-cultured with Nalm6 tumor cells at a 20:1 effector tumor ratio in the presence of 5 ng/mL IL-15, without and with the addition of 10 ng/mL TGF- β . Increased cytotoxicity was observed in all conditions while a greater increase was observed when TGF- β was added in the cell culture.

[56] **Fig. 12** depicts that CISH/TGFBR2 DKO NK cells demonstrate robust serial killing against Nalm6 cells over a tested period up to 20 days with multiple additions of Nalm6 cells relative to control NK cells.

[57] **Fig. 13** depicts that CISH/TGFBR2 DKO NK cells continually killed Nalm6 tumor cells for more than 8 days, whereas unedited NK cells had limited serial killing effect. Data are representative of NK cells from 6 unique donors in 2 independent experiments.

[58] **Fig. 14** depicts that CISH/TGFBR2 DKO NK cells produced increased levels of inflammatory cytokines (IFN- γ and TNF- α) throughout the serial-killing assay in the presence of TGF- β relative to unedited control NK cells.

[59] **Figs. 15A, 15B, and 15C** depict that CISH/TGFBR2 DKO NK cells demonstrated sustained serial-killing activity against numerous other hematologic tumor cell lines, *e.g.*, Raji (Burkitt's lymphoma) (**Fig. 15A**), RPMI8226 (multiple myeloma) (**Fig. 15B**) and THP-1 cells (acute monocytic leukemia) (**Fig. 15C**), in the presence of TGF- β . Data are representative of NK cells from 5 unique donors in 5 independent experiments.

DETAILED DESCRIPTION

[60] The present disclosure provides modified NK cells (or other lymphocytes) that are useful in NK cell therapy, *e.g.*, in the context of immunotherapeutic approaches, in combination with a therapeutic antibody, or antigen-binding portion thereof, to generate striking antibody-dependent cellular cytotoxicity (ADCC) effects, thereby surprisingly increasing the effectiveness of the modified NK cells in killing target cells, *e.g.* cancer cells. ADCC is a mechanism of cell-mediated immune defense, where an immune effector cell actively lyses a target cell after its membrane-surface antigens have been bound by specific antibodies. To participate in ADCC, the immune effector cells must express Fc-gamma receptors (Fc γ R) to be able to recognize the Fc region of the antibodies that bind to the target cells. Most immune effector cells have both activating and inhibitory Fc γ R. An advantage of using NK cells to target cancer cells via ADCC is that, unlike other effector cells, NK cells only have activating Fc γ Rs (*e.g.*, Fc γ R IIIa, also known as CD16a, and Fc γ R IIc, also known as CD32c) and are believed to be the most important effectors of ADCC in humans. Thus, the use of the modified NK cells disclosed herein and antibodies targeting cancer cell-specific antigens to elicit ADCC provides novel and surprisingly effective immunotherapies.

[61] Some aspects of the present disclosure provide compositions, methods, and strategies for the generation of modified NK cells. In some embodiments, such modified NK cells are generated by editing the genome of NK cells, *e.g.*, mature NK cells. In one embodiment, NK cells are obtained from a healthy donor, and then edited using the compositions and methods described herein to make modified NK cells. For example, NK cell expansion *ex vivo* is described at least in Myers and Miller, Exploring the NK cell platform for cancer immunotherapy, Nat Rev Clin Oncol (2020), <https://doi.org/10.1038/s41571-020-0426-7>, the entire contents of which are expressly incorporated herein by reference.

[62] In other embodiments, modified NK cells are generated by editing the genome of a cell from which an NK cell is derived, either *in vitro* or *in vivo*. In some embodiments, the cell from which an NK cell is derived is a stem cell, for example, a hematopoietic stem cell (HSC), or a pluripotent stem cells, such as, *e.g.*, an embryonic stem cell (ES cell) or an induced pluripotent stem cell (iPS cell). For example, in some embodiments, modified NK cells are generated by editing the genome of an ES cell, an iPS cell, or a hematopoietic stem cell, and subsequently differentiating the edited stem cell into an NK cell. In some embodiments, where the generation of modified NK cells involves differentiation of the modified NK cell from an iPS cell, the editing of the genome may take place at any suitable time during the generation, maintenance, or differentiation of the iPS cell. For example, where a donor cell is reprogrammed into an iPS cell, the donor cell, *e.g.*, a somatic cell such as, for example, a fibroblast cell or a T lymphocyte, may be subjected to the gene editing approaches described herein before reprogramming to an iPS cell, during the reprogramming procedure, or after the donor cell has been reprogrammed to an iPS cell.

[63] NK cells derived from iPS cells are also referred to herein as iNK cells. In some embodiments, the present disclosure provides compositions, methods, and strategies for generating iNK cells that have been derived from developmentally mature cells, also referred to as somatic cells, such as, for example, fibroblasts or peripheral blood cells.

[64] In some embodiments, the present disclosure provides compositions, methods, and strategies for generating iNK cells that have been derived from developmentally mature T cells (T cells that have undergone thymic selection). One hallmark of developmentally mature T cells is a rearranged T cell receptor locus. During T cell maturation, the TCR locus undergoes V(D)J rearrangements to generate complete V-domain exons. These rearrangements are retained throughout reprogramming of a T cells to an induced pluripotent stem (iPS) cell, and throughout differentiation of the resulting iPS cell to a somatic cell.

[65] One advantage of using T cells for the generation of iPS cells is that T cells can be edited with relative ease, *e.g.*, by CRISPR-based methods or other gene-editing methods.

[66] Another advantage of using T cells for the generation of iPS cells is that the rearranged TCR locus allows for genetic tracking of individual cells and their daughter cells. If the reprogramming, expansion, culture, and/or differentiation strategies involved in the generation of NK cells a clonal expansion of a single cell, the rearranged TCR locus can be used as a genetic marker unambiguously identifying a cell and its daughter cells. This, in turn, allows for the characterization of a cell population as truly clonal, or for the identification of mixed populations, or contaminating cells in a clonal population.

[67] A third advantage of using T cells in generating iNK cells carrying multiple edits is that certain karyotypic aberrations associated with chromosomal translocations are selected against in T cell culture. Such aberrations pose a concern when editing cells by CRISPR technology, and in particular when generating cells carrying multiple edits.

[68] A fourth advantage of using T cell derived iPS cells as a starting point for the derivation of therapeutic lymphocytes is that it allows for the expression of a pre-screened TCR in the lymphocytes, *e.g.*, via selecting the T cells for binding activity against a specific antigen, *e.g.*, a tumor antigen, reprogramming the selected T cells to iPS cells, and then deriving lymphocytes from these iPS cells that express the TCR (*e.g.*, T cells). This strategy would also allow for activating the TCR in other cell types, *e.g.*, by genetic or epigenetic strategies.

[69] A fifth advantage of using T cell derived iPS cells as a starting point for iNK differentiation is that the T cells retain at least part of their "epigenetic memory" throughout the reprogramming process, and thus subsequent differentiation of the same or a closely related cell type, such as iNK cells will be more efficient and/or result in higher quality cell populations as compared to approaches using non-related cells, such as fibroblasts, as a starting point for iNK derivation.

Definitions and Abbreviations

[70] Unless otherwise specified, each of the following terms have the meaning set forth in this section.

[71] The indefinite articles “a” and “an” refer to at least one of the associated noun, and are used interchangeably with the terms “at least one” and “one or more.”

[72] The conjunctions “or” and “and/or” are used interchangeably as non-exclusive disjunctions.

[73] “Subject” means a human or non-human animal. A human subject can be any age (*e.g.*, an infant, child, young adult, or adult), and may suffer from a disease, or may be in need of alteration of a gene or a combination of specific genes. Alternatively, the subject may be an animal, which term includes, but is not limited to, a mammal, and, more particularly, a non-human primate, a rodent (*e.g.*, a mouse, rat, hamster, *etc.*), a rabbit, a guinea pig, a dog, a cat, and so on. In certain embodiments of this disclosure, the subject is livestock, *e.g.*, a cow, a horse, a sheep, or a goat. In certain embodiments, the subject is poultry.

[74] The terms “treatment,” “treat,” and “treating,” refer to a clinical intervention aimed to reverse, alleviate, delay the onset of, or inhibit the progress, and/or prevent or delay the recurrence of a disease or disorder, or one or more symptoms thereof, as described herein. Treatment, *e.g.*, in the form of a modified NK cell or a population of modified NK cells as described herein, may be administered to a subject after one or more symptoms have developed and/or after a disease has been diagnosed. Treatment may be administered in the absence of symptoms, *e.g.*, to prevent or delay onset of a symptom or inhibit onset or progression of a disease. For example, treatment may be administered to a susceptible individual prior to the onset of symptoms (*e.g.*, in light of genetic or other susceptibility factors). Treatment may also be continued after symptoms have resolved, for example to prevent or delay their recurrence.

[75] “Prevent,” “preventing,” and “prevention” refer to the prevention of a disease in a mammal, *e.g.*, in a human, including (a) avoiding or precluding the disease; (b) affecting the predisposition toward the disease; or (c) preventing or delaying the onset of at least one symptom of the disease.

[76] The terms “polynucleotide”, “nucleotide sequence”, “nucleic acid”, “nucleic acid molecule”, “nucleic acid sequence”, and “oligonucleotide” refer to a series of nucleotide bases (also called “nucleotides”) in DNA and RNA, and mean any chain of two or more nucleotides. The polynucleotides, nucleotide sequences, nucleic acids *etc.* can be chimeric mixtures or derivatives or modified versions thereof, single-stranded or double-stranded. They can be modified at the base moiety, sugar moiety, or phosphate backbone, for example, to improve stability of the molecule, its hybridization parameters, *etc.* A nucleotide sequence typically carries genetic information, including, but not limited to, the information used by cellular machinery to make proteins and enzymes. These terms include double- or single-stranded genomic DNA, RNA, any synthetic and genetically manipulated polynucleotide, and both sense and antisense polynucleotides. These terms also include nucleic acids containing modified bases.

[77] Conventional IUPAC notation is used in nucleotide sequences presented herein, as shown in Table 1, below (*see also* Cornish-Bowden A, Nucleic Acids Res. 1985 May 10; 13(9):3021-30, incorporated by reference herein). It should be noted, however, that “T” denotes “Thymine or Uracil” in those instances where a sequence may be encoded by either DNA or RNA, for example in gRNA targeting domains.

Table 1: IUPAC nucleic acid notation

Character	Base
A	Adenine
T	Thymine or Uracil
G	Guanine
C	Cytosine
U	Uracil
K	G or T/U
M	A or C
R	A or G
Y	C or T/U
S	C or G
W	A or T/U
B	C, G or T/U
V	A, C or G
H	A, C or T/U
D	A, G or T/U
N	A, C, G or T/U

[78] The terms “protein,” “peptide” and “polypeptide” are used interchangeably to refer to a sequential chain of amino acids linked together via peptide bonds. The terms include individual proteins, groups or complexes of proteins that associate together, as well as fragments or portions, variants, derivatives and analogs of such proteins. Peptide sequences are presented herein using conventional notation, beginning with the amino or N-terminus on the left, and proceeding to the carboxyl or C-terminus on the right. Standard one-letter or three-letter abbreviations can be used.

[79] The term “variant” refers to an entity such as a polypeptide, polynucleotide or small molecule that shows significant structural identity with a reference entity but differs structurally from the reference entity in the presence or level of one or more chemical moieties as compared with the reference entity. In many embodiments, a variant also differs functionally from its reference entity. In general, whether a particular entity is properly considered to be a “variant” of a reference entity is based on its degree of structural identity with the reference entity.

[80] The term “endogenous,” as used herein in the context of nucleic acids (*e.g.*, genes, protein-encoding genomic regions, promoters), refers to a native nucleic acid or protein in its natural location, *e.g.*, within the genome of a cell. In contrast, the term “exogenous,” as used herein in the context of

nucleic acids, *e.g.*, expression constructs, cDNAs, indels, and nucleic acid vectors, refers to nucleic acids that have artificially been introduced into the genome of a cell using, for example, gene-editing or genetic engineering techniques, *e.g.*, CRISPR-based editing techniques.

[81] The terms “RNA-guided nuclease” and “RNA-guided nuclease molecule” are used interexchangeably herein. In some embodiments, the RNA-guided nuclease is a RNA-guided DNA endonuclease enzyme. In some embodiments, the RNA-guided nuclease is a CRISPR nuclease. Non-limiting examples of RNA-guided nucleases are listed in Table 2 below, and the methods and compositions disclosed herein can use any combination of RNA-guided nucleases disclosed herein, or known to those of ordinary skill in the art. Those of ordinary skill in the art will be aware of additional nucleases and nuclease variants suitable for use in the context of the present disclosure, and it will be understood that the present disclosure is not limited in this respect.

Table 2. RNA-Guided Nucleases

Nuclease	Length (a.a.)	PAM	Reference
SpCas9	1368	NGG	Cong <i>et al.</i> , Science. 2013;339(6121):819-23
SaCas9	1053	NNGRRT	Ran <i>et al.</i> , Nature. 2015;520(7546):186-91.
(KKH) SaCas9	1067	NNNRRT	Kleinstiver <i>et al.</i> , Nat Biotechnol. 2015;33(12):1293-1298
AsCpf1 (AsCas12a)	1353	TTTV	Zetsche <i>et al.</i> , Nat Biotechnol. 2017;35(1):31-34.
LbCpf1 (LbCas12a)	1274	TTTV	Zetsche <i>et al.</i> , Cell. 2015;163(3):759-71.
CasX	980	TTC	Burstein <i>et al.</i> , Nature. 2017;542(7640):237-241.
CasY	1200	TA	Burstein <i>et al.</i> , Nature. 2017;542(7640):237-241.
Cas12h1	870	RTR	Yan <i>et al.</i> , Science. 2019;363(6422):88-91.
Cas12i1	1093	TTN	Yan <i>et al.</i> , Science. 2019;363(6422):88-91.
Cas12c1	unknown	TG	Yan <i>et al.</i> , Science. 2019;363(6422):88-91.
Cas12c2	unknown	TN	Yan <i>et al.</i> , Science. 2019;363(6422):88-91.
eSpCas9	1423	NGG	Chen <i>et al.</i> , Nature. 2017;550(7676):407-410.
Cas9-HF1	1367	NGG	Chen <i>et al.</i> , Nature. 2017;550(7676):407-410.
HypaCas9	1404	NGG	Chen <i>et al.</i> , Nature. 2017;550(7676):407-410.
dCas9-FokI	1623	NGG	U.S. Patent No. 9,322,037
Sniper-Cas9	1389	NGG	Lee <i>et al.</i> , Nat Commun. 2018;9(1):3048.
xCas9	1786	NGG, NG, GAA, GAT	Wang <i>et al.</i> , Plant Biotechnol J. 2018; pbi.13053.
AaCas12b	1129	TTN	Teng <i>et al.</i> Cell Discov. 2018;4:63.
evoCas9	1423	NGG	Casini <i>et al.</i> , Nat Biotechnol. 2018;36(3):265-271.

SpCas9-NG	1423	NG	Nishimasu <i>et al.</i> , Science. 2018;361(6408):1259-1262.
VRQR	1368	NGA	Li <i>et al.</i> , The CRISPR Journal, 2018; 01:01
VRER	1372	NGCG	Kleinstiver <i>et al.</i> , Nature. 2016;529(7587):490-5.
NmeCas9	1082	NNNNGAT T	Amrani <i>et al.</i> , Genome Biol. 2018;19(1):214.
CjCas9	984	NNNNRYA C	Kim <i>et al.</i> , Nat Commun. 2017;8:14500.
BhCas12b	1108	ATTN	Strecker <i>et al.</i> , Nat Commun. 2019 Jan 22;10(1):212.
BhCas12b V4	1108	ATTN	Strecker <i>et al.</i> , Nat Commun. 2019 Jan 22;10(1):212.
CasΦ			Pausch <i>et al.</i> , Science 2020;369(6501):333-337.

[82] Additional suitable RNA-guided nucleases, *e.g.*, Cas9 and Cas12 nucleases, will be apparent to the skilled artisan in view of the present disclosure, and the disclosure is not limited by the exemplary suitable nucleases provided herein. In some embodiment, a suitable nuclease is a Cas9 or Cpf1 (Cas12a) nuclease. In some embodiments, the disclosure also embraces nuclease variants, *e.g.*, Cas9 or Cpf1 nuclease variants. A nuclease variant refers to a nuclease comprising an amino acid sequence characterized by one or more amino acid substitutions, deletions, or additions as compared to the wild type amino acid sequence of the nuclease. Suitable nucleases and nuclease variants may also include purification tags (*e.g.*, polyhistidine tags) and signaling peptides, *e.g.*, comprising or consisting of a nuclear localization signal sequence. Some non-limiting examples of suitable nucleases and nuclease variants are described in more detail elsewhere herein, and also include those described in PCT application PCT/US2019/22374, filed March 14, 2019, and entitled “*Systems and Methods for the Treatment of Hemoglobinopathies*,” the entire contents of which are incorporated herein by reference.

[83] In some embodiments, the RNA-guided nuclease is an *Acidaminococcus sp.* Cpf1 variant (AsCpf1 variant). Suitable Cpf1 nuclease variants, including suitable AsCpf1 variants will be known or apparent to those of ordinary skill in the art based on the present disclosure, and include, but are not limited to, the Cpf1 variants disclosed herein or otherwise known in the art. For example, in some embodiments, the RNA-guided nuclease is a *Acidaminococcus sp.* Cpf1 RR variant (AsCpf1-RR). In another embodiment, the RNA-guided nuclease is a Cpf1 RVR variant. For example, suitable Cpf1 variants include those having an M537R substitution, an H800A substitution, and/or an F870L substitution, or any combination thereof (numbering scheme according to AsCpf1 wild-type sequence). In some embodiments, the RNA-guided nuclease is an *Acidaminococcus sp.* Cpf1 variant

(AsCpf1 variant) having an M537R substitution, an H800A substitution, and an F870L substitution (numbering scheme according to AsCpf1 wild-type sequence).

[84] The term "hematopoietic stem cell," or "definitive hematopoietic stem cell" as used herein, refers to CD34+ stem cells capable of giving rise to both mature myeloid and lymphoid cell types including T cells, natural killer cells and B cells.

[85] As used herein, the terms "reprogramming" or "dedifferentiation" or "increasing cell potency" or "increasing developmental potency" refers to a method of increasing the potency of a cell or dedifferentiating the cell to a less differentiated state. For example, a cell that has an increased cell potency has more developmental plasticity (*i.e.*, can differentiate into more cell types) compared to the same cell in the non-reprogrammed state. In other words, a reprogrammed cell is one that is in a less differentiated state than the same cell in a non-reprogrammed state. In some embodiments, the term "reprogramming" refers to de-differentiating a somatic cell, or a multipotent stem cell, into a pluripotent stem cell, also referred to as an induced pluripotent stem cell, or iPS cell. Suitable methods for the generation of iPS cells from somatic or multipotent stem cells are well known to those of skill in the art.

[86] As used herein, the term "differentiation" is the process by which an unspecialized ("uncommitted") or less specialized cell acquires the features of a specialized cell such as, for example, a blood cell or a muscle cell. A differentiated or differentiation-induced cell is one that has taken on a more specialized ("committed") position within the lineage of a cell. For example, an iPS cell can be differentiated into various more differentiated cell types, for example, a neural or a hematopoietic stem cell, a lymphocyte, a cardiomyocyte, and other cell types, upon treatment with suitable differentiation factors in the cell culture medium. Suitable methods, differentiation factors, and cell culture media for the differentiation of pluri- and multipotent cell types into more differentiated cell types are well known to those of skill in the art. The term "committed", when applied to the process of differentiation, refers to a cell that has proceeded in the differentiation pathway to a point where, under normal circumstances, it will continue to differentiate into a specific cell type or subset of cell types, and cannot, under normal circumstances, differentiate into a different cell type or revert to a less differentiated cell type.

[87] As used herein, the terms "differentiation marker," "differentiation marker gene," or "differentiation gene," refers to genes or proteins whose expression are indicative of cell differentiation occurring within a cell, such as a pluripotent cell. Differentiation marker genes include, but are not limited to, the following genes: CD34, CD4, CD8, CD3, CD56 (NCAM), CD49, CD45; NK cell receptor (cluster of differentiation 16 (CD16)), natural killer group-2 member D (NKG2D), CD69, NKp30, NKp44, NKp46, CD158b, FOXA2, FGF5, SOX17, XIST, NODAL, COL3A1, OTX2, DUSP6, EOMES, NR2F2, NR0B1, CXCR4, CYP2B6, GAT A3, GATA4, ERBB4, GATA6, HOXC6, INHA, SMAD6, RORA, NIPBL, TNFSF11, CDH11, ZIC4, GAL, SOX3, PITX2, APOA2, CXCL5, CER1, FOXQ1, MLL5, DPP10, GSC, PCDH10, CTCFL, PCDH20, TSHZ1, MEGF10, MYC, DKK1,

BMP2, LEFTY2, HES1, CDX2, GNAS, EGR1, COL3A1, TCF4, HEPH, KDR, TOX, FOXA1, LCK, PCDH7, CD1D FOXG1, LEFTY1, TUJ1, T gene (Brachyury), ZIC1, GATA1, GATA2, HDAC4, HDAC5, HDAC7, HDAC9, NOTCH1, NOTCH2, NOTCH4, PAX5, RBPJ, RUNX1, STAT1 and STAT3.

[88] As used herein, the term "differentiation marker gene profile," or "differentiation gene profile," "differentiation gene expression profile," "differentiation gene expression signature," "differentiation gene expression panel," "differentiation gene panel," or "differentiation gene signature" refers to the expression or levels of expression of a plurality of differentiation marker genes.

[89] As used herein in the context of cellular developmental potential, the term "potency" or "developmental potency" refers to the sum of all developmental options accessible to the cell (*i.e.*, the developmental potency). The continuum of cell potency includes, but is not limited to, totipotent cells, pluripotent cells, multipotent cells, oligopotent cells, unipotent cells, and terminally differentiated cells.

[90] As used herein, the term "pluripotent" refers to the ability of a cell to form all lineages of the body or soma (*i.e.*, the embryo proper). For example, embryonic stem cells are a type of pluripotent stem cells that are able to form cells from each of the three germ layers, the ectoderm, the mesoderm, and the endoderm. Pluripotency is a continuum of developmental potencies ranging from the incompletely or partially pluripotent cell (*e.g.*, an epiblast stem cell or EpiSC), which is unable to give rise to a complete organism to the more primitive, more pluripotent cell, which is able to give rise to a complete organism (*e.g.*, an embryonic stem cell or an induced pluripotent stem cell).

[91] As used herein, the term "induced pluripotent stem cell" or, iPS cell refers to a stem cell obtained from a differentiated somatic, *e.g.*, adult, neonatal, or fetal cell by a process referred to as reprogramming into cells capable of differentiating into tissues of all three germ or dermal layers: mesoderm, endoderm, and ectoderm. IPS cells are not found in nature.

[92] As used herein, the term "embryonic stem cell" refers to pluripotent stem cells derived from the inner cell mass of the embryonic blastocyst. Embryonic stem cells are pluripotent and give rise during development to all derivatives of the three primary germ layers: ectoderm, endoderm and mesoderm. They do not contribute to the extra-embryonic membranes or the placenta, *i.e.*, are not totipotent.

[93] As used herein, the term "multipotent stem cell" refers to a cell that has the developmental potential to differentiate into cells of one or more germ layers (ectoderm, mesoderm and endoderm), but not all three. Thus, a multipotent cell can also be termed a "partially differentiated cell." Multipotent cells are well known in the art, and examples of multipotent cells include adult stem cells, such as for example, hematopoietic stem cells and neural stem cells. "Multipotent" indicates that a cell may form many types of cells in a given lineage, but not cells of other lineages. For example, a multipotent hematopoietic cell can form the many different types of blood cells (red, white, platelets,

etc.), but it cannot form neurons. Accordingly, the term "multipotency" refers to a state of a cell with a degree of developmental potential that is less than totipotent and pluripotent.

[94] Pluripotency can be determined, in part, by assessing pluripotency characteristics of the cells. Pluripotency characteristics include, but are not limited to: (i) pluripotent stem cell morphology; (ii) the potential for unlimited self-renewal; (iii) expression of pluripotent stem cell markers including, but not limited to SSEA1 (mouse only), SSEA3/4, SSEA5, TRA1-60/81, TRA1-85, TRA2-54, GCTM-2, TG343, TG30, CD9, CD29, CD133/prominin, CD140a, CD56, CD73, CD90, CD105, OCT4, NANOG, SOX2, CD30 and/or CD50; (iv) ability to differentiate to all three somatic lineages (ectoderm, mesoderm and endoderm); (v) teratoma formation consisting of the three somatic lineages; and (vi) formation of embryoid bodies consisting of cells from the three somatic lineages.

[95] As used herein, the term "pluripotent stem cell morphology" refers to the classical morphological features of an embryonic stem cell. Normal embryonic stem cell morphology is characterized by being round and small in shape, with a high nucleus-to-cytoplasm ratio, the notable presence of nucleoli, and typical intercell spacing.

[96] As used herein, the term "nutrient-depriving condition" refers to unfavorable growth or metabolic conditions where either a lower level of nutrients or a lack of nutrients is observed. Nutrient deprivation is one of the hallmark conditions of the tumor microenvironment. The rapid growth of the tumor leads to the development of a hypoxic and nutrient deprived microenvironment within the core of the tumor mass due to an insufficient blood supply. In some embodiments, the nutrient-depriving condition comprises a decreasing concentration of nutrients for cell metabolism, *e.g.*, glucose or glutamine. In some embodiments, the nutrient-depriving condition comprises a decreasing concentration of glucose, *e.g.*, a concentration of glucose from about 10 mM, about 9 mM, about 8 mM, about 7 mM, about 6 mM, about 5 mM, about 4 mM, about 3 mM, about 2 mM or about 1 mM to a concentration of glucose less than about 1 mM, *e.g.*, about 0.9 mM, about 0.8 mM, about 0.7 mM, about 0.6 mM, about 0.5 mM, about 0.4 mM, about 0.3 mM, about 0.2 mM or about 0.1 mM. In some embodiments, the nutrient-depriving condition comprises a decreasing concentration of glutamine, *e.g.*, a concentration of glutamine from about 10 mM, about 9 mM, about 8 mM, about 7 mM, about 6 mM, about 5 mM, about 4 mM, about 3 mM, about 2 mM or about 1 mM to a concentration of glutamine less than about 1 mM, *e.g.*, about 0.9 mM, about 0.8 mM, about 0.7 mM, about 0.6 mM, about 0.5 mM, about 0.4 mM, about 0.3 mM, about 0.2 mM or about 0.1 mM. In some embodiments, the nutrient-depriving condition comprises an increasing concentration of inhibitory metabolic, *e.g.*, lactate, *e.g.*, a concentration of lactate from about 0 mM, about 0.1 mM, about 0.2 mM, about 0.3 mM, about 0.4 mM, about 0.5 mM, about 0.6 mM, about 0.7 mM, about 0.8 mM, about 0.9 mM or about 1 mM to a concentration of lactate about 10 mM, about 15 mM, about 20 mM, about 25 mM, about 30 mM, about 35 mM, about 40 mM, about 45 mM or about 50 mM. In another embodiment, the nutrient-depriving condition comprises a decreasing pH, *e.g.*, from a pH about 7.5,

about 7.4, about 7.3, about 7.2, about 7.1 or about 7 to a pH about 6.9, about 6.8, about 6.7, about 6.6 or about 6.5.

[97] As used herein, the term “spare respiratory capacity” refers to a functional parameter for evaluation of mitochondrial reserve. Spare respiratory capacity is the difference between basal ATP production and its maximal activity. When cells are subjected to stress, energy demand increases, with more ATP required to maintain cellular functions. A cell with a larger spare respiratory capacity can produce more ATP and overcome more stress.

Genome Editing Systems

[98] The present disclosure relates to the generation of modified NK cells, *e.g.*, NK cells the genome of which has been modified, or that are derived from a multipotent or pluripotent stem cell, *e.g.*, an HSC, ES cell, or iPS cell, the genome of which has been modified. The NK cells and stem cells provided herein can be modified using any gene-editing technology known to those of ordinary skill in the art, including, for example, by using genome editing systems, *e.g.*, CRISPR.

[99] The term “genome editing system” refers to any system having RNA-guided DNA editing activity. Genome editing systems of the present disclosure include at least two components adapted from naturally occurring CRISPR systems: a guide RNA (gRNA) and an RNA-guided nuclease. These two components form a complex that is capable of associating with a specific nucleic acid sequence and editing the DNA in or around that nucleic acid sequence, for instance by making one or more of a single-strand break (an SSB or nick), a double-strand break (a DSB) and/or a point mutation.

[100] Naturally occurring CRISPR systems are organized evolutionarily into two classes and five types (Makarova *et al.* Nat Rev Microbiol. 2011 Jun; 9(6): 467–477 (Makarova), incorporated by reference herein), and while genome editing systems of the present disclosure may adapt components of any type or class of naturally occurring CRISPR system, the embodiments presented herein are generally adapted from Class 2, and type II or V CRISPR systems. Class 2 systems, which encompass types II and V, are characterized by relatively large, multidomain RNA-guided nuclease proteins (*e.g.*, Cas9 or Cpf1) and one or more guide RNAs (*e.g.*, a crRNA and, optionally, a tracrRNA) that form ribonucleoprotein (RNP) complexes that associate with (*i.e.* target) and cleave specific loci complementary to a targeting (or spacer) sequence of the crRNA. Genome editing systems according to the present disclosure similarly target and edit cellular DNA sequences, but differ significantly from CRISPR systems occurring in nature. For example, the unimolecular guide RNAs described herein do not occur in nature, and both guide RNAs and RNA-guided nucleases according to this disclosure may incorporate any number of non-naturally occurring modifications.

[101] Genome editing systems can be implemented (*e.g.* administered or delivered to a cell or a subject) in a variety of ways, and different implementations may be suitable for distinct applications. For instance, a genome editing system is implemented, in certain embodiments, as a protein/RNA

complex (a ribonucleoprotein, or RNP), which can be included in a pharmaceutical composition that optionally includes a pharmaceutically acceptable carrier and/or an encapsulating agent, such as a lipid or polymer micro- or nano-particle, micelle, liposome, *etc.* In certain embodiments, a genome editing system is implemented as one or more nucleic acids encoding the RNA-guided nuclease and guide RNA components described above (optionally with one or more additional components); in certain embodiments, the genome editing system is implemented as one or more vectors comprising such nucleic acids, for instance a viral vector such as an adeno-associated virus; and in certain embodiments, the genome editing system is implemented as a combination of any of the foregoing. Additional or modified implementations that operate according to the principles set forth herein will be apparent to the skilled artisan and are within the scope of this disclosure.

[102] It should be noted that the genome editing systems of the present disclosure can be targeted to a single specific nucleotide sequence, or may be targeted to — and capable of editing in parallel — two or more specific nucleotide sequences through the use of two or more guide RNAs. The use of multiple gRNAs is referred to as “multiplexing” throughout this disclosure, and can be employed to target multiple, unrelated target sequences of interest, or to form multiple SSBs or DSBs within a single target domain and, in some cases, to generate specific edits within such target domain. For example, International Patent Publication No. WO 2015/138510 by Maeder *et al.* (Maeder), which is incorporated by reference herein, describes a genome editing system for correcting a point mutation (C.2991+1655A to G) in the human CEP290 gene that results in the creation of a cryptic splice site, which in turn reduces or eliminates the function of the gene. The genome editing system of Maeder utilizes two guide RNAs targeted to sequences on either side of (*i.e.* flanking) the point mutation, and forms DSBs that flank the mutation. This, in turn, promotes deletion of the intervening sequence, including the mutation, thereby eliminating the cryptic splice site and restoring normal gene function.

[103] As another example, WO 2016/073990 by Cotta-Ramusino, *et al.* (“Cotta-Ramusino”), incorporated by reference herein, describes a genome editing system that utilizes two gRNAs in combination with a Cas9 nickase (a Cas9 that makes a single strand nick such as *S. pyogenes* D10A), an arrangement termed a “dual-nickase system.” The dual-nickase system of Cotta-Ramusino is configured to make two nicks on opposite strands of a sequence of interest that are offset by one or more nucleotides, which nicks combine to create a double strand break having an overhang (5’ in the case of Cotta-Ramusino, though 3’ overhangs are also possible). The overhang, in turn, can facilitate homology directed repair events in some circumstances. And, as another example, WO 2015/070083 by Palestrant *et al.* (“Palestrant”, incorporated by reference herein) describes a gRNA targeted to a nucleotide sequence encoding Cas9 (referred to as a “governing RNA”), which can be included in a genome editing system comprising one or more additional gRNAs to permit transient expression of a Cas9 that might otherwise be constitutively expressed, for example in some virally transduced cells. These multiplexing applications are intended to be exemplary, rather than limiting, and the skilled

artisan will appreciate that other applications of multiplexing are generally compatible with the genome editing systems described here.

[104] Genome editing systems can, in some instances, form double strand breaks that are repaired by cellular DNA double-strand break mechanisms such as NHEJ or HDR. These mechanisms are described throughout the literature, for example by Davis & Maizels, PNAS, 111(10):E924-932, March 11, 2014 (Davis) (describing Alt-HDR); Frit *et al.* DNA Repair 17(2014) 81-97 (Frit) (describing Alt-NHEJ); and Iyama and Wilson III, DNA Repair (Amst.) 2013-Aug; 12(8): 620-636 (Iyama) (describing canonical HDR and NHEJ pathways generally).

[105] Where genome editing systems operate by forming DSBs, such systems optionally include one or more components that promote or facilitate a particular mode of double-strand break repair or a particular repair outcome. For instance, Cotta-Ramusino also describes genome editing systems in which a single stranded oligonucleotide “donor template” is added; the donor template is incorporated into a target region of cellular DNA that is cleaved by the genome editing system, and can result in a change in the target sequence.

[106] In certain embodiments, genome editing systems modify a target sequence, or modify expression of a gene in or near the target sequence, without causing single- or double-strand breaks. For example, a genome editing system may include an RNA-guided nuclease fused to a functional domain that acts on DNA, thereby modifying the target sequence or its expression. As one example, an RNA-guided nuclease can be connected to (*e.g.* fused to) a cytidine deaminase functional domain, and may operate by generating targeted C-to-A substitutions. Exemplary nuclease/deaminase fusions are described in Komor *et al.* Nature 533, 420–424 (19 May 2016) (“Komor”), which is incorporated by reference. Alternatively, a genome editing system may utilize a cleavage-inactivated (*i.e.* a “dead”) nuclease, such as a dead Cas9 (dCas9), and may operate by forming stable complexes on one or more targeted regions of cellular DNA, thereby interfering with functions involving the targeted region(s) including, without limitation, mRNA transcription, chromatin remodeling, *etc.*

Guide RNA (gRNA) molecules

[107] The terms “guide RNA” and “gRNA” refer to any nucleic acid that promotes the specific association (or “targeting”) of an RNA-guided nuclease such as a Cas9 or a Cpf1 to a target sequence such as a genomic or episomal sequence in a cell. gRNAs can be unimolecular (comprising a single RNA molecule, and referred to alternatively as chimeric), or modular (comprising more than one, and typically two, separate RNA molecules, such as a crRNA and a tracrRNA, which are usually associated with one another, for instance by duplexing). gRNAs and their component parts are described throughout the literature, for instance in Briner *et al.* (Molecular Cell 56(2), 333-339, October 23, 2014 (Briner), which is incorporated by reference), and in Cotta-Ramusino.

[108] In bacteria and archaea, type II CRISPR systems generally comprise an RNA-guided nuclease protein such as Cas9, a CRISPR RNA (crRNA) that includes a 5' region that is complementary to a foreign sequence, and a trans-activating crRNA (tracrRNA) that includes a 5' region that is

complementary to, and forms a duplex with, a 3' region of the crRNA. While not intending to be bound by any theory, it is thought that this duplex facilitates the formation of — and is necessary for the activity of — the Cas9/gRNA complex. As type II CRISPR systems were adapted for use in gene editing, it was discovered that the crRNA and tracrRNA could be joined into a single unimolecular or chimeric guide RNA, in one non-limiting example, by means of a four nucleotide (*e.g.* GAAA) “tetraloop” or “linker” sequence bridging complementary regions of the crRNA (at its 3' end) and the tracrRNA (at its 5' end). (Mali *et al.* Science. 2013 Feb 15; 339(6121): 823–826 (“Mali”); Jiang *et al.* Nat Biotechnol. 2013 Mar; 31(3): 233–239 (“Jiang”); and Jinek *et al.*, 2012 Science Aug. 17; 337(6096): 816–821 (“Jinek”), all of which are incorporated by reference herein.)

[109] Guide RNAs, whether unimolecular or modular, include a “targeting domain” that is fully or partially complementary to a target domain within a target sequence, such as a DNA sequence in the genome of a cell where editing is desired. Targeting domains are referred to by various names in the literature, including without limitation “guide sequences” (Hsu *et al.*, Nat Biotechnol. 2013 Sep; 31(9): 827–832, (“Hsu”), incorporated by reference herein), “complementarity regions” (Cotta-Ramusino), “spacers” (Briner) and generically as “crRNAs” (Jiang). Irrespective of the names they are given, targeting domains are typically 10–30 nucleotides in length, and in certain embodiments are 16–24 nucleotides in length (for instance, 16, 17, 18, 19, 20, 21, 22, 23 or 24 nucleotides in length), and are at or near the 5' terminus of in the case of a Cas9 gRNA, and at or near the 3' terminus in the case of a Cpf1 gRNA.

[110] In addition to the targeting domains, gRNAs typically (but not necessarily, *e.g.*, as discussed below) include a plurality of domains that may influence the formation or activity of gRNA/Cas9 complexes. For instance, as mentioned above, the duplexed structure formed by first and second complementarity domains of a gRNA (also referred to as a repeat:anti-repeat duplex) interacts with the recognition (REC) lobe of Cas9 and can mediate the formation of Cas9/gRNA complexes. (Nishimasu *et al.*, Cell 156, 935–949, February 27, 2014 (Nishimasu 2014) and Nishimasu *et al.*, Cell 162, 1113–1126, August 27, 2015 (Nishimasu 2015), both incorporated by reference herein). It should be noted that the first and/or second complementarity domains may contain one or more poly-A tracts, which can be recognized by RNA polymerases as a termination signal. The sequence of the first and second complementarity domains are, therefore, optionally modified to eliminate these tracts and promote the complete *in vitro* transcription of gRNAs, for instance through the use of A-G swaps as described in Briner, or A-U swaps. These and other similar modifications to the first and second complementarity domains are within the scope of the present disclosure.

[111] Along with the first and second complementarity domains, Cas9 gRNAs typically include two or more additional duplexed regions that are involved in nuclease activity *in vivo* but not necessarily *in vitro*. (Nishimasu 2015). A first stem-loop one near the 3' portion of the second complementarity domain is referred to variously as the “proximal domain,” (Cotta-Ramusino) “stem loop 1” (Nishimasu 2014 and 2015) and the “nexus” (Briner). One or more additional stem loop structures

are generally present near the 3' end of the gRNA, with the number varying by species: *S. pyogenes* gRNAs typically include two 3' stem loops (for a total of four stem loop structures including the repeat:anti-repeat duplex), while *S. aureus* and other species have only one (for a total of three stem loop structures). A description of conserved stem loop structures (and gRNA structures more generally) organized by species is provided in Briner.

[112] While the foregoing description has focused on gRNAs for use with Cas9, it should be appreciated that other RNA-guided nucleases have been (or may in the future be) discovered or invented which utilize gRNAs that differ in some ways from those described to this point. For instance, Cpf1 ("CRISPR from Prevotella and Francisella 1") is a recently discovered RNA-guided nuclease that does not require a tracrRNA to function. (Zetsche *et al.*, 2015, Cell 163, 759–771 October 22, 2015 (Zetsche I), incorporated by reference herein). A gRNA for use in a Cpf1 genome editing system generally includes a targeting domain and a complementarity domain (alternately referred to as a "handle"). It should also be noted that, in gRNAs for use with Cpf1, the targeting domain is usually present at or near the 3' end, rather than the 5' end as described above in connection with Cas9 gRNAs (the handle is at or near the 5' end of a Cpf1 gRNA).

[113] Those of skill in the art will appreciate that, although structural differences may exist between gRNAs from different prokaryotic species, or between Cpf1 and Cas9 gRNAs, the principles by which gRNAs operate are generally consistent. Because of this consistency of operation, gRNAs can be defined, in broad terms, by their targeting domain sequences, and skilled artisans will appreciate that a given targeting domain sequence can be incorporated in any suitable gRNA, including a unimolecular or chimeric gRNA, or a gRNA that includes one or more chemical modifications and/or sequential modifications (substitutions, additional nucleotides, truncations, *etc.*). Thus, for economy of presentation in this disclosure, gRNAs may be described solely in terms of their targeting domain sequences.

[114] More generally, skilled artisans will appreciate that some aspects of the present disclosure relate to systems, methods and compositions that can be implemented using multiple RNA-guided nucleases. For this reason, unless otherwise specified, the term gRNA should be understood to encompass any suitable gRNA that can be used with any RNA-guided nuclease, and not only those gRNAs that are compatible with a particular species of Cas9 or Cpf1. By way of illustration, the term gRNA can, in certain embodiments, include a gRNA for use with any RNA-guided nuclease occurring in a Class 2 CRISPR system, such as a type II or type V or CRISPR system, or an RNA-guided nuclease derived or adapted therefrom.

[115] In some embodiments, the guide RNA used comprises a modification as compared to the standard gRNA scaffold. Such modifications may comprise, for example, chemical modifications of a part of the gRNA, *e.g.*, of a nucleobase or backbone moiety. In some embodiments, such a modification may also include the presence of a DNA nucleotide within the gRNA, *e.g.*, within or outside of the targeting domain. In some embodiments, the modification may include an extension of

the gRNA scaffold, *e.g.*, by addition of 1-100 nucleotides, including RNA and/or DNA nucleotides at the 3' or the 5' terminus of the guide RNA, *e.g.*, at the terminus distal to the targeting domain.

[116] Generally, gRNAs include the sugar group ribose, which is a 5-membered ring having an oxygen. Exemplary modified gRNAs can include, without limitation, replacement of the oxygen in ribose (*e.g.*, with sulfur (S), selenium (Se), or alkylene, such as, *e.g.*, methylene or ethylene); addition of a double bond (*e.g.*, to replace ribose with cyclopentenyl or cyclohexenyl); ring contraction of ribose (*e.g.*, to form a 4-membered ring of cyclobutane or oxetane); ring expansion of ribose (*e.g.*, to form a 6- or 7-membered ring having an additional carbon or heteroatom, such as for example, anhydrohexitol, altritol, mannitol, cyclohexanyl, cyclohexenyl, and morpholino that also has a phosphoramidate backbone). Although the majority of sugar analog alterations are localized to the 2' position, other sites are amenable to modification, including the 4' position. In certain embodiments, a gRNA comprises a 4'-S, 4'-Se or a 4'-C-aminomethyl-2'-O-Me modification.

[117] In certain embodiments, deaza nucleotides, *e.g.*, 7-deaza-adenosine, can be incorporated into the gRNA. In certain embodiments, O- and N-alkylated nucleotides, *e.g.*, N6-methyl adenosine, can be incorporated into the gRNA. In certain embodiments, one or more or all of the nucleotides in a gRNA are deoxynucleotides.

[118] In certain embodiments, gRNAs as used herein may be modified or unmodified gRNAs. In certain embodiments, a gRNA may include one or more modifications. In certain embodiments, the one or more modifications may include a phosphorothioate linkage modification, a phosphorodithioate (PS2) linkage modification, a 2'-O-methyl modification, or combinations thereof. In certain embodiments, the one or more modifications may be at the 5' end of the gRNA, at the 3' end of the gRNA, or combinations thereof.

[119] In certain embodiments, a gRNA modification may comprise one or more phosphorodithioate (PS2) linkage modifications.

[120] In some embodiments, a gRNA used herein includes one or more or a stretch of deoxyribonucleic acid (DNA) bases, also referred to herein as a "DNA extension." In some embodiments, a gRNA used herein includes a DNA extension at the 5' end of the gRNA, the 3' end of the gRNA, or a combination thereof. In certain embodiments, the DNA extension may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 DNA bases long. For example, in certain embodiments, the DNA extension may be 1, 2, 3, 4, 5, 10, 15, 20, or 25 DNA bases long. In certain embodiments, the DNA extension may include one or more DNA bases selected from adenine (A), guanine (G), cytosine (C), or thymine (T). In certain embodiments, the DNA extension includes the same DNA bases. For example, the DNA extension may include a stretch of adenine (A) bases. In certain embodiments, the DNA extension may include a stretch of thymine (T) bases. In certain

embodiments, the DNA extension includes a combination of different DNA bases. In certain embodiments, a DNA extension may comprise a sequence set forth in Table 3.

[121] In certain embodiments, a gRNA used herein includes a DNA extension as well as one or more chemical modification, *e.g.*, one or more phosphorothioate linkage modifications, one or more phosphorodithioate (PS2) linkage modifications, one or more 2'-O-methyl modifications, or combinations thereof. In certain embodiments, the one or more modifications may be at the 5' end of the gRNA, at the 3' end of the gRNA, or combinations thereof. In certain embodiments, a gRNA including a DNA extension may comprise a sequence set forth in Table 3 that includes a DNA extension. Without wishing to be bound by theory, it is contemplated that any DNA extension may be used herein, so long as it does not hybridize to the target nucleic acid being targeted by the gRNA. In some embodiments, a gRNA with a DNA extension exhibits an increase in editing at the target nucleic acid site relative to a gRNA which does not include such a DNA extension. In some embodiments, a gRNA with a DNA extension exhibits more effective delivery into NK cells and/or stem cells relative to a gRNA which does not include such an extension.

[122] In some embodiments, a gRNA used herein includes one or more or a stretch of ribonucleic acid (RNA) bases, also referred to herein as an "RNA extension." In some embodiments, a gRNA used herein includes an RNA extension at the 5' end of the gRNA, the 3' end of the gRNA, or a combination thereof. In certain embodiments, the RNA extension may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, 45, 46, 47, 48, 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, 99, or 100 RNA bases long. For example, in certain embodiments, the RNA extension may be 1, 2, 3, 4, 5, 10, 15, 20, or 25 RNA bases long. In certain embodiments, the RNA extension may include one or more RNA bases selected from adenine (rA), guanine (rG), cytosine (rC), or uracil (rU), in which the "r" represents RNA, 2'-hydroxy. In certain embodiments, the RNA extension includes the same RNA bases. For example, the RNA extension may include a stretch of adenine (rA) bases. In certain embodiments, the RNA extension includes a combination of different RNA bases. In certain embodiments, an RNA extension may comprise a sequence set forth in Table 3.

[123] In certain embodiments, a gRNA used herein includes an RNA extension as well as one or more chemical modifications, *e.g.*, one or more phosphorothioate linkage modifications, one or more phosphorodithioate (PS2) linkage modifications, one or more 2'-O-methyl modifications, or combinations thereof. In certain embodiments, the one or more modifications may be at the 5' end of the gRNA, at the 3' end of the gRNA, or combinations thereof. In certain embodiments, a gRNA including a RNA extension may comprise a sequence set forth in Table 3 that includes an RNA extension. gRNAs including an RNA extension at the 5' end of the gRNA may comprise a sequence

disclosed herein. gRNAs including an RNA extension at the 3' end of the gRNA may comprise a sequence disclosed herein.

[124] It is contemplated that gRNAs used herein may also include an RNA extension and a DNA extension. In certain embodiments, the RNA extension and DNA extension may both be at the 5' end of the gRNA, the 3' end of the gRNA, or a combination thereof. In certain embodiments, the RNA extension is at the 5' end of the gRNA and the DNA extension is at the 3' end of the gRNA. In certain embodiments, the RNA extension is at the 3' end of the gRNA and the DNA extension is at the 5' end of the gRNA.

[125] In some embodiments, a gRNA which includes a modification, *e.g.*, a DNA extension at the 5' end, and/or a chemical modification as disclosed herein, is complexed with a RNA-guided nuclease, *e.g.*, an AsCpf1 nuclease, to form an RNP, which is then employed to edit a target cell, *e.g.*, an NK cell.

[126] Exemplary suitable 5' extensions for Cpf1 guide RNAs are provided in the table below:

Table 3: gRNA 5' Extensions

SEQ ID NO:	5' extension sequence	5' modification
	rCrUrUrUrU	+5 RNA
1	rArArGrArCrCrUrUrUrU	+10 RNA
2	rArUrGrUrGrUrUrUrUrUrGrUrCrArArArArGrArCrCrUrUrUrU	+25 RNA
3	rArGrGrCrCrArGrCrUrUrGrCrCrGrGrUrUrUrUrUrArGrUrCrGrUrGrCrUrGrCrUrUrCrArUrGrUrGrUrUrUrUrGrUrCrArArArArGrArCrCrUrUrUrU	+60 RNA
	CTTTT	+5 DNA
4	AAGACCTTTT	+10 DNA
5	ATGTGTTTTTGTCAAAAGACCTTTT	+25 DNA
6	AGGCCAGCTTGCCGGTTTTTTAGTCGTGCTGCTTCATGTGTTT TTGTCAAAAGACCTTTT	+60 DNA
7	TTTTTGTCAAAAGACCTTTT	+20 DNA
8	GCTTCATGTGTTTTTGTCAAAAGACCTTTT	+30 DNA
9	GCCGGTTTTTTAGTCGTGCTGCTTCATGTGTTTTTGTCAAAAG ACCTTTT	+50 DNA
10	TAGTCGTGCTGCTTCATGTGTTTTTGTCAAAAGACCTTTT	+40 DNA
11	C*C*GAAGTTTTCTTCGGTTTT	+20 DNA + 2xPS
12	T*T*TTCCGAAGTTTTCTTCGGTTTT	+25 DNA + 2xPS
13	A*A*CGCTTTTTCCGAAGTTTTCTTCGGTTTT	+30 DNA + 2xPS
14	G*C*GTTGTTTTCAACGCTTTTTCCGAAGTTTTCTTCGGTTTT	+41 DNA + 2xPS
15	G*G*CTTCTTTTGAAGCCTTTTTGCGTTGTTTTCAACGCTTTTT CCGAAGTTTTCTTCGGTTTT	+62 DNA + 2xPS
16	A*T*GTGTTTTTGTCAAAAGACCTTTT	+25 DNA + 2xPS
17	AAAAAAAAAAAAAAAAAAAAAAAAA	+25 A
18	TTTTTTTTTTTTTTTTTTTTTTTTT	+25 T
19	mA*mU*rGrUrGrUrUrUrUrGrUrCrArArArArGrArCrCrUrUrUrU	+25 RNA + 2xPS
20	mA*mA*rArArArArArArArArArArArArArArArArArArArA	PolyA RNA + 2xPS

21	mU*mU*rUrUrUrUrUrUrUrUrUrUrUrUrUrUrUrUrUrUrUrUrU	PolyU RNA + 2xPS
All bases are in upper case Lowercase “r” represents RNA, 2’-hydroxy; bases not modified by an “r” are DNA All bases are linked via standard phosphodiester bonds except as noted: “*” represents phosphorothioate modification “PS” represents phosphorothioate modification		

[127] Additional suitable gRNA modifications will be apparent to those of ordinary skill in the art based on the present disclosure. Suitable gRNA modifications include, for example, those described in PCT application PCT/US2018/054027, filed on October 2, 2018, and entitled “*MODIFIED CPF1 GUIDE RNA*,” in PCT application PCT/US2015/000143, filed on December 3, 2015, and entitled “*GUIDE RNA WITH CHEMICAL MODIFICATIONS*,” in PCT application PCT/US2016/026028, filed April 5, 2016, and entitled “*CHEMICALLY MODIFIED GUIDE RNAS FOR CRISPR/CAS-MEDIATED GENE REGULATION*,” and in PCT application PCT/US2016/053344, filed on September 23, 2016, and entitled “*NUCLEASE-MEDIATED GENOME EDITING OF PRIMARY CELLS AND ENRICHMENT THEREOF*,” the entire contents of each of which are incorporated herein by reference.

gRNA design

[128] Methods for selection and validation of target sequences as well as off-target analyses have been described previously, *e.g.*, in Mali; Hsu; Fu *et al.*, 2014 Nat biotechnol 32(3): 279-84, Heigwer *et al.*, 2014 Nat methods 11(2):122-3; Bae *et al.* (2014) Bioinformatics 30(10): 1473-5; and Xiao A *et al.* (2014) Bioinformatics 30(8): 1180-1182. Each of these references is incorporated by reference herein. As a non-limiting example, gRNA design may involve the use of a software tool to optimize the choice of potential target sequences corresponding to a user’s target sequence, *e.g.*, to minimize total off-target activity across the genome. While off-target activity is not limited to cleavage, the cleavage efficiency at each off-target sequence can be predicted, *e.g.*, using an experimentally-derived weighting scheme. These and other guide selection methods are described in detail in Maeder and Cotta-Ramusino.

[129] In certain embodiments, one or more or all of the nucleotides in a gRNA are modified. Strategies for modifying a gRNA are described in WO2019/152519, published August 8, 2019, the entire contents of which are expressly incorporated herein by reference.

[130] Non-limiting examples of guide RNAs suitable for certain embodiments embraced by the present disclosure are provided herein, for example, in the Tables below. Those of ordinary skill in the art will be able to envision suitable guide RNA sequences for a specific nuclease, *e.g.*, a Cas9 or Cpf-1 nuclease, from the disclosure of the targeting domain sequence, either as a DNA or RNA sequence. For example, a guide RNA comprising a targeting sequence consisting of RNA nucleotides

would include the RNA sequence corresponding to the targeting domain sequence provided as a DNA sequence, and this contain uracil instead of thymidine nucleotides. For example, a guide RNA comprising a targeting domain sequence consisting of RNA nucleotides, and described by the DNA sequence TCTGCAGAAATGTTCCCCGT (SEQ ID NO:22) would have a targeting domain of the corresponding RNA sequence UCUGCAGAAAUGUCCCCGU (SEQ ID NO:23). As will be apparent to the skilled artisan, such a targeting sequence would be linked to a suitable guide RNA scaffold, *e.g.*, a crRNA scaffold sequence or a chimeric crRNA/tracrRNA scaffold sequence. Suitable gRNA scaffold sequences are known to those of ordinary skill in the art. For AsCpf1, for example, a suitable scaffold sequence comprises the sequence UAAUUUCUACUCUUGUAGAU (SEQ ID NO:24), added to the 5'-terminus of the targeting domain. In the example above, this would result in a Cpf1 guide RNA of the sequence UAAUUUCUACUCUUGUAGAUUCUGCAGAAAUGUCCCCGU (SEQ ID NO:25). Those of skill in the art would further understand how to modify such a guide RNA, *e.g.*, by adding a DNA extension (*e.g.*, in the example above, adding a 25-mer DNA extension as described herein would result, for example, in a guide RNA of the sequence ATGTGTTTTTGTCAAAAGACCTTTTrUrArArUrUrCrUrArCrUrCrUrUrGrUrArGrArUrUrCrUrGrCrArGrArArUrGrUrUrCrCrCrCrGrU (SEQ ID NO:26), ATGTGTTTTTGTCAAAAGACCTTTTrUrArArUrUrCrUrArCrUrCrUrUrGrUrArGrArUrArCrUrGrArCrArGrCrGrUrGrArArCrArGrGrUrArG (SEQ ID NO: 1164), or ATGTGTTTTTGTCAAAAGACCTTTTrUrArArUrUrCrUrArCrUrCrUrUrGrUrArGrArUrUrGrArUrGrUrGrArGrArUrUrUrCrCrArCrCrU (SEQ ID NO: 1166). It will be understood that the exemplary targeting sequences provided herein are not limiting, and additional suitable sequences, *e.g.*, variants of the specific sequences disclosed herein, will be apparent to the skilled artisan based on the present disclosure in view of the general knowledge in the art.

[131] In some embodiments the gRNA for use in the disclosure is a gRNA targeting TGFbetaR2 (TGFβR2 gRNA). In some embodiments, the gRNA targeting TGFbetaR2 is one or more of the gRNAs described in Table 4.

Table 4. TGFbetaR2 gRNAs

Name	gRNA Targeting Domain Sequence (DNA)	Length	SEQ ID NO:	Enzyme
TGFBR24326	CAGGACGATGTGCAGCGGCC	20	540	AsCpf1 RR
TGFBR24327	ACCGCACGTTTCAGAAGTCGG	20	541	AsCpf1 RR
TGFBR24328	ACAACGTGTGTAATTTTGTG	20	542	AsCpf1 RR
TGFBR24329	CAACTGTGTAAATTTTGTGA	20	543	AsCpf1 RR
TGFBR24330	ACCTGTGACAACCAGAAATC	20	544	AsCpf1 RR
TGFBR24331	CCTGTGACAACCAGAAATCC	20	545	AsCpf1 RR
TGFBR24332	TGTGGCTTCTCACAGATGGA	20	546	AsCpf1 RR
TGFBR24333	TCTGTGAGAAGCCACAGGAA	20	547	AsCpf1 RR

TGFBR24334	AAGCTCCCCTACCATGACTT	20	548	AsCpf1 RR
TGFBR24335	GAATAAAGTCATGGTAGGGG	20	549	AsCpf1 RR
TGFBR24336	AGAATAAAGTCATGGTAGGG	20	550	AsCpf1 RR
TGFBR24337	CTACCATGACTTTATTCTGG	20	551	AsCpf1 RR
TGFBR24338	TACCATGACTTTATTCTGGA	20	552	AsCpf1 RR
TGFBR24339	TAATGCACTTTGGAGAAGCA	20	553	AsCpf1 RR
TGFBR24340	TTCATAATGCACTTTGGAGA	20	554	AsCpf1 RR
TGFBR24341	AAGTGCATTATGAAGGAAAA	20	555	AsCpf1 RR
TGFBR24342	TGTGTTCCCTGTAGCTCTGAT	20	556	AsCpf1 RR
TGFBR24343	TGTAGCTCTGATGAGTGCAA	20	557	AsCpf1 RR
TGFBR24344	AGTGACAGGCATCAGCCTCC	20	558	AsCpf1 RR
TGFBR24345	AGTGGTGGCAGGAGGCTGAT	20	559	AsCpf1 RR
TGFBR24346	AGGTTGAACTCAGCTTCTGC	20	560	AsCpf1 RR
TGFBR24347	CAGGTTGAACTCAGCTTCTG	20	561	AsCpf1 RR
TGFBR24348	ACCTGGGAAACCGGCAAGAC	20	562	AsCpf1 RR
TGFBR24349	CGTCTTGCCGGTTTCCCAGG	20	563	AsCpf1 RR
TGFBR24350	GCGTCTTGCCGGTTTCCCAG	20	564	AsCpf1 RR
TGFBR24351	TGAGCTTCCGCGTCTTGCCG	20	565	AsCpf1 RR
TGFBR24352	GCGAGCACTGTGCCATCATC	20	566	AsCpf1 RR
TGFBR24353	GGATGATGGCACAGTGCTCG	20	567	AsCpf1 RR
TGFBR24354	AGGATGATGGCACAGTGCTC	20	568	AsCpf1 RR
TGFBR24355	CGTGTGCCAACAACATCAAC	20	569	AsCpf1 RR
TGFBR24356	GCTCAATGGGCAGCAGCTCT	20	570	AsCpf1 RR
TGFBR24357	ACCAGGGTGTCCAGCTCAAT	20	571	AsCpf1 RR
TGFBR24358	CACCAGGGTGTCCAGCTCAA	20	572	AsCpf1 RR
TGFBR24359	CCACCAGGGTGTCCAGCTCA	20	573	AsCpf1 RR
TGFBR24360	GCTTGGCCTTATAGACCTCA	20	574	AsCpf1 RR
TGFBR24361	GAGCAGTTTGAGACAGTGGC	20	575	AsCpf1 RR
TGFBR24362	AGAGGCATACTCCTCATAGG	20	576	AsCpf1 RR
TGFBR24363	CTATGAGGAGTATGCCTCTT	20	577	AsCpf1 RR
TGFBR24364	AAGAGGCATACTCCTCATAG	20	578	AsCpf1 RR
TGFBR24365	TATGAGGAGTATGCCTCTTG	20	579	AsCpf1 RR
TGFBR24366	GATTGATGTCTGAGAAGATG	20	580	AsCpf1 RR
TGFBR24367	CTCCTCAGCCGTCAGGAACT	20	581	AsCpf1 RR
TGFBR24368	GTTCTTGACGGCTGAGGAGC	20	582	AsCpf1 RR
TGFBR24369	GCTCCTCAGCCGTCAGGAAC	20	583	AsCpf1 RR
TGFBR24370	TGACGGCTGAGGAGCGGAAG	20	584	AsCpf1 RR
TGFBR24371	TCTTCCGCTCCTCAGCCGTC	20	585	AsCpf1 RR
TGFBR24372	AACTCCGTCTTCCGCTCCTC	20	586	AsCpf1 RR
TGFBR24373	CAACTCCGTCTTCCGCTCCT	20	587	AsCpf1 RR
TGFBR24374	CCAACTCCGTCTTCCGCTCC	20	588	AsCpf1 RR
TGFBR24375	ACGCCAAGGGCAACCTACAG	20	589	AsCpf1 RR
TGFBR24376	CGCCAAGGGCAACCTACAGG	20	590	AsCpf1 RR
TGFBR24377	AGCTGATGACATGCCGCGTC	20	591	AsCpf1 RR

TGFBR24378	GGGCGAGGGAGCTGCCCAGC	20	592	AsCpf1 RR
TGFBR24379	CGGGCGAGGGAGCTGCCCAG	20	593	AsCpf1 RR
TGFBR24380	CCGGGCGAGGGAGCTGCCCA	20	594	AsCpf1 RR
TGFBR24381	TCGCCCCGGGGGATTGCTCAC	20	595	AsCpf1 RR
TGFBR24382	ACATGGAGTGTGATCACTGT	20	596	AsCpf1 RR
TGFBR24383	CAGTGATCACACTCCATGTG	20	597	AsCpf1 RR
TGFBR24384	TGTGGGAGGCCCAAGATGCC	20	598	AsCpf1 RR
TGFBR24385	TGTGCACGATGGGCATCTTG	20	599	AsCpf1 RR
TGFBR24386	CGAGGATATTGGAGCTCTTG	20	600	AsCpf1 RR
TGFBR24387	ATATCCTCGTGAAGAACGAC	20	601	AsCpf1 RR
TGFBR24388	GACGCAGGGAAAGCCCAAAG	20	602	AsCpf1 RR
TGFBR24389	CTGCGTCTGGACCCTACTCT	20	603	AsCpf1 RR
TGFBR24390	TGCGTCTGGACCCTACTCTG	20	604	AsCpf1 RR
TGFBR24391	CAGACAGAGTAGGGTCCAGA	20	605	AsCpf1 RR
TGFBR24392	GCCAGCACGATCCCACCGCA	20	606	AsCpf1 RVR
TGFBR24393	AAGGAAAAAAAAAAGCCTGG	20	607	AsCpf1 RVR
TGFBR24394	ACACCAGCAATCCTGACTTG	20	608	AsCpf1 RVR
TGFBR24395	ACTAGCAACAAGTCAGGATT	20	609	AsCpf1 RVR
TGFBR24396	GCAACTCCCAGTGGTGGCAG	20	610	AsCpf1 RVR
TGFBR24397	TGTCATCATCATCTTCTACT	20	611	AsCpf1 RVR
TGFBR24398	GACCTCAGCAAAGCGACCTT	20	612	AsCpf1 RVR
TGFBR24399	AGGCCAAGCTGAAGCAGAAC	20	613	AsCpf1 RVR
TGFBR24400	AGGAGTATGCCTCTTGGAAG	20	614	AsCpf1 RVR
TGFBR24401	CCTCTTGGAAGACAGAGAAG	20	615	AsCpf1 RVR
TGFBR24402	TTCTCATGCTTCAGATTGAT	20	616	AsCpf1 RVR
TGFBR24403	CTCGTGAAGAACGACCTAAC	20	617	AsCpf1 RVR
TGFbR2036	GGCCGCTGCACATCGTCCTG	20	618	SpyCas9
TGFbR2037	GCGGGGTCTGCCATGGGTCG	20	619	SpyCas9
TGFbR2038	AGTTGCTCATGCAGGATTTC	20	620	SpyCas9
TGFbR2039	CCAGAATAAAGTCATGGTAG	20	621	SpyCas9
TGFbR2040	CCCCTACCATGACTTTATTC	20	622	SpyCas9
TGFbR2041	AAGTCATGGTAGGGGAGCTT	20	623	SpyCas9
TGFbR2042	AGTCATGGTAGGGGAGCTTG	20	624	SpyCas9
TGFbR2043	ATTGCACTCATCAGAGCTAC	20	625	SpyCas9
TGFbR2044	CCTAGAGTGAAGAGATTTCAT	20	626	SpyCas9
TGFbR2045	CCAATGAATCTCTTCACTCT	20	627	SpyCas9
TGFbR2046	AAAGTCATGGTAGGGGAGCT	20	628	SpyCas9
TGFbR2047	GTGAGCAATCCCCCGGGCGA	20	629	SpyCas9
TGFbR2048	GTCGTTCTTCACGAGGATAT	20	630	SpyCas9
TGFbR2049	GCCGCGTCAGGTACTCCTGT	20	631	SpyCas9
TGFbR2050	GACGCGGCATGTCATCAGCT	20	632	SpyCas9
TGFbR2051	GCTTCTGCTGCCGGTTAACG	20	633	SpyCas9
TGFbR2052	GTGGATGACCTGGCTAACAG	20	634	SpyCas9
TGFbR2053	GTGATCACACTCCATGTGGG	20	635	SpyCas9

TGFbR2054	GCCCATTGAGCTGGACACCC	20	636	SpyCas9
TGFbR2055	GCGGTCATCTTCCAGGATGA	20	637	SpyCas9
TGFbR2056	GGGAGCTGCCCAGCTTGCGC	20	638	SpyCas9
TGFbR2057	GTTGATGTTGTTGGCACACG	20	639	SpyCas9
TGFbR2058	GGCATCTTGGGCCTCCCACA	20	640	SpyCas9
TGFbR2059	GCGGCATGTCATCAGCTGGG	20	641	SpyCas9
TGFbR2060	GCTCCTCAGCCGTCAGGAAC	20	642	SpyCas9
TGFbR2061	GCTGGTGTATATTCTGATG	20	643	SpyCas9
TGFbR2062	CCGACTTCTGAACGTGCGGT	20	644	SpyCas9
TGFbR2063	TGCTGGCGATACGCGTCCAC	20	645	SpyCas9
TGFbR2064	CCCGACTTCTGAACGTGCGG	20	646	SpyCas9
TGFbR2065	CCACCGCACGTTCAGAAGTC	20	647	SpyCas9
TGFbR2066	TCACCCGACTTCTGAACGTG	20	648	SpyCas9
TGFbR2067	CCCACCGCACGTTCAGAAGT	20	649	SpyCas9
TGFbR2068	CGAGCAGCGGGGTCTGCCAT	20	650	SpyCas9
TGFbR2069	ACGAGCAGCGGGGTCTGCCA	20	651	SpyCas9
TGFbR2070	AGCGGGGTCTGCCATGGGTC	20	652	SpyCas9
TGFbR2071	CCTGAGCAGCCCCGACCCA	20	653	SpyCas9
TGFbR2072	CCATGGGTCTGGGGGCTGCTC	20	654	SpyCas9
TGFbR2073	AACGTGCGGTGGGATCGTGC	20	655	SpyCas9
TGFbR2074	GGACGATGTGCAGCGGCCAC	20	656	SpyCas9
TGFbR2075	GTCCACAGGACGATGTGCAG	20	657	SpyCas9
TGFbR2076	CATGGGTCTGGGGGCTGCTCA	20	658	SpyCas9
TGFbR2077	CAGCGGGGTCTGCCATGGGT	20	659	SpyCas9
TGFbR2078	ATGGGTCTGGGGGCTGCTCAG	20	660	SpyCas9
TGFbR2079	CGGGGTCTGCCATGGGTCTGG	20	661	SpyCas9
TGFbR2080	AGGAAGTCTGTGTGGCTGTA	20	662	SpyCas9
TGFbR2081	CTCCATCTGTGAGAAGCCAC	20	663	SpyCas9
TGFbR2082	ATGATAGTCACTGACAACAA	20	664	SpyCas9
TGFbR2083	GATGCTGCAGTTGCTCATGC	20	665	SpyCas9
TGFbR2084	ACAGCCACACAGACTTCCTG	20	666	SpyCas9
TGFbR2085	GAAGCCACAGGAAGTCTGTG	20	667	SpyCas9
TGFbR2086	TTCCTGTGGCTTCTCACAGA	20	668	SpyCas9
TGFbR2087	CTGTGGCTTCTCACAGATGG	20	669	SpyCas9
TGFbR2088	TCACAAAATTTACACAGTTG	20	670	SpyCas9
TGFbR2089	GACAACATCATCTTCTCAGA	20	671	SpyCas9
TGFbR2090	TCCAGAATAAAGTCATGGTA	20	672	SpyCas9
TGFbR2091	GGTAGGGGAGCTTGGGGTCA	20	673	SpyCas9
TGFbR2092	TTCTCCAAAGTGCATTATGA	20	674	SpyCas9
TGFbR2093	CATCTTCCAGAATAAAGTCA	20	675	SpyCas9
TGFbR2094	CACATGAAGAAAGTCTCACC	20	676	SpyCas9
TGFbR2095	TTCCAGAATAAAGTCATGGT	20	677	SpyCas9
TGFbR2096	TTTTCCTTCATAATGCACTT	20	678	SpyCas9
TGFBR24024	CACAGTTGTGGAAACTTGAC	20	679	AsCpf1

TGFBR24039	CCCAACTCCGTCTTCCGCTC	20	680	AsCpf1
TGFBR24040	GGCTTTCCTGCGTCTGGAC	20	681	AsCpf1
TGFBR24036	CTGAGGTCTATAAGGCCAAG	20	682	AsCpf1
TGFBR24026	TGATGTGAGATTTTCCACCT	20	683	AsCpf1
TGFBR38402	TGATGTGAGATTTTCCACCTG	21	1173	AsCpf1
TGFBR24038	CCTATGAGGAGTATGCCTCT	20	684	AsCpf1
TGFBR24033	AAGTGACAGGCATCAGCCTC	20	685	AsCpf1
TGFBR24028	CCATGACCCCAAGCTCCCCT	20	686	AsCpf1
TGFBR24031	CTTCATAATGCACTTTGGAG	20	687	AsCpf1
TGFBR24032	TTCATGTGTTTCTGTAGCTC	20	688	AsCpf1
TGFBR24029	TTCTGGAAGATGCTGCTTCT	20	689	AsCpf1
TGFBR24035	CCCACCAGGGTGTCCAGCTC	20	690	AsCpf1
TGFBR24037	AGACAGTGGCAGTCAAGATC	20	691	AsCpf1
TGFBR24041	CCTGCGTCTGGACCCTACTC	20	692	AsCpf1
TGFBR24025	CACAACTGTGTAAATTTTGT	20	693	AsCpf1
TGFBR24030	GAGAAGCAGCATCTTCCAGA	20	694	AsCpf1
TGFBR24027	TGGTTGTACAGGTGGAAAA	20	695	AsCpf1
TGFBR24034	CCAGGTTGAACTCAGCTTCT	20	696	AsCpf1
TGFBR24043	ATCACAAAATTTACACAGTTG	21	697	SauCas9
TGFBR24065	GGCATCAGCCTCCTGCCACCA	21	698	SauCas9
TGFBR24110	GTTAGCCAGGTCATCCACAGA	21	699	SauCas9
TGFBR24099	GCTGGGCAGCTCCCTCGCCCG	21	700	SauCas9
TGFBR24064	CAGGAGGCTGATGCCTGTAC	21	701	SauCas9
TGFBR24094	GAGGAGCGGAAGACGGAGTTG	21	702	SauCas9
TGFBR24108	CGTCTGGACCCTACTCTGTCT	21	703	SauCas9
TGFBR24058	TTTTTCCTTCATAATGCACTT	21	704	SauCas9
TGFBR24075	CCATTGAGCTGGACACCCTGG	21	705	SauCas9
TGFBR24057	CTTCTCCAAAGTGCATTATGA	21	706	SauCas9
TGFBR24103	GCCCAAGATGCCCATCGTGCA	21	707	SauCas9
TGFBR24060	TCATGTGTTCTGTAGCTCTG	21	708	SauCas9
TGFBR24048	GTGATGCTGCAGTTGCTCATG	21	709	SauCas9
TGFBR24087	TCTCATGCTTCAGATTGATGT	21	710	SauCas9
TGFBR24081	TCCCTATGAGGAGTATGCCTC	21	711	SauCas9
TGFBR24044	CATCACAAAATTTACACAGTT	21	712	SauCas9
TGFBR24077	ATTGAGCTGGACACCCTGGTG	21	713	SauCas9
TGFBR24080	CAGTCAAGATCTTCCCTATG	21	714	SauCas9
TGFBR24046	AGGATTTCTGGTTGTACAGG	21	715	SauCas9
TGFBR24101	TCCACAGTGATCACACTCCAT	21	716	SauCas9
TGFBR24079	AGCAGAACTTCAGAGCAGT	21	717	SauCas9
TGFBR24072	CCGGCAAGACGCGGAAGCTCA	21	718	SauCas9
TGFBR24074	GATGTCAGAGCGGTCATCTTC	21	719	SauCas9
TGFBR24062	TCATTGCACTCATCAGAGCTA	21	720	SauCas9
TGFBR24054	CTTCCAGAATAAAGTCATGGT	21	721	SauCas9
TGFBR24045	AGATTTTCCACCTGTGACAAC	21	722	SauCas9

TGFBR24049	ACTGCAGCATCACCTCCATCT	21	723	SauCas9
TGFBR24098	AGCTGGGCAGCTCCCTCGCCC	21	724	SauCas9
TGFBR24090	TGACGGCTGAGGAGCGGAAGA	21	725	SauCas9
TGFBR24076	CATTGAGCTGGACACCCTGGT	21	726	SauCas9
TGFBR24078	AGCAAAGCGACCTTTCCCCAC	21	727	SauCas9
TGFBR24067	CGCGTTAACCGGCAGCAGAAG	21	728	SauCas9
TGFBR24063	GAAATATGACTAGCAACAAGT	21	729	SauCas9
TGFBR24107	AGACAGAGTAGGGTCCAGACG	21	730	SauCas9
TGFBR24047	CAGGATTTCTGGTTGTACAG	21	731	SauCas9
TGFBR24096	CTCCTGTAGGTTGCCCTTGGC	21	732	SauCas9
TGFBR24105	ACAGAGTAGGGTCCAGACGCA	21	733	SauCas9
TGFBR24056	GCTTCTCCAAAGTGCATTATG	21	734	SauCas9
TGFBR24068	GCAGCAGAAGCTGAGTTCAAC	21	735	SauCas9
TGFBR24093	TGAGGAGCGGAAGACGGAGTT	21	736	SauCas9
TGFBR24055	CTTTGGAGAAGCAGCATCTTC	21	737	SauCas9
TGFBR24053	CTCCCCCTACCATGACTTTATT	21	738	SauCas9
TGFBR24106	GACAGAGTAGGGTCCAGACGC	21	739	SauCas9
TGFBR24092	CTGAGGAGCGGAAGACGGAGT	21	740	SauCas9
TGFBR24102	GGGCATCTTGGGCCTCCCACA	21	741	SauCas9
TGFBR24082	CCAAGAGGCATACTCCTCATA	21	742	SauCas9
TGFBR24051	AGAATGACGAGAACATAACAC	21	743	SauCas9
TGFBR24097	CCTGACGCGGCATGTCATCAG	21	744	SauCas9
TGFBR24073	AGCGAGCACTGTGCCATCATC	21	745	SauCas9
TGFBR24104	GCAGGTTAGGTCGTTCTTCAC	21	746	SauCas9
TGFBR24050	ACCTCCATCTGTGAGAAGCCA	21	747	SauCas9
TGFBR24052	TAAAGTCATGGTAGGGGAGCT	21	748	SauCas9
TGFBR24061	TCAGAGCTACAGGAACACATG	21	749	SauCas9
TGFBR24086	TCTCAGACATCAATCTGAAGC	21	750	SauCas9
TGFBR24066	CATCAGCCTCCTGCCACCACT	21	751	SauCas9
TGFBR24089	CGCTCCTCAGCCGTCAGGAAC	21	752	SauCas9
TGFBR24071	AACCTGGGAAACCGGCAAGAC	21	753	SauCas9
TGFBR24095	TCCACGCCAAGGGCAACCTAC	21	754	SauCas9
TGFBR24100	GAGGTGAGCAATCCCCCGGGC	21	755	SauCas9
TGFBR24069	CAGCAGAAGCTGAGTTCAACC	21	756	SauCas9
TGFBR24083	TCCAAGAGGCATACTCCTCAT	21	757	SauCas9
TGFBR24070	AGCAGAAGCTGAGTTCAACCT	21	758	SauCas9
TGFBR24088	CCAGTTCCTGACGGCTGAGGA	21	759	SauCas9
TGFBR24085	AGGAGTATGCCTCTTGGAAGA	21	760	SauCas9
TGFBR24084	TTCCAAGAGGCATACTCCTCA	21	761	SauCas9
TGFBR24042	CAACTGTGTAAATTTTGTGAT	21	762	SauCas9
TGFBR24059	TGAAGGAAAAAAAAAAGCCTG	21	763	SauCas9
TGFBR24091	CGTCTTCCGCTCCTCAGCCGT	21	764	SauCas9
TGFBR24109	CCAGGTCATCCACAGACAGAG	21	765	SauCas9
TGFBR2736	GCCTAGAGTGAAGAGATTCAT	21	766	SpyCas9

TGFBR2737	GTTCTCCAAAGTGCATTATGA	21	767	SpyCas9
TGFBR2738	GCATCTTCCAGAATAAAGTCA	21	768	SpyCas9

[132] In some embodiments the gRNA for use in the disclosure is a gRNA targeting CISH (CISH gRNA). In some embodiments, the gRNA targeting CISH is one or more of the gRNAs described in Table 5.

Table 5. CISH gRNAs

Name	gRNA Targeting Domain Sequence (DNA)	Length	SEQ ID NO:	Enzyme
CISH0873	CAACCGTCTGGTGGCCGACG	20	769	SpyCas9
CISH0874	CAGGATCGGGGCTGTCGCTT	20	770	SpyCas9
CISH0875	TCGGGCCTCGCTGGCCGTAA	20	771	SpyCas9
CISH0876	GAGGTAGTCGGCCATGCGCC	20	772	SpyCas9
CISH0877	CAGGTGTTGTCTGGGCCTCGC	20	773	SpyCas9
CISH0878	GGAGGTAGTCGGCCATGCGC	20	774	SpyCas9
CISH0879	GGCATACTCAATGCGTACAT	20	775	SpyCas9
CISH0880	CCGCCTTGTCATCAACCGTC	20	776	SpyCas9
CISH0881	AGGATCGGGGCTGTCGCTTC	20	777	SpyCas9
CISH0882	CCTTGTCATCAACCGTCTGG	20	778	SpyCas9
CISH0883	TACTCAATGCGTACATTGGT	20	779	SpyCas9
CISH0884	GGGTTCATTACGGCCAGCG	20	780	SpyCas9
CISH0885	GGCACTGCTTCTGCGTACAA	20	781	SpyCas9
CISH0886	GGTTGATGACAAGGCGGCAC	20	782	SpyCas9
CISH0887	TGCTGGGGCCTTCCTCGAGG	20	783	SpyCas9
CISH0888	TTGCTGGCTGTGGAGCGGAC	20	784	SpyCas9
CISH0889	TTCTCCTACCTTCGGGAATC	20	785	SpyCas9
CISH0890	GACTGGCTTGGGCAGTTCCA	20	786	SpyCas9
CISH0891	CATGCAGCCCTTGCTGCTG	20	787	SpyCas9
CISH0892	AGCAAAGGACGAGGTCTAGA	20	788	SpyCas9
CISH0893	GCCTGCTGGGGCCTTCCTCG	20	789	SpyCas9
CISH0894	CAGACTCACCAGATTCCCGA	20	790	SpyCas9
CISH0895	ACCTCGTCCTTTGCTGGCTG	20	791	SpyCas9
CISH0896	CTCACCAGATTCCCGAAGGT	20	792	SpyCas9
CISH7048	TACGCAGAAGCAGTGCCCGC	20	793	AsCpf1
CISH7049	AGGTGTACAGCAGTGGCTGG	20	794	AsCpf1
CISH7050	GGTGTACAGCAGTGGCTGGT	20	795	AsCpf1
CISH7051	CGGATGTGGTCAGCCTTGTG	20	796	AsCpf1
CISH7052	CACTGACAGCGTGAACAGGT	20	797	AsCpf1
CISH7053	ACTGACAGCGTGAACAGGTA	20	798	AsCpf1
CISH7054	GCTCACTCTCTGTCTGGGCT	20	799	AsCpf1
CISH7055	CTGGCTGTGGAGCGGACTGG	20	800	AsCpf1
CISH7056	GCTCTGACTGTACGGGGCAA	20	801	AsCpf1 RR
CISH7057	AGCTCTGACTGTACGGGGCA	20	802	AsCpf1 RR

CISH7058	ACAGTACCCCTTCCAGCTCT	20	803	AsCpf1 RR
CISH7059	CGTCGGCCACCAGACGGTTG	20	804	AsCpf1 RR
CISH7060	CCAGCCACTGCTGTACACCT	20	805	AsCpf1 RR
CISH7061	ACCCCGGCCCTGCCTATGCC	20	806	AsCpf1 RR
CISH7062	GGTATCAGCAGTGCAGGAGG	20	807	AsCpf1 RR
CISH7063	GATGTGGTCAGCCTTGTGCA	20	808	AsCpf1 RR
CISH7064	GGATGTGGTCAGCCTTGTGC	20	809	AsCpf1 RR
CISH7065	GGCCACGCATCCTGGCCTTT	20	810	AsCpf1 RR
CISH7066	GAAAGGCCAGGATGCGTGGC	20	811	AsCpf1 RR
CISH7067	ACTGCTTGTCCAGGCCACGC	20	812	AsCpf1 RR
CISH7068	TCTGGACTCCAAGTCTTGT	20	813	AsCpf1 RR
CISH7069	GTCTGGACTCCAAGTCTTG	20	814	AsCpf1 RR
CISH7070	GCTTCCGTCTGGACTCCAAC	20	815	AsCpf1 RR
CISH7071	GACGGAAGCTGGAGTCGGCA	20	816	AsCpf1 RR
CISH7072	CGCTGTCAGTGAAAACCACT	20	817	AsCpf1 RR
CISH7073	CTGACAGCGTGAACAGGTAG	20	818	AsCpf1 RR
CISH38401	ACTGACAGCGTGAACAGGTAG	21	1174	AsCpf1 RR
CISH7074	TTACGGCCAGCGAGGCCCGA	20	819	AsCpf1 RR
CISH7075	ATTACGGCCAGCGAGGCCCG	20	820	AsCpf1 RR
CISH7076	GGAATCTGGTGAGTCTGAGG	20	821	AsCpf1 RR
CISH7077	CCCTCAGACTCACCAGATTC	20	822	AsCpf1 RR
CISH7078	CGAAGGTAGGAGAAGGTCTT	20	823	AsCpf1 RR
CISH7079	GAAGGTAGGAGAAGGTCTTG	20	824	AsCpf1 RR
CISH7080	GCACCTTTGGCTCACTCTCT	20	825	AsCpf1 RR
CISH7081	TCGAGGAGGTGGCAGAGGGT	20	826	AsCpf1 RR
CISH7082	TGGAAGTGCCCAAGCCAGTC	20	827	AsCpf1 RR
CISH7083	AGGGACGGGGCCCACAGGGG	20	828	AsCpf1 RR
CISH7084	GGGACGGGGCCCACAGGGGC	20	829	AsCpf1 RR
CISH7085	CTCCACAGCCAGCAAAGGAC	20	830	AsCpf1 RR
CISH7086	CAGCCAGCAAAGGACGAGGT	20	831	AsCpf1 RR
CISH7087	CTGCCTTCTAGACCTCGTCC	20	832	AsCpf1 RR
CISH7088	CCTAAGGAGGATGCGCCTAG	20	833	AsCpf1 RVR
CISH7089	TGGCCTCCTGCACTGCTGAT	20	834	AsCpf1 RVR
CISH7090	AGCAGTGCAGGAGGCCACAT	20	835	AsCpf1 RVR
CISH7091	CCGACTCCAGCTTCCGTCTG	20	836	AsCpf1 RVR
CISH7092	GGGGTTCCATTACGGCCAGC	20	837	AsCpf1 RVR
CISH7093	CACAGCAGATCCTCCTCTGG	20	838	AsCpf1 RVR
CISH7094	ATTGCCCCGTACAGTCAGAG	21	839	SauCas9
CISH7095	CCCGTACAGTCAGAGCTGGA	21	840	SauCas9
CISH7096	TGGTGGAGGAGCAGGCAGTG	21	841	SauCas9
CISH7097	TCCTTAGGCATAGGCAGGGC	21	842	SauCas9
CISH7098	CGGCCCTGCCTATGCCTAAG	21	843	SauCas9
CISH7099	TAGGCATAGGCAGGGCCGGG	21	844	SauCas9
CISH7100	AGGCAGGGCCGGGGTGGGAG	21	845	SauCas9

CISH7101	GCAGGATCGGGGCTGTCGCT	21	846	SauCas9
CISH7102	CTGCACAAGGCTGACCACAT	21	847	SauCas9
CISH7103	TGCACAAGGCTGACCACATC	21	848	SauCas9
CISH7104	CTGACCACATCCGGAAGGC	21	849	SauCas9
CISH7105	GGCCACGCATCCTGGCCTTT	21	850	SauCas9
CISH7106	GCGTGGCCTGGACAAGCAGT	21	851	SauCas9
CISH7107	GACAAGCAGTTGGAGTCCAG	21	852	SauCas9
CISH7108	GTTGGAGTCCAGACGGAAGC	21	853	SauCas9
CISH7109	ATGCGTACATTGGTGGGGCC	21	854	SauCas9
CISH7110	TGGCCCCACCAATGTACGCA	21	855	SauCas9
CISH7111	GCTACCTGTTACGCTGTCA	21	856	SauCas9
CISH7112	TGACAGCGTGAACAGGTAGC	21	857	SauCas9
CISH7113	GTCGGGCCTCGCTGGCCGTA	21	858	SauCas9
CISH7114	GCACTTGCTAGGCTGGTAT	21	859	SauCas9
CISH7115	GGGAATCTGGTGAGTCTGAG	21	860	SauCas9
CISH7116	CTCACCAGATTCCCGAAGGT	21	861	SauCas9
CISH7117	CTCCTACCTTCGGGAATCTG	21	862	SauCas9
CISH7118	CAAGACCTTCTCCTACCTTC	21	863	SauCas9
CISH7119	CCAAGACCTTCTCCTACCTT	21	864	SauCas9
CISH7120	GCCAAGACCTTCTCCTACCT	21	865	SauCas9
CISH7121	TATGCACAGCAGATCCTCCT	21	866	SauCas9
CISH7122	CAAAGGTGCTGGACCCAGAG	21	867	SauCas9
CISH7123	GGCTCACTCTCTGTCTGGGC	21	868	SauCas9
CISH7124	AGGGTACCCCAGCCCAGACA	21	869	SauCas9
CISH7125	AGAGGGTACCCCAGCCCAGA	21	870	SauCas9
CISH7126	GTACCCTCTGCCACCTCCTC	21	871	SauCas9
CISH7127	CCTTCCTCGAGGAGGTGGCA	21	872	SauCas9
CISH7128	ATGACTGGCTTGGGCAGTTC	21	873	SauCas9
CISH7129	GGCCCCTGTGGGCCCCGTCC	21	874	SauCas9
CISH7130	AGGACGAGGTCTAGAAGGCA	21	875	SauCas9

RNA-guided nucleases

[133] RNA-guided nucleases according to the present disclosure include, but are not limited to, naturally-occurring Class 2 CRISPR nucleases such as Cas9, and Cpf1, as well as other nucleases derived or obtained therefrom. In functional terms, RNA-guided nucleases are defined as those nucleases that: (a) interact with (*e.g.*, complex with) a gRNA; and (b) together with the gRNA, associate with, and optionally cleave or modify, a target region of a DNA that includes (i) a sequence complementary to the targeting domain of the gRNA and, optionally, (ii) an additional sequence referred to as a “protospacer adjacent motif,” or “PAM,” which is described in greater detail below. As the following examples will illustrate, RNA-guided nucleases can be defined, in broad terms, by their PAM specificity and cleavage activity, even though variations may exist between individual RNA-guided nucleases that share the same PAM specificity or cleavage activity. Skilled artisans will

appreciate that some aspects of the present disclosure relate to systems, methods and compositions that can be implemented using any suitable RNA-guided nuclease having a certain PAM specificity and/or cleavage activity. For this reason, unless otherwise specified, the term RNA-guided nuclease should be understood as a generic term, and not limited to any particular type (*e.g.* Cas9 vs. Cpf1), species (*e.g.* *S. pyogenes* vs. *S. aureus*) or variation (*e.g.*, full-length vs. truncated or split; naturally-occurring PAM specificity vs. engineered PAM specificity, *etc.*) of RNA-guided nuclease.

[134] The PAM sequence takes its name from its sequential relationship to the “protospacer” sequence that is complementary to gRNA targeting domains (or “spacers”). Together with protospacer sequences, PAM sequences define target regions or sequences for specific RNA-guided nuclease / gRNA combinations.

[135] Various RNA-guided nucleases may require different sequential relationships between PAMs and protospacers. For example, Cas9 nucleases recognize PAM sequences that are 3' of the protospacer, while

[136] Cpf1, on the other hand, generally recognizes PAM sequences that are 5' of the protospacer.

[137] In addition to recognizing specific sequential orientations of PAMs and protospacers, RNA-guided nucleases can also recognize specific PAM sequences. *S. aureus* Cas9, for instance, recognizes a PAM sequence of NNGRRT or NNGRRV, wherein the N residues are immediately 3' of the region recognized by the gRNA targeting domain. *S. pyogenes* Cas9 recognizes NGG PAM sequences. And *F. novicida* Cpf1 recognizes a TTN PAM sequence. PAM sequences have been identified for a variety of RNA-guided nucleases, and a strategy for identifying novel PAM sequences has been described by Shmakov *et al.*, 2015, Molecular Cell 60, 385–397, November 5, 2015. It should also be noted that engineered RNA-guided nucleases can have PAM specificities that differ from the PAM specificities of reference molecules (for instance, in the case of an engineered RNA-guided nuclease, the reference molecule may be the naturally occurring variant from which the RNA-guided nuclease is derived, or the naturally occurring variant having the greatest amino acid sequence homology to the engineered RNA-guided nuclease).

[138] In addition to their PAM specificity, RNA-guided nucleases can be characterized by their DNA cleavage activity: naturally-occurring RNA-guided nucleases typically form DSBs in target nucleic acids, but engineered variants have been produced that generate only SSBs (discussed above) Ran & Hsu, *et al.*, Cell 154(6), 1380–1389, September 12, 2013 (Ran), incorporated by reference herein), or that that do not cut at all.

Cas9

[139] Crystal structures have been determined for *S. pyogenes* Cas9 (Jinek 2014), and for *S. aureus* Cas9 in complex with a unimolecular guide RNA and a target DNA (Nishimasu 2014; Anders 2014; and Nishimasu 2015).

[140] A naturally occurring Cas9 protein comprises two lobes: a recognition (REC) lobe and a nuclease (NUC) lobe; each of which comprise particular structural and/or functional domains. The

REC lobe comprises an arginine-rich bridge helix (BH) domain, and at least one REC domain (*e.g.* a REC1 domain and, optionally, a REC2 domain). The REC lobe does not share structural similarity with other known proteins, indicating that it is a unique functional domain. While not wishing to be bound by any theory, mutational analyses suggest specific functional roles for the BH and REC domains: the BH domain appears to play a role in gRNA:DNA recognition, while the REC domain is thought to interact with the repeat:anti-repeat duplex of the gRNA and to mediate the formation of the Cas9/gRNA complex.

[141] The NUC lobe comprises a RuvC domain, an HNH domain, and a PAM-interacting (PI) domain. The RuvC domain shares structural similarity to retroviral integrase superfamily members and cleaves the non-complementary (*i.e.* bottom) strand of the target nucleic acid. It may be formed from two or more split RuvC motifs (such as RuvC I, RuvCII, and RuvCIII in *S. pyogenes* and *S. aureus*). The HNH domain, meanwhile, is structurally similar to HNN endonuclease motifs, and cleaves the complementary (*i.e.* top) strand of the target nucleic acid. The PI domain, as its name suggests, contributes to PAM specificity.

[142] While certain functions of Cas9 are linked to (but not necessarily fully determined by) the specific domains set forth above, these and other functions may be mediated or influenced by other Cas9 domains, or by multiple domains on either lobe. For instance, in *S. pyogenes* Cas9, as described in Nishimasu 2014, the repeat:antirepeat duplex of the gRNA falls into a groove between the REC and NUC lobes, and nucleotides in the duplex interact with amino acids in the BH, PI, and REC domains. Some nucleotides in the first stem loop structure also interact with amino acids in multiple domains (PI, BH and REC1), as do some nucleotides in the second and third stem loops (RuvC and PI domains).

Cpf1

[143] The crystal structure of *Acidaminococcus sp.* Cpf1 in complex with crRNA and a double-stranded (ds) DNA target including a TTTN PAM sequence has been solved by Yamano *et al.* (Cell. 2016 May 5; 165(4): 949–962 (Yamano), incorporated by reference herein). Cpf1, like Cas9, has two lobes: a REC (recognition) lobe, and a NUC (nuclease) lobe. The REC lobe includes REC1 and REC2 domains, which lack similarity to any known protein structures. The NUC lobe, meanwhile, includes three RuvC domains (RuvC-I, -II and -III) and a BH domain. However, in contrast to Cas9, the Cpf1 REC lobe lacks an HNH domain, and includes other domains that also lack similarity to known protein structures: a structurally unique PI domain, three Wedge (WED) domains (WED-I, -II and -III), and a nuclease (Nuc) domain.

[144] While Cas9 and Cpf1 share similarities in structure and function, it should be appreciated that certain Cpf1 activities are mediated by structural domains that are not analogous to any Cas9 domains. For instance, cleavage of the complementary strand of the target DNA appears to be mediated by the Nuc domain, which differs sequentially and spatially from the HNH domain of Cas9.

Additionally, the non-targeting portion of Cpf1 gRNA (the handle) adopts a pseudoknot structure, rather than a stem loop structure formed by the repeat:antirepeat duplex in Cas9 gRNAs.

Modifications of RNA-guided nucleases

[145] The RNA-guided nucleases described above have activities and properties that can be useful in a variety of applications, but the skilled artisan will appreciate that RNA-guided nucleases can also be modified in certain instances, to alter cleavage activity, PAM specificity, or other structural or functional features.

[146] Turning first to modifications that alter cleavage activity, mutations that reduce or eliminate the activity of domains within the NUC lobe have been described above. Exemplary mutations that may be made in the RuvC domains, in the Cas9 HNH domain, or in the Cpf1 Nuc domain are described in Ran and Yamano, as well as in Cotta-Ramusino. In general, mutations that reduce or eliminate activity in one of the two nuclease domains result in RNA-guided nucleases with nickase activity, but it should be noted that the type of nickase activity varies depending on which domain is inactivated. As one example, inactivation of a RuvC domain or of a Cas9 HNH domain results in a nickase.

[147] Modifications of PAM specificity relative to naturally occurring Cas9 reference molecules has been described by Kleinstiver *et al.* for both *S. pyogenes* (Kleinstiver *et al.*, Nature. 2015 Jul 23;523(7561):481-5 (Kleinstiver I) and *S. aureus* (Kleinstiver *et al.*, Nat Biotechnol. 2015 Dec; 33(12): 1293–1298 (Kleinstiver II)). Kleinstiver *et al.* have also described modifications that improve the targeting fidelity of Cas9 (Nature, 2016 January 28; 529, 490-495 (Kleinstiver III)). Each of these references is incorporated by reference herein.

[148] RNA-guided nucleases have been split into two or more parts, as described by Zetsche *et al.* (Nat Biotechnol. 2015 Feb;33(2):139-42 (Zetsche II), incorporated by reference), and by Fine *et al.* (Sci Rep. 2015 Jul 1;5:10777 (Fine), incorporated by reference).

[149] RNA-guided nucleases can be, in certain embodiments, size-optimized or truncated, for instance via one or more deletions that reduce the size of the nuclease while still retaining gRNA association, target and PAM recognition, and cleavage activities. In certain embodiments, RNA guided nucleases are bound, covalently or non-covalently, to another polypeptide, nucleotide, or other structure, optionally by means of a linker. Exemplary bound nucleases and linkers are described by Guilinger *et al.*, Nature Biotechnology 32, 577–582 (2014), which is incorporated by reference for all purposes herein.

[150] RNA-guided nucleases also optionally include a tag, such as, but not limited to, a nuclear localization signal to facilitate movement of RNA-guided nuclease protein into the nucleus. In certain embodiments, the RNA-guided nuclease can incorporate C- and/or N-terminal nuclear localization signals. Nuclear localization sequences are known in the art and are described in Maeder and elsewhere.

[151] The foregoing list of modifications is intended to be exemplary in nature, and the skilled artisan will appreciate, in view of the instant disclosure, that other modifications may be possible or desirable in certain applications. For brevity, therefore, exemplary systems, methods and compositions of the present disclosure are presented with reference to particular RNA-guided nucleases, but it should be understood that the RNA-guided nucleases used may be modified in ways that do not alter their operating principles. Such modifications are within the scope of the present disclosure.

[152] Exemplary suitable nuclease variants include, but are not limited to, AsCpf1 variants comprising an M537R substitution, an H800A substitution, and/or an F870L substitution, or any combination thereof (numbering scheme according to AsCpf1 wild-type sequence). Other suitable modifications of the AsCpf1 amino acid sequence are known to those of ordinary skill in the art. Some exemplary sequences of wild-type AsCpf1 and AsCpf1 variants are provided below.

[153] His-AsCpf1-sNLS-sNLS H800A amino acid sequence (SEQ ID NO:1142)

MGHHHHHHGSTQFEGFTNLYQVSKTLRFELIPQGKTLKHIQEQQFIEEDKARNNDHYKELKPIIDRIYK
 TYADQCLQLVQLDWNLSAAIDSYRKEKTEETRNLALIEEQATYRNAIHDYFIGRTDNLDAINKRHA
 E IYKGLFKAELFNGKVLKQLGTVTTTEHENALLRSFDKFTTYFSGFYENRKNVFS AEDISTAIPHRI
 VQDNFPKFKENCHIFTRLITAVPSLREHFENVKKAIGIFVSTSIEEVFSFPFYNQLLTQTQIDLYNQL
 LGGISREAGTEKIKGLNEVLNLAIQKNDETAHIIASLPHRFIPLFKQILSDRNTLSFIIEEFKSDEEVI
 QSFCKYKTLRLNENVLETAELFNELNLSIDLTHIFISHKKLETISSALCDHWDTLRNALYERRISELT
 GKITKSAKEKVQRSKLEDINLQEIISAAGKELSEAFKQKTSEILSHAHAAALDQPLPTTLKKQEEKEIL
 KSQDLSLLGLYHLLDWFVAVDESNEVDPEFSARLTGIKLEMEPSLSFYNKARNYATKKPYSVEKFKLNF
 QMPTLASGWDVNKEKNNGAILFVKNGLYYLGIMPKQKGRYKALSFEPTTEKTSEGFDKMYDYFDPDAK
 MIPKCSTQLKAVTAHFQTHHTPILLSNNFIEPLEITKEIYDLNNPEKEPKKFQTAYAKKTGDQKGYRE
 ALCKWIDFTRDFLSKYTKTTSIDLSSLRPSSQYKDLGEYYAELNPLLYHISFQRIAEKEIMDAVETGK
 LYLFIYQYNKDFAKGHHGKPNLHTLYWTGLFSPENLAKTSIKLNGQAELFYRPKSRMKRMAARLGEKML
 NKKLKDQKTPIPDTLYQELYDYVNHRLSHDLSDEARALLPNVITKEVSHEIIKDRRFTSDKFFFHVP
 ITLNYQAANSPSKFNQVRNAYLKEHPETPIIGIDRGERNLIYITVIDSTGKILEQRSNTIQQFDYQKK
 LDNREKERVAAARQAWSVVGTIKDLKQGYLSQVIHEIVDLMIHYQAVVLENLNFQFKSKRTGIAEKAV
 YQQFEKMLIDKLNCLVLKDYPAEKVGGLNPNYQLTDQFTSFAMGTQSGFLFYVPAPYTSKIDPLTGF
 VDPFVWKTIKNHESRKHFLEGFDFLHYDVKTGDFILHFKMNRNLSFQRLGPGFMPAWDIVFEKNETQF
 DAKGTPFIAGKRIVPVIEHNRFTGRYRDLYPANELIALLEEKGIVFRDGSNILPKLLENDSDHAIDTM
 VALIRSVLQMRNSNAATGEDYINSPVRDLNGVCFDSRFQNPWPMDADANGAYHIALKGQLLLNHLKE
 SKDLKLQNGISNQDWLAYIQELRNGSPKKKRKVGSPKKKRKV

[154] Cpf1 variant 1 amino acid sequence (SEQ ID NO:1143)

MTQFEGFTNLYQVSKTLRFELIPQGKTLKHIQEQQFIEEDKARNNDHYKELKPIIDRIYKTYADQCLQL
 VQLDWNLSAAIDSYRKEKTEETRNLALIEEQATYRNAIHDYFIGRTDNLDAINKRHA E IYKGLFKA
 ELFNGKVLKQLGTVTTTEHENALLRSFDKFTTYFSGFYENRKNVFS AEDISTAIPHRI VQDNFPKF
 KENCHIFTRLITAVPSLREHFENVKKAIGIFVSTSIEEVFSFPFYNQLLTQTQIDLYNQLLGGISREAGTE
 KIKGLNEVLNLAIQKNDETAHIIASLPHRFIPLFKQILSDRNTLSFIIEEFKSDEEVIQSFCKYKTL
 LRNENVLETAELFNELNLSIDLTHIFISHKKLETISSALCDHWDTLRNALYERRISELTGKITKSAKEK
 VQRSKLEDINLQEIISAAGKELSEAFKQKTSEILSHAHAAALDQPLPTTLKKQEEKEILKSQDLSLLG
 LYHLLDWFVAVDESNEVDPEFSARLTGIKLEMEPSLSFYNKARNYATKKPYSVEKFKLNFQRPTLASG
 WDNKEKNNGAILFVKNGLYYLGIMPKQKGRYKALSFEPTTEKTSEGFDKMYDYFDPDAKMIPKCSTQL
 KAVTAHFQTHHTPILLSNNFIEPLEITKEIYDLNNPEKEPKKFQTAYAKKTGDQKGYREALCKWIDF
 TRDFLSKYTKTTSIDLSSLRPSSQYKDLGEYYAELNPLLYHISFQRIAEKEIMDAVETGKLYLFQIYNK
 DFAKGHHGKPNLHTLYWTGLFSPENLAKTSIKLNGQAELFYRPKSRMKRMAHRLGEKMLNKKLKDQKT
 PIPDTLYQELYDYVNHRLSHDLSDEARALLPNVITKEVSHEIIKDRRFTSDKFLFHVPITLNYQAANS

PSKFNQVRNAYLKEHPETPIIGIDRGERNLIYITVIDSTGKILEQRSNTIQQFDYQKKLDNREKERV
 AARQAWSVVGTIKDLKQGYLSQVIHEIVDLMIHYQAVVLENLNFQFKSKRTGIAEKAVYQQFEKMLI
 DKLNCLVLKDYPAEKVGGVLPYQLTDQFTSFAMGTQSGFLFYVPAPYTSKIDPLTGFDVDFVWKT
 KNHESRKHFLEGFDFLHYDVKTGDFILHFKMNRNLSFQRLPGFMPAWDIVFEKNETQFQDAKGTFFIA
 GKRIVPVIENHRFTGRYRDLYPANELIALLEEKGIVFRDGSNILPKLLENDSDSHADTMVALIRSVLQ
 MRNSNAATGEDYINSPVRDLNGVCFDSRFQNPPEWPMADANGAYHIALKGQLLNHLKESKDLKLQNG
 ISNQDWLAYIQELRNGRSSDDEATADSQHAAPPKKKRKVGSGSGSGSGSGSGSGSGSGSGSLEHHH
 HHH

[155] Cpf1 variant 2 amino acid sequence (SEQ ID NO:1144)

MTQFEGFTNLYQVSKTLRFELIPQGKTLKHIQEQQFIEEDKARNHYKELKPIIDRIYKTYADQCLQL
 VQLDWNLSAAIDSYRKEKTEETRNLALIEEQATYRNAIHDYFIGRTDNLTDAINKRHAEIYKGLFKA
 LFNGKVLKQLGTVTTEHENALLRSFDKFTTYFSGFYENRKNVSAEDISTAIPHRIVQDNFPKFEN
 CHIFTRLITAVPSLREHFENVKKAIGIFVSTSIIEEVFSFPFYNQLLTQTQIDLYNQLLGGISREAGTE
 KIKGLNEVLNLAIQKNDETAHIASLPHRFIPLFKQILSDRNTLSFILLEEFKSDEEVIQSFCYKTL
 RNENVLETAALFNELENSIDLTHIFISHKKLETISSALCDHWDTLRNALYERRISELTGKITKSAKEK
 VQRLKHEDINLQEIISAAGKELSEAFKQKTSEILSHAHAAALDQPLPTTLKKQEEKEILKSQLD
 SLLGLYHLDDWFAVDESNEVDPEFSARLTGIKLEMEPSLSFYNKARNYATKKPYSVEKFKLNFQMP
 TLASGWDVNKEKNNGAILFVKNGLYYLGIMPKQKGRYKALSFEPTTEKTSEGFDKMYDYFDPAAKM
 IPKCSTQLKAVTAHFQTHHTPILLSNNFIEPLEITKEIYDLNNPEKEPKKFQAYAKKTGDQKGY
 REALCKWIDFTRDFLSKYTKTTSIDLSSLRPSSQYKDLGEYYAELNPLLYHISFQRIAEKEIMDA
 VETGKLYLFQIYNKDFAKGHHGKPNLHTLYWTGLFSPENLAKTSIKLNGQAEFYRPKSRMKRMAH
 RLGEKMLNKKLKDQKTPIPDITLYQELYDYVNHRLSHDLSDEARALLPNVITKEVSHEI
 IKDRRFTSDKFFHFVPIITLNYQAANSPSKFNQVRNAYLKEHPETPIIGIDRGERNLIYITVIDST
 GKILEQRSNTIQQFDYQKKLDNREKERV AARQAWSVVGTIKDLKQGYLSQVIHEIVDLMIHYQAVV
 LENLNFQFKSKRTGIAEKAVYQQFEKMLI DKLNCLVLKDYPAEKVGGVLPYQLTDQFTSFAMGTQ
 SGFLFYVPAPYTSKIDPLTGFDVDFVWKT KNHESRKHFLEGFDFLHYDVKTGDFILHFKMNRNLS
 FQRLPGFMPAWDIVFEKNETQFQDAKGTFFIA GKRIVPVIENHRFTGRYRDLYPANELIALLEEK
 GIVFRDGSNILPKLLENDSDSHADTMVALIRSVLQ MRNSNAATGEDYINSPVRDLNGVCFDSRFQ
 NPPEWPMADANGAYHIALKGQLLNHLKESKDLKLQNG ISNQDWLAYIQELRNGRSSDDEATADSQ
 HAAPPKKKRKVGSGSGSGSGSGSGSGSGSGSGSLEHHH HHH

[156] Cpf1 variant 3 amino acid sequence (SEQ ID NO:1145)

MTQFEGFTNLYQVSKTLRFELIPQGKTLKHIQEQQFIEEDKARNHYKELKPIIDRIYKTYADQCLQL
 VQLDWNLSAAIDSYRKEKTEETRNLALIEEQATYRNAIHDYFIGRTDNLTDAINKRHAEIYKGLFKA
 LFNGKVLKQLGTVTTEHENALLRSFDKFTTYFSGFYENRKNVSAEDISTAIPHRIVQDNFPKFEN
 CHIFTRLITAVPSLREHFENVKKAIGIFVSTSIIEEVFSFPFYNQLLTQTQIDLYNQLLGGISREAGTE
 KIKGLNEVLNLAIQKNDETAHIASLPHRFIPLFKQILSDRNTLSFILLEEFKSDEEVIQSFCYKTL
 RNENVLETAALFNELENSIDLTHIFISHKKLETISSALCDHWDTLRNALYERRISELTGKITKSAKEK
 VQRLKHEDINLQEIISAAGKELSEAFKQKTSEILSHAHAAALDQPLPTTLKKQEEKEILKSQLD
 SLLGLYHLDDWFAVDESNEVDPEFSARLTGIKLEMEPSLSFYNKARNYATKKPYSVEKFKLNFQRP
 TLASGWDVNKEKNNGAILFVKNGLYYLGIMPKQKGRYKALSFEPTTEKTSEGFDKMYDYFDPAAKM
 IPKCSTQLKAVTAHFQTHHTPILLSNNFIEPLEITKEIYDLNNPEKEPKKFQAYAKKTGDQKGY
 REALCKWIDFTRDFLSKYTKTTSIDLSSLRPSSQYKDLGEYYAELNPLLYHISFQRIAEKEIMDA
 VETGKLYLFQIYNKDFAKGHHGKPNLHTLYWTGLFSPENLAKTSIKLNGQAEFYRPKSRMKRMAA
 RLGEKMLNKKLKDQKTPIPDITLYQELYDYVNHRLSHDLSDEARALLPNVITKEVSHEI
 IKDRRFTSDKFLFHFVPIITLNYQAANSPSKFNQVRNAYLKEHPETPIIGIDRGERNLIYITVIDST
 GKILEQRSNTIQQFDYQKKLDNREKERV AARQAWSVVGTIKDLKQGYLSQVIHEIVDLMIHYQAVV
 LENLNFQFKSKRTGIAEKAVYQQFEKMLI DKLNCLVLKDYPAEKVGGVLPYQLTDQFTSFAMGTQ
 SGFLFYVPAPYTSKIDPLTGFDVDFVWKT KNHESRKHFLEGFDFLHYDVKTGDFILHFKMNRNLS
 FQRLPGFMPAWDIVFEKNETQFQDAKGTFFIA GKRIVPVIENHRFTGRYRDLYPANELIALLEEK
 GIVFRDGSNILPKLLENDSDSHADTMVALIRSVLQ MRNSNAATGEDYINSPVRDLNGVCFDSRFQ
 NPPEWPMADANGAYHIALKGQLLNHLKESKDLKLQNG

[160] Cpf1 variant 7 amino acid sequence (SEQ ID NO:1149)

[161] Exemplary AsCpf1 wild-type amino acid sequence (SEQ ID NO:1150):

PIPDITLYQELYDYVNHRLSHDLSEARALLPNVITKEVSHEI IKDRRFTSDKFFFHVPITLNYQAANS
 PSKFNQVRVNAVYLKEHPETPIIGIDRGERNLIYITVIDSTGKILEQSLNTIQQFDYQKKLDNREKERV
 AARQAWSVVGTIKDLKQGYLSQVIHEIVDLMIHYQAVVLENLNFQFKSKRTGIAEKAVYQQFEKMLI
 DKLNLCLVLKDYPAEKVGGLNPNYQLTDQFTSFAKMGTSQGLFYVPAPYTSKIDPLTGFDVDFVWKT
 KNHESRKHFLEGFDFLHYDVKTGDFILHFKMNRNLSFQRLPGFMPAWDIVFEKNETQFQDAKGTPIA
 GKRIVPVIENHRFTGRYRDLYPANELIALLEEKGIVFRDGSNILPKLLENDSDSHADTMVALIRSVLQ
 MRNSNAATGEDYINSPVRDLNGVCFDSRFQNPWPMDADANGAYHIALKGQLLLNHLKESKDLKLQNG
 ISNQDWLAYIQELRN

Nucleic acids encoding RNA-guided nucleases

[162] Nucleic acids encoding RNA-guided nucleases, *e.g.*, Cas9, Cpf1 or functional fragments thereof, are provided herein. Exemplary nucleic acids encoding RNA-guided nucleases have been described previously (see, *e.g.*, Cong 2013; Wang 2013; Mali 2013; Jinek 2012).

[163] In some cases, a nucleic acid encoding an RNA-guided nuclease can be a synthetic nucleic acid sequence. For example, the synthetic nucleic acid molecule can be chemically modified. In certain embodiments, an mRNA encoding an RNA-guided nuclease will have one or more (*e.g.*, all) of the following properties: it can be capped; polyadenylated; and substituted with 5-methylcytidine and/or pseudouridine.

[164] Synthetic nucleic acid sequences can also be codon optimized, *e.g.*, at least one non-common codon or less-common codon has been replaced by a common codon. For example, the synthetic nucleic acid can direct the synthesis of an optimized messenger mRNA, *e.g.*, optimized for expression in a mammalian expression system, *e.g.*, described herein. Examples of codon optimized Cas9 coding sequences are presented in Cotta-Ramusino.

[165] In addition, or alternatively, a nucleic acid encoding an RNA-guided nuclease may comprise a nuclear localization sequence (NLS). Nuclear localization sequences are known in the art.

[166] As an example, the nucleic acid sequence for Cpf1 variant 4 is set forth below as SEQ ID NO:1175:

ATGACCCAGTTTGAAGGTTTACCAATCTGTATCAGGTTAGCAAAACCCCTGCGTTTTGAACTGATTCC
 GCAGGGTAAACCCCTGAAACATATTCAAGAACAGGGCTTCATCGAAGAGGATAAAGCACGTAACGATC
 ACTACAAAGAACTGAAACCGATTATCGACCGCATCTATAAAACCTATGCAGATCAGTGTCTGCAGCTG
 GTTCAGCTGGATTGGGAAAATCTGAGCGCAGCAATTGATAGTTATCGCAAAGAAAAACCGAAGAAAC
 CCGTAATGCACTGATTGAAGAACAGGCAACCTATCGTAATGCCATCCATGATTATTTTATTGGTCGTA
 CCGATAATCTGACCGATGCAATTAACAAACGTCACGCCGAAATCTATAAAGGCCTGTTTAAAGCCGAA
 CTGTTTAAATGGCAAAGTTCTGAAACAGCTGGGCACCGTTACCACCACCGAACATGAAAATGCACTGCT
 GCGTAGCTTTGATAAATTCACCACCTATTTTACGCGGCTTTTATGAGAATCGCAAAAACGTGTTTAGCG
 CAGAAGATATTAGCACCGCAATTCGCGATCGTATTGTGCAGGATAATTTCCCGAAATTCAGAGAAAC
 TGCCACATTTTTTACCCTGCTGATTACCGCAGTTCCGAGCCTGCGTGAACATTTTGAAAACGTAAAAA
 AGCCATCGGCATCTTTGTTAGCACCAGCATTGAAGAAGTTTTAGCTTCCCGTTTTACAATCAGCTGC
 TGACCCAGACCCAGATTGATCTGTATAACCAACTGCTGGGTGGTATTAGCCGTGAAGCAGGCACCGAA
 AAAATCAAAGGTCTGAATGAAGTGTGAATCTGGCCATTGAGAAAAATGATGAAACCGCACATATTAT
 TGCAAGCCTGCCGCATCGTTTTATTCCGCTGTTCAAACAAATTCTGAGCGATCGTAATACCCTGAGCT
 TTATTCTGGAAGAATTCAAATCCGATGAAGAGGTGATTCAGAGCTTTTGCAAATACAAAACGCTGCTG
 CGCAATGAAAATGTTCTGGAAACTGCCGAAGCACTGTTTAACGAACTGAATAGCATTGATCTGACCCA
 CATCTTTATCAGCCACAAAAAAGTGAAGCACTTTTCAAGCGCACTGTGTGATCATTGGGATACCCTGC
 GTAATGCCCTGTATGAACGTCGTATTAGCGAACTGACCGGTAAAATTACCAAAAGCGCGAAAGAAAA
 GTTCAGCGCAGTCTGAAACATGAGGATATTAATCTGCAAGAGATTATTAGCGCAGCCGGTAAAGAACT

GTCAGAAGCATTTTAAACAGAAAACCAGCGAAATTCTGTACATGCACATGCAGCACTGGATCAGCCGC
 TGCCGACCACCCTGAAAAACAAGAAGAAAAAGAAATCCTGAAAAGCCAGCTGGATAGCCTGCTGGGT
 CTGTATCATCTGCTGGACTGGTTTGCAGTTGATGAAAGCAATGAAGTTGATCCGGAATTTAGCGCACG
 TCTGACCGGCATTAACTGGAAATGGAACCGAGCCTGAGCTTTTATAACAAAGCCCGTAATTATGCCA
 CCAAAAAACCGTATAGCGTCGAAAAATTCAAACCTGAACCTTCAGCGTCCGACCCTGGCAAGCGTTGG
 GATGTTAATAAAGAAAAAACAACGGTGCCATCCTGTTCTGTGAAAAATGGCCTGTATTATCTGGGTAT
 TATGCCGAAACAGAAAGGTCGTTATAAAGCGCTGAGCTTTGAACCGACGGAAAAAACAGTGAAGGTT
 TTGATAAAATGTACTACGACTATTTTCCGGATGCAGCCAAAATGATTCCGAAATGTAGCACCAGCTG
 AAAGCAGTTACCGCACATTTTCAGACCCATACCACCCCGATTCTGCTGAGCAATAACTTTATTGAACC
 GCTGGAAATCACCAAAGAGATCTACGATCTGAATAACCCGGAAGAGCCGAAAAAATTCAGACCCG
 CATATGCAAAAAAACCGGTGATCAGAAAGGTTATCGTGAAGCGCTGTGTAAATGGATTGATTTACC
 CGTGATTTTCTGAGCAAATACACCAAACACCAGTATCGATCTGAGCAGCCTGCGTCCGAGCAGCCA
 GTATAAAGATCTGGGCGAATATTATGCAGAACTGAATCCGCTGCTGTATCATATTAGCTTTCAGCGTA
 TTGCCGAGAAAGAAATCATGGACGCAGTTGAAACCGGTAAACTGTACCTGTTCCAGATCTACAATAAA
 GATTTTGCCAAAGGCCATCATGGCAAACCGAATCTGCATACCCTGTATTGGACCGGTCTGTTTAGCCC
 TGAAAATCTGGCAAAAACCTCGATTAACTGAATGGTCAGGCGGAACTGTTTTATCGTCCGAAAAGCC
 GTATGAAACGTATGGCAGCTCGTCTGGGTGAAAAATGCTGAACAAAAAAGTGAAGACCAGAAAACC
 CCGATCCCGGATACACTGTATCAAGAACTGTATGATTATGTGAACCATCGTCTGAGCCATGATCTGAG
 TGATGAAGCACGTGCCCTGCTGCCGAATGTTATTACCAAAGAAGTTAGCCACGAGATCATTAAAGATC
 GTCGTTTTACCAGCGACAAATTCCTGTTTCATGTGCCGATTACCCTGAATTATCAGGCAGCAAATAGC
 CCGAGCAAATTTAACCAGCGTGTTAATGCATATCTGAAAGAACATCCAGAAACGCCGATTATTGGTAT
 TGATCGTGGTGAACGTAACCTGATTTATATCACCGTTATTGATAGCACCGGCAAAATCCTGGAACAGC
 GTAGCCTGAATACCATTACAGCAGTTTGATTACCAGAAAAAAGTGGATAATCGCGAGAAAGAACGTGTT
 GCAGCACGTCAGGCATGGTCAGTTGTTGGTACAATTAAGACCTGAAACAGGGTTATCTGAGCCAGGT
 TATTCATGAAATTGTGGATCTGATGATTCACTATCAGGCCGTTGTTGTGCTGGAAAACCTGAATTTTG
 GCTTTAAAGCAAACGTACCGGCATTGCAGAAAAAGCAGTTTATCAGCAGTTCGAGAAAAATGCTGATT
 GACAACTGAATTGCCTGGTGTGTAAGATTATCCGGCTGAAAAAGTTGGTGGTGTCTGAATCCGTA
 TCAGCTGACCGATCAGTTTACCAGCTTTGCAAAAATGGGCACCCAGAGCGGATTTCTGTTTTATGTTT
 CGGCACCGTATACGAGCAAAATGATCCGCTGACCGTTTTGTTGATCCGTTTGTGTTGGAAAACCATC
 AAAAACCATGAAAGCCGCAAAACATTTTCTGGAAGTTTTGATTTTCTGCATTACGACGTTAAACGGG
 TGATTTTCATCCTGCATTTTAAATGAATCGCAATCTGAGTTTTTCAGCGTGGCCTGCCTGGTTTTATGC
 CTGCATGGGATATTGTGTTTGAAGAAAACGAAACACAGTTTCGATGCAAAAGGCACCCCGTTTTATTGCA
 GGTAAACGTATTGTTCCGGTGATTGAAAATCATCGTTTTACCGGTGCTTATCGCGATCTGTATCCGGC
 AAATGAACTGATCGCACTGCTGGAAGAGAAAGTATTGTTTTCTGTGATGGCTCAAACATTCTGCCGA
 AACTGCTGGAATAATGATGATAGCCATGCAATTGATACCATGGTTGCACTGATTTCGTAGCGTTCTGCAG
 ATGCGTAATAGCAATGCAGCAACCGGTGAAGATTACATTAATAGTCCGGTTTCGTGATCTGAATGGTGT
 TTGTTTTGATAGCCGTTTTTCAAGATCCGGAATGGCCGATGGATGCAGATGCAATGGTGCATATCATA
 TTGCACTGAAAGGACAGCTGCTGCTGAACCACCTGAAAGAAAGCAAAGATCTGAACTGCAAAACGGC
 ATTAGCAATCAGGATTGGCTGGCATATATCCAAGAACTGCGTAACGGTCGTAGCAGTGATGATGAAGC
 AACCGCAGATAGCCAGCATGCAGCACCGCCTAAAAAGAAACGTAAAGTT

Functional analysis of candidate molecules

[167] Candidate RNA-guided nucleases, gRNAs, and complexes thereof, can be evaluated by standard methods known in the art. See, *e.g.* Cotta-Ramusino. The stability of RNP complexes may be evaluated by differential scanning fluorimetry, as described below.

Differential Scanning Fluorimetry (DSF)

[168] The thermostability of ribonucleoprotein (RNP) complexes comprising gRNAs and RNA-guided nucleases can be measured via DSF. The DSF technique measures the thermostability of a protein, which can increase under favorable conditions such as the addition of a binding RNA molecule, *e.g.*, a gRNA.

[169] A DSF assay can be performed according to any suitable protocol, and can be employed in any suitable setting, including without limitation (a) testing different conditions (*e.g.* different stoichiometric ratios of gRNA: RNA-guided nuclease protein, different buffer solutions, *etc.*) to identify optimal conditions for RNP formation; and (b) testing modifications (*e.g.* chemical modifications, alterations of sequence, *etc.*) of an RNA-guided nuclease and/or a gRNA to identify those modifications that improve RNP formation or stability. One readout of a DSF assay is a shift in melting temperature of the RNP complex; a relatively high shift suggests that the RNP complex is more stable (and may thus have greater activity or more favorable kinetics of formation, kinetics of degradation, or another functional characteristic) relative to a reference RNP complex characterized by a lower shift. When the DSF assay is deployed as a screening tool, a threshold melting temperature shift may be specified, so that the output is one or more RNPs having a melting temperature shift at or above the threshold. For instance, the threshold can be 5-10°C (*e.g.* 5°, 6°, 7°, 8°, 9°, 10°) or more, and the output may be one or more RNPs characterized by a melting temperature shift greater than or equal to the threshold.

[170] Two non-limiting examples of DSF assay conditions are set forth below:

[171] To determine the best solution to form RNP complexes, a fixed concentration (*e.g.* 2 µM) of Cas9 in water+10x SYPRO Orange® (Life Technologies cat#S-6650) is dispensed into a 384 well plate. An equimolar amount of gRNA diluted in solutions with varied pH and salt is then added. After incubating at room temperature for 10' and brief centrifugation to remove any bubbles, a Bio-Rad CFX384™ Real-Time System C1000 Touch™ Thermal Cycler with the Bio-Rad CFX Manager software is used to run a gradient from 20°C to 90°C with a 1°C increase in temperature every 10 seconds.

[172] The second assay consists of mixing various concentrations of gRNA with fixed concentration (*e.g.* 2 µM) Cas9 in optimal buffer from assay 1 above and incubating (*e.g.* at RT for 10') in a 384 well plate. An equal volume of optimal buffer + 10x SYPRO Orange® (Life Technologies cat#S-6650) is added and the plate sealed with Microseal® B adhesive (MSB-1001). Following brief centrifugation to remove any bubbles, a Bio-Rad CFX384™ Real-Time System C1000 Touch™ Thermal Cycler with the Bio-Rad CFX Manager software is used to run a gradient from 20°C to 90°C with a 1°C increase in temperature every 10 seconds.

Genome Editing Strategies

[173] The genome editing systems described above are used, in various embodiments of the present disclosure, to generate edits in (*i.e.* to alter) targeted regions of DNA within or obtained from a cell. Various strategies are described herein to generate particular edits, and these strategies are generally described in terms of the desired repair outcome, the number and positioning of individual edits (*e.g.* SSBs or DSBs), and the target sites of such edits.

[174] Genome editing strategies that involve the formation of SSBs or DSBs are characterized by repair outcomes including: (a) deletion of all or part of a targeted region; (b) insertion into or

replacement of all or part of a targeted region; or (c) interruption of all or part of a targeted region. This grouping is not intended to be limiting, or to be binding to any particular theory or model, and is offered solely for economy of presentation. Skilled artisans will appreciate that the listed outcomes are not mutually exclusive and that some repairs may result in other outcomes. The description of a particular editing strategy or method should not be understood to require a particular repair outcome unless otherwise specified.

[175] Replacement of a targeted region generally involves the replacement of all or part of the existing sequence within the targeted region with a homologous sequence, for instance through gene correction or gene conversion, two repair outcomes that are mediated by HDR pathways. HDR is promoted by the use of a donor template, which can be single-stranded or double stranded, as described in greater detail below. Single or double stranded templates can be exogenous, in which case they will promote gene correction, or they can be endogenous (*e.g.* a homologous sequence within the cellular genome), to promote gene conversion. Exogenous templates can have asymmetric overhangs (*i.e.* the portion of the template that is complementary to the site of the DSB may be offset in a 3' or 5' direction, rather than being centered within the donor template), for instance as described by Richardson *et al.* (Nature Biotechnology 34, 339–344 (2016), (Richardson), incorporated by reference). In instances where the template is single stranded, it can correspond to either the complementary (top) or non-complementary (bottom) strand of the targeted region.

Gene constructs

[176] In some aspects, the present disclosure provides complex editing strategies, and resulting modified cells having complex genomic alterations, that allow for the generation of advanced NK cell products for clinical applications, *e.g.*, for immunooncology therapeutic approaches.

[177] In some embodiments, the genomic alterations are introduced by use of one or more HDR expression constructs. In some embodiments, the genomic alterations are introduced by use of one or more HDR expression constructs. In some embodiments, the one or more HDR expression constructs comprise one or more donor HDR templates. In some embodiments, the one or more donor HDR templates comprise one or more expression cassettes encoding one or more cDNAs. In some embodiments, the donor HDR template comprises one expression cassette. In some embodiments, the donor HDR template comprises two expression cassettes. In some embodiments, the donor HDR template comprises three expression cassettes. In some embodiments, the donor HDR template comprises four expression cassettes. In some embodiments, the donor HDR template comprises five expression cassettes. In some embodiments, the donor HDR template comprises six expression cassettes. In some embodiments, the donor HDR template comprises seven expression cassettes. In some embodiments, the donor HDR template comprises eight expression cassettes. In some embodiments, the donor HDR template comprises nine expression cassettes. In some embodiments, the donor HDR template comprises ten expression cassettes. In some embodiments, the one or more

expression cassette is monocistronic. In some embodiments, the one or more expression cassette is bicistronic.

[178] In some embodiments, the one or more expression cassettes comprise one cDNA. In some embodiments, the one or more expression cassettes comprise two cDNAs. In some embodiments, the one or more expression cassettes comprise three cDNAs. In some embodiments, the one or more expression cassettes comprise four cDNAs. In some embodiments, the one or more expression cassettes comprise five cDNAs. In some embodiments, the one or more expression cassettes comprise six cDNAs. In some embodiments, the one or more expression cassettes comprise seven cDNAs. In some embodiments, the one or more expression cassettes comprise eight cDNAs. In some embodiments, the one or more expression cassettes comprise nine cDNAs. In some embodiments, the one or more expression cassettes comprise ten cDNAs. In some embodiments, the one or more expression cassettes comprise one or more cDNAs separated by a 2A sequence. In some embodiments, the one or more expression cassettes comprise two cDNAs separated by a 2A sequence. In some embodiments, the one or more expression cassettes comprise three cDNAs separated by a 2A sequence.

[179] In some embodiments, the HDR expression construct comprises one or more cDNAs driven by a heterologous promoter.

[180] In some embodiments, the HDR expression construct comprises one or more donor templates for inserting an inactivating mutation in a target gene, wherein the gene product has less, or no, function (being partially or wholly inactivated). In some embodiments, the HDR expression construct comprises one or more donor templates for inserting an inactivating mutation in a target gene, wherein the gene product has no function (wholly inactivated).

[181] Gene conversion and gene correction are facilitated, in some cases, by the formation of one or more nicks in or around the targeted region, as described in Ran and Cotta-Ramusino. In some cases, a dual-nickase strategy is used to form two offset SSBs that, in turn, form a single DSB having an overhang (*e.g.* a 5' overhang).

[182] Interruption and/or deletion of all or part of a targeted sequence can be achieved by a variety of repair outcomes. As one example, a sequence can be deleted by simultaneously generating two or more DSBs that flank a targeted region, which is then excised when the DSBs are repaired, as is described in Maeder for the LCA10 mutation. As another example, a sequence can be interrupted by a deletion generated by formation of a double strand break with single-stranded overhangs, followed by exonucleolytic processing of the overhangs prior to repair.

[183] One specific subset of target sequence interruptions is mediated by the formation of an indel within the targeted sequence, where the repair outcome is typically mediated by NHEJ pathways (including Alt-NHEJ). NHEJ is referred to as an “error prone” repair pathway because of its association with indel mutations. In some cases, however, a DSB is repaired by NHEJ without alteration of the sequence around it (a so-called “perfect” or “scarless” repair); this generally requires

the two ends of the DSB to be perfectly ligated. Indels, meanwhile, are thought to arise from enzymatic processing of free DNA ends before they are ligated that adds and/or removes nucleotides from either or both strands of either or both free ends.

[184] Because the enzymatic processing of free DSB ends may be stochastic in nature, indel mutations tend to be variable, occurring along a distribution, and can be influenced by a variety of factors, including the specific target site, the cell type used, the genome editing strategy used, *etc.* Even so, it is possible to draw limited generalizations about indel formation: deletions formed by repair of a single DSB are most commonly in the 1-50 bp range, but can reach greater than 100-200 bp. Insertions formed by repair of a single DSB tend to be shorter and often include short duplications of the sequence immediately surrounding the break site. However, it is possible to obtain large insertions, and in these cases, the inserted sequence has often been traced to other regions of the genome or to plasmid DNA present in the cells.

[185] Indel mutations – and genome editing systems configured to produce indels – are useful for interrupting target sequences, for example, when the generation of a specific final sequence is not required and/or where a frameshift mutation would be tolerated. They can also be useful in settings where particular sequences are preferred, insofar as the certain sequences desired tend to occur preferentially from the repair of an SSB or DSB at a given site. Indel mutations are also a useful tool for evaluating or screening the activity of particular genome editing systems and their components. In these and other settings, indels can be characterized by (a) their relative and absolute frequencies in the genomes of cells contacted with genome editing systems and (b) the distribution of numerical differences relative to the unedited sequence, *e.g.* ± 1 , ± 2 , ± 3 , *etc.* As one example, in a lead-finding setting, multiple gRNAs can be screened to identify those gRNAs that most efficiently drive cutting at a target site based on an indel readout under controlled conditions. Guides that produce indels at or above a threshold frequency, or that produce a particular distribution of indels, can be selected for further study and development. Indel frequency and distribution can also be useful as a readout for evaluating different genome editing system implementations or formulations and delivery methods, for instance by keeping the gRNA constant and varying certain other reaction conditions or delivery methods.

Multiplex Strategies

[186] While exemplary strategies discussed above have focused on repair outcomes mediated by single DSBs, genome editing systems according to this disclosure may also be employed to generate two or more DSBs, either in the same locus or in different loci. Strategies for editing that involve the formation of multiple DSBs, or SSBs, are described in, for instance, Cotta-Ramusino. In some embodiments, where multiple edits are made in the genome of an NK cell, or a cell that an NK cell is derived from, the edits are made at the same time or in close temporal proximity. In some such embodiments, two or more genomic edits are effected by two or more different RNA-guided nucleases. For example, one of the genomic edits may be effected by saCas9 (in connection with the

respective saCas9 guide RNA), and a different genomic edit may be effected by Cpf1 (in connection with the respective Cpf1 guide RNA). In some embodiments, using different RNA-guided nucleases in the context of multiplex genomic editing approaches is advantageous as compared to using the same RNA-guided nuclease for two or more edits, *e.g.*, in that it allows to decrease the likelihood or frequency of undesirable effects, such as, for example, off-target cutting, and the occurrence of genomic translocations.

Donor template design

[187] Donor template design is described in detail in the literature, for instance in Cotta-Ramusino. DNA oligomer donor templates (oligodeoxynucleotides or ODNs), which can be single stranded (ssODNs) or double-stranded (dsODNs), can be used to facilitate HDR-based repair of DSBs, and are particularly useful for introducing alterations into a target DNA sequence, inserting a new sequence into the target sequence, or replacing the target sequence altogether.

[188] Whether single-stranded or double stranded, donor templates generally include regions that are homologous to regions of DNA within or near (*e.g.* flanking or adjoining) a target sequence to be cleaved. These homologous regions are referred to here as “homology arms,” and are illustrated schematically below:

[5' homology arm] — [replacement sequence] — [3' homology arm].

[189] The homology arms can have any suitable length (including 0 nucleotides if only one homology arm is used), and 3' and 5' homology arms can have the same length, or can differ in length. The selection of appropriate homology arm lengths can be influenced by a variety of factors, such as the desire to avoid homologies or microhomologies with certain sequences such as Alu repeats or other very common elements. For example, a 5' homology arm can be shortened to avoid a sequence repeat element. In other embodiments, a 3' homology arm can be shortened to avoid a sequence repeat element. In some embodiments, both the 5' and the 3' homology arms can be shortened to avoid including certain sequence repeat elements. In addition, some homology arm designs can improve the efficiency of editing or increase the frequency of a desired repair outcome. For example, Richardson *et al.* Nature Biotechnology 34, 339–344 (2016) (Richardson), which is incorporated by reference, found that the relative asymmetry of 3' and 5' homology arms of single stranded donor templates influenced repair rates and/or outcomes.

[190] Replacement sequences in donor templates have been described elsewhere, including in Cotta-Ramusino *et al.* A replacement sequence can be any suitable length (including zero nucleotides, where the desired repair outcome is a deletion), and typically includes one, two, three or more sequence modifications relative to the naturally-occurring sequence within a cell in which editing is desired. One common sequence modification involves the alteration of the naturally-occurring sequence to repair a mutation that is related to a disease or condition of which treatment is desired. Another common sequence modification involves the alteration of one or more sequences that are complementary to, or code for, the PAM sequence of the RNA-guided nuclease or the

targeting domain of the gRNA(s) being used to generate an SSB or DSB, to reduce or eliminate repeated cleavage of the target site after the replacement sequence has been incorporated into the target site.

[191] Where a linear ssODN is used, it can be configured to (i) anneal to the nicked strand of the target nucleic acid, (ii) anneal to the intact strand of the target nucleic acid, (iii) anneal to the plus strand of the target nucleic acid, and/or (iv) anneal to the minus strand of the target nucleic acid. An ssODN may have any suitable length, *e.g.*, about, at least, or no more than 150-200 nucleotides (*e.g.*, 150, 160, 170, 180, 190, or 200 nucleotides).

[192] It should be noted that a template nucleic acid can also be a nucleic acid vector, such as a viral genome or circular double stranded DNA, *e.g.*, a plasmid. Nucleic acid vectors comprising donor templates can include other coding or non-coding elements. For example, a template nucleic acid can be delivered as part of a viral genome (*e.g.* in an AAV or lentiviral genome) that includes certain genomic backbone elements (*e.g.* inverted terminal repeats, in the case of an AAV genome) and optionally includes additional sequences coding for a gRNA and/or an RNA-guided nuclease. In certain embodiments, the donor template can be adjacent to, or flanked by, target sites recognized by one or more gRNAs, to facilitate the formation of free DSBs on one or both ends of the donor template that can participate in repair of corresponding SSBs or DSBs formed in cellular DNA using the same gRNAs. Exemplary nucleic acid vectors suitable for use as donor templates are described in Cotta-Ramusino.

[193] Whatever format is used, a template nucleic acid can be designed to avoid undesirable sequences. In certain embodiments, one or both homology arms can be shortened to avoid overlap with certain sequence repeat elements, *e.g.*, Alu repeats, LINE elements, *etc.*

Quantitative measurement of on-target gene editing

[194] It should be noted that the genome editing systems of the present disclosure allow for the detection and quantitative measurement of on-target gene editing outcomes, including targeted integration. The compositions and methods described herein can rely on the use of donor templates comprising a 5' homology arm, a cargo, a one or more priming sites, a 3' homology arm, and optionally stuffer sequence. For example, International Patent Publication No. WO2019/014564 by Ramusino *et al.* (Ramusino), which is incorporated by reference herein in its entirety, describes compositions and methods which allow for the quantitative analysis of on-target gene editing outcomes, including targeted integration events, by embedding one or more primer binding sites (*i.e.*, priming sites) into a donor template that are substantially identical to a priming site present at the targeted genomic DNA locus (*i.e.*, the target nucleic acid). The priming sites are embedded into the donor template such that, when homologous recombination of the donor template with a target nucleic acid occurs, successful targeted integration of the donor template integrates the priming sites from the donor template into the target nucleic acid such that at least one amplicon can be generated in order to quantitatively determine the on-target editing outcomes.

[195] In some embodiments, the target nucleic acid comprises a first priming site (P1) and a second priming site (P2), and the donor template comprises a cargo sequence, a first priming site (P1'), and a second priming site (P2'), wherein P2' is located 5' from the cargo sequence, wherein P1' is located 3' from the cargo sequence (*i.e.*, A1--P2'--N--P1'--A2), wherein P1' is substantially identical to P1, and wherein P2' is substantially identical to P2. After accurate homology-driven targeted integration, three amplicons are produced using a single PCR reaction with two oligonucleotide primers. The first amplicon, Amplicon X, is generated from the primer binding sites originally present in the genomic DNA (P1 and P2), and may be sequenced to analyze on-target editing events that do not result in targeted integration (*e.g.*, insertions, deletions, gene conversion). The remaining two amplicons are mapped to the 5' and 3' junctions after homology-driven targeted integration. The second amplicon, Amplicon Y, results from the amplification of the nucleic acid sequence between P1 and P2' following a targeted integration event at the target nucleic acid, thereby amplifying the 5' junction. The third amplicon, Amplicon Z, results from the amplification of the nucleic acid sequence between P1' and P2 following a targeted integration event at the target nucleic acid, thereby amplifying the 3' junction. Sequencing of these amplicons provides a quantitative assessment of targeted integration at the target nucleic acid, in addition to information about the fidelity of the targeted integration. To avoid any biases inherent to amplicon size, stuffer sequence may optionally be included in the donor template to keep all three expected amplicons the same length.

Implementation of genome editing systems: delivery, formulations, and routes of administration

[196] As discussed above, the genome editing systems of this disclosure can be implemented in any suitable manner, meaning that the components of such systems, including without limitation the RNA-guided nuclease, gRNA, and optional donor template nucleic acid, can be delivered, formulated, or administered in any suitable form or combination of forms that results in the transduction, expression or introduction of a genome editing system and/or causes a desired repair outcome in a cell, tissue or subject. The genome editing systems according to this disclosure can incorporate multiple gRNAs, multiple RNA-guided nucleases, and other components such as proteins, and a variety of implementations will be evident to the skilled artisan based on the principles illustrated in systems of the disclosure. In some embodiments the genome editing system of the disclosure are delivered into cells as an ribonucleoprotein (RNP) complex. In some embodiments, one or more RNP complexes are delivered to the cell sequentially in any order, or simultaneously.

[197] Nucleic acids encoding the various elements of a genome editing system according to the present disclosure can be administered to subjects or delivered into cells by art-known methods or as described herein. For example, RNA-guided nuclease-encoding and/or gRNA-encoding DNA, as well as donor template nucleic acids can be delivered by, *e.g.*, vectors (*e.g.*, viral or non-viral vectors), non-vector based methods (*e.g.*, using naked DNA or DNA complexes), or a combination thereof. In some embodiments the genome editing system of the disclosure are delivered by AAV.

[198] Nucleic acids encoding genome editing systems or components thereof can be delivered directly to cells as naked DNA or RNA, for instance by means of transfection or electroporation, or can be conjugated to molecules (*e.g.*, N-acetylgalactosamine) promoting uptake by the target cells (*e.g.*, erythrocytes, HSCs). In some embodiments the genome editing system of the disclosure are delivered into cells by electroporation.

[199] One promising solution to improve cell therapy processes consists on the direct delivery of active proteins into human cells. A protein delivery agent, the Feldan Shuttle, is a protein-based delivery agent, which is designed for cell therapy (Del'Guidice et al., PLoS One. 2018 Apr 4;13(4):e0195558; incorporated in its entirety herein by reference). In some embodiments the genome editing system of the disclosure are delivered into cells by the Feldan Shuttle.

[200] The modified cells of the disclosure can be administered by any known routes of administration known to a person of skill in the art, at the time of filing this application. In some embodiments the modified cells of the disclosure are administered intravenously (IV). In some embodiments the modified NK cells of the disclosure are administered intravenously (IV).

[201] As used herein, “dose” refers to a specific quantity of a pharmacologically active material for administration to a subject for a given time. Unless otherwise specified, the doses recited refer to NK cells having complex genomic alterations, that allow for the generation of advanced NK cell products for clinical applications. In some embodiments, a dose of modified NK cells refers to an effective amount of modified NK cells. For example, in some embodiments a dose or effective amount of modified NK cells refers to about $1 \times 10^9 - 5 \times 10^9$ modified NK cells, or about $2 \times 10^9 - 5 \times 10^9$ modified NK cells per dose. In some embodiments a dose or effective amount of modified NK cells refers to about $3 \times 10^9 - 5 \times 10^9$ modified NK cells, or about $4 \times 10^9 - 5 \times 10^9$ modified NK cells per dose.

Generation of modified iNK cells

[202] Some aspects of this disclosure relate to the generation of genetically modified NK cells that are derived from stem cells, *e.g.*, from multipotent cells, such as, *e.g.*, HSCs, or from pluripotent stem cells, such as, *e.g.*, ES cells or iPS cells. In some embodiments, where genetically modified iNK cells are derived from iPS cells, the iPS cells are derived from a somatic donor cell. In some embodiments, where genetically modified iNK cells are derived from iPS cells, the iPS cells are derived from a multipotent donor cell, *e.g.*, from an HSC.

[203] The genomic edits present in the final iNK cell can be made at any stage of the process of reprogramming the donor cell to the iPS cell state, during the iPS cell state, and/or at any stage of the process of differentiating the iPS cell to an iNK state, *e.g.*, at an intermediary state, such as, for example, an iPS cell-derived HSC state, or even up to or at the final iNK cell state. In some embodiments, one or more genomic edits present in a modified iNK cell provided herein is made before reprogramming the donor cell to the iPS cell state. In some embodiments, all edits present in a modified iNK cell provided herein are made at the same time, in close temporal proximity, and/or at

the same cell stage of the reprogramming/differentiation process, *e.g.*, at the donor cell stage, during the reprogramming process, at the iPS cell stage, or during the differentiation process. In some embodiments, two or more edits present in a modified iNK cell provided herein are made at different times and/or at different cell stages of the reprogramming/differentiation process. For example, in some embodiments, an edit is made at the donor cell stage and a different edit is made at the iPS cell stage; in some embodiments, an edit is made at the reprogramming stage and a different edit is made at the iPS cell stage. These examples are provided to illustrate some of the strategies provided herein, and are not meant to be limiting.

[204] A variety of cell types can be used as a donor cell that can be subjected to the reprogramming, differentiation, and genomic editing strategies provided herein for the derivation of modified iNK cells. The donor cell to be subjected to the reprogramming, differentiation, and genomic editing strategies provided herein can be any suitable cell type. For example, the donor cell can be a pluripotent stem cell or a differentiated cell, *e.g.*, a somatic cell, such as, for example, a fibroblast or a T lymphocyte.

[205] In some embodiments, the donor cell is a human cell. In some embodiments, the donor cell is a non-human primate cell. In some embodiments, the donor cell is a mammalian cell. In some embodiments, the donor cell is a somatic cell. In some embodiments, the donor cell is a stem or progenitor cell. In certain embodiments, the donor cell is not part of a human embryo and its derivation does not involve the destruction of a human embryo.

[206] In some embodiments, iNK cells, and methods of deriving such iNK cells, having one or more genomic alterations (*e.g.*, a knock-out of a gene undesirable for immunooncology therapeutic approaches, and/or a knock-in of an exogenous nucleic acid, *e.g.* an expression construct encoding a gene product desirable for immunooncology therapeutic approaches) are provided herein. In some embodiments, the iNK cells are derived from an iPS cell, which in turn is derived from a somatic donor cell. Any suitable somatic cell can be used in the generation of iPS cells, and in turn, the generation of iNK cells. Suitable strategies for deriving iPS cells from various somatic donor cell types have been described and are known in the art. In some embodiments, the somatic donor cell is a fibroblast cell. In some embodiments, the somatic donor cell is a mature T cell.

[207] For example, in some embodiments, the somatic donor cell, from which an iPS cell, and subsequently an iNK cell is derived, is a developmentally mature T cell (a T cell that has undergone thymic selection). One hallmark of developmentally mature T cells is a rearranged T cell receptor locus. During T cell maturation, the TCR locus undergoes V(D)J rearrangements to generate complete V-domain exons. These rearrangements are retained throughout reprogramming of a T cells to an induced pluripotent stem (iPS) cell, and throughout differentiation of the resulting iPS cell to a somatic cell.

[208] In certain embodiments, the somatic donor cell is a CD8⁺ T cell, a CD8⁺ naïve T cell, a CD4⁺ central memory T cell, a CD8⁺ central memory T cell, a CD4⁺ effector memory T cell, a CD4⁺

effector memory T cell, a CD4⁺ T cell, a CD4⁺ stem cell memory T cell, a CD8⁺ stem cell memory T cell, a CD4⁺ helper T cell, a regulatory T cell, a cytotoxic T cell, a natural killer T cell, a CD4⁺ naïve T cell, a TH17 CD4⁺ T cell, a TH1 CD4⁺ T cell, a TH2 CD4⁺ T cell, a TH9 CD4⁺ T cell, a CD4⁺ Foxp3⁺ T cell, a CD4⁺ CD25⁺ CD127⁻ T cell, or a CD4⁺ CD25⁺ CD127⁻ Foxp3⁺ T cell.

[209] One advantage of using T cells for the generation of iPS cells is that T cells can be edited with relative ease, *e.g.*, by CRISPR-based methods or other gene-editing methods. Another advantage of using T cells for the generation of iPS cells is that the rearranged TCR locus allows for genetic tracking of individual cells and their daughter cells. If the reprogramming, expansion, culture, and/or differentiation strategies involved in the generation of NK cells a clonal expansion of a single cell, the rearranged TCR locus can be used as a genetic marker unambiguously identifying a cell and its daughter cells. This, in turn, allows for the characterization of a cell population as truly clonal, or for the identification of mixed populations, or contaminating cells in a clonal population.

[210] A third advantage of using T cells in generating iNK cells carrying multiple edits is that certain karyotypic aberrations associated with chromosomal translocations are selected against in T cell culture. Such aberrations pose a concern when editing cells by CRISPR technology, and in particular when generating cells carrying multiple edits.

[211] A fourth advantage of using T cell derived iPS cells as a starting point for the derivation of therapeutic lymphocytes is that it allows for the expression of a pre-screened TCR in the lymphocytes, *e.g.*, via selecting the T cells for binding activity against a specific antigen, *e.g.*, a tumor antigen, reprogramming the selected T cells to iPS cells, and then deriving lymphocytes from these iPS cells that express the TCR (*e.g.*, T cells). This strategy would also allow for activating the TCR in other cell types, *e.g.*, by genetic or epigenetic strategies.

[212] A fifth advantage of using T cell derived iPS cells as a starting point for iNK differentiation is that the T cells retain at least part of their "epigenetic memory" throughout the reprogramming process, and thus subsequent differentiation of the same or a closely related cell type, such as iNK cells will be more efficient and/or result in higher quality cell populations as compared to approaches using non-related cells, such as fibroblasts, as a starting point for iNK derivation.

[213] In certain embodiments, the donor cell being manipulated, *e.g.*, the cell being reprogrammed and/or the cell, the genome of which is being edited, is a long term hematopoietic stem cell, a short term hematopoietic stem cell, a multipotent progenitor cell, a lineage restricted progenitor cell, a lymphoid progenitor cell, a myeloid progenitor cell, a common myeloid progenitor cell, an erythroid progenitor cell, a megakaryocyte erythroid progenitor cell, a retinal cell, a photoreceptor cell, a rod cell, a cone cell, a retinal pigmented epithelium cell, a trabecular meshwork cell, a cochlear hair cell, an outer hair cell, an inner hair cell, a pulmonary epithelial cell, a bronchial epithelial cell, an alveolar epithelial cell, a pulmonary epithelial progenitor cell, a striated muscle cell, a cardiac muscle cell, a muscle satellite cell, a neuron, a neuronal stem cell, a mesenchymal stem cell, an induced pluripotent stem (iPS) cell, an embryonic stem cell, a fibroblast, a monocyte-derived macrophage or dendritic

cell, a megakaryocyte, a neutrophil, an eosinophil, a basophil, a mast cell, a reticulocyte, a B cell, *e.g.*, a progenitor B cell, a Pre B cell, a Pro B cell, a memory B cell, a plasma B cell, a gastrointestinal epithelial cell, a biliary epithelial cell, a pancreatic ductal epithelial cell, an intestinal stem cell, a hepatocyte, a liver stellate cell, a Kupffer cell, an osteoblast, an osteoclast, an adipocyte, a preadipocyte, a pancreatic islet cell (*e.g.*, a beta cell, an alpha cell, a delta cell), a pancreatic exocrine cell, a Schwann cell, or an oligodendrocyte.

[214] In certain embodiments, the donor cell is a circulating blood cell, *e.g.*, a reticulocyte, megakaryocyte erythroid progenitor (MEP) cell, myeloid progenitor cell (CMP/GMP), lymphoid progenitor (LP) cell, hematopoietic stem/progenitor cell (HSC), or endothelial cell (EC). In certain embodiments, the donor cell is a bone marrow cell (*e.g.*, a reticulocyte, an erythroid cell (*e.g.*, erythroblast), an MEP cell, myeloid progenitor cell (CMP/GMP), LP cell, erythroid progenitor (EP) cell, HSC, multipotent progenitor (MPP) cell, endothelial cell (EC), hemogenic endothelial (HE) cell, or mesenchymal stem cell). In certain embodiments, the donor cell is a myeloid progenitor cell (*e.g.*, a common myeloid progenitor (CMP) cell or granulocyte macrophage progenitor (GMP) cell). In certain embodiments, the donor cell is a lymphoid progenitor cell, *e.g.*, a common lymphoid progenitor (CLP) cell. In certain embodiments, the donor cell is an erythroid progenitor cell (*e.g.*, an MEP cell). In certain embodiments, the donor cell is a hematopoietic stem/progenitor cell (*e.g.*, a long term HSC (LT-HSC), short term HSC (ST-HSC), MPP cell, or lineage restricted progenitor (LRP) cell). In certain embodiments, the donor cell is a CD34⁺ cell, CD34⁺CD90⁺ cell, CD34⁺CD38⁻ cell, CD34⁺CD90⁺CD49f⁺CD38⁻CD45RA⁻ cell, CD105⁺ cell, CD31⁺, or CD133⁺ cell, or a CD34⁺CD90⁺CD133⁺ cell. In certain embodiments, the donor cell is an umbilical cord blood CD34⁺ HSPC, umbilical cord venous endothelial cell, umbilical cord arterial endothelial cell, amniotic fluid CD34⁺ cell, amniotic fluid endothelial cell, placental endothelial cell, or placental hematopoietic CD34⁺ cell. In certain embodiments, the donor cell is a mobilized peripheral blood hematopoietic CD34⁺ cell (after the patient is treated with a mobilization agent, *e.g.*, G-CSF or Plerixafor). In certain embodiments, the donor cell is a peripheral blood endothelial cell.

[215] In some embodiments, the donor cell is a dividing cell. In other embodiments, the donor cell is a non-dividing cell.

[216] In some embodiments, the modified iNK cells resulting from the methods and strategies of reprogramming, differentiating, and editing provided herein, are administered to a subject in need thereof, *e.g.*, in the context of an immunooncology therapeutic approach. In some embodiments, donor cells, or any cells of any stage of the reprogramming, differentiating, and editing strategies provided herein can be maintained in culture or stored (*e.g.*, frozen in liquid nitrogen) using any suitable method known in the art, *e.g.*, for subsequent characterization or administration to a subject in need thereof.

Cell Reprogramming

[217] A cell that has an increased cell potency has more developmental plasticity (*i.e.*, can differentiate into more cell types) compared to the same cell in the non-reprogrammed state. In other words, a reprogrammed cell is one that is in a less differentiated state than the same cell in a non-reprogrammed state.

[218] The reprogramming of the cells of the disclosure can be performed by utilizing several methods. Examples of some methods for reprogramming somatic cells of the disclosure are described in, but are not limited to, Valamehr *et al.* WO2017/078807 (“Valamehr”) and Mendlein *et al.* WO2010/108126 (“Mendlein”), which are hereby incorporated by reference in their entireties.

[219] Briefly, a method for directing differentiation of pluripotent stem cells into cells of a definitive hematopoietic lineage, may comprise: (i) contacting pluripotent stem cells with a composition comprising a BMP activator, and optionally bFGF, to initiate differentiation and expansion of mesodermal cells from the pluripotent stem cells; (ii) contacting the mesodermal cells with a composition comprising a BMP activator, bFGF, and a GSK3 inhibitor, wherein the composition is optionally free of TGF β receptor/ALK inhibitor, to initiate differentiation and expansion of mesodermal cells having definitive HE potential from the mesodermal cells; (iii) contacting the mesodermal cells having definitive HE potential with a composition comprising a ROCK inhibitor; one or more growth factors and cytokines selected from the group consisting of bFGF, VEGF, SCF, IGF, EPO, IL6, and IL11; and optionally, a Wnt pathway activator, wherein the composition is optionally free of TGF β receptor/ALK inhibitor, to initiate differentiation and expansion of definitive hemogenic endothelium from pluripotent stem cell-derived mesodermal cells having definitive hemogenic endothelium potential; and optionally, subjecting pluripotent stem cells, pluripotent stem cell-derived mesodermal cells, mesodermal cells having hemogenic endothelium, and/or definitive hemogenic endothelium under low oxygen tension between about 2% to about 10%.

[220] In some embodiments of the method for directing differentiation of pluripotent stem cells into cells of a hematopoietic lineage, the method further comprises contacting pluripotent stem cells with a composition comprising a MEK inhibitor, a GSK3 inhibitor, and a ROCK inhibitor, wherein the composition is free of TGF β receptor/ALK inhibitors, to seed and expand the pluripotent stem cells. In some embodiments, the pluripotent stem cells are iPSCs. In some embodiments, the iPSCs are naïve iPSCs. In some embodiments, the iPSC comprises one or more genetic imprints, and wherein the one or more genetic imprints comprised in the iPSC are retained in the pluripotent stem cell derived hematopoietic cells differentiated therefrom.

[221] In some embodiments of the method for directing differentiation of pluripotent stem cells into cells of a hematopoietic lineage, the differentiation of the pluripotent stem cells into cells of hematopoietic lineage is void of generation of embryoid bodies, and is in a monolayer culturing form.

[222] In some embodiments of the above method, the obtained pluripotent stem cell-derived definitive hemogenic endothelium cells are CD34+. In some embodiments, the obtained definitive

hemogenic endothelium cells are CD34+CD43-. In some embodiments, the definitive hemogenic endothelium cells are CD34+CD43-CXCR4-CD73-. In some embodiments, the definitive hemogenic endothelium cells are CD34+ CXCR4-CD73-. In some embodiments, the definitive hemogenic endothelium cells are CD34+CD43-CD93-. In some embodiments, the definitive hemogenic endothelium cells are CD34+ CD93-.

[223] In some embodiments of the above method, the method further comprises (i) contacting pluripotent stem cell-derived definitive hemogenic endothelium with a composition comprising a ROCK inhibitor; one or more growth factors and cytokines selected from the group consisting of VEGF, bFGF, SCF, Flt3L, TPO, and IL7; and optionally a BMP activator; to initiate the differentiation of the definitive hemogenic endothelium to pre-T cell progenitors; and optionally, (ii) contacting the pre-T cell progenitors with a composition comprising one or more growth factors and cytokines selected from the group consisting of SCF, Flt3L, and IL7, but free of one or more of VEGF, bFGF, TPO, BMP activators and ROCK inhibitors, to initiate the differentiation of the pre-T cell progenitors to T cell progenitors or T cells. In some embodiments of the method, the pluripotent stem cell-derived T cell progenitors are CD34+CD45+CD7+. In some embodiments of the method, the pluripotent stem cell-derived T cell progenitors are CD45+CD7+.

[224] In yet some embodiments of the above method for directing differentiation of pluripotent stem cells into cells of a hematopoietic lineage, the method further comprises: (i) contacting pluripotent stem cell-derived definitive hemogenic endothelium with a composition comprising a ROCK inhibitor; one or more growth factors and cytokines selected from the group consisting of VEGF, bFGF, SCF, Flt3L, TPO, IL3, IL7, and IL15; and optionally, a BMP activator, to initiate differentiation of the definitive hemogenic endothelium to pre-NK cell progenitor; and optionally, (ii) contacting pluripotent stem cells-derived pre-NK cell progenitors with a composition comprising one or more growth factors and cytokines selected from the group consisting of SCF, Flt3L, IL3, IL7, and IL15, wherein the medium is free of one or more of VEGF, bFGF, TPO, BMP activators and ROCK inhibitors, to initiate differentiation of the pre-NK cell progenitors to NK cell progenitors or NK cells. In some embodiments, the pluripotent stem cell-derived NK progenitors are CD3-CD45+CD56+CD7+. In some embodiments, the pluripotent stem cell-derived NK cells are CD3-CD45+CD56+, and optionally further defined by NKp46+, CD57+ and CD16+.

[225] In yet some embodiments of the above method for directing differentiation of pluripotent stem cells into NK cells, the method further comprises knocking out the gene Nrg1 in the pluripotent stem cells.

[226] In some embodiments, the disclosure provides a method for generating pluripotent stem cell-derived T lineage cells, which comprises: (i) contacting pluripotent stem cells with a composition comprising a BMP activator, and optionally bFGF, to initiate differentiation and expansion of mesodermal cells from pluripotent stem cells; (ii) contacting the mesodermal cells with a composition comprising a BMP activator, bFGF, and a GSK3 inhibitor, but free of TGFβ receptor/ALK inhibitor,

to initiate differentiation and expansion of the mesodermal cells having definitive HE potential from the mesodermal cells; (iii) contacting mesodermal cells having definitive HE potential with a composition comprising a ROCK inhibitor; one or more growth factors and cytokines selected from the group consisting of bFGF, VEGF, SCF, IGF, EPO, IL6, and IL11; and optionally, a Wnt pathway activator; wherein the composition is free of TGF β receptor/ALK inhibitor, to initiate differentiation and expansion of definitive hemogenic endothelium from mesodermal cells having definitive HE potential; (iv) contacting definitive hemogenic endothelium with a composition comprising a ROCK inhibitor; one or more growth factors and cytokines selected from the group consisting of VEGF, bFGF, SCF, Flt3L, TPO, and IL7; and optionally a BMP activator; to initiate differentiation of the definitive hemogenic endothelium to pre-T cell progenitors; and (v) contacting the pre-T cell progenitors with a composition comprising one or more growth factors and cytokines selected from the group consisting of SCF, Flt3L, and IL7, wherein the composition is free of one or more of VEGF, bFGF, TPO, BMP activators and ROCK inhibitors; to initiate differentiation of the pre-T cell progenitors to T cell progenitors or T cells; and optionally, the seeded pluripotent stem cells, mesodermal cells, mesodermal cells having definitive HE potential, and/or definitive hemogenic endothelium may be subject to low oxygen tension between about 2% to about 10%. In some embodiments, group II of the above method further comprises: contacting iPSCs with a composition comprising a MEK inhibitor, a GSK3 inhibitor, and a ROCK inhibitor, but free of TGF β receptor/ALK inhibitors, to seed and expand pluripotent stem cells; and/or wherein the pluripotent stem cells. In some embodiments, the pluripotent stem cells are iPSCs. In some embodiments, the iPSCs are naïve iPSC. In some embodiments of the method, the differentiation of the pluripotent stem cells into T cell lineages is void of generation of embryoid bodies, and is in a monolayer culturing format.

[227] In some embodiments, the disclosure provides a method for generating pluripotent stem cell-derived NK lineage cells, which comprises: (i) contacting pluripotent stem cells with a composition comprising a BMP activator, and optionally bFGF, to initiate differentiation and expansion of mesodermal cells from the pluripotent stem cells; (ii) contacting mesodermal cells with a composition comprising a BMP activator, bFGF, and a GSK3 inhibitor, and optionally free of TGF β receptor/ALK inhibitor, to initiate differentiation and expansion of mesodermal cells having definitive HE potential from mesodermal cells; (iii) contacting mesodermal cells having definitive HE potential with a composition comprising one or more growth factors and cytokines selected from the group consisting of bFGF, VEGF, SCF, IGF, EPO, IL6, and IL11; a ROCK inhibitor; optionally a Wnt pathway activator; and optionally free of TGF β receptor/ALK inhibitor, to initiate differentiation and expansion of pluripotent stem cell-derived definitive hemogenic endothelium from the pluripotent stem cell-derived mesodermal cells having definitive HE potential; (iv) contacting pluripotent stem cell-derived definitive hemogenic endothelium with a composition comprising a ROCK inhibitor; one or more growth factors and cytokines selected from the group consisting of VEGF, bFGF, SCF, Flt3L,

TPO, IL3, IL7, and IL15, and optionally, a BMP activator, to initiate differentiation of the pluripotent stem cell-derived definitive hemogenic endothelium to pre-NK cell progenitors; and (v) contacting pluripotent stem cell-derived pre-NK cell progenitors with a composition comprising one or more growth factors and cytokines selected from the group consisting of SCF, Flt3L, IL3, IL7, and IL15, but free of one or more of VEGF, bFGF, TPO, BMP activators and ROCK inhibitors, to initiate differentiation of the pluripotent stem cell-derived pre-NK cell progenitors to pluripotent stem cell-derived NK cell progenitors or NK cells; and optionally, subjecting seeded pluripotent stem cells, pluripotent stem cell-derived-mesodermal cells, and/or definitive hemogenic endothelium under low oxygen tension between about 2% to about 10%. In some embodiments, the method for generating pluripotent stem cell-derived NK lineage cells of group II further comprises contacting iPSCs with a composition comprising a MEK inhibitor, a GSK3 inhibitor, and a ROCK inhibitor, but free of TGF β receptor/ALK inhibitors, to seed and expand the iPSCs. In some embodiments, the iPSCs are naïve iPSCs. In some embodiments, the method for generating pluripotent stem cell-derived NK lineage cells is void of generation of embryoid bodies, and is in a monolayer culturing format.

[228] In some embodiments, the disclosure provides a method for generating pluripotent stem cell-derived definitive hemogenic endothelium, the method comprises: (i) contacting iPSCs with a composition comprising a BMP activator, and optionally bFGF, to initiate differentiation and expansion of pluripotent stem cell-derived mesodermal cells from pluripotent stem cells; (ii) contacting pluripotent stem cell-derived mesodermal cells with a composition comprising a BMP activator, bFGF, and a GSK3 inhibitor, and optionally free of TGF β receptor/ALK inhibitor, to initiate differentiation and expansion of pluripotent stem cell-derived mesodermal cells having definitive HE potential from pluripotent stem cell-derived mesodermal cells; (iii) contacting pluripotent stem cell-derived mesodermal cells having definitive HE potential with a composition comprising one or more growth factors and cytokines selected from the group consisting of bFGF, VEGF, SCF, IGF, EPO, IL6, and IL11; a ROCK inhibitor; and optionally a Wnt pathway activator, and optionally free of TGF β receptor/ALK inhibitor, to initiate differentiation and expansion of pluripotent stem cell-derived definitive hemogenic endothelium from the pluripotent stem cell-derived mesodermal cells having definitive HE potential; and optionally, subjecting seeded pluripotent stem cells, pluripotent stem cell-derived mesodermal cells, and/or definitive hemogenic endothelium under low oxygen tension between about 2% to about 10%. In some embodiments, the above method for generating pluripotent stem cell-derived definitive hemogenic endothelium, further comprises: contacting iPSCs with a composition comprising a MEK inhibitor, a GSK3 inhibitor, and a ROCK inhibitor, but free of TGF β receptor/ALK inhibitors, to seed and expand the iPSCs; and/or wherein the iPSCs are naïve iPSCs. In some embodiments, the iPSC comprises one or more genetic imprints, and wherein the one or more genetic imprints comprised in the iPSC are retained in the pluripotent stem cell derived definitive hemogenic endothelium cells differentiated therefrom. In some embodiments,

the above method of differentiating iPSCs into cells of a definitive hemogenic endothelium is void of generation of embryoid bodies, and is in monolayer culturing format.

[229] In some embodiments, the disclosure provides a method for generating pluripotent stem cell-derived multipotent progenitors of hematopoietic lineage, comprising: (i) contacting iPSCs with a composition comprising a BMP activator, and optionally bFGF, to initiate differentiation and expansion of pluripotent stem cell-derived mesodermal cells from iPSCs; (ii) contacting pluripotent stem cell-derived mesodermal cells with a composition comprising a BMP activator, bFGF, and a GSK3 inhibitor, but free of TGF β receptor/ALK inhibitor, to initiate differentiation and expansion of the mesodermal cells having definitive HE potential from the mesodermal cells; (iii) contacting mesodermal cells having definitive HE potential with a composition comprising a ROCK inhibitor; one or more growth factors and cytokines selected from the group consisting of bFGF, VEGF, SCF, IGF, EPO, IL6, and IL11; and optionally, a Wnt pathway activator, wherein the composition is free of TGF β receptor/ALK inhibitor, to initiate differentiation and expansion of definitive hemogenic endothelium from mesodermal cells having definitive HE potential; (iv) contacting definitive hemogenic endothelium with a composition comprising a BMP activator, a ROCK inhibitor, one or more growth factors and cytokines selected from the group consisting of TPO, IL3, GM-CSF, EPO, bFGF, VEGF, SCF, IL6, Flt3L and IL11, to initiate differentiation of definitive hemogenic endothelium to pre-HSC; and (v) contacting pre-HSC with a composition comprising a BMP activator, one or more growth factors and cytokines selected from the group consisting of TPO, IL3, GM-CSF, EPO, bFGF, VEGF, SCF, IL6, and IL11, but free of ROCK inhibitor, to initiate differentiation of the pre-HSC to hematopoietic multipotent progenitors; and optionally, subjecting seeded pluripotent stem cells, mesodermal cells, and/or definitive hemogenic endothelium under low oxygen tension between about 2% to about 10%. In some embodiments, the above method for generating pluripotent stem cell-derived hematopoiesis multipotent progenitors further comprises contacting pluripotent stem cells with a composition comprising a MEK inhibitor, a GSK3 inhibitor, and a ROCK inhibitor, but free of TGF β receptor/ALK inhibitors, to seed and expand the pluripotent stem cells. In some embodiments, the pluripotent stem cells are iPSCs. In some embodiments, the iPSCs are naïve iPSCs. In some embodiments, the iPSC comprises one or more genetic imprints, and wherein the one or more genetic imprints comprised in the iPSC are retained in the pluripotent stem cell derived hematopoietic multipotent progenitor cells differentiated therefrom. In some embodiments, the differentiation of the pluripotent stem cells into hematopoiesis multipotent progenitors using the above method is void of generation of embryoid bodies, and is in monolayer culturing format.

[230] In some embodiments, the disclosure provides a composition comprising: one or more cell populations generated from the culture platform disclosed herein: pluripotent stem cells-derived (i) CD34⁺ definitive hemogenic endothelium (iCD34), wherein the iCD34 cells have capacity to differentiate into multipotent progenitor cells, T cell progenitors, NK cell progenitors, T cells, NK cells, NKT cells and B cells, and wherein the iCD34 cells are CD34⁺CD43⁻; (ii) definitive

hemogenic endothelium (iHE), wherein the iHE cells are CD34+, and at least one of CD43-, CD93-, CXCR4-, CD73-, and CXCR4-CD73-; (iii) pluripotent stem cell-derived definitive HSCs, wherein the iHSC is CD34+CD45+; (iv) hematopoietic multipotent progenitor cells, wherein the iMPP cells are CD34+CD45+; (v) T cell progenitors, wherein the T cell progenitors are CD34+CD45+CD7+ or CD34-CD45+CD7+; (vi) T cells, wherein the T cells are CD45+CD3+CD4+ or CD45+CD3+CD8+; (vii) NK cell progenitors, wherein the NK cell progenitors are CD45+CD56+CD7+; (viii) NK cells, wherein the NK cells are CD3-CD45+CD56+, and optionally further defined by NKp46+, CD57+, and CD16+; (ix) NKT cells, wherein the NKT cells are CD45+V α 24J α 18+CD3+; and (x) B cells, wherein the B cells are CD45+CD19+.

[231] In some embodiments, the disclosure provides one or more cell lines, or clonal cells generated using the methods disclosed herein: pluripotent stem cell-derived (i) CD34+ definitive hemogenic endothelium (iCD34), wherein the iCD34 cells have capacity to differentiate into multipotent progenitor cells, T cell progenitors, NK cell progenitors, T cells, NK cells, and NKT cells, and wherein the iCD34 cells are CD34+CD43-; (ii) definitive hemogenic endothelium (iHE), wherein the iHE cell line or clonal cells are CD34+, and at least one of CD43-, CD93-, CXCR4-, CD73-, and CXCR4-CD73-; (iii) definitive HSCs, wherein the iHSCs is CD34+CD45+; (iv) hematopoietic multipotent progenitor cells (iMPP), wherein the iMPP cells are CD34+CD45+; (v) T cell progenitors, wherein the T cell progenitors are CD34+CD45+CD7+ or CD34-CD45+CD7+; (vi) T cells, wherein the T cells are CD45+CD3+CD4+ or CD45+CD3+CD8+; (vii) NK cell progenitors, wherein the NK cell progenitors are CD45+CD56+CD7+; (viii) NK cells, wherein the NK cells are CD3-CD45+CD56+, and optionally further defined by NKp46+, CD57+, and CD16+; (ix) NKT cells, wherein the NKT cells are CD45+V α 24J α 18+CD3+; and (x) B cells, wherein the B cells are CD45+CD19+.

[232] In some embodiments, the present disclosure provides a method of promoting hematopoietic self-renewal, reconstitution or engraftment using one or more of cell populations, cell lines or clonal cells generated using methods as disclosed: pluripotent stem cell-derived (i) CD34+ definitive hemogenic endothelium (iCD34), wherein the iCD34 cells have capacity to differentiate into multipotent progenitor cells, T cell progenitors, NK cell progenitors, T cells NK cells and NKT cells, and wherein the iCD34 cells are CD34+CD43-; (ii) definitive hemogenic endothelium (iHE), wherein the iHE cell line or clonal cells are CD34+, and at least one of CD43-, CD93-, CXCR4-, CD73-, and CXCR4-CD73-; (iii) definitive HSCs, wherein the iHSCs are CD34+CD45+; (iv) hematopoietic multipotent progenitor cells, wherein the iMPP cells are CD34+CD45+; (v) T cell progenitors, wherein the T cell progenitors are CD34+CD45+CD7+ or CD34-CD45+CD7+; (vi) T cells, wherein the T cells are CD45+CD3+CD4+ or CD45+CD3+CD8+; (vii) NK cell progenitors, wherein the NK cell progenitors are CD45+CD56+CD7+; (viii) NK cells, wherein the NK cells are CD3-CD45+CD56+, and optionally further defined by NKp46+, CD57+, and CD16+; (ix) NKT cells,

wherein the NKT cells are CD45+V α 24J α 18+CD3+; and (x) B cells, wherein the B cells are CD45+CD19+.

[233] In some embodiments, the present disclosure provides a method of generating hematopoietic lineage cells with enhanced therapeutic properties, and the method comprises: obtaining iPSCs comprising one or more genetic imprints; and directing differentiation of iPSCs to hematopoietic lineage cells. The step of directed differentiation further comprises: (i) contacting the pluripotent stem cells with a composition comprising a BMP pathway activator, and optionally bFGF, to obtain mesodermal cells; and (ii) contacting the mesodermal cells with a composition comprising a BMP pathway activator, bFGF, and a WNT pathway activator, to obtain mesodermal cells having definitive hemogenic endothelium (HE) potential, wherein the mesodermal cells having definitive hemogenic endothelium (HE) potential are capable of providing hematopoietic lineage cells. Preferably, the mesodermal cells and mesodermal cells having definitive HE potential are obtained in steps (i) and (ii) without the step of forming embryoid bodies, and the obtained hematopoietic lineage cells comprise definitive hemogenic endothelium cells, hematopoietic stem and progenitor cells (HSC), hematopoietic multipotent progenitor cell (MPP), pre-T cell progenitor cells, pre-NK cell progenitor cells, T cell progenitor cells, NK cell progenitor cells, T cells, NK cells, NKT cells, or B cells. Moreover, the hematopoietic lineage cells retain the genetic imprints comprised in the iPSCs for directed differentiation.

[234] In some embodiments, the step of directed differentiation of the above method further comprises: (i) contacting the mesodermal cells having definitive HE potential with a composition comprising bFGF and a ROCK inhibitor to obtain definitive HE cells; (ii) contacting the definitive HE cells with a composition comprising a BMP activator, and optionally a ROCK inhibitor, and one or more growth factors and cytokines selected from the group consisting of TPO, IL3, GM-CSF, EPO, bFGF, VEGF, SCF, IL6, Flt3L and IL11 to obtain hematopoietic multipotent progenitor cells (MPP); (iii) contacting the definitive HE cells with a composition comprising one or more growth factors and cytokines selected from the group consisting of SCF, Flt3L, and IL7; and optionally one or more of a BMP activator, a ROCK inhibitor, TPO, VEGF and bFGF to obtain pre-T cell progenitors, T cell progenitors, and/or T cells; or (iv) contacting the definitive HE cells with a composition comprising one or more growth factors and cytokines selected from the group consisting of SCF, Flt3L, TPO, IL7 and IL15, and optionally one or more of a BMP activator, a ROCK inhibitor, VEGF and bFGF to obtain pre-NK cell progenitors, NK cell progenitors, and/or NK cells.

[235] Briefly, the method may comprise reprogramming a mature source T or B cell to obtain induced pluripotent stem cells (iPSCs); and detecting the presence, in the iPSCs or the hematopoietic lineage cells derived therefrom, of a specific V(D)J recombination that is same as the one comprised in the mature T or B cell for generating the iPSC. In some embodiments, the above method further comprises isolating iPSCs or hematopoietic lineage cells comprising the same V(D)J recombination as that of the mature source T or B cell. In some embodiments, the above method comprises, prior to

reprogramming the source cells, obtaining a mature source T or B cell for reprogramming; and determining V(D)J recombination comprised in immunoglobulins (Ig) or T cell receptors (TCR) that is specific to the mature source T or B cell.

[236] A “pluripotency factor,” or “reprogramming factor,” refers to an agent capable of increasing the developmental potency of a cell, either alone or in combination with other agents. Pluripotency factors include, without limitation, polynucleotides, polypeptides, and small molecules capable of increasing the developmental potency of a cell. Exemplary pluripotency factors include, for example, transcription factors and small molecule reprogramming agents.

[237] A number of various cell types from all three germ layers have been shown to be suitable for somatic cell reprogramming, including, but not limited to liver and stomach (Aoi et al., 2008); pancreatic β cells (Stadtfeld et al., 2008); mature B lymphocytes (Hanna et al., 2008); human dermal fibroblasts (Takahashi et al., 2007; Yu et al., 2007; Lowry et al., 2008; Aasen et al., 2008); meningiocytes (Qin et al., 2008); neural stem cells (DiSteffano et al., 2008); and neural progenitor cells (Eminli et al., 2008). Thus, the present disclosure contemplates, in part, methods to reprogram and/or program cells from any cell lineage.

[238] The present disclosure contemplates, in part, to alter the potency of a cell by contacting the cell with one or more repressors and/or activators to modulate the epigenetic state, chromatin structure, transcription, mRNA splicing, post-transcriptional modification, mRNA stability and/or half-life, translation, post-translational modification, protein stability and/or half-life and/or protein activity of a component of a cellular pathway associated with determining or influencing cell potency.

[239] Thus, in various embodiments, the present disclosure uses predictable and highly controlled methods for gene expression, as discussed elsewhere herein, that enable the reprogramming or de-differentiation and programming or differentiation of somatic cells *ex vivo* or *in vivo*. As, noted above, the intentional genetic engineering of cells, however, is not preferred, since it alters the cellular genome and would likely result in genetic or epigenetic abnormalities. In contrast, the compositions and methods of the present disclosure provide repressors and/or activators that non-genetically alter the potency of a cell by mimicking the cell's endogenous developmental potency pathways to achieve reprogramming and/or programming of the cell.

Small Molecules in Reprogramming

[240] Reprogramming of somatic cells into induced pluripotent stem cells has also been achieved by retroviral infection of defined genes (*e.g.*, Oct-3/4, Sox-2, Klf-4, c-Myc, and Lin28, and the like) in combination with small molecules.

[241] In some embodiments, the present disclosure provides a method of altering the potency of a cell that comprises contacting the cell with one or more repressors and/or activators or a composition comprising the same, wherein said one or more repressors and/or activators modulates at least one component of a cellular pathway associated with the potency of the cell, thereby altering the potency of the cell. In particular embodiments, the one or more repressors and/or activators modulate one or

more components of a cellular pathway associated with the potency of the cell and thereby alter the potency of the cell. In certain embodiments, the one or more repressors and/or activators modulate one or more components of one or more cellular pathways associated with the potency of the cell and thereby alter the potency of the cell. In certain related embodiments, the modulation of the component(s) is synergistic and increases the overall efficacy of altering the potency of a cell. The potency of the cell can be altered, compared to the ground potency state, to a more potent state (*e.g.*, from a differentiated cell to a multipotent, pluripotent, or totipotent cell) or a less potent state (*e.g.*, from a totipotent, pluripotent, or multipotent cell to a differentiated somatic cell). In still yet other embodiments, the potency of a cell may be altered more than once. For example, a cell may first be reprogrammed to a more potent state, then programmed to a particular somatic cell.

[242] In another embodiment, the methods of the present disclosure provide for increasing the potency a cell, wherein the cell is reprogrammed or dedifferentiated to a totipotent state, comprising contacting the cell with a composition comprising one or more repressors and/or activators, wherein the one or more repressors and/or activators modulates at least one component of a cellular pathway associated with the totipotency of the cell, thereby increasing the potency of the cell to a totipotent state.

[243] In a particular embodiment, a method of increasing the potency a cell to a pluripotent state comprises contacting the cell with one or more repressors and/or activators, wherein the one or more repressors and/or activators modulates at least one component of a cellular pathway associated with the potency of the cell, thereby increasing the potency of the cell to a pluripotent state.

[244] In another particular embodiment, a method of increasing the potency a cell to a multipotent state comprises contacting the cell with one or more repressors and/or activators, wherein the one or more repressors and/or activators modulates at least one component of a cellular pathway associated with the potency of the cell, thereby increasing the potency of the cell to a multipotent state.

[245] In certain embodiments, a method of increasing the potency of a cell further comprises a step of contacting the totipotent cell, the pluripotent cell or the multipotent cell with a second composition, wherein the second composition modulates the at least one component of a cellular potency pathway to decrease the totipotency, pluripotency or multipotency of the cell and differentiate the cell to a mature somatic cell.

[246] In another related embodiment, the present disclosure provides a method of reprogramming a cell that comprises contacting the cell with a composition comprising one or more repressors and/or activators, wherein the one or more repressors and/or activators modulates at least one component of a cellular pathway or pathways associated with the reprogramming of a cell, thereby reprogramming the cell.

[247] In other embodiments, the present disclosure provides a method of dedifferentiating a cell to a more potent state, comprising contacting the cell with the composition comprising one/or more activators, wherein the one or more repressors and/or activators modulates at least one component of a

cellular pathway or pathways associated with the dedifferentiation of the cell to the more potent state, thereby dedifferentiating the cell to an impotent state.

[248] According to various embodiments of the present disclosure a repressor can be an antibody or an antibody fragment, an intrabody, a transbody, a DNAzyme, an ssRNA, a dsRNA, an mRNA, an antisense RNA, a ribozyme, an antisense oligonucleotide, a pri-miRNA, an shRNA, an antagomir, an aptamer, an siRNA, a dsDNA, a ssDNA; a polypeptide or an active fragment thereof, a peptidomimetic, a peptoid, or a small organic molecule. Polypeptide-based repressors include, but are not limited to fusion polypeptides. Polypeptide-based repressors also include transcriptional repressors, which can further be fusion polypeptides and/or artificially designed transcriptional repressors as described elsewhere herein.

[249] According to other various embodiments, an activator can be an antibody or an antibody fragment, an mRNA, a bifunctional antisense oligonucleotide, a dsDNA, a polypeptide or an active fragment thereof, a peptidomimetic, a peptoid, or a small organic molecule.

[250] In some embodiments, repressors modulate at least one component of a cellular potency pathway by a) repressing the at least one component; b) de-repressing a repressor of the at least one component; or c) repressing an activator of the at least one component. In related embodiments, one or more repressors can modulate at least one component of a pathway associated with the potency of a cell by a) de-repressing the at least one component; b) repressing a repressor of the at least one component; or c) de-repressing an activator of the at least one component.

[251] In certain embodiments, one or more repressors modulates at least one component of a cellular pathway associated with the potency of a cell by a) repressing a histone methyltransferase or repressing the at least one component's epigenetic state, chromatin structure, transcription, mRNA splicing, post-transcriptional modification, mRNA stability and/or half-life, translation, post-translational modification, protein stability and/or half-life and/or protein activity; or b) de-repressing a demethylase or activating the at least one component's epigenetic state, chromatin structure, transcription, mRNA splicing, post-transcriptional modification, mRNA stability and/or half-life, translation, post-translational modification, protein stability and/or half-life and/or protein activity.

[252] In related embodiments, activators modulate at least one component of a cellular pathway associated with the potency of a cell by a) activating the at least one component; b) activating a repressor of a repressor of the at least one component; or c) activating an activator of the at least one component.

[253] In certain embodiments, one or more activators modulates at least one component by a) activating a histone demethylase or activating the at least one component's epigenetic state, chromatin structure, transcription, mRNA splicing, post-transcriptional modification, mRNA stability and/or half-life, translation, post-translational modification, protein stability and/or half-life and/or protein activity; or b) activating a repressor of a histone methyltransferase or activating a repressor of the at least one component's epigenetic state, chromatin structure, transcription, mRNA splicing, post-

transcriptional modification, mRNA stability and/or half-life, translation, post-translational modification, protein stability and/or half-life and/or protein activity.

[254] In various other embodiments, the present disclosure contemplates, in part, a method of reprogramming a cell, comprising contacting the cell with one or more repressors, wherein the one or more repressors modulates at least one component of a cellular pathway associated with the reprogramming of a cell, thereby reprogramming the cell.

[255] In various other embodiments, the present disclosure contemplates, in part, a method of reprogramming a cell, comprising contacting the cell with a composition comprising one or more activators, wherein the one or more activators modulates at least one component of a cellular pathway associated with the reprogramming of a cell, thereby re-programming the cell.

[256] While some exemplary methods for reprogramming/NK cell differentiation are provided herein, these are exemplary and not meant to limit the scope of the present disclosure. Additional suitable methods for reprogramming/NK cell differentiation will be apparent to those of skill in the art based on the present disclosure in view of the knowledge in the art.

[257] Methods for culturing NK cells on feeder layers or with feeder cells are described in detail in, for *e.g.*, EP3184109 by Valamehr *et al.* (“Valamehr”) incorporated in its entirety herein by reference.

[258] In general, any type of NK cell population can be cultured using a variety of methods and devices. Selection of culture apparatus is usually based on the scale and purpose of the culture. Scaling up of cell culture preferably involves the use of dedicated devices. Apparatus for large scale, clinical grade NK cell production is detailed, for example, in Spanholtz *et al.* (PLoS ONE 2010;5:e9221) and Sutlu *et al.* (Cytotherapy 2010, Early Online 1-12).

[259] The methods described hereinabove for *ex vivo* culturing NK cells populations can result, inter alia, in a cultured population of NK cells.

Types of Edits

[260] Some aspects of the present disclosure provide complex editing strategies, and resulting NK cells having complex genomic alterations, that allow for the generation of advanced NK cell products for clinical applications, *e.g.*, for immunooncology therapeutic approaches. In some embodiments, the modified NK cells provided herein can serve as an off-the-shelf clinical solution for patients having, or having been diagnosed with, a hyperproliferative disease, such as, for example, a cancer. In some embodiments, the modified NK cells exhibit an enhanced survival, proliferation, NK cell response level, NK cell response duration, resistance against NK cell exhaustion, and/or target recognition as compared to non-modified NK cells. For example, the modified NK cells provided herein may comprise genomic edits that result in: a loss-of-function in TGF beta receptor 2 (TGFbetaR2) and/or a loss-of-function of CISH in the modified NK cell.

[261] The modified NK cells may exhibit one or more edits in their genome that results in a loss-of-function in a target gene, and/or one or more modifications that results in a gain-of-function, or an

overexpression, of a gene product, *e.g.*, of a protein, from an exogenous nucleic acid construct, *e.g.*, from an expression construct comprising a cDNA encoding for the gene product that is integrated into the genome of the modified NK cell or provided in an extrachromosomal manner, *e.g.*, in the form of an episomal expression construct.

[262] A loss-of-function of a target gene is characterized by a decrease in the expression of a target gene based on a genomic modification, *e.g.*, an RNA-guided nuclease-mediated cut in the target gene that results in an inactivation, or in diminished expression or function, of the encoded gene product.

[263] A gain-of-function of a gene product is characterized by an increased expression (also referred to herein as overexpression) of a gene product, *e.g.*, of a protein, in a cell, which can include, for example, an increased expression level of the gene product, or expression of the gene product in a cell that does not express the gene product endogenously, *e.g.*, from an endogenous gene.

[264] In some embodiments, increased expression of a gene product is effected by introducing an exogenous nucleic acid construct that encodes the gene product into a cell, *e.g.*, an exogenous nucleic acid construct that comprises a cDNA encoding the gene product under the control of a heterologous promoter. In some embodiments, the exogenous nucleic acid construct is integrated into a specific locus, *e.g.*, via HDR-mediated gene editing, as described in more detail elsewhere herein. Methods for effecting loss-of-function edits as well as methods for effecting increased expression of gene products, *e.g.*, via RNA-guided nuclease technology are well known to one of ordinary skill in the art.

[265] The present disclosure embraces modified NK cells exhibiting any of the edits and/or increased expression of gene products listed in TABLE 4 and TABLE 5 combined, as well as any combination of such edits and/or increased expression of gene products listed in these tables.

[266] It is to be understood that the exemplary embodiments provided herein are meant to illustrate some examples of NK cells embraced by the present disclosure. Additional configurations are embraced that are not described here in detail for the sake of brevity, but such embodiments will be immediately apparent to those of skill in the art based on the present disclosure.

Knock-Ins and Knock-Outs

[267] In some embodiments, a modified cell may express one or more of a loss of function in TGFbetaR2 and/or a loss of function in CISH.

[268] As used herein, the term “express” or “expression” refers to the process to produce a polypeptide, including transcription and translation. Expression may be, *e.g.*, increased by a number of approaches, including: increasing the number of genes encoding the polypeptide, increasing the transcription of the gene (such as by placing the gene under the control of a constitutive promoter), increasing the translation of the gene, knocking out of a competitive gene, or a combination of these and/or other approaches.

[269] As used herein, “knock-in” refers to the addition of a target gene into a genetic locus of a cell.

[270] As used herein, the term “knock-out” refers to an inactivating mutation in a target gene, wherein the product of the target gene comprises a loss of function.

[271] As used herein, the term “loss of function” refers to an inactivating mutation in a target gene, wherein the gene product has less, or no, function (being partially or wholly inactivated). As used herein the term “complete loss of function” refers to an inactivating mutation in a target gene, wherein the gene product has no function (wholly inactivated).

[272] As used herein, the term “TGFβRII” or “TGFbetaR2” refers to a transmembrane protein that has a protein kinase domain, forms a heterodimeric complex with TGF-beta receptor type-1, and binds TGF-beta. This receptor/ligand complex phosphorylates proteins, which then enter the nucleus and regulate the transcription of genes related to cell proliferation, cell cycle arrest, wound healing, immunosuppression, and tumorigenesis. Exemplary sequences of TGFβRII are set forth in KR710923.1, NM_001024847.2, and NM_003242.5.

[273] As used herein, the term “CISH” refers to the Cytokine Inducible SH2 Containing Protein, for *e.g.*, see Delconte *et al.*, Nat Immunol. 2016 Jul;17(7):816-24; incorporated in its entirety herein by reference. Exemplary sequences for CISH are set forth as NG_023194.1.

[274] As used herein, the term “IL-15/IL15RA” or “Interleukin-15” (IL-15) refers to a cytokine with structural similarity to Interleukin-2 (IL-2). Like IL-2, IL-15 binds to and signals through a complex composed of IL-2/IL-15 receptor beta chain (CD122) and the common gamma chain (gamma-C, CD132). IL-15 is secreted by mononuclear phagocytes (and some other cells) following infection by virus(es). This cytokine induces cell proliferation of natural killer cells; cells of the innate immune system whose principal role is to kill virally infected cells. IL-15 Receptor alpha (IL15RA) specifically binds IL15 with very high affinity, and is capable of binding IL-15 independently of other subunits. It is suggested that this property allows IL-15 to be produced by one cell, endocytosed by another cell, and then presented to a third party cell. IL15RA is reported to enhance cell proliferation and expression of apoptosis inhibitor BCL2L1/BCL2-XL and BCL2. Exemplary sequences of IL-15 are provided in NG_029605.2, and exemplary sequences of IL-15RA are provided in NM_002189.4.

[275] IL-15 is a key cytokine in promoting NK cell growth and homeostatic maintenance of memory T cells. IL-15 and its receptor chain, IL-15Ra, are essential for NK survival and do not stimulate regulatory T cells. IL-15/IL-15Ra binds to the beta and gamma subunits of IL-2 receptor and thereby activates JAK1/3 and STAT5. In some embodiments, the modified cell of the disclosure (for *e.g.*, an NK cell) expresses an exogenous IL-15/IL-15Ra. In some embodiments, the exogenous IL-15/IL-15Ra is expressed as a membrane-bound IL15.IL15Ra complex, as described in Imamura *et al.*, Blood. 2014 Aug 14;124(7):1081-8 and Hurton LV *et al.*, PNAS, 2016; incorporated in their entirety herein by reference. In some embodiments, the exogenous IL-15/IL-15Ra is expressed as a soluble IL15Ra.IL15 complex, as described in Mortier E *et al.*, JBC 2006; Bessard A, Mol Cancer Ther 2009; and Desbois M, JI 2016; incorporated in their entirety herein by reference. In some embodiments, the modified cell of the disclosure (for *e.g.*, an NK cell) expresses a membrane-bound IL15.IL15Ra complex and a soluble IL15Ra.IL15 complex. In some embodiments, the modified cell of the disclosure (for *e.g.*, an NK cell) express a membrane-bound form of IL15.IL15Ra complex with

a cleavable linker. A knockout of CISH is associated with further promoting the IL-15 signaling, as described in Delconte P, Nat Immunol 2016; incorporated in its entirety herein by reference. In some embodiments, the modified cell of the disclosure (for *e.g.*, an NK cell) expresses a loss of function in CISH. In some embodiments, the modified cell of the disclosure (for *e.g.*, an NK cell) express exogenous IL-15/IL-15Ra and a loss of function in CISH.

[276] The disclosure specifically encompasses variants of the above genes, including variants having at least 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% percent identity to the above-identified gene sequences. As used herein, the term “percent (%) sequence identity” or “percent (%) identity,” also including “homology,” is defined as the percentage of amino acid residues or nucleotides in a candidate sequence that are identical with the amino acid residues or nucleotides in the reference sequences after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Optimal alignment of the sequences for comparison may be produced, besides manually, by means of the local homology algorithm of Smith and Waterman, 1981, *Adv. App. Math.* 2, 482, by means of the local homology algorithm of Needleman and Wunsch, 1970, *J. Mol. Biol.* 48, 443, by means of the similarity search method of Pearson and Lipman, 1988, *Proc. Natl. Acad. Sci. USA* 85, 2444, or by means of computer programs which use these algorithms (GAP, BESTFIT, FASTA, BLAST P, BLAST N and TFASTA in Wisconsin Genetics Software Package, Genetics Computer Group, 575 Science Drive, Madison, Wis.).

[277] Knock-ins and knock-outs can be effected by genome editing technologies known to those of skill in the art and include CRISPR/Cas technologies. Single-cut as well as multiplex editing strategies are suitable to achieve the desired product configurations provided herein, and such strategies are described herein or otherwise known to those of ordinary skill in the art.

[278] In some embodiments, exemplary modified cells, *e.g.*, modified pluripotent cells or differentiated progeny thereof, *e.g.*, iNK cells or other modified lymphocyte types, are evaluated for their ability to escape the immune system of a non-autologous host, *e.g.*, a patient in need of immunotherapy. In some embodiments, such an evaluation includes an *in vitro* assay. Suitable *in vitro* assays for such evaluations are known to those of ordinary skill in the relevant art, and include, without limitation, mixed lymphocyte reactivity (MLR) assays. This assay and other suitable assays are described, *e.g.*, in Abbas et al., *Cellular and Molecular Immunology*, 7th edition, ISBN 9781437735734, the entire contents of which are incorporated herein by reference. Other suitable assays will be apparent to the skilled artisan in view of the present disclosure.

Methods of Use

[279] A variety of diseases may be ameliorated by introducing the modified cells of the invention to a subject. Examples of diseases are, including but not limited to, cancer, including but not limited to solid tumors, including but not limited to, tumor of the brain, prostate, breast, lung, colon, uterus, skin, liver, bone, pancreas, ovary, testes, bladder, kidney, head, neck, stomach, cervix, rectum, larynx, or

esophagus; and hematological malignancies, including but not limited to, acute and chronic leukemias, lymphomas, multiple myeloma and myelodysplastic syndromes.

[280] Particular embodiments of the present invention are directed to methods of treating a subject in need thereof by administering to the subject a composition comprising any of the cells described herein. In particular embodiments, the terms “treating,” “treatment,” and the like are used herein to generally mean obtaining a desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease and/or may be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the disease. “Treatment” as used herein covers any treatment of a disease in a mammal, and includes: preventing the disease from occurring in a subject which may be predisposed to the disease but has not yet been diagnosed as having it; inhibiting the disease, *i.e.*, arresting its development; or relieving the disease, *i.e.*, causing regression of the disease. The therapeutic agent or composition may be administered before, during or after the onset of disease or injury. The treatment of ongoing disease, where the treatment stabilizes or reduces the undesirable clinical symptoms of the patient, is of particular interest.

[281] In particular embodiments, the subject has a disease, condition, and/or an injury that can be treated, ameliorated, and/or improved by a cell therapy. Some embodiments contemplate that a subject in need of cell therapy is a subject with an injury, disease, or condition, whereby a cell therapy, *e.g.*, a therapy in which a cellular material is administered to the subject, can treat, ameliorate, improve, and/or reduce the severity of at least one symptom associated with the injury, disease, or condition. Certain embodiments contemplate that a subject in need of cell therapy, includes, but is not limited to, a candidate for bone marrow or stem cell transplantation, a subject who has received chemotherapy or irradiation therapy, a subject who has or is at risk of having a hyperproliferative disorder or a cancer, *e.g.* a hyperproliferative disorder or a cancer of hematopoietic system, a subject having or at risk of developing a tumor, *e.g.*, a solid tumor, a subject who has or is at risk of having a viral infection or a disease associated with a viral infection.

[282] According, the embodiments described herein further provide pharmaceutical compositions comprising the cells made by the methods and composition disclosed herein, wherein the pharmaceutical compositions further comprise a pharmaceutically acceptable medium. In some embodiments, the pharmaceutical composition comprises the NK cells made by the methods and composition disclosed herein.

[283] Additionally, the embodiments described herein provide therapeutic use of the above pharmaceutical compositions by introducing the composition to a subject suitable for adoptive cell therapy, wherein the subject has a solid tumor; a hematological malignancy; an autoimmune disorder; or an infection associated with viral, bacterial, fungal and/or helminth infections, including but not limited to, HIV, RSV, EBV, CMV, adenovirus, or BK polyomavirus infections.

[284] Particular embodiments described herein are also directed to methods of treating a subject in need thereof by administering to the subject a composition comprising any of the cells described herein with one or more antibodies, or fragments thereof, to induce and/or increase an antibody-dependent cellular cytotoxicity (ADCC) effect in the subject. In some embodiments, the modified NK cells described herein exhibit greater ADCC activity when administered with one or more antibodies, or fragments thereof, to a subject in need thereof, *e.g.*, a subject with a cancer, relative to unmodified NK cells that are administered with the same one or more antibodies, or fragments thereof, to a subject in need thereof. In some embodiments, the modified NK cells described herein kill a greater number of cancer cells when administered with one or more antibodies, or fragments thereof, to a subject in need thereof, *e.g.*, a subject with cancer, relative to unmodified NK cells that are administered with the same one or more antibodies, or fragments thereof, to a subject.

Cancers

[285] Cancers that are suitable therapeutic targets of the present disclosure include cancer cells from the bladder, blood, bone, bone marrow, brain, breast, colon, esophagus, eye, gastrointestinal, gum, head, kidney, liver, lung, nasopharynx, neck, ovary, prostate, skin, stomach, testis, tongue, or uterus. In addition, the cancer may specifically be of the following histological type, though it is not limited to these: neoplasm, malignant; carcinoma; carcinoma, undifferentiated; giant and spindle cell carcinoma; small cell carcinoma; papillary carcinoma; squamous cell carcinoma; lymphoepithelial carcinoma; basal cell carcinoma; pilomatrix carcinoma; transitional cell carcinoma; papillary transitional cell carcinoma; adenocarcinoma; gastrinoma, malignant; cholangiocarcinoma; hepatocellular carcinoma; combined hepatocellular carcinoma and cholangiocarcinoma; trabecular adenocarcinoma; adenoid cystic carcinoma; adenocarcinoma in adenomatous polyp; adenocarcinoma, familial polyposis coli; solid carcinoma; carcinoid tumor, malignant; branchiolo-alveolar adenocarcinoma; papillary adenocarcinoma; chromophobe carcinoma; acidophil carcinoma; oxyphilic adenocarcinoma; basophil carcinoma; clear cell adenocarcinoma; granular cell carcinoma; follicular adenocarcinoma; papillary and follicular adenocarcinoma; nonencapsulating sclerosing carcinoma; adrenal cortical carcinoma; endometroid carcinoma; skin appendage carcinoma; apocrine adenocarcinoma; sebaceous adenocarcinoma; ceruminous adenocarcinoma; mucoepidermoid carcinoma; cystadenocarcinoma; papillary cystadenocarcinoma; papillary serous cystadenocarcinoma; mucinous cystadenocarcinoma; mucinous adenocarcinoma; signet ring cell carcinoma; infiltrating duct carcinoma; medullary carcinoma; lobular carcinoma; inflammatory carcinoma; paget's disease, mammary; acinar cell carcinoma; adenosquamous carcinoma; adenocarcinoma w/squamous metaplasia; thymoma, malignant; ovarian stromal tumor, malignant; thecoma, malignant; granulosa cell tumor, malignant; androblastoma, malignant; sertoli cell carcinoma; leydig cell tumor, malignant; lipid cell tumor, malignant; paraganglioma, malignant; extra-mammary paraganglioma, malignant; pheochromocytoma; glomangiosarcoma; malignant melanoma; amelanotic melanoma; superficial spreading melanoma; malig melanoma in giant pigmented nevus; epithelioid cell melanoma; blue

nevus, malignant; sarcoma; fibrosarcoma; fibrous histiocytoma, malignant; myxosarcoma; liposarcoma; leiomyosarcoma; rhabdomyosarcoma; embryonal rhabdomyosarcoma; alveolar rhabdomyosarcoma; stromal sarcoma; mixed tumor, malignant; mullerian mixed tumor; nephroblastoma; hepatoblastoma; carcinosarcoma; mesenchymoma, malignant; brenner tumor, malignant; phyllodes tumor, malignant; synovial sarcoma; mesothelioma, malignant; dysgerminoma; embryonal carcinoma; teratoma, malignant; struma ovarii, malignant; choriocarcinoma; mesonephroma, malignant; hemangiosarcoma; hemangioendothelioma, malignant; kaposi's sarcoma; hemangiopericytoma, malignant; lymphangiosarcoma; osteosarcoma; juxtacortical osteosarcoma; chondrosarcoma; chondroblastoma, malignant; mesenchymal chondrosarcoma; giant cell tumor of bone; ewing's sarcoma; odontogenic tumor, malignant; ameloblastic odontosarcoma; ameloblastoma, malignant; ameloblastic fibrosarcoma; pinealoma, malignant; chordoma; glioma, malignant; ependymoma; astrocytoma; protoplasmic astrocytoma; fibrillary astrocytoma; astroblastoma; glioblastoma; oligodendroglioma; oligodendroblastoma; primitive neuroectodermal; cerebellar sarcoma; ganglioneuroblastoma; neuroblastoma; retinoblastoma; olfactory neurogenic tumor; meningioma, malignant; neurofibrosarcoma; neurilemmoma, malignant; granular cell tumor, malignant; malignant lymphoma; Hodgkin's disease; Hodgkin's lymphoma; paragranuloma; malignant lymphoma, small lymphocytic; malignant lymphoma, large cell, diffuse; malignant lymphoma, follicular; mycosis fungoides; other specified non-Hodgkin's lymphomas; malignant histiocytosis; multiple myeloma; mast cell sarcoma; immunoproliferative small intestinal disease; leukemia; lymphoid leukemia; plasma cell leukemia; erythroleukemia; lymphosarcoma cell leukemia; myeloid leukemia; basophilic leukemia; eosinophilic leukemia; monocytic leukemia; mast cell leukemia; megakaryoblastic leukemia; myeloid sarcoma; and hairy cell leukemia.

[286] In some embodiments, the cancer is head and neck cancer.

[287] In some embodiments, the cancer is a breast cancer. In another embodiment, the cancer is colon cancer. In another embodiment, the cancer is gastric cancer. In another embodiment, the cancer is RCC. In another embodiment, the cancer is non-small cell lung cancer (NSCLC).

[288] In some embodiments, solid cancer indications that can be treated with the modified NK cells provided herein, either alone or in combination with one or more additional cancer treatment modality, include: bladder cancer, hepatocellular carcinoma, prostate cancer, ovarian/uterine cancer, pancreatic cancer, mesothelioma, melanoma, glioblastoma, HPV-associated and/or HPV-positive cancers such as cervical and HPV+ head and neck cancer, oral cavity cancer, cancer of the pharynx, thyroid cancer, gallbladder cancer, and soft tissue sarcomas.

[289] In some embodiments, hematological cancer indications that can be treated with the modified NK cells provided herein, either alone or in combination with one or more additional cancer treatment modality, include: ALL, CLL, NHL, DLBCL, AML, CML, multiple myeloma (MM).

[290] As used herein, the term “cancer” (also used interchangeably with the terms, “hyperproliferative” and “neoplastic”) refers to cells having the capacity for autonomous growth, *i.e.*,

an abnormal state or condition characterized by rapidly proliferating cell growth. Cancerous disease states may be categorized as pathologic, *i.e.*, characterizing or constituting a disease state, *e.g.*, malignant tumor growth, or may be categorized as non-pathologic, *i.e.*, a deviation from normal but not associated with a disease state, *e.g.*, cell proliferation associated with wound repair. The term is meant to include all types of cancerous growths or oncogenic processes, metastatic tissues or malignantly transformed cells, tissues, or organs, irrespective of histopathologic type or stage of invasiveness. The term “cancer” includes malignancies of the various organ systems, such as those affecting lung, breast, thyroid, lymphoid, gastrointestinal, and genito-urinary tract, as well as adenocarcinomas which include malignancies such as most colon cancers, renal-cell carcinoma, prostate cancer and/or testicular tumors, non-small cell carcinoma of the lung, cancer of the small intestine and cancer of the esophagus. The term “carcinoma” is art recognized and refers to malignancies of epithelial or endocrine tissues including respiratory system carcinomas, gastrointestinal system carcinomas, genitourinary system carcinomas, testicular carcinomas, breast carcinomas, prostatic carcinomas, endocrine system carcinomas, and melanomas. Exemplary carcinomas include those forming from tissue of the cervix, lung, prostate, breast, head and neck, colon and ovary. The term “carcinoma” also includes carcinosarcomas, *e.g.*, which include malignant tumors composed of carcinomatous and sarcomatous tissues. An “adenocarcinoma” refers to a carcinoma derived from glandular tissue or in which the tumor cells form recognizable glandular structures. The term “sarcoma” is art recognized and refers to malignant tumors of mesenchymal derivation.

[291] Examples of cellular proliferative and/or differentiative disorders of the lung include, but are not limited to, tumors such as bronchogenic carcinoma, including paraneoplastic syndromes, bronchioloalveolar carcinoma, neuroendocrine tumors, such as bronchial carcinoid, miscellaneous tumors, metastatic tumors, and pleural tumors, including solitary fibrous tumors (pleural fibroma) and malignant mesothelioma.

[292] Examples of cellular proliferative and/or differentiative disorders of the breast include, but are not limited to, proliferative breast disease including, *e.g.*, epithelial hyperplasia, sclerosing adenosis, and small duct papillomas; tumors, *e.g.*, stromal tumors such as fibroadenoma, phyllodes tumor, and sarcomas, and epithelial tumors such as large duct papilloma; carcinoma of the breast including in situ (noninvasive) carcinoma that includes ductal carcinoma in situ (including Paget's disease) and lobular carcinoma in situ, and invasive (infiltrating) carcinoma including, but not limited to, invasive ductal carcinoma, invasive lobular carcinoma, medullary carcinoma, colloid (mucinous) carcinoma, tubular carcinoma, and invasive papillary carcinoma, and miscellaneous malignant neoplasms. Disorders in the male breast include, but are not limited to, gynecomastia and carcinoma.

[293] Examples of cellular proliferative and/or differentiative disorders involving the colon include, but are not limited to, tumors of the colon, such as non-neoplastic polyps, adenomas, familial syndromes, colorectal carcinogenesis, colorectal carcinoma, and carcinoid tumors.

[294] Examples of cancers or neoplastic conditions, in addition to the ones described above, include, but are not limited to, a fibrosarcoma, myosarcoma, liposarcoma, chondrosarcoma, osteogenic sarcoma, chordoma, angiosarcoma, endotheliosarcoma, lymphangiosarcoma, lymphangioendotheliosarcoma, synovioma, mesothelioma, Ewing's tumor, leiomyosarcoma, rhabdomyosarcoma, gastric cancer, esophageal cancer, rectal cancer, pancreatic cancer, ovarian cancer, prostate cancer, uterine cancer, cancer of the head and neck, skin cancer, brain cancer, squamous cell carcinoma, sebaceous gland carcinoma, papillary carcinoma, papillary adenocarcinoma, cystadenocarcinoma, medullary carcinoma, bronchogenic carcinoma, renal cell carcinoma, hepatoma, bile duct carcinoma, choriocarcinoma, seminoma, embryonal carcinoma, Wilm's tumor, cervical cancer, testicular cancer, small cell lung carcinoma, non-small cell lung carcinoma, bladder carcinoma, epithelial carcinoma, glioma, astrocytoma, medulloblastoma, craniopharyngioma, ependymoma, pinealoma, hemangioblastoma, acoustic neuroma, oligodendroglioma, meningioma, melanoma, neuroblastoma, retinoblastoma, leukemia, lymphoma, or Kaposi sarcoma.

[295] Contemplated useful secondary or adjunctive therapeutic agents in this context include, but are not limited to: chemotherapeutic agents include alkylating agents such as thiotepea and CYTOXAN® cyclophosphamide; alkyl sulfonates such as busulfan, improsulfan and piposulfan; aziridines such as benzodopa, carboquone, meturedopa, and uredopa; ethylenimines and methylamelamines including altretamine, triethylenemelamine, trietylenephosphoramidate, triethylenethiophosphoramidate and trimethylolomelamine; acetogenins (especially bullatacin and bullatacinone); delta-9-tetrahydrocannabinol (dronabinol, MARINOL®); beta-lapachone; lapachol; colchicines; betulinic acid; a camptothecin (including the synthetic analogue topotecan (HYCAMTIN®), CPT-11 (irinotecan, CAMPTOSAR®), acetylcamptothecin, scoplectin, and 9-aminocamptothecin); bryostatin; callystatin; CC-1065 (including its adozelesin, carzelesin and bizelesin synthetic analogues); podophyllotoxin; podophyllinic acid; teniposide; cryptophycins (particularly cryptophycin 1 and cryptophycin 8); dolastatin; duocarmycin (including the synthetic analogues, KW-2189 and CB1-TM1); eleutherobin; pancratistatin; a sarcodictyin; spongistatin; nitrogen mustards such as chlorambucil, chlornaphazine, cholophosphamide, estramustine, ifosfamide, mechlorethamine, mechlorethamine oxide hydrochloride, melphalan, novembichin, phenesterine, prednimustine, trofosfamide, uracil mustard; nitrosureas such as carmustine, chlorozotocin, fotemustine, lomustine, nimustine, and ranimustine; antibiotics such as the enediyne antibiotics (*e.g.*, calicheamicin, especially calicheamicin gammaII and calicheamicin omegall (see, *e.g.*, Agnew, Chem. Intl. Ed. Engl., 33: 183-186 (1994)); dynemicin, including dynemicin A; an esperamicin; as well as neocarzinostatin chromophore and related chromoprotein enediyne antiobiotic chromophores), aclacinomysins, actinomycin, authramycin, azaserine, bleomycins, cactinomycin, carabycin, caminomycin, carzinophilin, chromomycinis, dactinomycin, daunorubicin, detorubicin, 6-diazo-5-oxo-L-norleucine, doxorubicin (including ADRIAMYCIN®, morpholino-doxorubicin, cyanomorpholino-doxorubicin, 2-pyrrolino-doxorubicin, doxorubicin HCl liposome injection

(DOXIL®) and deoxydoxorubicin), epirubicin, esorubicin, idarubicin, marcellomycin, mitomycins such as mitomycin C, mycophenolic acid, nogalamycin, olivomycins, peplomycin, potfiromycin, puromycin, quelamycin, rodorubicin, streptonigrin, streptozocin, tubercidin, ubenimex, zinostatin, zorubicin; anti-metabolites such as methotrexate, gemcitabine (GEMZAR®), tegafur (UFTORAL®), capecitabine (XELODA®), an epothilone, and 5-fluorouracil (5-FU); folic acid analogues such as denopterin, methotrexate, pteropterin, trimetrexate; purine analogs such as fludarabine, 6-mercaptopurine, thiamiprine, thioguanine; pyrimidine analogs such as ancitabine, azacitidine, 6-azauridine, carmofur, cytarabine, dideoxyuridine, doxifluridine, enocitabine, floxuridine; androgens such as calusterone, dromostanolone propionate, epitio stanol, mepitio stanane, testolactone; anti-adrenals such as aminogluthethimide, mitotane, trilostane; folic acid replenisher such as frolinic acid; aceglatone; aldophosphamide glycoside; aminolevulinic acid; eniluracil; amsacrine; bestrabucil; bisantrene; edatraxate; defofamine; demecolcine; diaziquone; elformithine; elliptinium acetate; etoglucid; gallium nitrate; hydroxyurea; lentinan; lonidainine; maytansinoids such as maytansine and ansamitocins; mitoguazone; mitoxantrone; mopidanmol; nitraerine; pentostatin; phenamet; pirarubicin; losoxantrone; 2-ethylhydrazide; procarbazine; PSK® polysaccharide complex (JHS Natural Products, Eugene, Oreg.); razoxane; rhizoxin; sizofuran; spirogermanium; tenuazonic acid; triaziquone; 2,2',2''-trichlorotriethylamine; trichothecenes (especially T-2 toxin, verracurin A, roridin A and anguidine); urethan; vindesine (ELDISINE®, FILDESIN®); dacarbazine; mannomustine; mitobronitol; mitolactol; pipobroman; gacytosine; arabinoside ("Ara-C"); thiotepa; taxoids, *e.g.*, paclitaxel (TAXOL®), albumin-engineered nanoparticle formulation of paclitaxel (ABRAXANET™), and doxetaxel (TAXOTERE®); chloranbucil; 6-thioguanine; mercaptopurine; methotrexate; platinum analogs such as cisplatin and carboplatin; vinblastine (VELBAN®); platinum; etoposide (VP-16); ifosfamide; mitoxantrone; vincristine (ONCOVIN®); oxaliplatin; leucovovin; vinorelbine (NAVELBINE®); novantrone; edatrexate; daunomycin; aminopterin; cyclosporine, sirolimus, rapamycin, rapalogs, ibandronate; topoisomerase inhibitor RFS 2000; difluoromethylornithine (DMFO); retinoids such as retinoic acid; CHOP, an abbreviation for a combined therapy of cyclophosphamide, doxorubicin, vincristine, and prednisolone, and FOLFOX, an abbreviation for a treatment regimen with oxaliplatin (ELOXATIN™) combined with 5-FU, leucovovin; anti-estrogens and selective estrogen receptor modulators (SERMs), including, for example, tamoxifen (including NOLVADEX® tamoxifen), raloxifene (EVISTA®), droloxifene, 4-hydroxytamoxifen, trioxifene, keoxifene, LY117018, onapristone, and toremifene (FARESTON®); anti-progesterones; estrogen receptor down-regulators (ERDs); estrogen receptor antagonists such as fulvestrant (FASLODEX®); agents that function to suppress or shut down the ovaries, for example, leutinizing hormone-releasing hormone (LHRH) agonists such as leuprolide acetate (LUPRON® and ELIGARD®), goserelin acetate, buserelin acetate and triptorelin; other anti-androgens such as flutamide, nilutamide and bicalutamide; and aromatase inhibitors that inhibit the enzyme aromatase, which regulates estrogen production in the adrenal glands, such as, for example, 4(5)-imidazoles, aminogluthethimide, megestrol

acetate (MEGASE®), exemestane (AROMASIN®), formestanie, fadrozole, vorozole (RIVISOR®), letrozole (FEMARA®), and anastrozole (ARIMIDEX®); bisphosphonates such as clodronate (for example, BONEFOS® or OSTAC®), etidronate (DIDROCAL®), NE-58095, zoledronic acid/zoledronate (ZOMETA®), alendronate (FOSAMAX®), pamidronate (AREDIA®), tiludronate (SKELID®), or risedronate (ACTONEL®); troxacitabine (a 1,3-dioxolane nucleoside cytosine analog); aptamers, described for example in U.S. Pat. No. 6,344,321, which is herein incorporated by reference in its entirety; anti HGF monoclonal antibodies (*e.g.*, AV299 from Aveo, AMG102, from Amgen); truncated mTOR variants (*e.g.*, CGEN241 from Compugen); protein kinase inhibitors that block mTOR induced pathways (*e.g.*, ARQ197 from Arqule, XL880 from Exelexis, SGX523 from SGX Pharmaceuticals, MP470 from Supergen, PF2341066 from Pfizer); vaccines such as THERATOPE® vaccine and gene therapy vaccines, for example, ALLOVECTIN® vaccine, LEUVECTIN® vaccine, and VAXID® vaccine; topoisomerase 1 inhibitor (*e.g.*, LURTOTECAN®); rmRH (*e.g.*, ABARELIX®); lapatinib ditosylate (an ErbB-2 and EGFR dual tyrosine kinase small-molecule inhibitor also known as GW572016); COX-2 inhibitors such as celecoxib (CELEBREX®; 4-(5-(4-methylphenyl)-3-(trifluoromethyl)-1H-pyrazol-1-yl) benzenesulfonamide; and pharmaceutically acceptable salts, acids or derivatives of any of the above.

[296] Other compounds that are effective in treating cancer are known in the art and described herein that are suitable for use with the compositions and methods of the present disclosure are described, for example, in the “Physicians Desk Reference, 62nd edition. Oradell, N.J.: Medical Economics Co., 2008 “, Goodman & Gilman's “The Pharmacological Basis of Therapeutics, Eleventh Edition. McGraw-Hill, 2005”, “Remington: The Science and Practice of Pharmacy, 20th Edition. Baltimore, Md.: Lippincott Williams & Wilkins, 2000.”, and “The Merck Index, Fourteenth Edition. Whitehouse Station, N.J.: Merck Research Laboratories, 2006”, incorporated herein by reference in relevant parts.

Antibody-Dependent Cellular Cytotoxicity (ADCC)

[297] The present disclosure provides modified NK cells (or other lymphocytes) that are useful in NK cell therapy, *e.g.*, in the context of immunotherapeutic approaches, particularly in combination with an antibody, or antigen-binding portion thereof, to generate striking antibody-dependent cellular cytotoxicity (ADCC) effects, thereby surprisingly increasing the effectiveness of the modified NK cells in killing target cells, *e.g.* cancer cells. ADCC is a mechanism of cell-mediated immune defense, where an immune effector cell actively lyses a target cell after its membrane-surface antigens have been bound by specific antibodies. To participate in ADCC, the immune effector cells must express Fc-gamma receptors (FcγR) to be able to recognize the Fc region of the antibodies that bind to the target cells. Most immune effector cells have both activating and inhibitory FcγR. An advantage of using NK cells to target cancer cells via ADCC is that, unlike other effector cells, NK cells only have activating FcγRs (*e.g.*, FcγR IIIa, also known as CD16a, and FcγR IIc, also known as CD32c) and are believed to be the most important effectors of ADCC in humans. Thus, the use of the modified NK

cells disclosed herein and antibodies targeting cancer cell-specific antigens to elicit ADCC provides novel and surprisingly effective immunotherapies.

[298] In one embodiment, the molecule comprising an Fc domain that binds cancer cells, *e.g.*, antibody, or antigen-binding portion thereof, binds an antigen on a cancer cell, or a “cancer antigen.” In one embodiment, the antigen on the cancer cell is epidermal growth factor receptor (EGFR), HER2, CD20, PD-L1, PD-1 (PEMBRO and NIVO), CTLA-4 (IPI), CD73, TIGIT, GD2, VEGF-A, VEGFR-2, PDGFR-2, PDGFR α , RANKL, CD19, CD3. In one embodiment, the antibody is cetuximab, trastuzumab, rituximab, pertuzumab, panitumumab, necitumumab, dinutuximab, bevacizumab, ramucirumab, olaratumab, ipilimumab, nivolumab, blinatumomab, alemtuzumab, bevacizumab, brentuximab, cetuximab, gemtuzumab, ipilimumab, ofatumumab, panitumumab, rituximab, tositumomab, inotuzumab, glembatumumab, lovortuzumab or trastuzumab, or an antigen-binding portion thereof. Additional antibodies include adecatumumab, afutuzumab, bavituximab, belimumab, bivatuzumab, cantuzumab, citatuzumab, cixutumumab, conatumumab, dacetuzumab, elotuzumab, etaracizumab, farletuzumab, figitumumab, iratumumab, labetuzumab, lexatumumab, lintuzumab, lucatumumab, mapatumumab, matuzumab, milatuzumab, necitumumab, nimotuzumab, olaratumab, oportuzumab, pertuzumab, pritumumab, ranibizumab, robatumumab, sibrotuzumab, siltuximab, tacatuzumab, tigatuzumab, tucotuzumab, veltuzumab votumumab, and zalutumumab, or an antigen-binding portion thereof.

In one embodiment, the antibody is cetuximab, or an antigen-binding portion thereof. In one embodiment, the antibody is trastuzumab, or an antigen-binding portion thereof. In one embodiment, the antibody is rituximab, or an antigen-binding portion thereof. In one embodiment, the antibody is pertuzumab, or an antigen-binding portion thereof. In one embodiment, the antibody is panitumumab, or an antigen-binding portion thereof. In one embodiment, the antibody is necitumumab, or an antigen-binding portion thereof. In one embodiment, the antibody is dinutuximab, or an antigen-binding portion thereof. In one embodiment, the antibody is bevacizumab, or an antigen-binding portion thereof. In one embodiment, the antibody is ramucirumab, or an antigen-binding portion thereof. In one embodiment, the antibody is olaratumab, or an antigen-binding portion thereof. In one embodiment, the antibody is ipilimumab, or an antigen-binding portion thereof. In one embodiment, the antibody is nivolumab, or an antigen-binding portion thereof. In one embodiment, the antibody is blinatumomab, or an antigen-binding portion thereof. In one embodiment, the antibody is alemtuzumab, or an antigen-binding portion thereof. In one embodiment, the antibody is bevacizumab, or an antigen-binding portion thereof. In one embodiment, the antibody is brentuximab, or an antigen-binding portion thereof. In one embodiment, the antibody is gemtuzumab, or an antigen-binding portion thereof. In one embodiment, the antibody is ipilimumab, or an antigen-binding portion thereof. In one embodiment, the antibody is ofatumumab,

or an antigen-binding portion thereof. In one embodiment, the antibody is panitumumab, or an antigen-binding portion thereof. In one embodiment, the antibody is tositumomab, or an antigen-binding portion thereof. In one embodiment, the antibody is inotuzumab, or an antigen-binding portion thereof. In one embodiment, the antibody is glembatumumab, or an antigen-binding portion thereof. In one embodiment, the antibody is lovortuzumab, or an antigen-binding portion thereof. In one embodiment, the antibody is adecatumumab, or an antigen-binding portion thereof. In one embodiment, the antibody is afutuzumab, or an antigen-binding portion thereof. In one embodiment, the antibody is bavixumab, or an antigen-binding portion thereof. In one embodiment, the antibody is bivatuzumab, or an antigen-binding portion thereof. In one embodiment, the antibody is cantuzumab, or an antigen-binding portion thereof. In one embodiment, the antibody is citatuzumab, or an antigen-binding portion thereof. In one embodiment, the antibody is cixutumumab, or an antigen-binding portion thereof. In one embodiment, the antibody is conatumumab, or an antigen-binding portion thereof. In one embodiment, the antibody is dacetuzumab, or an antigen-binding portion thereof. In one embodiment, the antibody is elotuzumab, or an antigen-binding portion thereof. In one embodiment, the antibody is etaracizumab, or an antigen-binding portion thereof. In one embodiment, the antibody is farletuzumab, or an antigen-binding portion thereof. In one embodiment, the antibody is figitumumab, or an antigen-binding portion thereof. In one embodiment, the antibody is iratumumab, or an antigen-binding portion thereof. In one embodiment, the antibody is labetuzumab, or an antigen-binding portion thereof. In one embodiment, the antibody is lexatumumab, or an antigen-binding portion thereof. In one embodiment, the antibody is lintuzumab, or an antigen-binding portion thereof. In one embodiment, the antibody is lucatumumab, or an antigen-binding portion thereof. In one embodiment, the antibody is mapatumumab, or an antigen-binding portion thereof. In one embodiment, the antibody is matuzumab, or an antigen-binding portion thereof. In one embodiment, the antibody is milatuzumab, or an antigen-binding portion thereof. In one embodiment, the antibody is necitumumab, or an antigen-binding portion thereof. In one embodiment, the antibody is nimotuzumab, or an antigen-binding portion thereof. In one embodiment, the antibody is olaratumab, or an antigen-binding portion thereof. In one embodiment, the antibody is oportuzumab, or an antigen-binding portion thereof. In one embodiment, the antibody is pertuzumab, or an antigen-binding portion thereof. In one embodiment, the antibody is primumab, or an antigen-binding portion thereof. In one embodiment, the antibody is ranibizumab, or an antigen-binding portion thereof. In one embodiment, the antibody is robatumumab, or an antigen-binding portion thereof.

binding portion thereof. In one embodiment, the antibody is sibrotuzumab, or an antigen-binding portion thereof. In one embodiment, the antibody is siltuximab, or an antigen-binding portion thereof. In one embodiment, the antibody is tacatuzumab, or an antigen-binding portion thereof. In one embodiment, the antibody is tigatuzumab, or an antigen-binding portion thereof. In one embodiment, the antibody is tucotuzumab, or an antigen-binding portion thereof. In one embodiment, the antibody is veltuzumab, or an antigen-binding portion thereof. In one embodiment, the antibody is votumumab, or an antigen-binding portion thereof. In one embodiment, the antibody is zalutumumab, or an antigen-binding portion thereof.

[299] All publications, patents and patent applications cited herein, whether supra or infra, are hereby incorporated by reference in their entirety.

[300] Throughout this specification, unless the context requires otherwise, the words “comprise”, “comprises” and “comprising” will be understood to imply the inclusion of a stated step or element or group of steps or elements but not the exclusion of any other step or element or group of steps or elements. By “consisting of is meant including, and limited to, whatever follows the phrase “consisting of:” Thus, the phrase “consisting of indicates that the listed elements are required or mandatory, and that no other elements may be present. By “consisting essentially of is meant including any elements listed after the phrase, and limited to other elements that do not interfere with or contribute to the activity or action specified in the disclosure for the listed elements. Thus, the phrase “consisting essentially of indicates that the listed elements are required or mandatory, but that no other elements are optional and may or may not be present depending upon whether or not they affect the activity or action of the listed elements.

[301] The various embodiments described above can be combined to provide further embodiments. All of the U.S. patents, U.S. patent application publications, U.S. patent applications, foreign patents, foreign patent applications and non-patent publications referred to in this specification and/or listed in the Application Data Sheet are incorporated herein by reference, in their entirety. The contents of database entries, *e.g.*, NCBI nucleotide or protein database entries provided herein, are incorporated herein in their entirety. Where database entries are subject to change over time, the contents as of the filing date of the present application are incorporated herein by reference. Aspects of the embodiments can be modified, if necessary to employ concepts of the various patents, applications and publications to provide yet further embodiments.

[302] These and other changes can be made to the embodiments in light of the above-detailed description. In general, in the following claims, the terms used should not be construed to limit the claims to the specific embodiments disclosed in the specification and the claims, but should be construed to include all possible embodiments along with the full scope of equivalents to which such claims are entitled. Accordingly, the claims are not limited by the disclosure.

EXAMPLES

[303] The following Examples are merely illustrative and are not intended to limit the scope or content of the disclosure in any way.

Example 1: CRISPR-EngCas12a demonstrated efficient editing of CISH and TGFBR2 in NK cells, and edited NK cells exhibited improved effector functions

[304] Natural killer (NK) cells distinguish tumor from healthy tissue via multiple mechanisms, including recognition of stress ligands and loss of MHC class I expression. However, effector function of allogeneic NK cells can be diminished by the lack of functional persistence, as well as tumor-intrinsic immunosuppressive mechanisms, such as production of TGF- β . Described herein is a next-generation allogeneic NK cell therapy using CRISPR-Cas12a gene editing to enhance NK cell function through knockout of the CISH and TGFBR2 genes. Knockout of CISH, a negative regulator of IL-2/IL-15 signaling, improves NK cell effector function, while knockout of the TGF- β receptor gene, TGFBR2, renders NK cells resistant to TGF- β mediated suppression.

[305] Specifically, NK cells derived from healthy human donor NK cells were edited using engineered Cas12a ("EngCas12a"; Cpf1 variant 4 amino acid sequence (SEQ ID NO:1146)). CD3-depleted peripheral blood mononuclear cells were thawed into IL-15-containing NK MACS media and cultured for 14 days in GREX plates. CRISPR-EngCas12a gene editing was performed by ribonucleoprotein electroporation and cells were cultured for an additional 72 hours prior to analysis or functional assays.

[306] The following guide RNA sequences were used for editing of CISH and TGFBR2.

Table 6: gRNA sequences

gRNA (Target)	5' DNA Extension Sequence	Cas12A-binding Sequence (RNA)	Targeting Domain Sequence (RNA)
CISH8401	ATGTGTTTTGTCAAAA GACCTTTT (SEQ ID NO:5)	UAAUUUCUACUCUUGUAGAU (SEQ ID NO:24)	ACUGACAGCGUGAACAGGUAG (SEQ ID NO:1169)
	Full Length gRNA Sequence		
	5' ATGTGTTTTGTCAAAAAGACCTTTT UAAUUUCUACUCUUGUAGAUACUGACAGCGUGAACAGGU AG 3' (SEQ ID NO:1170)		
TGFBR238 402	ATGTGTTTTGTCAAAA GACCTTTT (SEQ ID NO:5)	UAAUUUCUACUCUUGUAGAU (SEQ ID NO:24)	UGAUGUGAGAUUUUCCACCUG (SEQ ID NO:1171)
	Full Length gRNA Sequence		
	5' ATGTGTTTTGTCAAAAAGACCTTTT UAAUUUCUACUCUUGUAGAUUGAUGUGAGAUUUUCCACC UG-3' (SEQ ID NO:1172)		

[307] Indel analysis was performed by polymerase chain reaction amplification of the genomic region surrounding the CRISPR-EngCas12a cut site for each target followed by next-generation sequencing (NGS) and comparison to a reference genome to obtain percentage editing (indels).

[308] As demonstrated in **Fig. 1A** and **1B**, robust single and double-gene editing of TGFBR2 and CISH was achieved in NK cells. Greater than 80% indels at both targets in NK cells in both single and double gene knockout (KO, DKO) contexts were achieved.

[309] Phosphoflow cytometry assay was performed to determine the phosphorylated state of STAT5 (pSTAT5) and SMAD2/3 (pSMAD2/3) in NK cells. Knockout (KO) of CISH increased pSTAT5 (**Fig. 2A**) and pSTAT3 levels (data not shown) upon IL-15 stimulation, and KO of TGFBR2 decreased pSMAD2/3 levels upon TGF- β stimulation (**Fig. 2B**) in both single and double KO NK cells, as compared to unedited NK cells. These data suggest that double KO of CISH and TGFBR2 by CRISPR-EngCas12a increased NK cells' sensitivity to IL-15 and resistance to TGF- β mediated immunosuppression.

[310] Spheroids were formed by seeding 5,000 SK-OV-3 or PC-3 cells in 96 well ultra low attachment plates. Spheroids were incubated at 37°C before addition of effector cells and 10ng/mL TGF- β . AlphaLISA was performed to analyze for TNF- α and IFN- γ secretion after co-culturing of effector cells with tumor spheroids and TGF- β for 120 hrs.

[311] As shown in **Figs. 3A-3D**, double KO (DKO) of CISH and TGFBR2 by CRISPR-EngCas12a increased the secretion of inflammatory cytokines TNF- α and IFN- γ at each of the E:T ratios tested in both SK-OV-3 and PC-3 cells as compared to unedited NK cells.

[312] These results demonstrate efficient editing of healthy NK cells by CRISPR-EngCas12a, and editing at CISH and TGFBR2 enhanced effector functions of NK cells.

Example 2: CISH/TGFBR2 DKO NK cells exhibit enhanced anti-tumor activity and antibody-dependent cellular cytotoxicity (ADCC) *in vitro*

[313] Spheroids were formed by seeding 5,000 NucLight Red labeled SK-OV-3 cells in 96 well ultra low attachment plates. Spheroids were incubated at 37°C before addition of effector cells and 10ng/mL TGF- β , followed by imaging of every 2 hours on the Incucyte S3 system for up to 120 hours. Data shown are normalized to the red object intensity at time of effector addition. Normalization of spheroid curves maintains the same efficacy patterns observed in non-normalized data.

[314] As depicted in **Figs. 4A-4D**, both single knockouts (TGFBR2 KO and CISH NK) demonstrated improved cytotoxicity against tumor targets in the presence of exogenous TGF- β relative to unedited control NK cells ($p < 0.0001$ for both single KOs). Furthermore, CISH KO NK cells unexpectedly perform killing at similar level to TGFBR2 KO NK cells, suggesting that knocking out CISH also helped NK cells overcome TGF- β immunosuppression. CISH/TGFBR2 DKO NK cells demonstrated superior rapid and sustained killing of ovarian tumor spheroids SK-OV-3 compared to either single knockouts or unedited control NK cells at the range of tested E:T ratios ($n = 7$ independent experiments, 4 unique NK cell donors, $p < 0.0001$), demonstrating additive effects of simultaneously

targeting both pathways. The unedited, single KO and double KO NK cells also killed PC-3 prostate tumor spheroids in a similar trend (data not shown).

[315] These data suggest that CISH/TGFBR2 DKO NK cells are very effective at targeting multiple types of tumors.

[316] Additionally, killing of SK-OV-3 tumor spheroids by the NK cells were examined in the presence of trastuzumab, a monoclonal antibody targeting HER2. The addition of trastuzumab (10 μ g/ml) surprisingly increased killing by the unedited NK cells at a low E:T ratio of 1.25:1 to a great extent, and trastuzumab also significantly enhanced killing by the already effective DKO NK cells (see **Fig. 5**), which resulted in the greatest amount of tumor spheroid killing. This data shows that trastuzumab and NK cells have a strong antibody-dependent cellular toxicity (ADCC), and the combination of trastuzumab and NK cells, particularly the CISH/TGFBR2 DKO NK cells, has the potential to be an effective oncotherapy. The CISH/TGFBR2 DKO cells also killed the greatest amount of PC-3 prostate tumor spheroids in the presence of certuximab in a similar trend (*i.e.*, more than unedited NK cells or single CISH KO or TGFBR2 KO cells in the presence of certuximab; data not shown).

Example 3: CISH/TGFBR2 DKO NK cells exhibit enhanced anti-tumor activity *in vivo*

[317] In an *in vivo* NSG mouse xenograft model, 0.5 or 1 million fLuc-SK-OV-3 cells (expressing luciferase) were injected intraperitoneally (i.p.). At 7 days post tumor cell injection, 10 million cells of either unedited control NK cells or DKO NK cells were injected via i.p. Bioluminescence imaging using the IVIS system was performed weekly to monitor tumor burden.

[318] A single dose of DKO NK cells reduced tumor burden more effectively than unedited control NK cells (**Fig. 6A** and **6B**), leading to a statistically significant increase in median survival time and lower tumor burden (**Figs. 6C-6D**)

[319] This result suggests that the CISH/TGFBR2 DKO NK cells are promising as cell-based medicine for cancer.

Example 4: Antibody-dependent-cellular cytotoxicity (ADDC) further enhanced anti-tumor activity by CISH/TGFBR2 DKO NK *in vivo*

[320] NSG mice (n=8 per group) were inoculated via i.p. with 0.5 million luciferase-expressing SK-OV-3 cells. On day 6 post-tumor inoculation, tumor bearing mice were randomized into groups with comparable tumor burden. A day later, mice were injected via i.p with 2.5 mpk isotype, 2.5 mpk trastuzumab, 10 million unedited CD56+ NK cells, 10 million DKO CD56+ NK cells or the combination of DKO CD56+ NK cells with trastuzumab.

[321] **Figs. 7A** and **7C** again show that DKO NK cells were significantly more effective at controlling tumor growth and increased lifespan of mice. Trastuzumab significantly increased these effects of DKO NK treatments, as shown in **Figs. 7B** and **7D**.

[322] This data show that traszutumab can mediate ADCC and promote tumor killing by the DKO NK cells *in vivo*, and strongly suggest that combination therapy of traszutumab and the DKO NK cells can be very effective treatment for cancers, such as ovarian cancer.

Example 5: ADCC effect was also observed in combination treatment of rituximab and NK cells in a serial killing assay

[323] A 2D Heme Restimulation/Serial Killing Assay was used to determine the endurance of NK cells in serial tumor killing. Specifically, 200 thousand unedited control NK cells or CISH/TGFBR2 DKO NK cells were seeded in each well. 10 thousand Raji tumor cells (a hematological malignant cell line) were added to the NK cells at the beginning of the assay, and subsequently 5 thousand tumor cells and IL-15 were spiked into each well every 48 hours. Surviving tumor cells were quantified by normalized total red object area (see **Fig. 8A**).

[324] Rituximab alone did not kill tumor cells without the presence of NK cells (data not shown). For unedited NK cells, the addition of rituximab improved tumor cell killing in both the absence and presence of TGF- β (**Fig. 8B**, left 2 panels). DKO NK cells were already much more effective than unedited NK cells in killing tumor cells (**Fig. 8B**, comparing top 2 panels), and the addition of rituximab further enhanced tumor cell killing by DKO NK cells (**Fig. 8B**, right 2 panels). NK cells were still effective at killing the tumor cells after 7 days in this serial killing assay.

[325] This experiment shows that rituximab mediates ADCC in the Raji cell killing by NK cells. The combination of rituximab and CISH/TGFBR2 DKO NK cells were most effective at serially killing tumor cells in the presence or absence of TGF- β for at least 7 days in this assay, suggesting that this is an effective combination therapy for cancers, such as hematologic cancer.

[326] Overall, the experimental results showing that CISH/TGFBR2 DKO NK cells exhibit improved ADCC and effector function in the presence of different therapeutic antibodies for cancer, including trastuzumab and certuximab (Examples 2 and 4) and rituximab (Example 5), show that the CISH/TGFBR2 DKO cells could be combined with a variety of cancer treating antibodies to improve treatment outcomes for a variety of cancers.

Example 6: Functional Characterization of CISH/TGFBR2 DKO NK cells Reveals Increased Granzyme B and Degranulation Supporting Improved Serial Killing Capacity

[327] As described above, CISH/TGFBR2 DKO NK cells have increased effector function and are resistant to TGF- β inhibition. These combined activities enable this healthy donor derived NK cell therapy to kill tumor cells more efficiently and for a longer duration than control NK cells in the presence of TGF- β .

[328] To further investigate the mechanism by which CISH/TGFBR2 double knockout (DKO) NK cells (produced as described in Example 1) have increased serial killing capacity, the transcriptional changes contributed by each gene edit was first explored with a focus on transcripts critical for NK cell effector function and metabolism using Nanostring analysis. Unedited, mock electroporated, and control edited (targeting a biologically irrelevant site) NK cells were included as controls in addition

to *CISH* and *TGFBR2* single and double gene knockout (KO) NK cells to interrogate the potential impact of electroporation and double-stranded DNA breaks on NK cell function. All samples included in the analysis were cultured for 3 days in IL-15 (10 ng/mL) post-electroporation.

Interestingly, no significant transcriptional changes were detected in all control conditions, while samples that contained *CISH* editing clearly upregulated transcripts relevant for NK cell effector function, including contents of cytolytic granules (*GZMB*, *GZMA*, and *GZMH*) (**Fig. 9A**).

Furthermore, an average of 22 fold more *GZMB* transcript was expressed in *CISH*/*TGFBR2* DKO NK cells than control NK cells as measured by RT-qPCR in four unique NK cell donors (**Fig. 9B**).

[329] Next, whether the increase in cytolytic signature could be one potential mechanism whereby *CISH*/*TGFBR2* DKO NK cells were functionally superior relative to control NK cells was tested. Consistent with this hypothesis, *CISH*/*TGFBR2* DKO NK cells showed significantly higher levels of CD107a, a marker of degranulation, after 14hrs of co-culture with SKOV-3 tumor cells, suggesting that *CISH*/*TGFBR2* DKO NK cells had an increased capacity to degranulate relative to control NK cells. To determine the presence of granzyme proteins within tumor cells post engagement with NK cells, a novel *GzmB* reporter gene was developed and lentiviral vectors were used to introduce this reporter into tumor cell lines (SK-OV-3::*GzmB*). SK-OV-3 tumor cells were transduced with the reporter, and then co-cultured with *CISH*/*TGFBR2* DKO NK cells or control NK cells. 10⁶ NK cells were co-cultured with 5000 SK-OV-3::*GzmB* cells labelled with NucLight Red; and imaged every 2 hours on the Incucyte S3 system for up to 36 hours (**Fig. 9D**). *GzmB* activity was identified 4 hours sooner in the SK-OV-3 tumor cells transduced with the *GzmB* reporter that were co-cultured with the *CISH*/*TGFBR2* DKO NK cells relative to transduced tumor cells co-cultured with control NK cells. In addition, *CISH*/*TGFBR2* DKO NK cells affected 80% more SK-OV-3 tumor cells with granzyme B compared to control NK cells over a 36-hour period (**Figs. 9C and 9E**). Significantly, these data demonstrated that *CISH*/*TGFBR2* DKO NK cells not only released *GzmB* more rapidly than control NK cells, but also the amount of *GzmB* degranulated was greater as well (relative to control NK cells), confirming that enhanced degranulation is a key mechanism by which *CISH*/*TGFBR2* DKO NK cells have superior functional capacity relative to control NK cells.

[330] Together, these data demonstrate that *CISH*/*TGFBR2* DKO NK cells expressed high levels of *GzmB* and had more rapid and enhanced degranulation activity than unedited NK cells, suggesting this as a potential mechanism by which *CISH*/*TGFBR2* DKO NK cells demonstrate superior cytotoxicity during in vitro killing of SK-OV-3 tumor targets.

Example 7: *CISH*/*TGFBR2* DKO NK cells Demonstrate Superior Function During Tumor Target Killing in Nutrient Deprived Conditions Through Increased Spare Respiratory Capacity.

[331] Natural killer (NK) cells distinguish tumor from healthy tissue via multiple mechanisms, including recognition of stress ligands and loss of MHC class I expression. As described above, *CISH*/*TGFBR2* DKO NK cells were produced via CRISPR-Cas12a mediated *CISH* and *TGFBR2*

double gene knockout in NK cells derived from healthy donors (see Example 1). These cells demonstrated resistance to TGF- β inhibition and increased tumor control both *in vitro* and *in vivo*.

[332] Anti-tumor activity by effector cells requires significant energy expenditure and is constrained by nutrients available in the tumor microenvironment (TME). The TME is known to be nutrient-deprived due to active tumor cell metabolism leading to competition for essential nutrients with infiltrating effector cells, while at the same time being enriched in immunosuppressive metabolites such as lactic acid due to Warburg Metabolism. To explore whether CISH/TGFBR2 DKO NK cells are functional in such hostile metabolic conditions, the metabolic microenvironment was modelled in the established SK-OV-3 ovarian tumor spheroid model.

[333] To model this hostile microenvironment *in vitro*, SK-OV-3 ovarian tumor spheroids were generated in decreasing concentrations of glucose (10-0.5mM, *e.g.*, 10 mM (control), 5 mM, 2.5 mM, 1.0 mM or 0.5 mM) or glutamine (2-0.1mM, *e.g.*, 2 mM (control), 1 mM, 0.5 mM or 0.1 mM), two important fuels for NK cell metabolism, as well as increasing concentrations of inhibitory metabolite lactate (0-50mM, *e.g.*, 0.0 mM (control), 25 mM or 50 mM), or decreasing pH (7.2-6.5, *e.g.*, 7.2 (control), 6.9, 6.7, or 6.5). Each of these metabolic conditions are known to suppress effector cell function, and the system was further stressed by performing spheroid cells co-cultures in the absence of TGF- β at a 10:1 effector:target ratio (**Fig. 10A**). In all of the above conditions, SK-OV-3 tumor spheroids formed at similar rates relative to spheroids formed in standard culture media. Significantly, it was found that in each of these conditions, CISH/TGFBR2 DKO NK cells demonstrated rapid and sustained tumor killing in the absence of critical nutrients or in unfavorable growth conditions relative to control unedited NK cells.

[334] To further model the complexity of the metabolic conditions in the TME, a multifactorial matrix of metabolic conditions was created where deprivation of multiple nutrients was combined in the presence of lactate and/or acidic cell culture media. Specifically, the cytotoxicity of NK cells was assayed with SK-OV3-tumor spheroid in the presence of 10 ng/mL TGF- β at a 5:1 effector:target ratio (**Fig. 10B**). The cytotoxicity of NK cells was also assayed with SK-OV3-tumor spheroid that were selectively evolved to grow in nutrient-deprived and/or high lactate media in the presence of 10 ng/mL TGF- β at a 10:1 effector:target ratio at 100 hours (**Fig. 10C**) or at varying effector:target ratios (**Fig. 10D**) at 100 hours. Remarkably, in all the matrixed conditions tested, it was surprisingly found that CISH/TGFBR2 DKO NK cells demonstrated increased cytotoxicity against SK-OV-3 spheroids relative to control NK cells, suggesting a clear and robust metabolic advantage of CISH/TGFBR2 DKO NK cells over control NK cells. A corresponding increase in the concentrations of IFN- γ and TNF- α was further observed by CISH/TGFBR2 DKO NK cells in all of these conditions relative to control NK cells.

[335] Given that mitochondrial respiration is key to NK cell persistence and function, the mitochondrial function of CISH/TGFBR2 DKO NK cells was next interrogated. CISH/TGFBR2 DKO NK cells consistently demonstrated greater spare respiratory capacity (SRC) relative to control

NK cells after overnight IL-15 starvation, suggesting enhanced mitochondrial reserve as a result of *CISH* and *TGFBR2* knockout (**Fig. 10E**). SRC is a function of mitochondrial mass and fitness. A cell with a larger SRC can produce more ATP and overcome more stress, including oxidative stress. Similar results were observed in NK cells cultured with IL-15 overnight. The increase in SRC likely enables *CISH*/*TGFBR2* DKO NK cells to meet enhanced energy demands necessary to mediate effector function in metabolically challenging conditions, thus sustaining superior cytotoxic capacity and cytokine production.

[336] In summary, a complex multifactorial *in vitro* tumor spheroid model was developed to more realistically probe the TME likely to be encountered *in vivo*. These data demonstrate that enhanced metabolic function of CRISPR-Cas12a *CISH* and *TGFBR2* gene edited NK cells results in superior cytotoxicity during *in vitro* killing of SK-OV-3 spheroids in metabolically unfavorable conditions that are similar to those experienced by effector cells in tumors. These data further demonstrate the potential of *CISH*/*TGFBR2* DKO NK cells as a novel cell therapy for cancer.

Example 8. *CISH*/*TGFBR2* DKO NK cells Demonstrated Enhanced Anti-Tumor Activity and Sustained Serial Killing Against Other Tumor Cell Lines

[337] The anti-tumor activity of *CISH*/*TGFBR2* double knockout NK cells (produced as described in Example 1) was further tested against numerous other tumor cells lines, such as Nalm6 tumor cells and other hematologic tumor cell lines.

[338] **Figs. 11A and 11B** depict that *CISH*/*TGFBR2* double knockout NK cells exhibited enhanced anti-tumor activity against Nalm6 tumor cells in the presence of TGF- β compared to control unedited NK cells. *CISH*/*TGFBR2* DKO NK cells, or unedited control NK cells, were co-cultured with Nalm6 tumor cells at a 20:1 effector tumor ratio in the presence of 5 ng/mL IL-15, without and with the addition of 10 ng/mL TGF- β . Increased cytotoxicity was observed in all conditions while a greater increase was observed when TGF- β was added in the cell culture.

[339] In addition, as shown in **Fig. 12** and **Fig. 13**, *CISH*/*TGFBR2* DKO NK cells continually killed Nalm6 tumor cells for more than 8 days in an *in vitro* serial killing assay, whereas the unedited NK cells had limited serial killing effect. Nalm6 tumor cells (5×10^3 cells) were added to the NK cells every 48 hours in the presence of 5 ng/mL IL-15 and 10 ng/mL TGF- β in this assay. Supernatant from this assay were harvested every 48 hours, and *CISH*/*TGFBR2* DKO NK cells were shown to produce higher levels of IFN- γ and TNF- α versus unedited NK cells over the duration of the assay (**Fig. 14**), suggesting that *CISH*/*TGFBR2* DKO NK cells can continue to produce these inflammatory cytokines even after serial killing.

[340] Other hematologic tumor cell lines, such as Raji (Burkitt's lymphoma), RPMI8226 (multiple myeloma) and THP-1 (acute monocytic leukemia) cells, were also tested in the serial killing assay. As shown in **Figs. 15A-15C**, *CISH*/*TGFBR2* DKO NK cells demonstrated sustained serial killing activity against each of these tumor cell lines in the presence of TGF- β , and the *CISH*/*TGFBR2* DKO NK cells continually killed the cells of each of these tumor cell lines for more than 8 days.

[341] These data suggest that CISH/TGFBR2 DKO NK cells are very effective at targeting multiple types of tumors.

CLAIMS

1. A method of inducing antibody-dependent cell-mediated cytotoxicity (ADCC) of a cancer cell, the method comprising contacting the cancer cell with a modified natural killer (NK) cell and an antibody, or an antigen-binding portion thereof, wherein the modified NK cell exhibits a loss of function of transforming growth factor beta receptor 2 (TGF β R2) and cytokine inducible SH2 containing protein (CISH), thereby inducing ADCC of the cancer cell.
2. The method of claim 1, wherein the contacting is in a subject.
3. A method of treating cancer in a subject, the method comprising administering to the subject a modified natural killer (NK) cell and an antibody, or an antigen-binding portion thereof, wherein the modified NK cell exhibits a loss of function of transforming growth factor beta receptor 2 (TGF β R2) and cytokine inducible SH2 containing protein (CISH), wherein the administering induces ADCC of a cancer cell in the subject, thereby treating the cancer in the subject.
4. The method of any one of the previous claims, wherein the administration increases ADCC by at least about 20%, at least about 25%, at least about 50%, at least about 75%, at least about 100%, at least about 2-fold, at least about 5-fold or at least about 10-fold as compared to ADCC of a cancer cell using an unmodified NK cell and the antibody.
5. The method of any one of claims 2-4, wherein the administering decreases tumor volume in the subject by at least about 10%, at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, or at least about 80%, or at least about 90% by about 10 days or about 20 days after administering.
6. The method of claim 5, where in the administering decreases tumor volume in the subject for at least about 10 days, at least about 20 days, at least about 30 days, at least about 40 days after the administering.
7. The method of any one of claims 2-6, wherein the administering increases the survival time of the subject.
8. The method of claim 7, wherein the survival time of the subject is increased by at least about two-fold as compared to a subject who has not be administered the modified NK cell and the antibody, by at least about two-fold as compared to a subject who has been administered the antibody alone, and/or by at least about 50% as compared to a subject who has been administered the modified NK cell alone.

9. The method of claim 1, wherein the contacting is *in vitro*.
10. The method of claim 9, wherein the modified NK cell comprises an increase in level of TNF α by at least about two fold as compared to a control level expression of TNF α , wherein the control level of TNF α is a level of TNF α produced by an unmodified NK cell under the same conditions.
11. The method of claim 10, wherein the modified NK cell comprises an increase in level of TNF α by at least about three fold as compared to the control level expression of TNF α .
12. The method of any one of claims 9-11, wherein the modified NK cell comprises an increase in level of IFN γ by at least about two fold as compared to a control level expression of IFN γ , wherein the control level of IFN γ is a level of IFN γ produced by an unmodified NK cell under the same conditions.
13. The method of claim 12, wherein the modified NK cell comprises an increase in level of IFN γ by at least about three fold as compared to the control level expression of IFN γ .
14. The method of any one of claims 9-13, wherein the modified NK cell comprises an increase in level of a cytolytic granule by at least about two fold as compared to a control level expression of the cytolytic granule, wherein the cytolytic granule is selected from the group consisting of GZMB, GZMA and GZMH; optionally wherein the control level of cytolytic granule is a level of cytolytic granule produced by an unmodified NK cell under the same conditions.
15. The method of claim 14, wherein the modified NK cell comprises an increase in level of the cytolytic granule by at least about three fold as compared to the control level expression of the cytolytic granule.
16. The method of any one of claims 9-15, wherein the modified NK cell comprises an increase in production rate of a cytolytic granule by at least about two fold as compared to a control production rate of the cytolytic granule, wherein the cytolytic granule is selected from the group consisting of GZMB, GZMA and GZMH; optionally wherein the control production rate of cytolytic granule is a production rate of cytolytic granule by an unmodified NK cell under the same conditions.
17. The method of claim 16, wherein the modified NK cell comprises an increase in production rate of the cytolytic granule by at least about three fold as compared to the control production rate of the cytolytic granule.

18. The method of any one of claims 9-17, wherein the modified NK cell comprises an increase in level of CD107a by at least about two fold as compared to a control level expression of CD107a; optionally wherein the control level of CD107a is a level of CD107a in an unmodified NK cell under the same conditions.

19. The method of claim 18, wherein the modified NK cell comprises an increase in level of CD107a by at least about three fold as compared to the control level expression of CD107a.

20. The method of any one of claims 9-19, wherein the modified NK cell comprises a decrease in normalized total integrated red object intensity in a tumor spheroid assay by at least about 20% as compared to a control level of normalized total integrated red object intensity, wherein the control level of normalized total integrated red object intensity is a level of normalized total integrated red object intensity produced using an unmodified NK cell under the same conditions.

21. The method of claim 20, wherein the modified NK cell comprises a decrease in normalized total integrated red object intensity in the tumor spheroid assay by at least about 25%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 75%, or about 100% as compared to the control level of normalized total integrated red object intensity.

22. The method of any one of the previous claims, wherein the modified NK cell comprises an increase in cytotoxicity activity under a nutrient-depriving condition by at least about 20% as compared to a control level of cytotoxicity activity, optionally wherein the control level of cytotoxicity activity is a cytotoxicity level of an unmodified NK cell under the same conditions.

23. The method of claim 22, wherein the modified NK cell comprises an increase in cytotoxicity activity under the nutrient-depriving condition by at least about 25%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 75% or about 100% as compared to the control level of cytotoxicity activity.

24. The method of any one of the previous claims, wherein the modified NK cell comprises an increase in spare respiratory capacity by at least 20% as compared to a control level of spare respiratory capacity, optionally wherein the control level of spare respiratory capacity is a level of spare respiratory capacity of an unmodified NK cell under the same conditions.

25. The method of claim 24, wherein the modified NK cell comprises an increase in spare respiratory capacity by at least about 25%, at least about 30%, at least about 40%, at least about 50%,

at least about 60%, at least about 75% or about 100% as compared to the control level of spare respiratory capacity.

26. The method of any one of the previous claims, wherein the antibody, or antigen-binding portion thereof, binds an antigen on the cancer cell.

27. The method of claim 26, wherein the antigen on the cancer cell is epidermal growth factor receptor (EGFR), HER2, CD20, PD-L1, PD-1 (PEMBRO and NIVO), CTLA-4 (IPI), CD73, or TIGIT.

28. The method of claim 27, wherein the antibody is cetuximab, rituximab, or trastuzumab, or an antigen-binding portion thereof.

29. The method of any one of the previous claims, wherein the modified NK cell is administered concurrently with the antibody, or the antigen-binding portion thereof.

30. The method of any one of claims 1-28, wherein the antibody, or antigen-binding portion thereof, is administered prior to the modified NK cell, or wherein the modified NK cell is administered prior to the antibody, or the antigen-binding portion thereof.

31. The method of any one of the previous claims, wherein the cancer cell is a head and neck cancer cell, breast cancer cell, colorectal cancer cell, gastric cancer cell, renal cell carcinoma (RCC) cell, or non-small cell lung cancer (NSCLC) cell, solid tumor cell, bladder cancer cell, hepatocellular carcinoma cell, prostate cancer cell, ovarian/uterine cancer cell, pancreatic cancer cell, mesothelioma cell, melanoma cell, glioblastoma cell, cervical cancer cell, oral cavity cancer cell, cancer of the pharynx, thyroid cancer cell, gallbladder cancer cell, soft tissue sarcoma, or a hematological cancer cell.

Fig. 1A

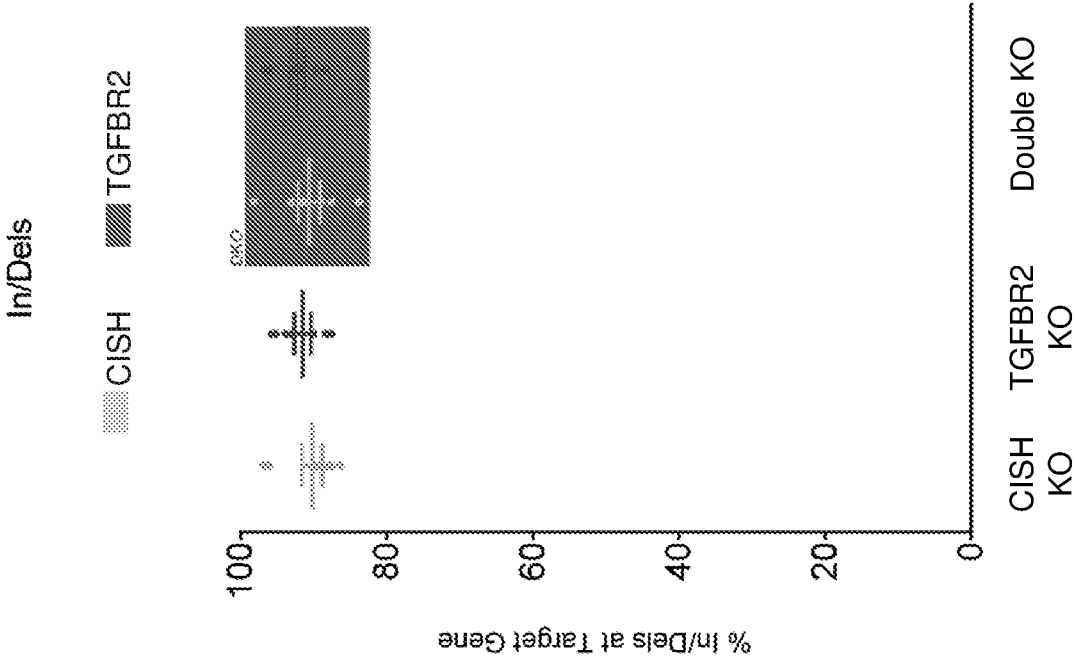


Fig. 1B

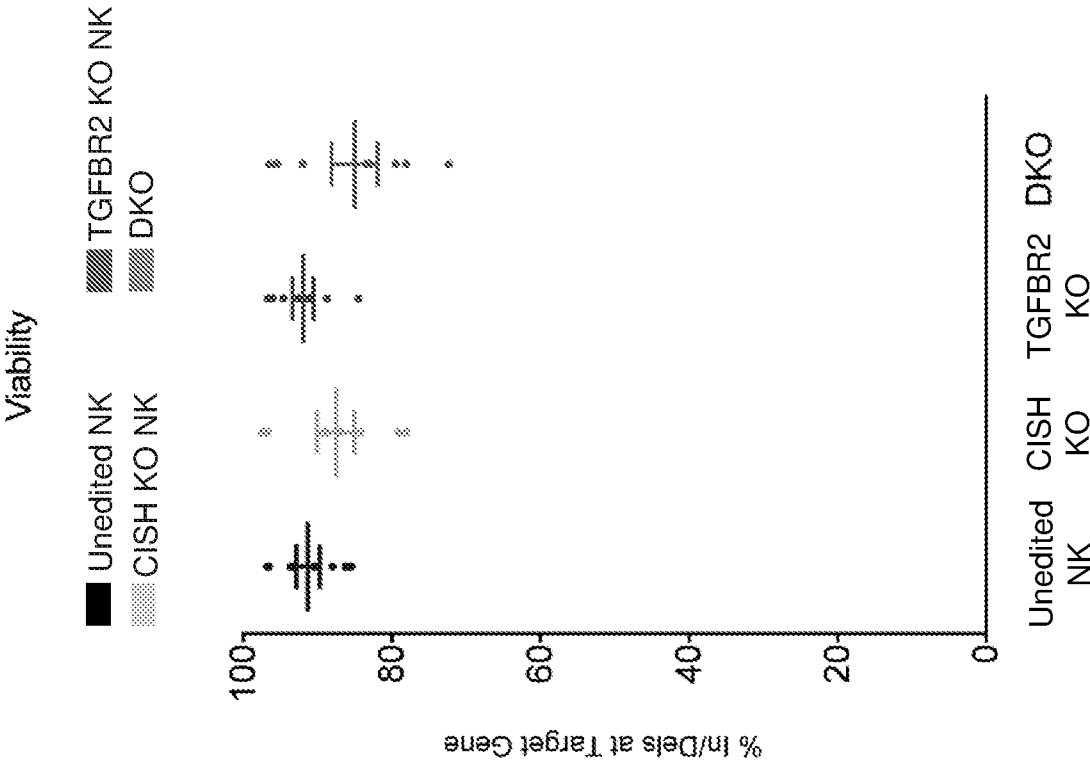


Fig. 2A

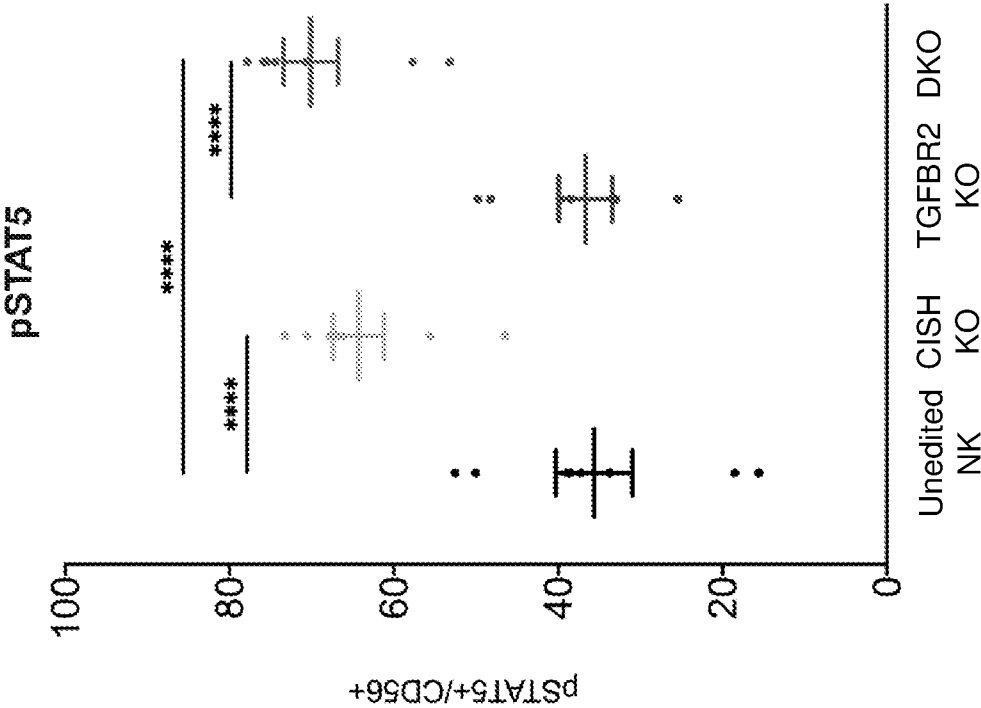


Fig. 2B

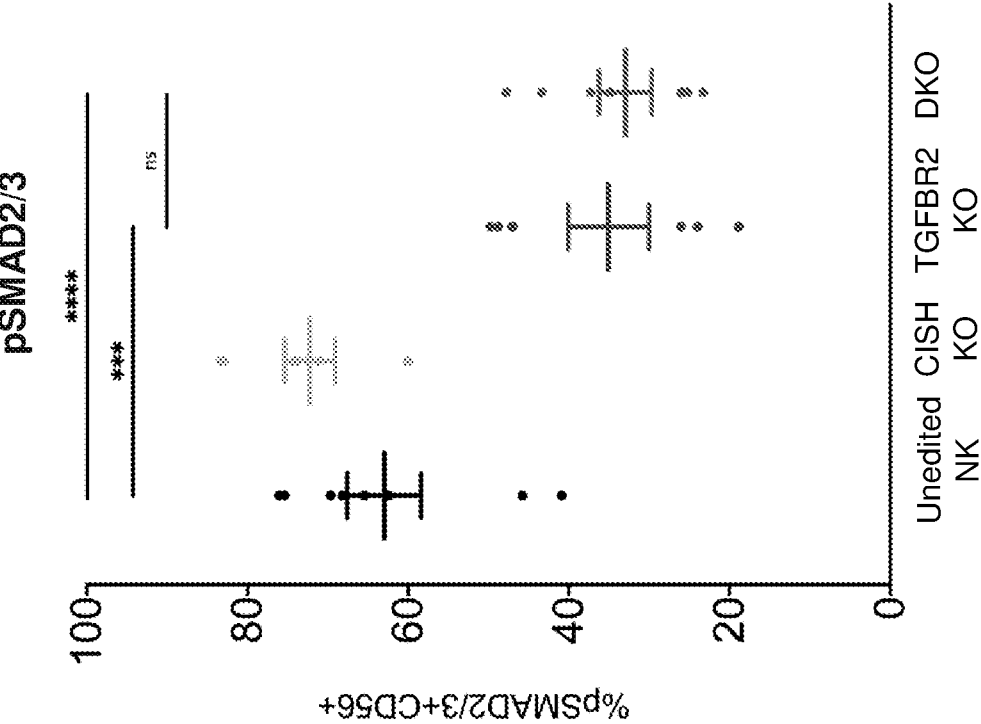


Fig. 3A

SK-OV-3
(6 donors, 6 independent experiments)

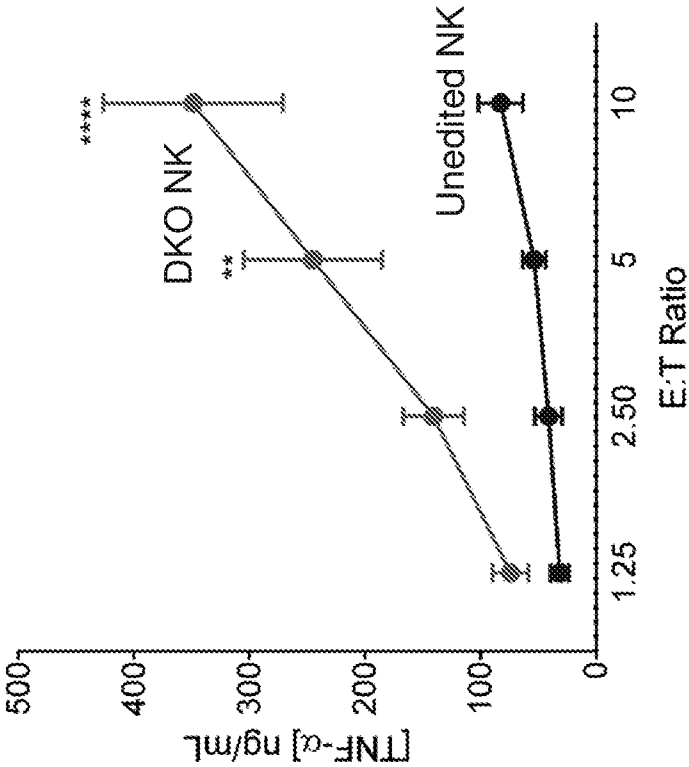


Fig. 3B

SK-OV-3
(6 donors, 6 independent experiments)

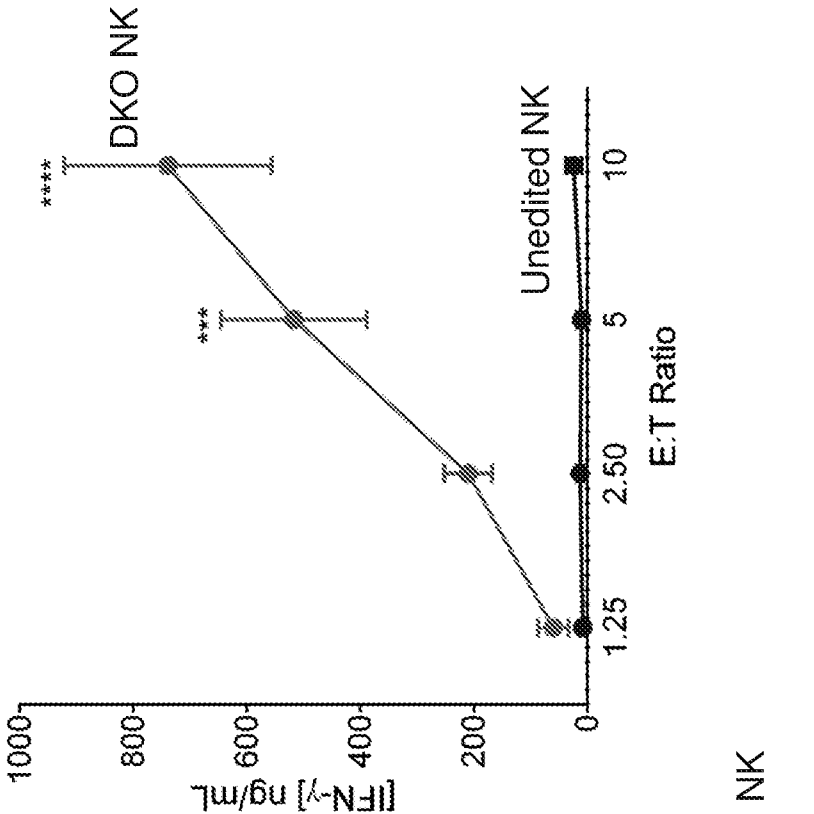


Fig. 3C

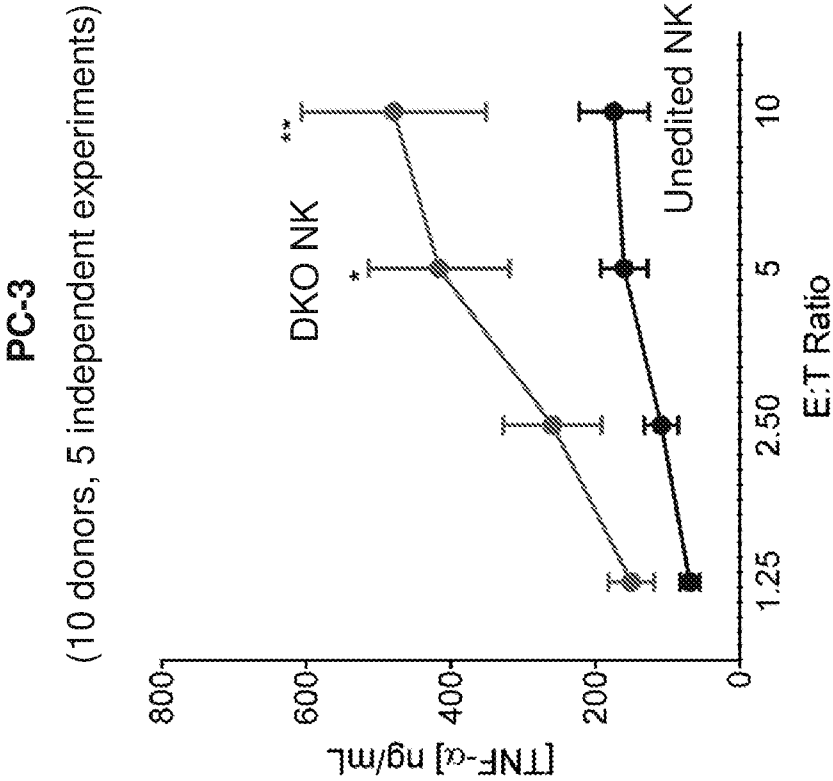


Fig. 3D

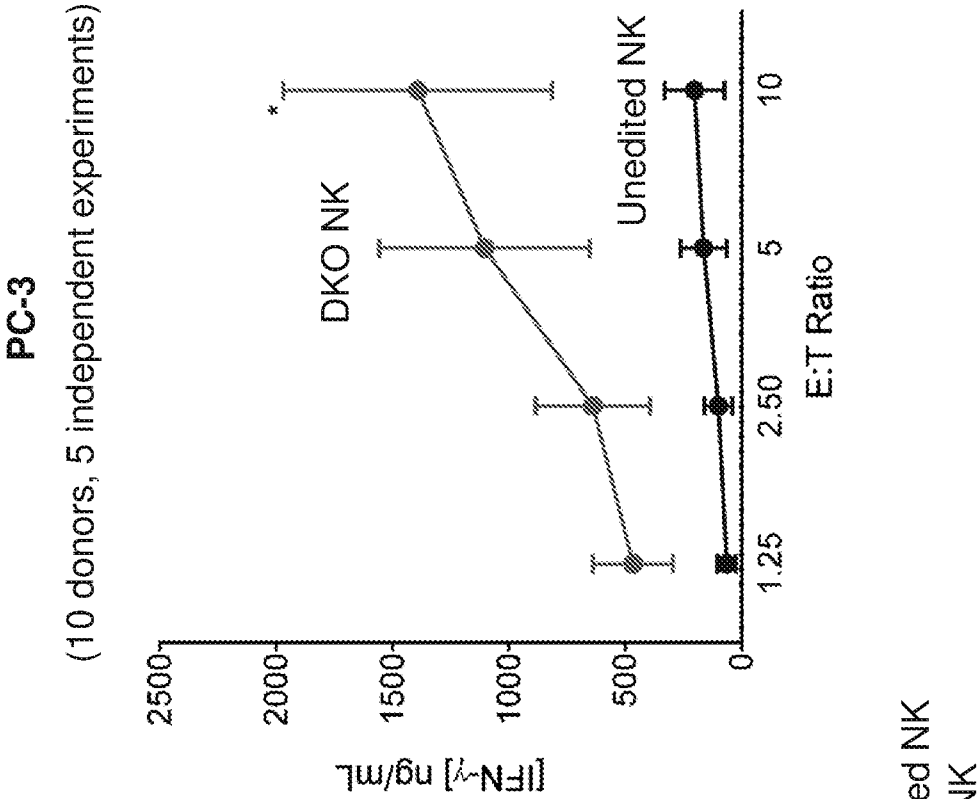


Fig. 4A

SK-OV-3

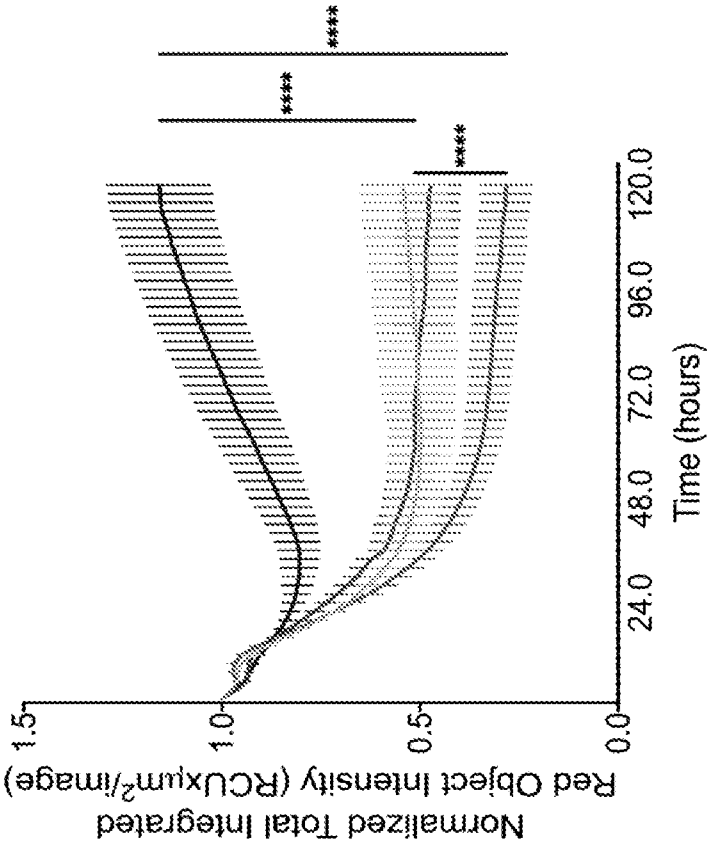
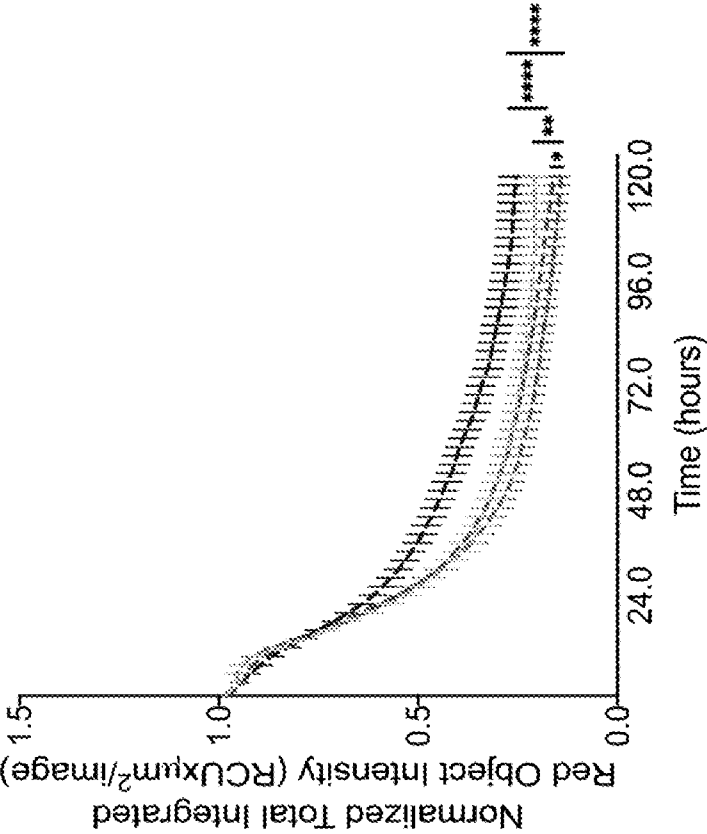


Fig. 4B

SK-OV-3

+10μg/mL trastuzumab



- Unedited NK
- CISH KO NK
- TGFB2 KO NK
- DKO NK

Fig. 4C

SK-OV-3

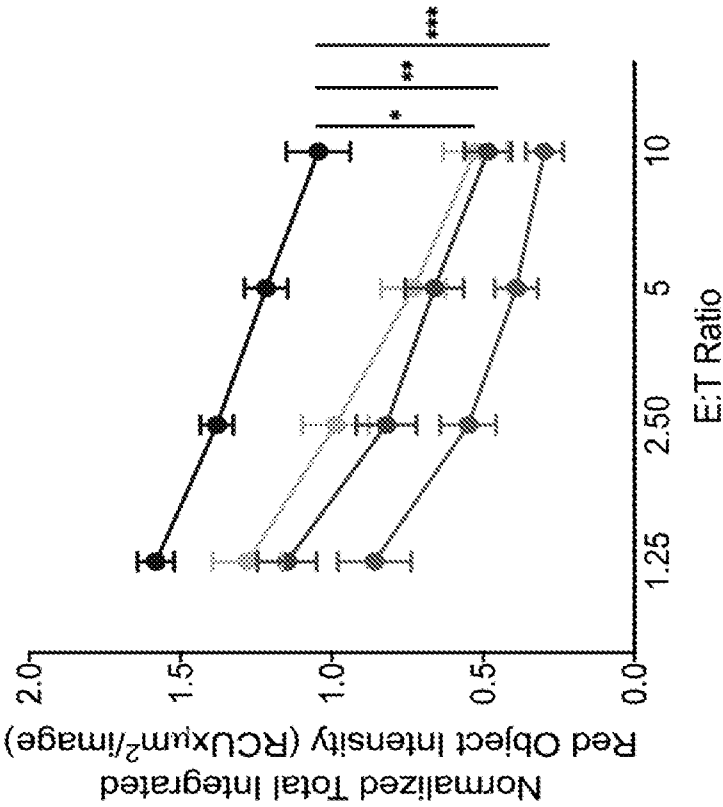


Fig. 4D

SK-OV-3

+10 μ g/mL trastuzumab

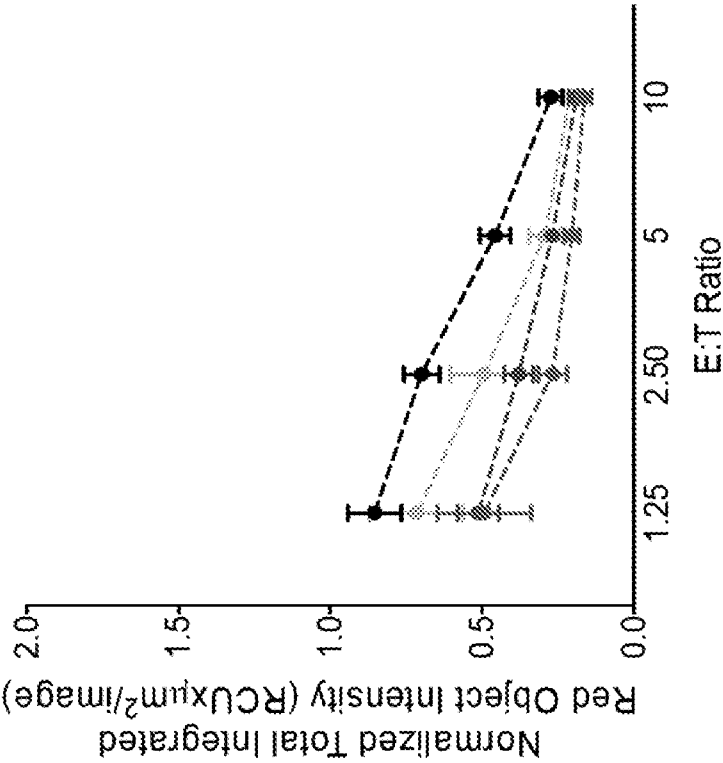


Fig. 5

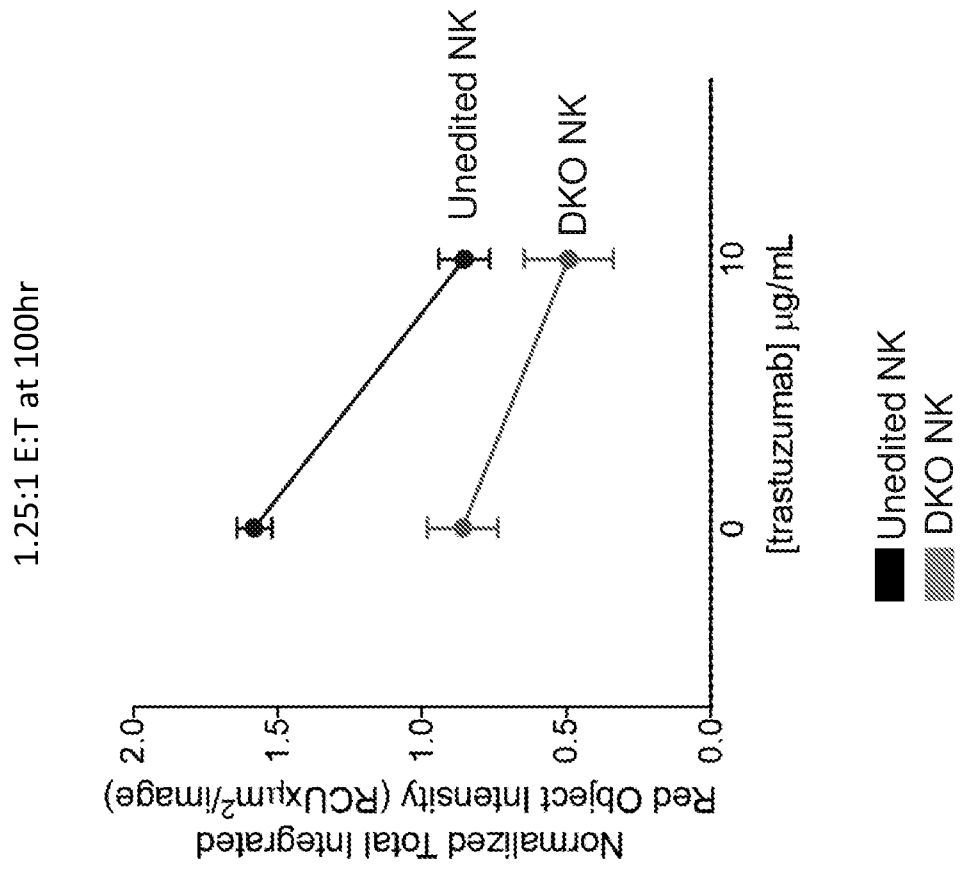


Fig. 6A

SK-OV-3
0.5 million cells i.p.

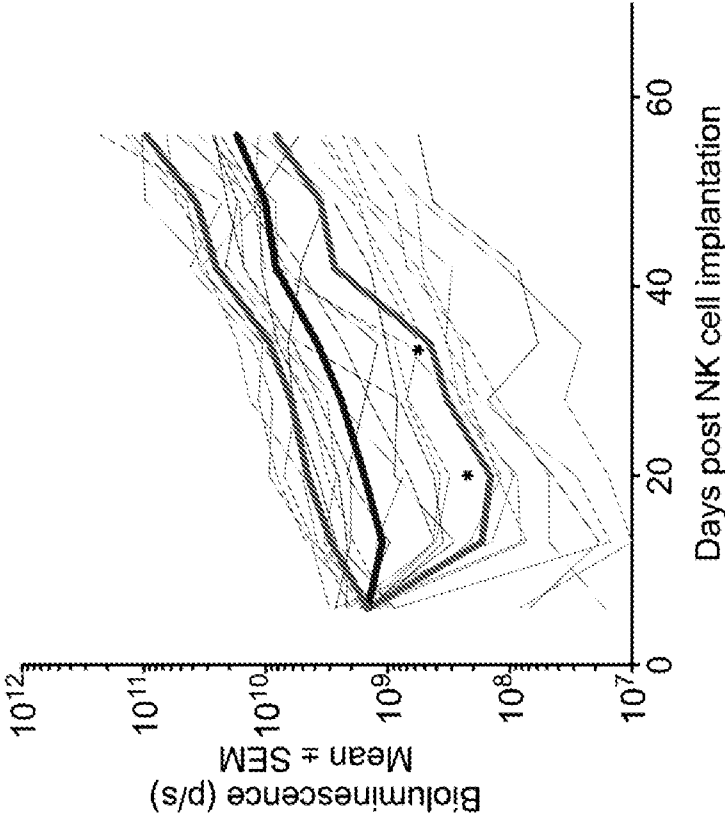


Fig. 6B

SK-OV-3
1 million cells i.p.

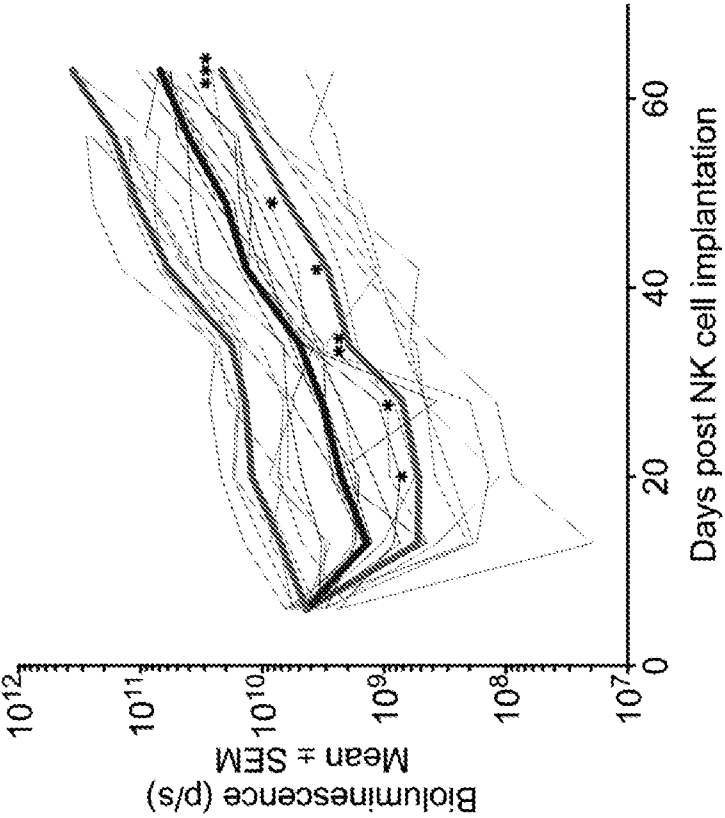


Fig. 6D

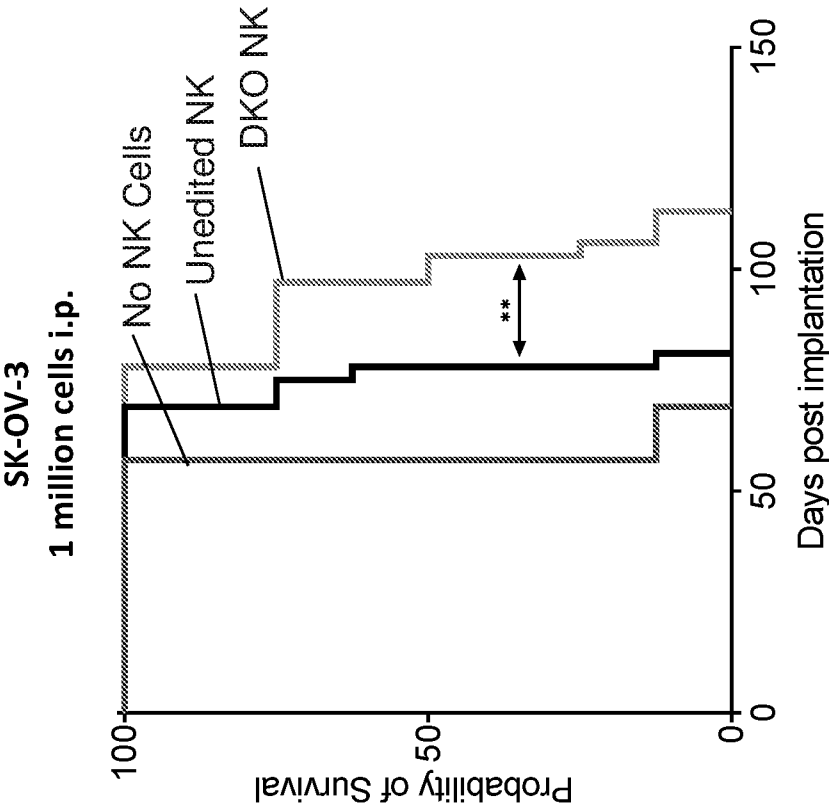
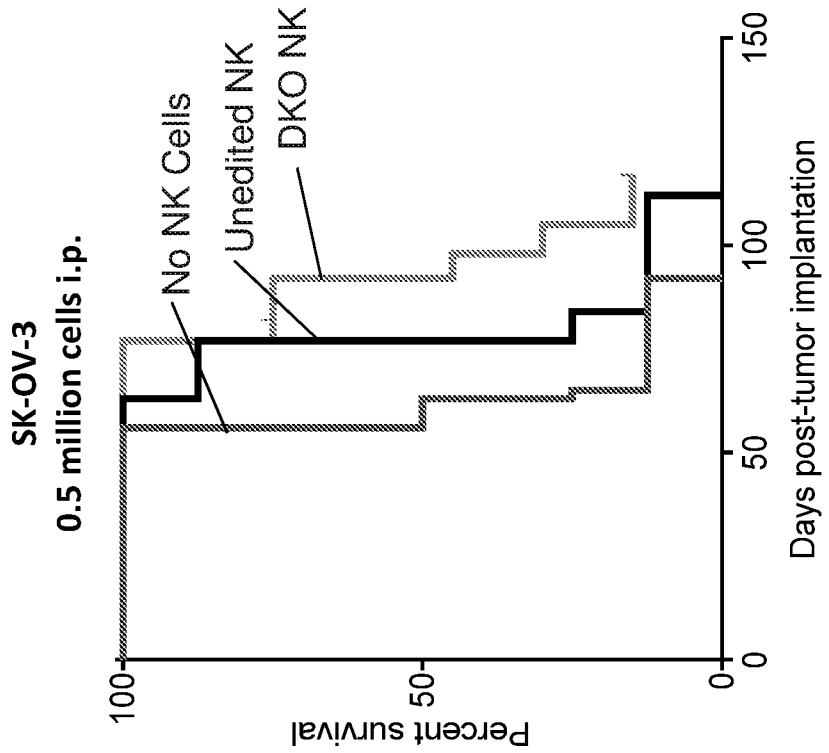


Fig. 6C



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Fig. 7B

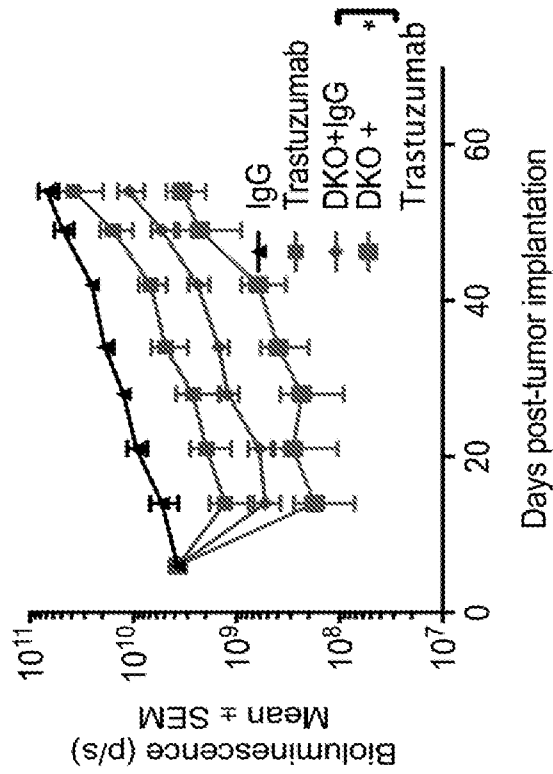


Fig. 7A

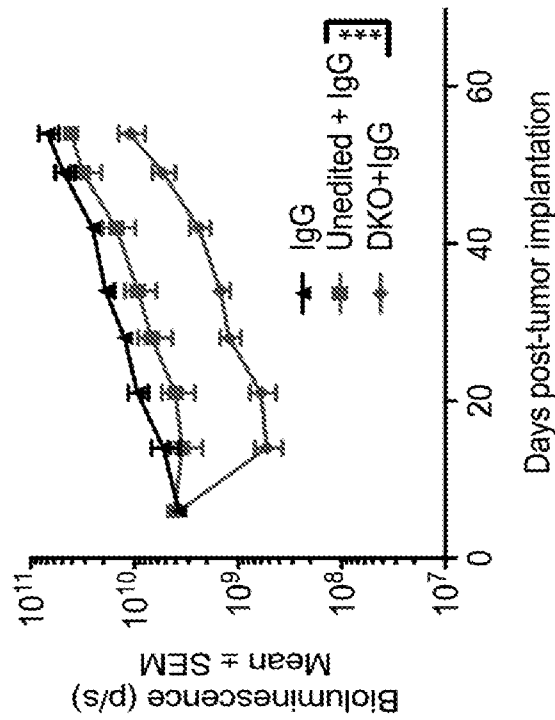


Fig. 7C

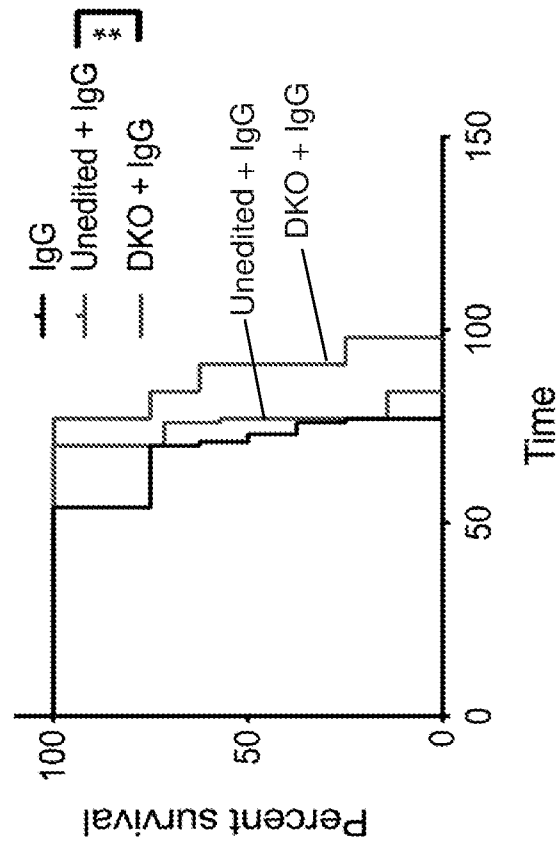


Fig. 7D

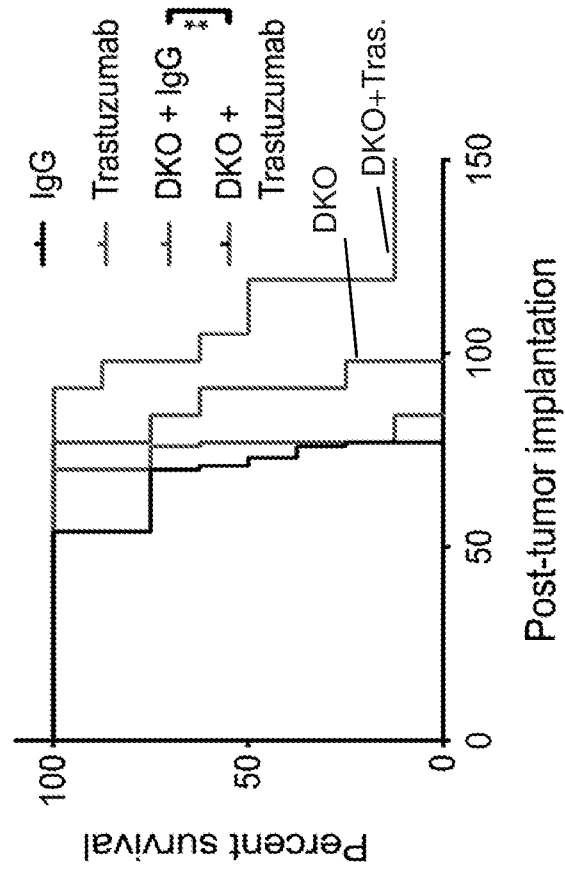


Fig. 8A

2D Heme Restim/Serial Killing Assay

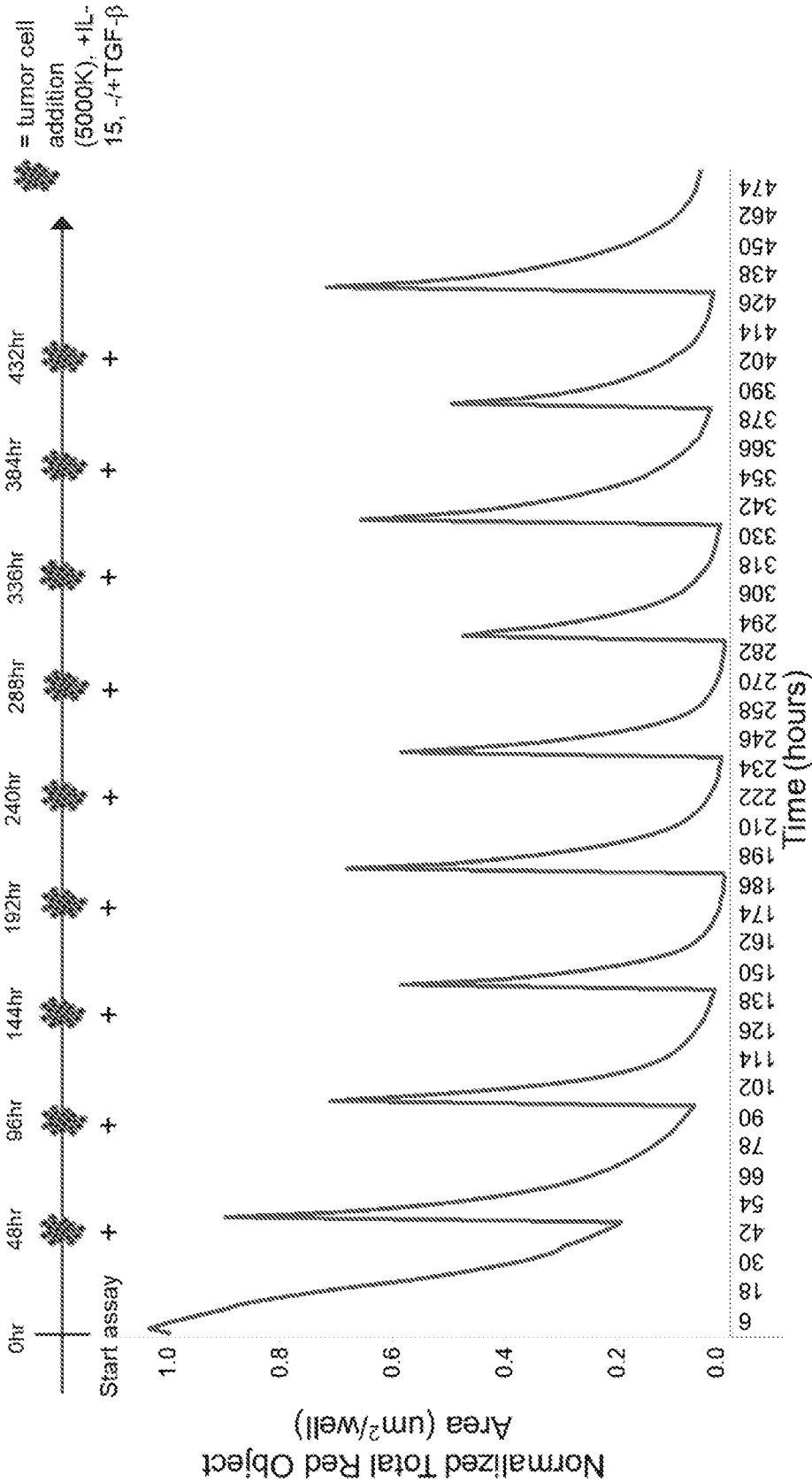


Fig. 8B

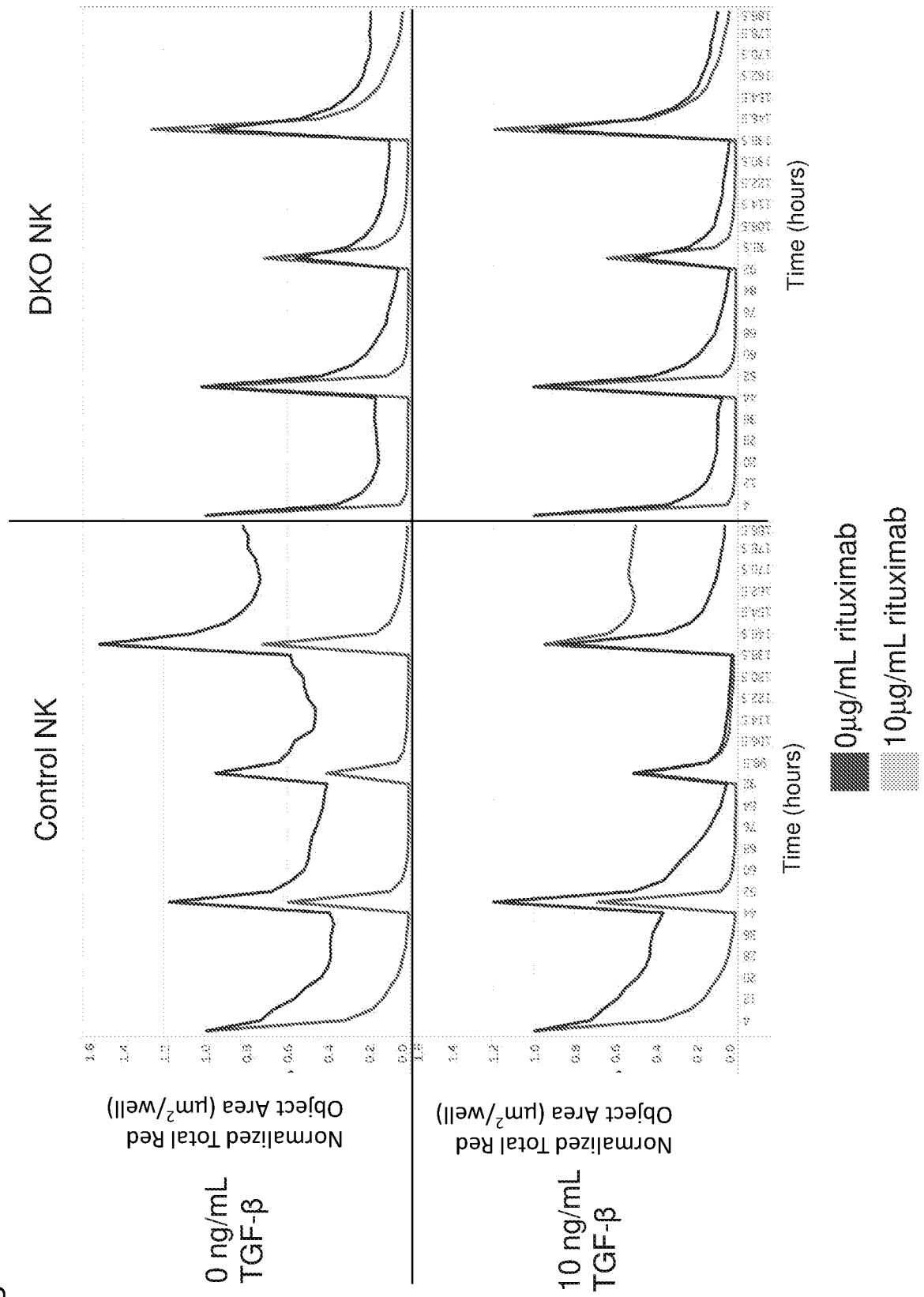


FIG. 9A

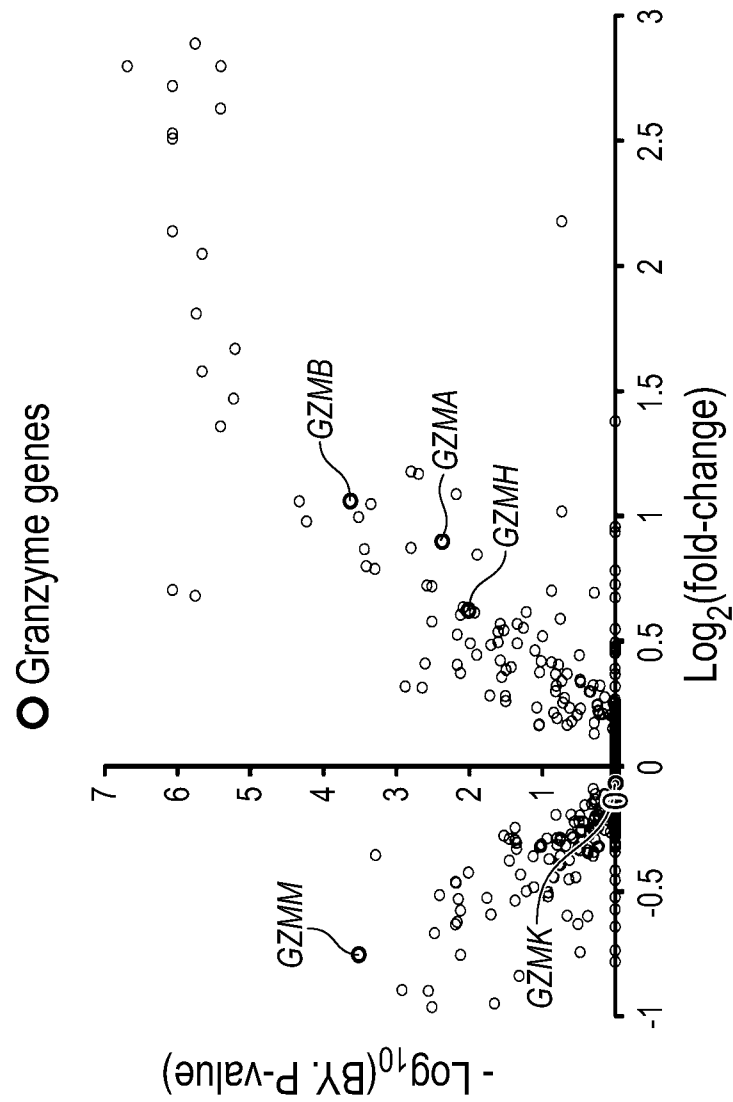


FIG. 9B

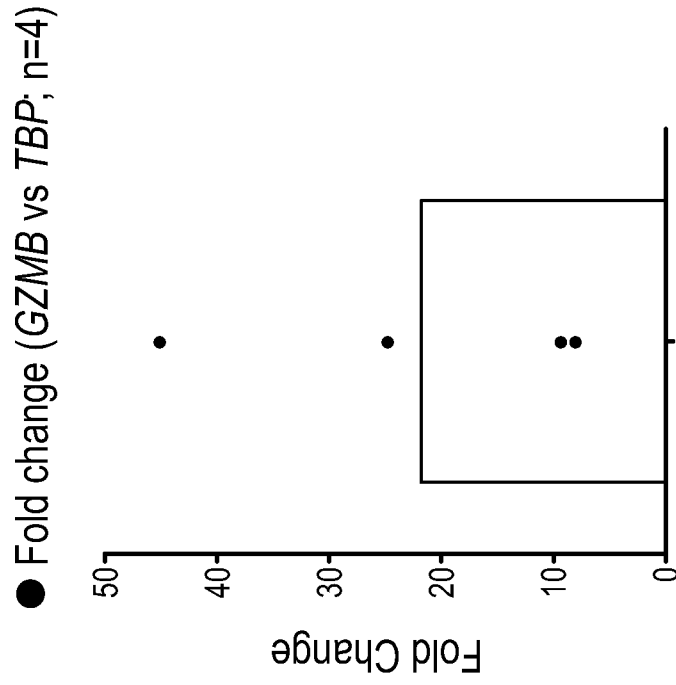


Fig. 9C

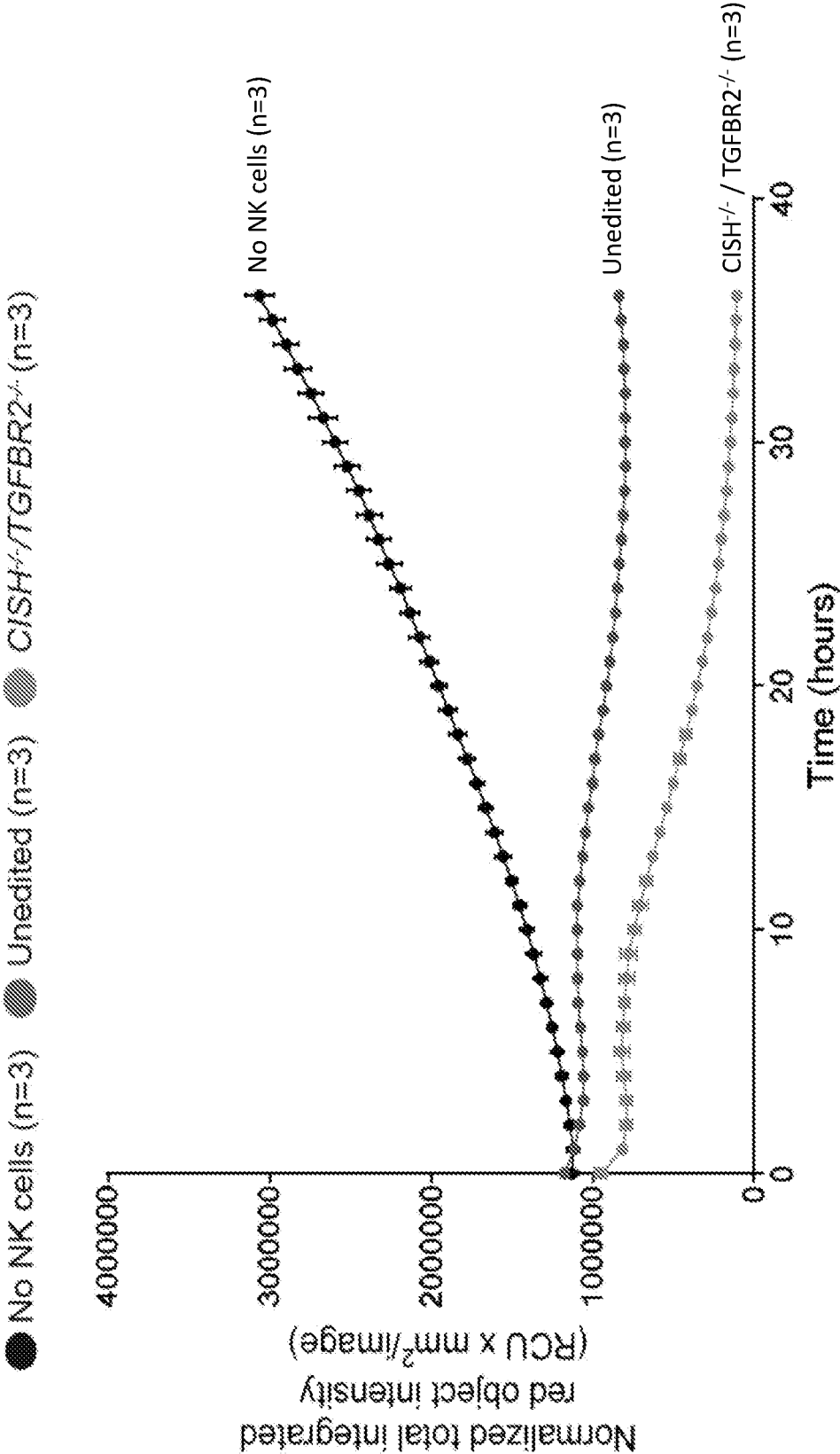


FIG. 9D

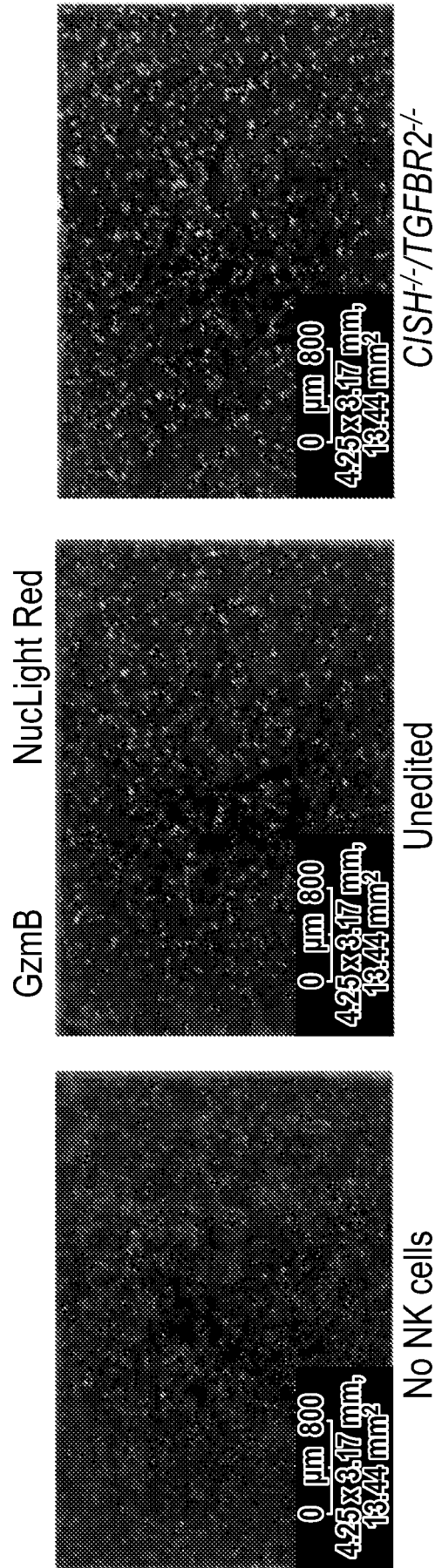


Fig. 9E

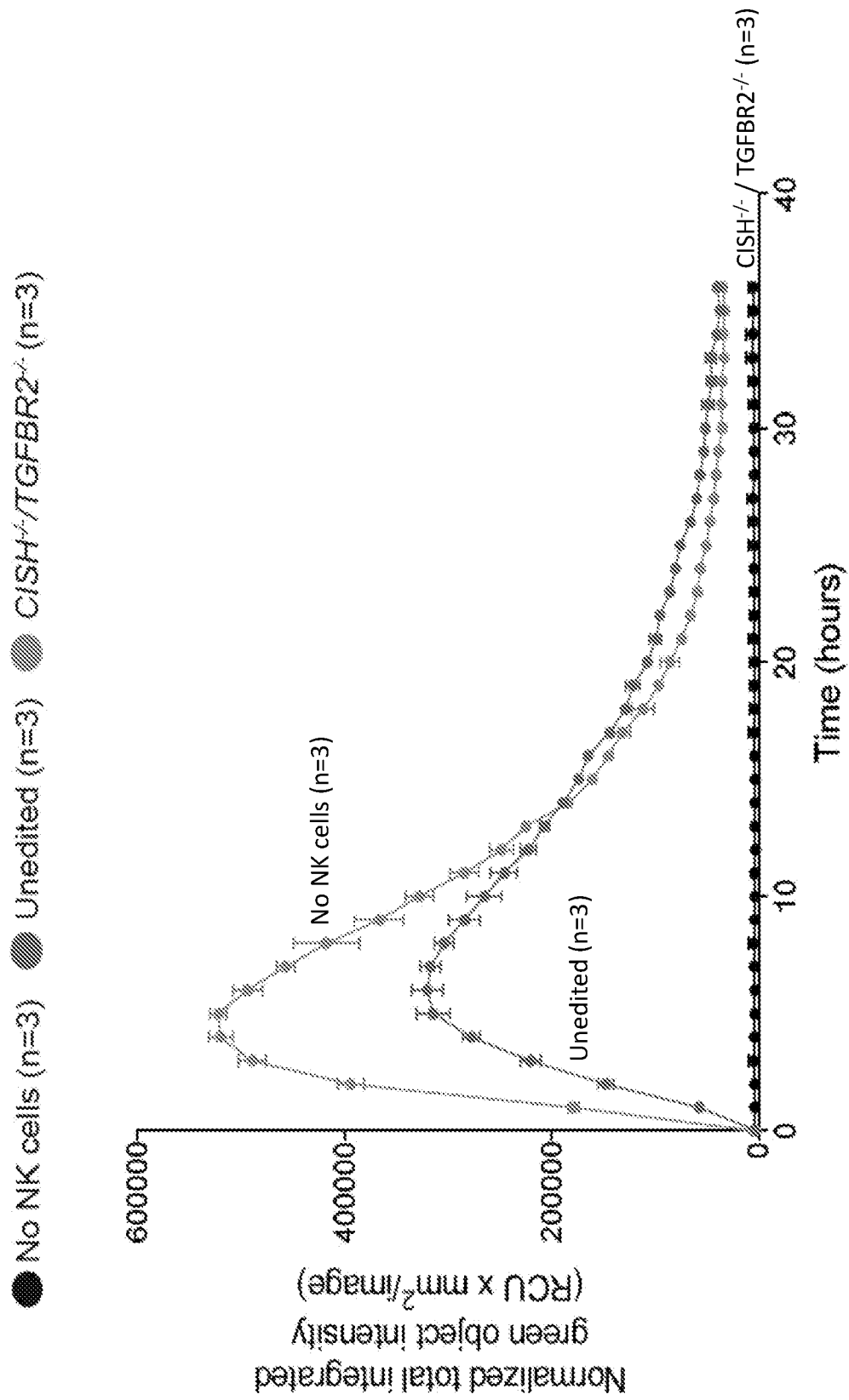


FIG. 10A

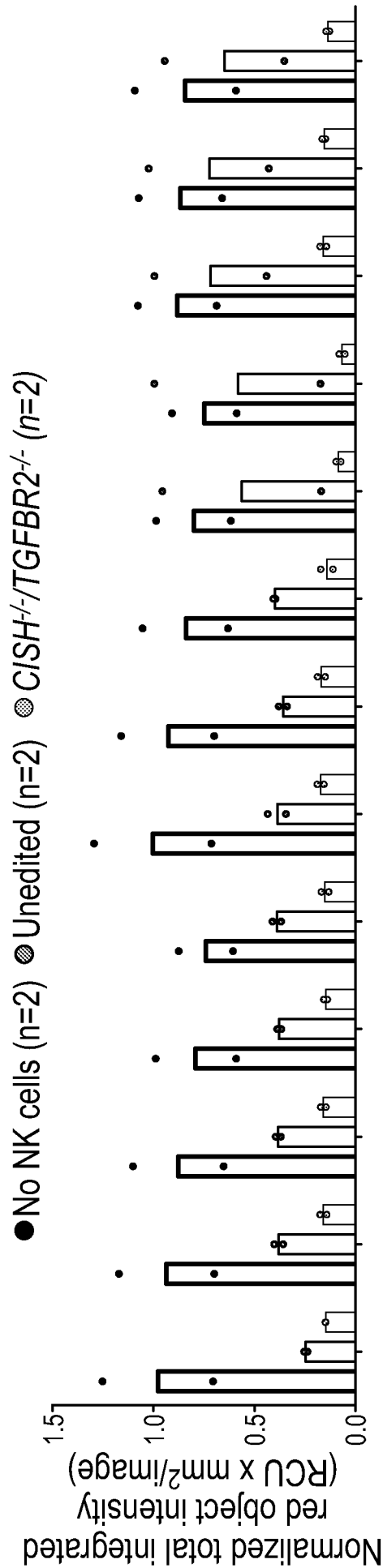


FIG. 10B

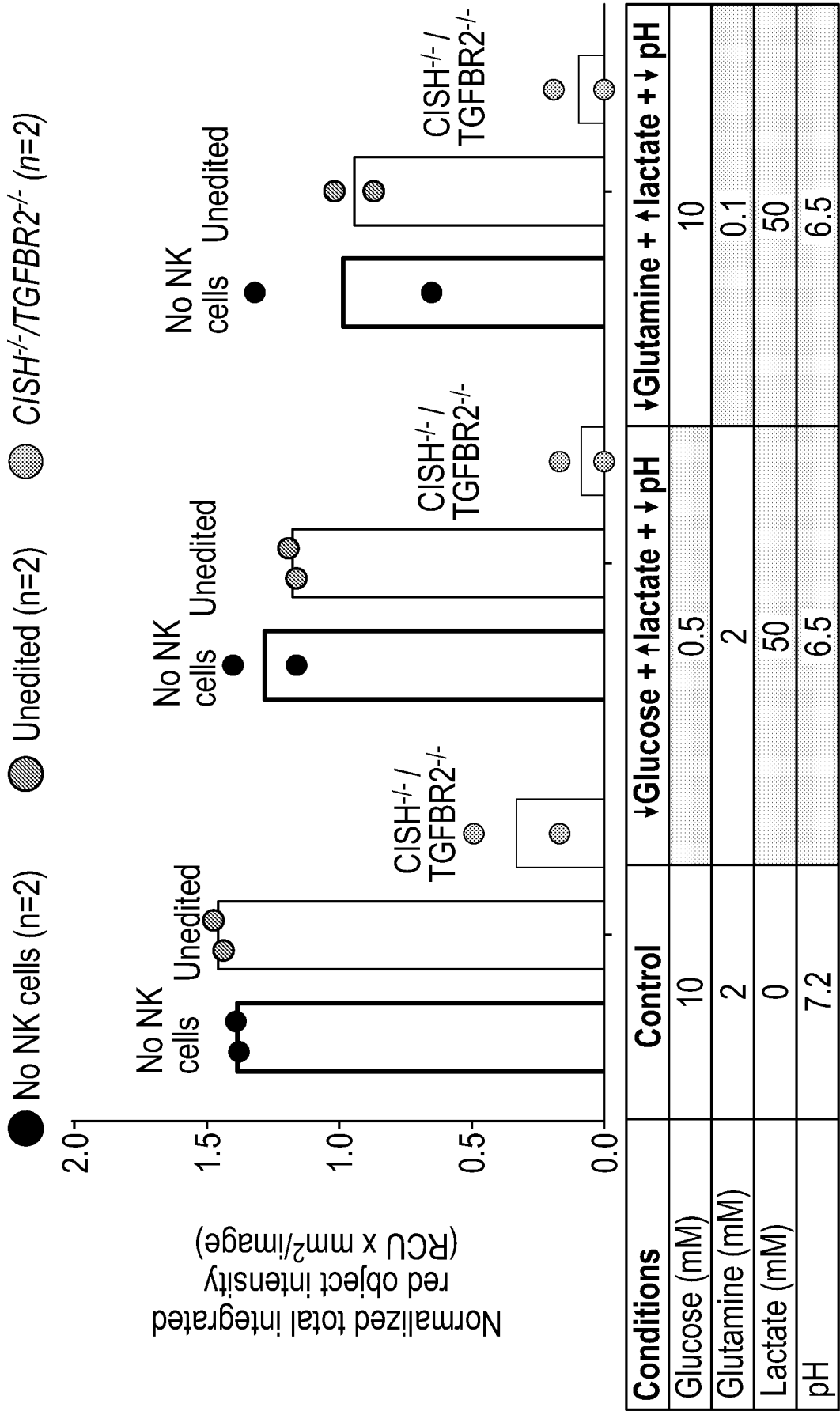
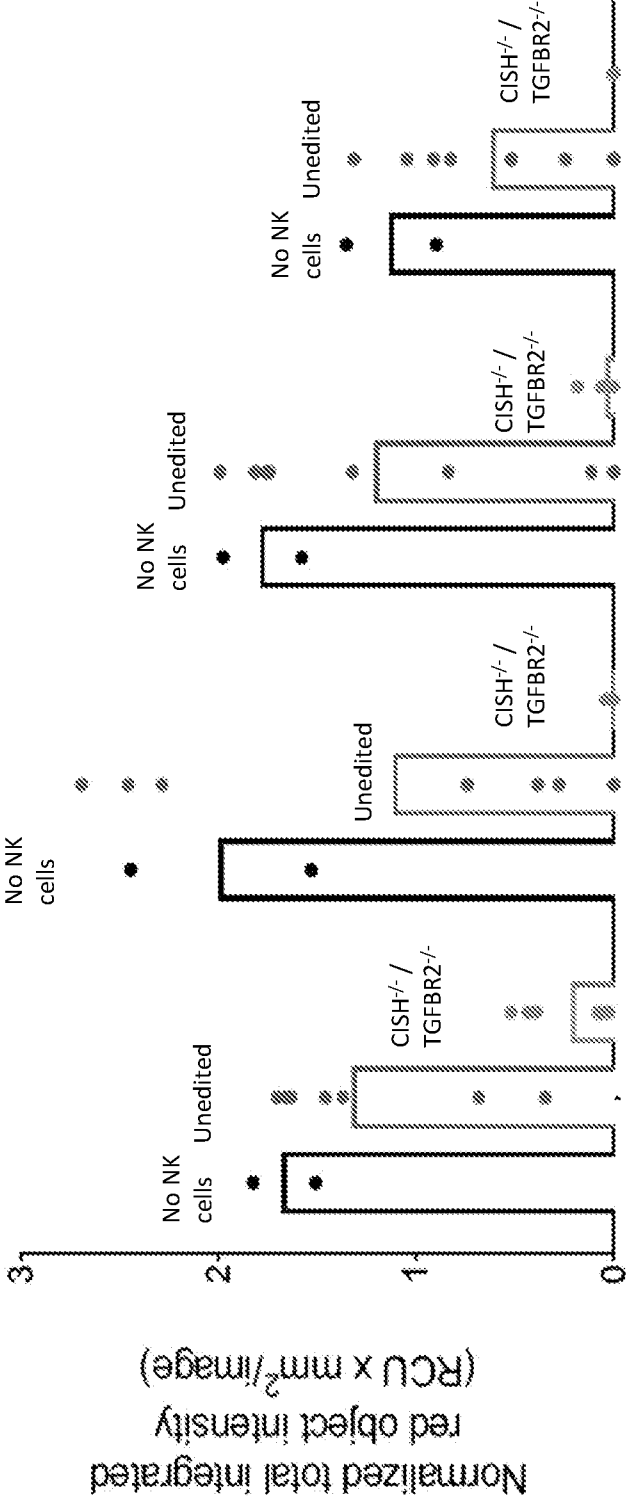


Fig. 10C

● No NK cells (n=2) ● Unedited (n=8) ● CISH^{-/-}/TGFB^{R2}^{-/-} (n=8)



EC ₅₀ Unedited	35.24	0.57	4.47	1.03
EC ₅₀ CISH ^{-/-} /TGFB ^{R2} ^{-/-}	1.92	0.04	0.34	0.06
Conditions	Control	Glutamine	Lactate	Glucose + Lactate
Glucose (mM)	10	10	10	0.5
Glutamine (mM)	2	0.1	2	2
Lactate (mM)	0	0	50	50
pH	7.2	7.2	7.2	7.2

Fig. 10D ● No NK cells (n=2) ● Unedited (n=8) ● CISH^{-/-}/TGFB^{R2-/-} (n=8)

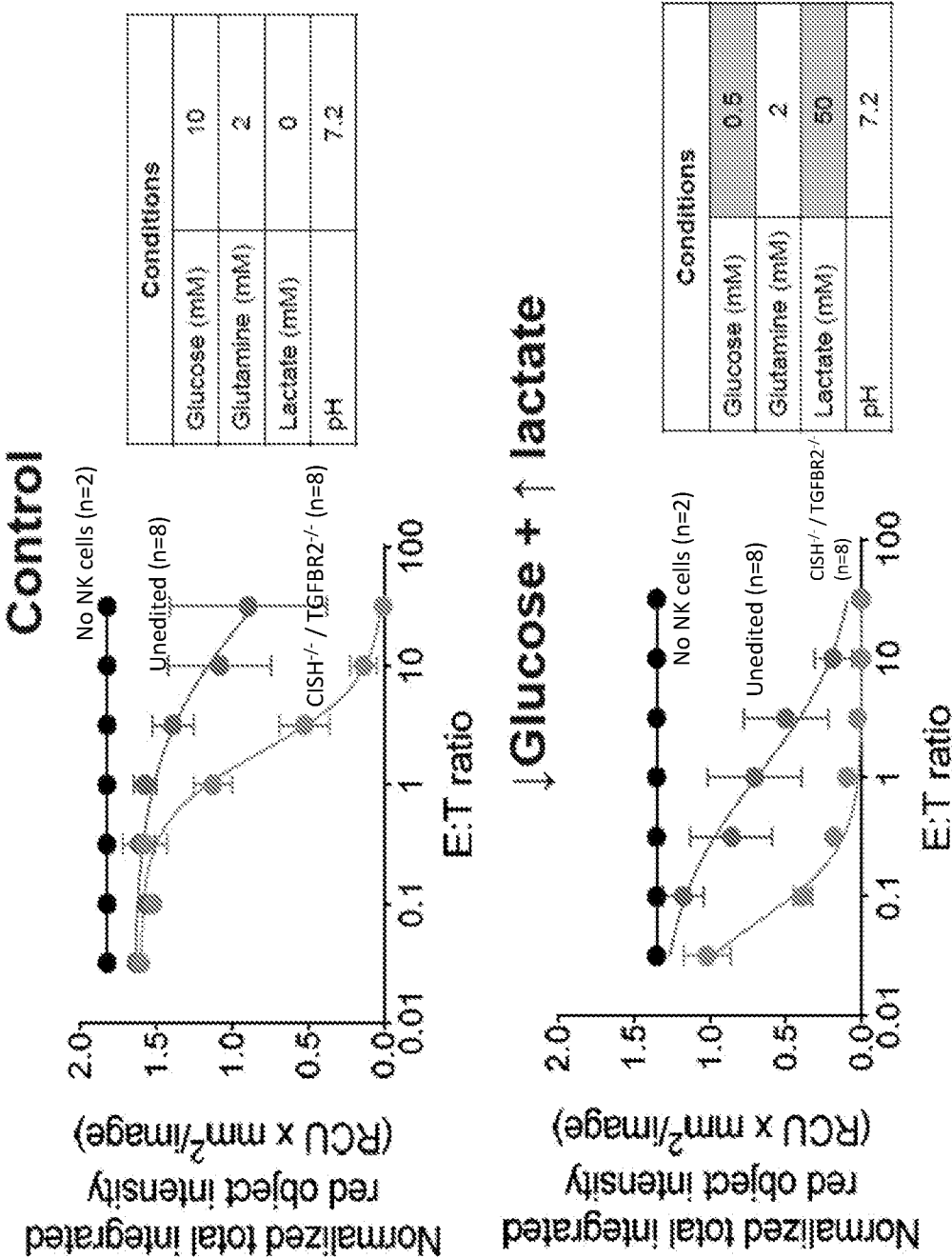


Fig. 10E

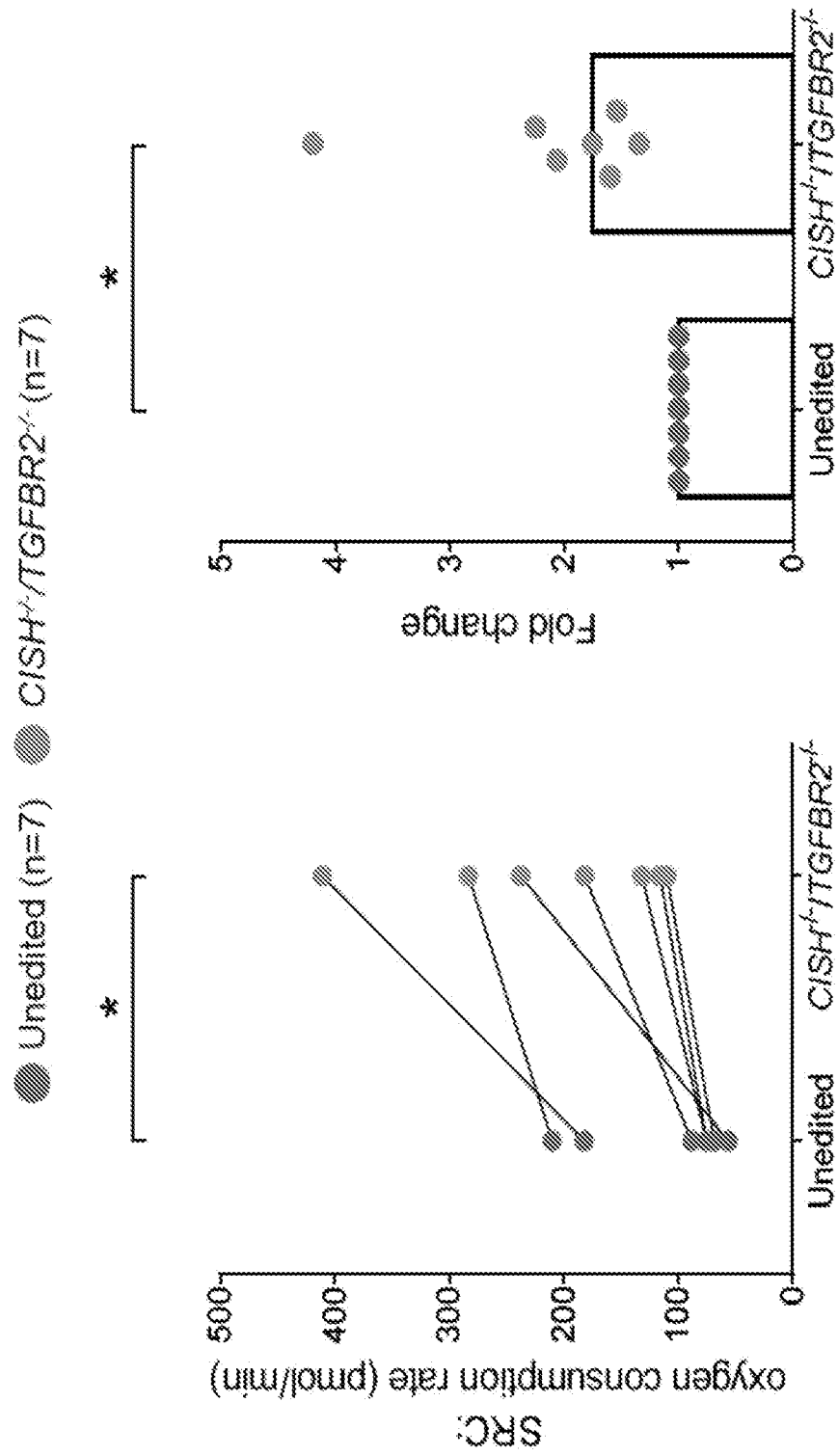


Fig. 11A

Increased cytotoxicity
in the absence of TGF-β

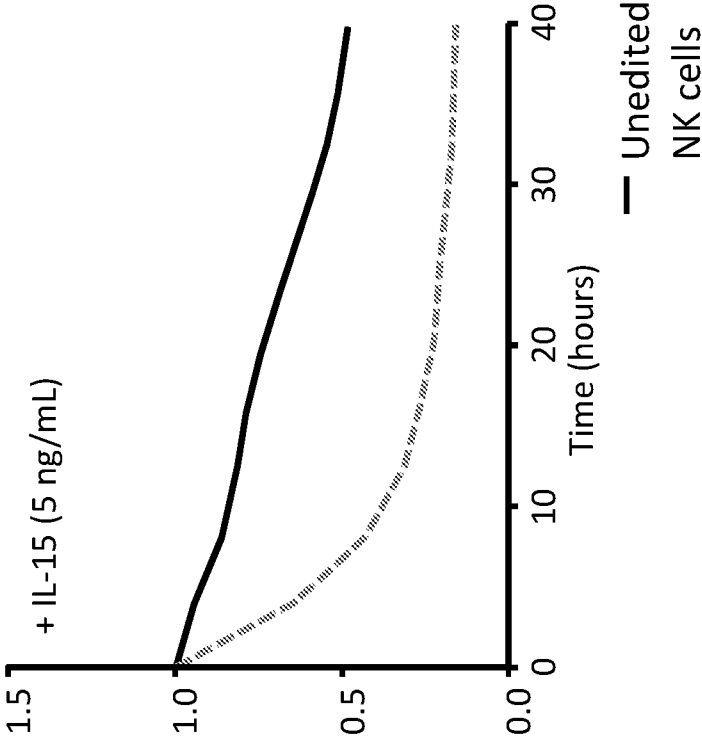


Fig. 11B

Increased cytotoxicity
in the presence of TGF-β

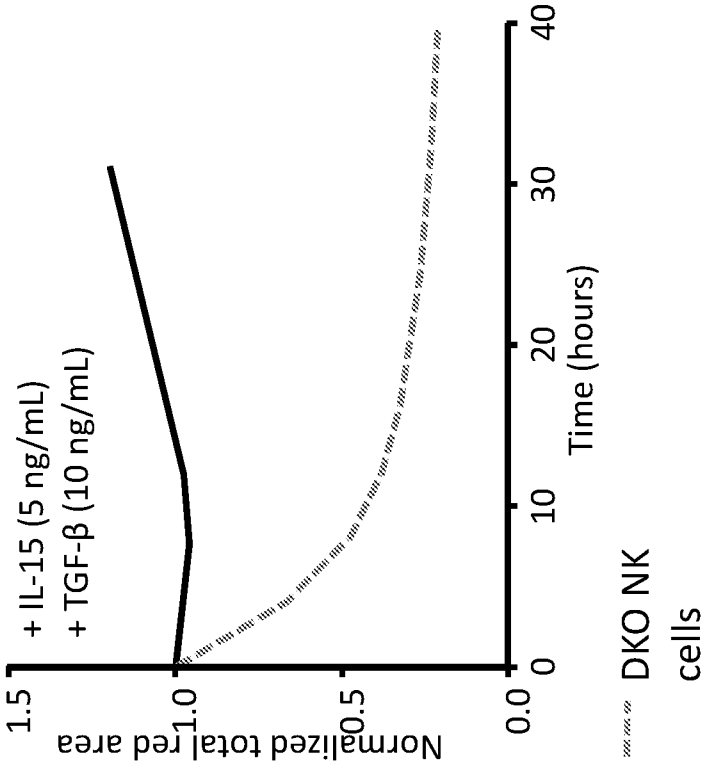
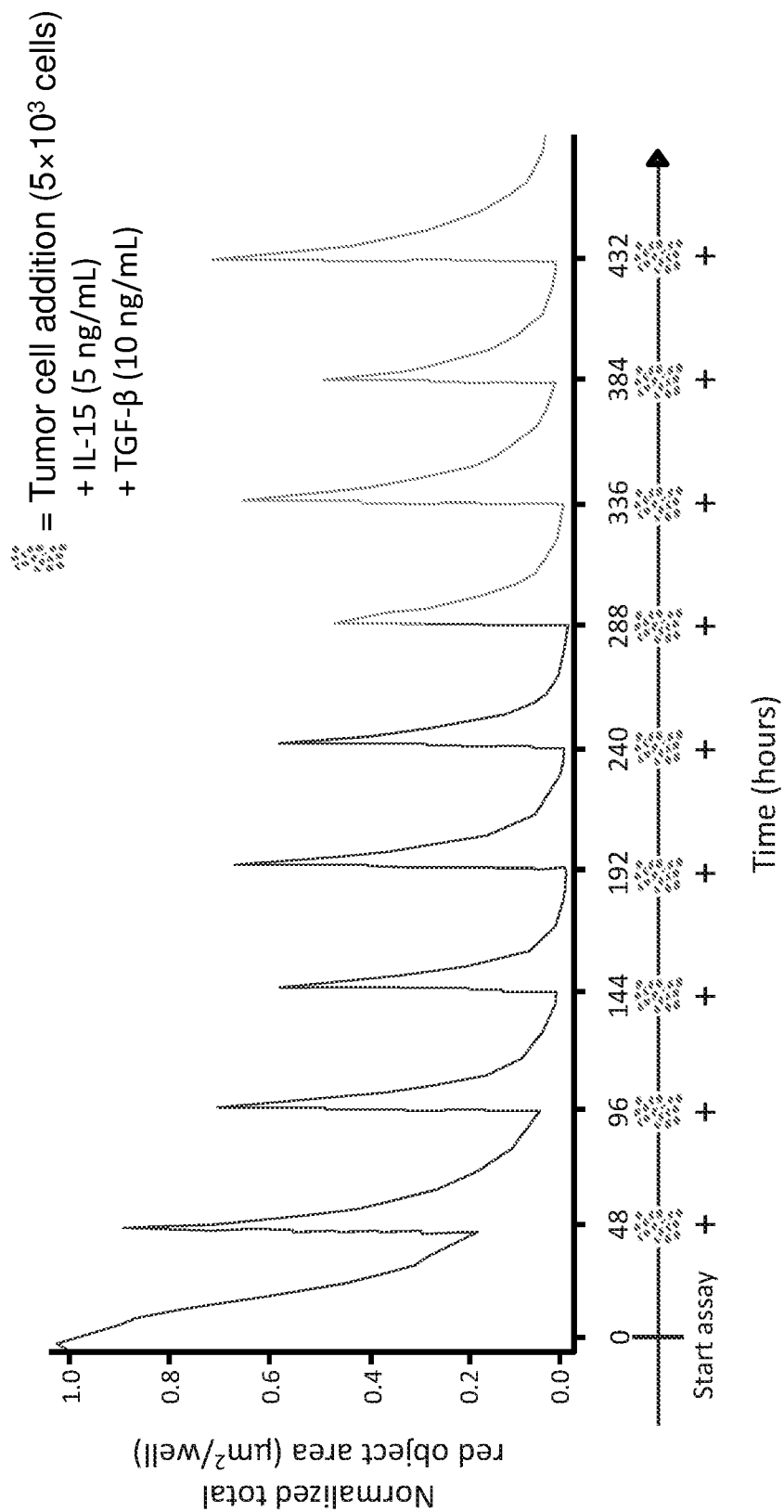


Fig. 12



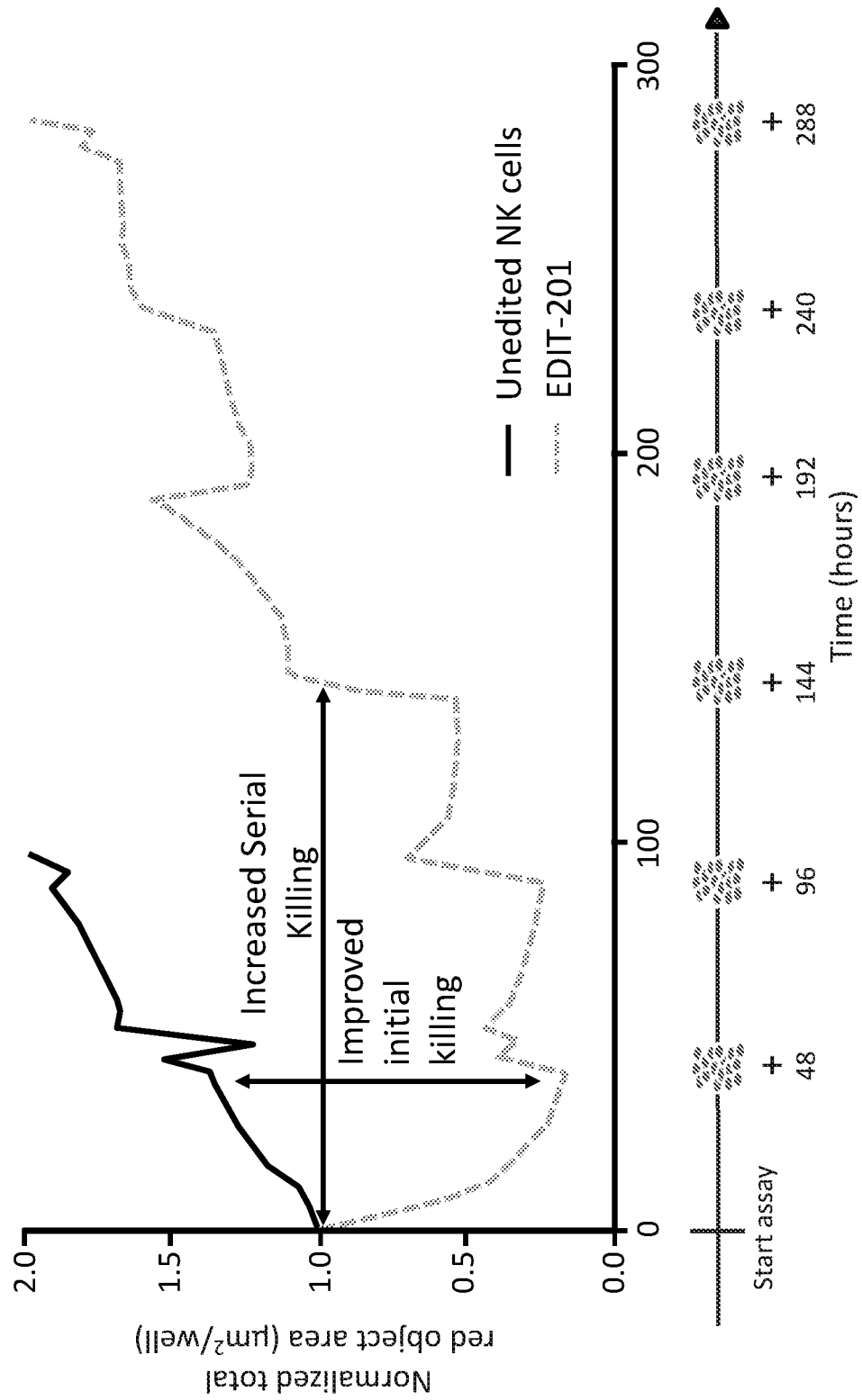


Fig. 13

Fig. 14

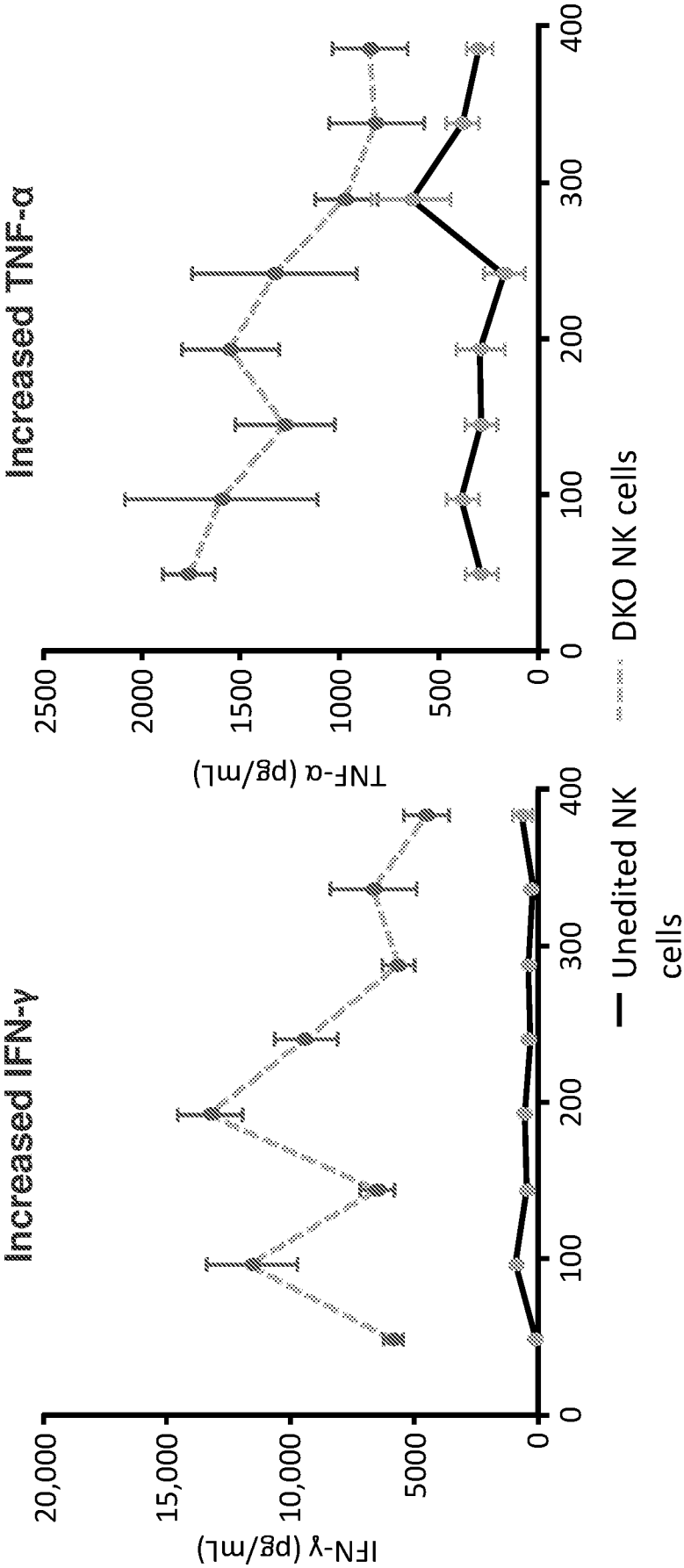


Fig. 15A

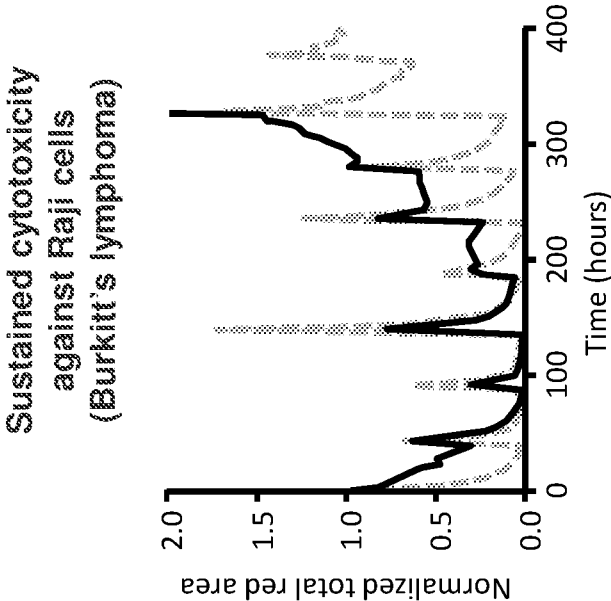


Fig. 15B

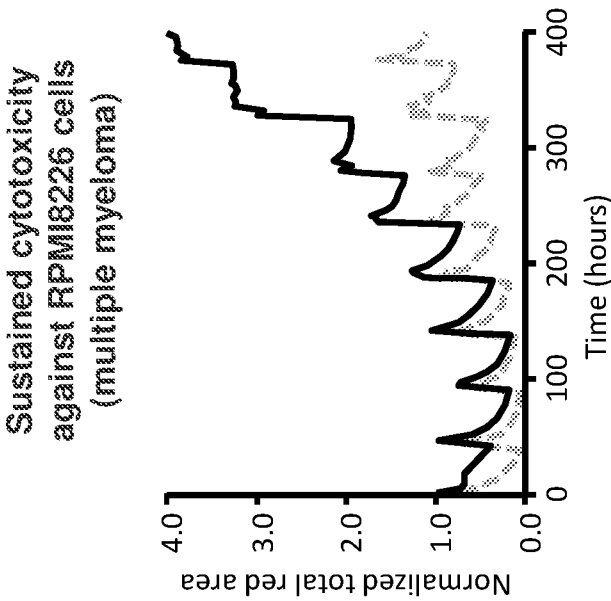
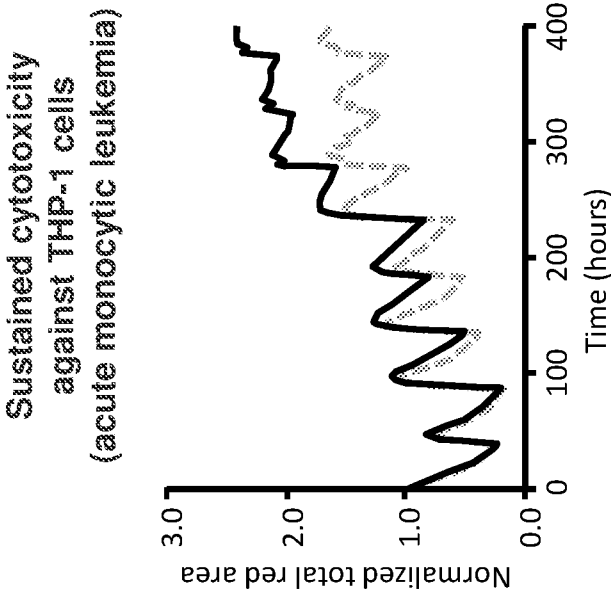


Fig. 15C



— Unedited NK cells
..... DKO NK cells