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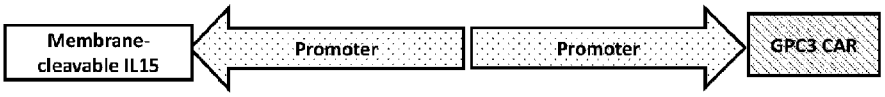

FIG. 11

(57) Abstract: Described herein are immunoresponsive cells engineered to express cytokines, chimeric receptors, and synthetic transcription factor systems. Also described herein are nucleic acids, cells, and methods directed to the same.

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ARMED CHIMERIC RECEPTORS AND METHODS OF USE THEREOF

CROSS REFERENCE

This application claims the benefit of U.S. Provisional Patent Application No. 63/211,468, filed June 16, 2021, and U.S. Provisional Patent Application No. 63/305,155, filed January 31, 2022, both of which are hereby incorporated by reference in their entirety for all purposes.

BACKGROUND

Cell-based therapy platforms provide promising avenues for treating a variety of diseases. One such promising platform is CAR-T based therapies in the treatment of cancer. Given their promise, improvements in cell-based therapies are needed. An active area of exploration is engineering cell-based therapies to produce and/or secrete effector molecules such as cytokines, a process referred to as armoring, that enhance the cell-based therapy. For example, unarmored CAR-T therapies have poor efficacy in solid tumors and armoring can impact the entire cancer immunity cycle and boost the activity of CAR-T. However, uncontrolled or unregulated armoring strategies can have negative impacts on treatment, such as off-target effects and toxicity in subjects. Thus, additional methods of controlling and regulating the armoring of cell-based therapies, such as regulating production and/or secretion of payload effector molecules, are required.

SUMMARY

Provided herein, in some embodiments, is a cell-based therapy platform involving regulated armoring of the cell-based therapy, such as regulated secretion of payload effector molecules. Also provided herein, in some embodiments, is a combinatorial cell-based immunotherapy involving regulated armoring for the targeted treatment of cancer, such as ovarian cancer, breast cancer, colon cancer, lung cancer, and pancreatic cancer.

The therapy provided herein, however, can limit systemic toxicity of armoring. For example, the immunotherapy provided herein can be tumor-specific and effective while limiting systemic toxicity and/or other off-target effects due to armoring. These therapies deliver proteins of interest, such as immunomodulatory effector molecules, in a regulated manner, including regulation of secretion kinetics, cell state specificity, and cell or tissue specificity. The design of the delivery vehicle is optimized to improve overall function in cell-based therapies, such as

cancer therapy, including, but not limited to, optimization of the membrane-cleavage sites, promoters, linkers, signal peptides, delivery methods, combination, regulation, and order of the immunomodulatory effector molecules.

Non-limiting examples of effector molecules encompassed by the present disclosure include cytokines, antibodies, chemokines, nucleotides, peptides, enzymes, and oncolytic viruses. For example, cells may be engineered to express and secrete in a regulated manner at least one, two, three or more of the following effector molecules: IL-12, IL-16, IFN-β, IFN-γ, IL-2, IL-15, IL-7, IL-36γ, IL-18, IL-1β, IL-21, OX40-ligand, CD40L, anti-PD-1 antibodies, anti-PD-L1 antibodies, anti-CTLA-4 antibodies, anti-TGFβ antibodies, anti-TNFR2, MIP1α (CCL3), MIP1β (CCL5), CCL21, CpG oligodeoxynucleotides, and anti-tumor peptides (*e.g.*, anti-microbial peptides having anti-tumor activity, see, *e.g.*, Gaspar, D. *et al. Front Microbiol.* 2013; 4: 294; Chu, H. *et al.* PLoS One. 2015; 10(5): e0126390, and website:aps.unmc.edu/AP/main.php).

Provide for herein is an immunoresponsive cell comprising: (a) a first engineered nucleic acid comprising a first expression cassette comprising a first promoter operably linked to a first, exogenous polynucleotide sequence encoding a first cytokine, and a second expression cassette comprising a second promoter operably linked to a second exogenous polynucleotide sequence encoding a chimeric antigen receptor (CAR) that binds to GPC3; and (b) a second engineered nucleic acid comprising a third expression cassette comprising a synthetic transcription factor-responsive promoter operably linked to a third exogenous polynucleotide sequence encoding a second cytokine, and a fourth expression cassette comprising a fourth promoter operably linked to a fourth exogenous polynucleotide sequence encoding an activation-conditional control polypeptide (ACP), wherein the ACP comprises a synthetic transcription factor comprising a DNA-binding domain and a transcriptional effector domain, wherein the ACP is capable of inducing expression of the third exogenous polynucleotide sequence by binding to the ACP-responsive promoter, wherein at least one of the first exogenous polynucleotide sequence and the third exogenous polynucleotide sequence encodes a membrane-cleavable chimeric protein, oriented from N-terminal to C-terminal, having the formula: **S – C – MT** or **MT – C – S** wherein S comprises a secretable effector molecule comprising the first and/or second cytokine, C comprises a protease cleavage site, and MT comprises a cell membrane tethering domain, and wherein **S – C – MT** or **MT – C – C** is configured to be expressed as a single polypeptide.

In some aspects, provided herein is an engineered nucleic acid comprising: a first expression cassette comprising a first promoter operably linked to a first, exogenous polynucleotide sequence encoding IL15, and a second expression cassette comprising a second

promoter operably linked to a second exogenous polynucleotide sequence encoding a chimeric antigen receptor (CAR) that binds to GPC3, wherein the first exogenous polynucleotide sequence encodes a membrane-cleavable chimeric protein, oriented from N-terminal to C-terminal, having the formula: S – C – MT or MT – C – S wherein S comprises a secretable effector molecule comprising the IL15, C comprises a protease cleavage site, and MT comprises a cell membrane tethering domain, and wherein S – C – MT or MT – C – S is configured to be expressed as a single polypeptide.

In another aspect, provided herein is an engineered nucleic acid comprising: a first expression cassette comprising a synthetic transcription factor-responsive promoter operably linked to a first exogenous polynucleotide sequence encoding an IL12p70 fusion protein, and a second expression cassette comprising a second promoter operably linked to a second exogenous polynucleotide sequence encoding an activation-conditional control polypeptide (ACP), wherein the ACP comprises a synthetic transcription factor comprising a DNA-binding domain and a transcriptional effector domain, wherein the ACP is capable of inducing expression of the first exogenous polynucleotide sequence by binding to the ACP-responsive promoter, wherein the first exogenous polynucleotide sequence encodes a membrane-cleavable chimeric protein, oriented from N-terminal to C-terminal, having the formula: S – C – MT or MT – C – S

wherein S comprises a secretable effector molecule comprising the IL12p70 fusion protein, C comprises a protease cleavage site, and MT comprises a cell membrane tethering domain, and wherein S – C – MT or MT – C – S is configured to be expressed as a single polypeptide.

In some aspects, the first expression cassette is configured to be transcribed in an opposite orientation relative to transcription of the second expression cassette. In some aspects, the first expression cassette and the second expression cassette are oriented within the first engineered nucleic acid in a head-to-head directionality. In some aspects, the first expression cassette is configured to be transcribed in a same orientation relative to the transcription of the second expression cassette. In some aspects, the first expression cassette and the second expression cassette are oriented within the first engineered nucleic acid in a head-to-tail directionality.

In another aspect, provided herein is an engineered nucleic acid comprising: (a) a first engineered nucleic acid comprising a first expression cassette comprising a first promoter operably linked to a first exogenous polynucleotide sequence encoding a chimeric antigen receptor (CAR) that binds to GPC3 and a second exogenous polynucleotide sequence encoding a first cytokine; and (b) a second engineered nucleic acid comprising a second expression cassette comprising a synthetic transcription factor-responsive promoter operably linked to a third

exogenous polynucleotide sequence encoding a second cytokine, and a third expression cassette comprising a third promoter operably linked to fourth exogenous polynucleotide sequence encoding an activation-conditional control polypeptide (ACP), wherein the ACP comprises a synthetic transcription factor comprising a DNA-binding domain and a transcriptional effector domain, wherein the ACP is capable of inducing expression of the third exogenous polynucleotide sequence by binding to the ACP-responsive promoter, wherein at least one of the second exogenous polynucleotide sequence and the third exogenous polynucleotide sequence encodes a membrane-cleavable chimeric protein, oriented from N-terminal to C-terminal, having the formula: S – C – MT or MT – C – S wherein S comprises a secretable effector molecule comprising the first and/or second cytokine, C comprises a protease cleavage site, and MT comprises a cell membrane tethering domain, and wherein S – C – MT or MT – C – S is configured to be expressed as a single polypeptide. In some aspects, transcription of the second expression cassette is oriented in the opposite direction relative to transcription of the third expression cassette within the first engineered nucleic acid. In some aspects, the second expression cassette and the third expression cassette are oriented within the second engineered nucleic acid in a head-to-head directionality.

In some aspects, the first promoter comprises a constitutive promoter, an inducible promoter, or a synthetic promoter. In some aspects, the first promoter is a constitutive promoter selected from the group consisting of: CAG, HLP, CMV, EFS, SFFV, SV40, MND, PGK, UbC, hEF1aV1, hCAGG, hEF1aV2, hACTb, heIF4A1, hGAPDH, hGRP78, hGRP94, hHSP70, hKINb, and hUBIb.

In some aspects, the second promoter comprises a constitutive promoter, an inducible promoter, or a synthetic promoter. In some aspects, the second promoter is a constitutive promoter selected from the group consisting of: CAG, HLP, CMV, EFS, SFFV, SV40, MND, PGK, UbC, hEF1aV1, hCAGG, hEF1aV2, hACTb, heIF4A1, hGAPDH, hGRP78, hGRP94, hHSP70, hKINb, and hUBIb.

In some aspects, the third expression cassette is configured to be transcribed in an opposite orientation relative to transcription of the fourth expression cassette within the second engineered nucleic acid. In some aspects, the third expression cassette and the fourth expression cassette are oriented within the second engineered nucleic acid in a head-to-head directionality. In some aspects, the third expression cassette and the fourth expression cassette are oriented within the second engineered nucleic acid in a tail-to-tail directionality.

In some aspects, the fourth promoter comprises a constitutive promoter, an inducible promoter, or a synthetic promoter. In some aspects, the fourth promoter is a constitutive promoter selected from the group consisting of: CAG, HLP, CMV, EFS, SFFV, SV40, MND,

PGK, UbC, hEF1aV1, hCAGG, hEF1aV2, hACTb, heIF4A1, hGAPDH, hGRP78, hGRP94, hHSP70, hKINb, and hUBIb.

Also provided herein is an immunoresponsive cell comprising: a first engineered nucleic acid comprising a first expression cassette comprising a first promoter operably linked to a first exogenous polynucleotide sequence encoding a first cytokine and a second exogenous polynucleotide sequence encoding a chimeric antigen receptor (CAR) that binds to GPC3, and a second expression cassette comprising a synthetic transcription factor-responsive promoter operably linked to a third exogenous polynucleotide sequence encoding a second cytokine; and a second engineered nucleic acid comprising a third expression cassette comprising a third promoter operably linked to fourth exogenous polynucleotide sequence encoding an activation-conditional control polypeptide (ACP), wherein the ACP comprises a synthetic transcription factor comprising a DNA-binding domain and a transcriptional effector domain, wherein the ACP is capable of inducing expression of the third exogenous polynucleotide sequence by binding to the ACP-responsive promoter, wherein the ACP comprises a synthetic transcription factor, wherein at least one of the first exogenous polynucleotide sequence and the third exogenous polynucleotide sequence encodes a membrane-cleavable chimeric protein, oriented from N-terminal to C-terminal, having the formula: **S – C – MT** or **MT – C – S** wherein S comprises a secretable effector molecule comprising the first and/or second cytokine, C comprises a protease cleavage site, and MT comprises a cell membrane tethering domain, and wherein **S – C – MT** or **MT – C – S** is configured to be expressed as a single polypeptide.

In some aspects, transcription of the first expression cassette is oriented in the opposite direction relative to transcription of the second expression cassette within the first engineered nucleic acid. In some aspects, the first expression cassette and the second expression cassette are oriented within the first engineered nucleic acid in a head-to-head directionality. In some aspects, the first expression cassette is configured to be transcribed in a same orientation relative to transcription of the second expression cassette. In some aspects, the first expression cassette and the second expression cassette are oriented within the first engineered nucleic acid in a head-to-tail directionality.

In some aspects, the first promoter comprises a constitutive promoter, an inducible promoter, or a synthetic promoter. In some aspects, the first promoter is a constitutive promoter selected from the group consisting of: CAG, HLP, CMV, EFS, SFFV, SV40, MND, PGK, UbC, hEF1aV1, hCAGG, hEF1aV2, hACTb, heIF4A1, hGAPDH, hGRP78, hGRP94, hHSP70, hKINb, and hUBIb.

In some aspects, the first exogenous polynucleotide sequence and the second exogenous polynucleotide sequence are separated by a linker polynucleotide sequence. In some aspects, the

linker polynucleotide sequence is operably associated with the translation of the first cytokine and the CAR as separate polypeptides. In some aspects, the linker polynucleotide sequence encodes one or more 2A ribosome skipping elements. In some aspects, the one or more 2A ribosome skipping elements are each selected from the group consisting of: P2A, T2A, E2A, and F2A. In some aspects, the one or more 2A ribosome skipping elements comprises an E2A/T2A. In some embodiments, the E2A/T2A comprises the amino acid sequence of SEQ ID NO: 281. In some aspects, the linker polynucleotide sequence encodes an Internal Ribosome Entry Site (IRES). In some aspects, the linker polynucleotide sequence encodes a cleavable polypeptide. In some aspects, the cleavable polypeptide comprises a furin polypeptide sequence.

In some aspects, the third promoter comprises a constitutive promoter, an inducible promoter, or a synthetic promoter. In some aspects, the third promoter is a constitutive promoter selected from the group consisting of: CAG, HLP, CMV, EFS, SFFV, SV40, MND, PGK, UbC, hEF1aV1, hCAGG, hEF1aV2, hACTb, heIF4A1, hGAPDH, hGRP78, hGRP94, hHSP70, hKINb, and hUBIb.

In some aspects, the first cytokine is IL-15. In some embodiments, the IL-15 comprises the amino acid sequence of SEQ ID NO: 285.

In some aspects, the second cytokine is selected from the group consisting of: IL12, an IL12p70 fusion protein, IL18, and IL21. In some aspects, the second cytokine is the IL12p70 fusion protein. In some embodiments, the IL12p70 fusion protein comprises the amino acid sequence of SEQ ID NO: 293.

In some aspects, the first cytokine is IL12 or an IL12p70 fusion protein. In some aspects, the second cytokine is selected from the group consisting of: IL15, IL18, and IL21.

In some aspects, the protease cleavage site is selected from the group consisting of: a Type 1 transmembrane protease cleavage site, a Type II transmembrane protease cleavage site, a GPI anchored protease cleavage site, an ADAM8 protease cleavage site, an ADAM9 protease cleavage site, an ADAM10 protease cleavage site, an ADAM12 protease cleavage site, an ADAM15 protease cleavage site, an ADAM17 protease cleavage site, an ADAM19 protease cleavage site, an ADAM20 protease cleavage site, an ADAM21 protease cleavage site, an ADAM28 protease cleavage site, an ADAM30 protease cleavage site, an ADAM33 protease cleavage site, a BACE1 protease cleavage site, a BACE2 protease cleavage site, a SIP protease cleavage site, an MT1-MMP protease cleavage site, an MT3-MMP protease cleavage site, an MT5-MMP protease cleavage site, a furin protease cleavage site, a PCSK7 protease cleavage site, a matriptase protease cleavage site, a matriptase-2 protease cleavage site, an MMP9 protease cleavage site, and an NS3 protease cleavage site. In some aspects, the protease cleavage site is cleavable by a protease selected from the group consisting of: a Type 1 transmembrane

protease, a Type II transmembrane protease, a GPI anchored protease, an ADAM8 protease, an ADAM9 protease, an ADAM10 protease, an ADAM12 protease, an ADAM15 protease, an ADAM17 protease, an ADAM19 protease, an ADAM20 protease, an ADAM21 protease, an ADAM28 protease, an ADAM30 protease, an ADAM33 protease, a BACE1 protease, a BACE2 protease, a SIP protease, an MT1-MMP protease, an MT3-MMP protease, an MT5-MMP protease, a furin protease, a PCSK7 protease, a matriptase protease, a matriptase-2 protease, an MMP9 protease, and an NS3 protease.

In some aspects, the protease cleavage site is cleavable by an ADAM17 protease. In some aspects, the protease cleavage site comprises a first region having the amino acid sequence of PRAE (SEQ ID NO: 176). In some aspects, the protease cleavage site comprises a second region having the amino acid sequence of KGG (SEQ ID NO: 177). In some aspects, the first region is located N-terminal to the second region. In some aspects, the protease cleavage site comprises the amino acid sequence of PRAEX1X2KGG (SEQ ID NO: 178), wherein X1 is A, Y, P, S, or F, and wherein X2 is V, L, S, I, Y, T, or A. In some aspects, the protease cleavage site comprises the amino acid sequence of PRAEAVKGG (SEQ ID NO: 179). In some aspects, the protease cleavage site comprises the amino acid sequence of PRAEALKGG (SEQ ID NO: 180). In some aspects, the protease cleavage site comprises the amino acid sequence of PRAEYSKGG (SEQ ID NO: 181). In some aspects, the protease cleavage site comprises the amino acid sequence of PRAEPIKGG (SEQ ID NO: 182). In some aspects, the protease cleavage site comprises the amino acid sequence of PRAEAYKGG (SEQ ID NO: 183). In some aspects, the protease cleavage site comprises the amino acid sequence of PRAESSKGG (SEQ ID NO: 184). In some aspects, the protease cleavage site comprises the amino acid sequence of PRAEFTKGG (SEQ ID NO: 185). In some aspects, the protease cleavage site comprises the amino acid sequence of PRAEAAKGG (SEQ ID NO: 186). In some aspects, the protease cleavage site comprises the amino acid sequence of DEPHYSQRR (SEQ ID NO: 187). In some aspects, the protease cleavage site comprises the amino acid sequence of PPLGPIFNPG (SEQ ID NO: 188). In some aspects, the protease cleavage site comprises the amino acid sequence of PLAQAYRSS (SEQ ID NO: 189). In some aspects, the protease cleavage site comprises the amino acid sequence of TPIDSSFNPD (SEQ ID NO: 190). In some aspects, the protease cleavage site comprises the amino acid sequence of VTPEPIFSLI (SEQ ID NO: 191). In some aspects, the protease cleavage site comprises the amino acid sequence of ITQGLAVSTISSFF (SEQ ID NO: 198). In some aspects, the protease cleavage site is comprised within a peptide linker. In some aspects, the protease cleavage site is N-terminal to a peptide linker. In some embodiments, the peptide linker comprises a glycine-serine (GS) linker.

In some aspects, the cell membrane tethering domain comprises a transmembrane-intracellular domain or a transmembrane domain. In some aspects, the transmembrane-intracellular domain and/or transmembrane domain is derived from PDGFR-beta, CD8, CD28, CD3zeta-chain, CD4, 4-1BB, OX40, ICOS, CTLA-4, PD-1, LAG-3, 2B4, LNGFR, NKG2D, EpoR, TNFR2, B7-1, or BTLA. In some aspects, the transmembrane-intracellular domain and/or transmembrane domain is derived from B7-1. In some embodiments, the transmembrane-intracellular domain and/or transmembrane domain comprises the amino acid sequence of SEQ ID NO: 219. In some aspects, the cell membrane tethering domain comprises a cell surface receptor, or a cell membrane-bound portion thereof.

In some aspects, the cell membrane tethering domain comprises a post-translational modification tag, or motif capable of post-translational modification to modify the chimeric protein to include a post-translational modification tag, wherein the post-translational modification tag is capable of association with a cell membrane. In some aspects, the post-translational modification tag comprises a lipid-anchor domain, optionally wherein the lipid-anchor domain is selected from the group consisting of: a GPI lipid-anchor, a myristoylation tag, and a palmitoylation tag.

In some aspects, when expressed in a cell, the secretable effector molecule (*e.g.*, any of the cytokines described herein) is tethered to a cell membrane of the cell. In some aspects, when expressed in a cell expressing a protease capable of cleaving the protease cleavage site, the secretable effector molecule is released from the cell membrane. In some aspects, the protease is expressed on the cell membrane of the cell.

In some aspects, the protease expressed on the cell membrane is endogenous to the cell. In some aspects, the protease is selected from the group consisting of: a Type 1 transmembrane protease, a Type II transmembrane protease, a GPI anchored protease, an ADAM8 protease, an ADAM9 protease, an ADAM10 protease, an ADAM12 protease, an ADAM15 protease, an ADAM17 protease, an ADAM19 protease, an ADAM20 protease, an ADAM21 protease, an ADAM28 protease, an ADAM30 protease, an ADAM33 protease, a BACE1 protease, a BACE2 protease, a SIP protease, an MT1-MMP protease, an MT3-MMP protease, an MT5-MMP protease, a furin protease, a PCSK7 protease, a matriptase protease, a matriptase-2 protease, and an MMP9 protease. In some aspects, the protease is an ADAM17 protease.

In some aspects, the protease expressed on the cell membrane is heterologous to the cell. In some aspects, the protease is hepatitis C virus (HCV) nonstructural protein 3 (NS3). In some aspects, the protease cleavage site comprises an NS3 protease cleavage site. In some aspects, the NS3 protease cleavage site comprises a NS3/NS4A, a NS4A/NS4B, a NS4B/NS5A, or a NS5A/NS5B junction cleavage site. In some aspects, the protease can be repressed by a protease

inhibitor. In some aspects, the protease inhibitor is selected from the group consisting of: simeprevir, danoprevir, asunaprevir, ciluprevir, boceprevir, sovaprevir, paritaprevir, telaprevir, grazoprevir, glecaprevir, and voxiloprevir. In some aspects, expression and/or localization of the protease is capable of regulation. In some aspects, the expression and/or localization is regulated by a cell state of the cell.

In some aspects, the first exogenous polynucleotide sequence encodes a membrane-cleavable chimeric protein. In some aspects, the first exogenous polynucleotide sequence further comprises a polynucleotide sequence encoding a secretion signal peptide. In some aspects, the second exogenous polynucleotide sequence encodes a membrane-cleavable chimeric protein. In some aspects, the second exogenous polynucleotide sequence further comprises a polynucleotide sequence encoding a secretion signal peptide. In some aspects, the secretion signal peptide is derived from a protein selected from the group consisting of: IL-12, Trypsinogen-2, Gaussia Luciferase, CD5, IgKVII, VSV-G, prolactin, serum albumin preproprotein, azurocidin preproprotein, osteonectin (BM40), CD33, IL-6, IL-8, CCL2, TIMP2, VEGFB, osteoprotegerin, serpin-E1, GROalpha, CXCL12, IL-21, CD8, GMCSFRa, NKG2D, and IgE. In some aspects, the secretion signal peptide is derived from GMCSFRa. In some aspects, the secretion signal peptide comprises the amino acid sequence of SEQ ID NO: 216. In some aspects, wherein the secretion signal peptide is derived from IgE. In some embodiments, the secretion signal peptide comprises the amino acid sequence of SEQ ID NO: 218. In some aspects, the third exogenous polynucleotide sequence further comprises a polynucleotide sequence encoding a secretion signal peptide. In some aspects, the secretion signal peptide is operably associated with the second cytokine. In some aspects, the secretion signal peptide is native to the second cytokine. In some aspects, the secretion signal peptide is non-native to the second cytokine.

In some aspects, the third exogenous polynucleotide sequence encodes a membrane-cleavable chimeric protein. In some aspects, the first expression cassette further comprises a polynucleotide sequence encoding a secretion signal peptide. In some aspects, the secretion signal peptide is operably associated with the first cytokine. In some aspects, the secretion signal peptide is native to the first cytokine. In some aspects, the secretion signal peptide is non-native to the first cytokine.

In some aspects, the first exogenous polynucleotide sequence encodes a first membrane-cleavable chimeric protein and the third exogenous polynucleotide sequence encodes a second membrane-cleavable chimeric protein. In some aspects, the second exogenous polynucleotide sequence encodes a first membrane-cleavable chimeric protein and the third exogenous polynucleotide sequence encodes a second membrane-cleavable chimeric protein.

In some aspects, the engineered nucleic acid is a single-stranded or double-stranded nucleic acid selected from the group consisting of: a DNA, cDNA, an RNA, an mRNA, and a naked plasmid.

In some aspects, the exogenous polynucleotide sequences encoded by the expression cassette further comprise a 3'untranslated region (UTR) comprising an mRNA-destabilizing element that is operably linked to the exogenous polynucleotide sequence. In some aspects, the mRNA-destabilizing element comprises an AU-rich element and/or a stem-loop destabilizing element (SLDE). In some aspects, the mRNA-destabilizing element comprises an AU-rich element. In some aspects, the AU-rich element includes at least two overlapping motifs of the sequence ATTTA (SEQ ID NO: 209). In some aspects, the AU-rich element comprises ATTTATTTATTTATTTATTTA (SEQ ID NO: 210). In some aspects, the mRNA-destabilizing element comprises a stem-loop destabilizing element (SLDE). In some aspects, the SLDE comprises CTGTTTAATATTTAAACAG (SEQ ID NO: 211). In some aspects, the mRNA-destabilizing element comprises at least one AU-rich element and at least one SLDE. In some aspects, the AuSLDE sequence comprises ATTTATTTATTTATTTATTTAacatcggttccCTGTTTAATATTTAAACAG (SEQ ID NO: 212). In some aspects, the mRNA-destabilizing element comprises a 2X AuSLDE. In some aspects, the 2X AuSLDE sequence is provided as ATTTATTTATTTATTTATTTAacatcggttccCTGTTTAATATTTAAACAGtgcggtaagcATTTATTTATTTATTTATTTAacatcggttccCTGTTTAATATTTAAACAG (SEQ ID NO: 213).

In some aspects, the CAR comprises an antigen-binding domain comprising a heavy chain variable (VH) region and a light chain variable (VL) region, wherein the VH comprises: a heavy chain complementarity determining region 1 (CDR-H1) having the amino acid sequence of KNAMN (SEQ ID NO: 199), a heavy chain complementarity determining region 2 (CDR-H2) having the amino acid sequence of RIRNKTNNYATYYADSVKA (SEQ ID NO: 200), and a heavy chain complementarity determining region 3 (CDR-H3) having the amino acid sequence of GNSFAY (SEQ ID NO: 201), and wherein the VL comprises: a light chain complementarity determining region 1 (CDR-L1) having the amino acid sequence of KSSQSLLYSSNQKNYLA (SEQ ID NO: 202), a light chain complementarity determining region 2 (CDR-L2) having the amino acid sequence of WASSRES (SEQ ID NO: 203), and a light chain complementarity determining region 3 (CDR-L3) having the amino acid sequence of QQYYNYPLT (SEQ ID NO: 204).

In some aspects, the VH region comprises an amino acid sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to the amino acid sequence of

EVQLVETGGGMVQPEGSLKLSCAASGFTFNKNAMNWVRQAPGKGLEWVARIRNKTN NYATYYADSVKARFTISRDDSQSMLYLQMNNLKIEDTAMYYCVAGNSFA YWGQGTLVTVSA (SEQ ID NO: 205) or EVQLVESGGGLVQPGGSLRLSCAASGFTFNKNAMNWVRQAPGKGLEWVGRIRNKTNN YATYYADSVKARFTISRDDSKNSLYLQMNSLKTEDTAVYYCVAGNSFAYWGQGTLVT VSA (SEQ ID NO: 206). In some embodiments, the VH region comprises an amino acid sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to the amino acid sequence of SEQ ID NO: 206.

In some aspects, the VL region comprises an amino acid sequence with at least 90 %, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to the amino acid sequence of DIVMSQSPSSLVVSIGEKVTMTCKSSQSLLYSSNQKNYLAWYQQKPGQSPKLLIYWASS RESGVPDRFTGSGSGTDFTLTISSVKAEDLAVYYCQQYYNYPLTFGAGTKLELK (SEQ ID NO: 207), or DIVMTQSPDSLAVSLGERATINCKSSQSLLYSSNQKNYLAWYQQKPGQPPKLLIYWASS RESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQQYYNYPLTFGQGTKLEIK (SEQ ID NO: 208). In some embodiments, the VL region comprises an amino acid sequence with at least 90 %, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to the amino acid sequence of SEQ ID NO: 2NO: 208.

In some aspects, the antigen-binding domain comprises a single chain variable fragment (scFv). In some aspects, the VH and VL are separated by a peptide linker. In some aspects, the scFv comprises the structure VH-L-VL or VL-L-VH, wherein VH is the heavy chain variable domain, L is the peptide linker, and VL is the light chain variable domain. In some aspects, the peptide linker comprises a glycine-serine (GS) linker. In some embodiments, the GS linker comprises the amino acid sequence of (GGGGS)3 (SEQ ID NO: 223).

In some aspects, the CAR comprises one or more intracellular signaling domains, and each of the one or more intracellular signaling domains is selected from the group consisting of: a CD3zeta-chain intracellular signaling domain, a CD97 intracellular signaling domain, a CD11a-CD18 intracellular signaling domain, a CD2 intracellular signaling domain, an ICOS intracellular signaling domain, a CD27 intracellular signaling domain, a CD154 intracellular signaling domain, a CD8 intracellular signaling domain, an OX40 intracellular signaling domain, a 4-1BB intracellular signaling domain, a CD28 intracellular signaling domain, a ZAP40 intracellular signaling domain, a CD30 intracellular signaling domain, a GITR

intracellular signaling domain, an HVEM intracellular signaling domain, a DAP10 intracellular signaling domain, a DAP12 intracellular signaling domain, a MyD88 intracellular signaling domain, a 2B4 intracellular signaling domain, a CD16a intracellular signaling domain, a DNAM-1 intracellular signaling domain, a KIR2DS1 intracellular signaling domain, a KIR3DS1 intracellular signaling domain, a NKp44 intracellular signaling domain, a NKp46 intracellular signaling domain, a FceRlg intracellular signaling domain, a NKG2D intracellular signaling domain, and an EAT-2 intracellular signaling domain. In some aspects, the one or more intracellular signaling domains comprises an OX40 intracellular signaling domain. In some aspects, the OX40 intracellular signaling domain comprises the amino acid sequence of SEQ ID NO: 269. In some aspects, the one or more intracellular signaling domains comprises a CD28 intracellular signaling domain. In some aspects, the CD28 intracellular signaling domain comprises the amino acid sequence of SEQ ID NO: 267. In some aspects, the one or more intracellular signaling domains comprises a CD3z intracellular signaling domain. In some aspects, the CD3z intracellular signaling domain comprises an amino acid sequence of SEQ ID NO: 277 or SEQ ID NO: 279.

In some aspects, the CAR comprises a transmembrane domain, and the transmembrane domain is selected from the group consisting of: a CD8 transmembrane domain, a CD28 transmembrane domain a CD3zeta-chain transmembrane domain, a CD4 transmembrane domain, a 4-1BB transmembrane domain, an OX40 transmembrane domain, an ICOS transmembrane domain, a CTLA-4 transmembrane domain, a PD-1 transmembrane domain, a LAG-3 transmembrane domain, a 2B4 transmembrane domain, a BTLA transmembrane domain, an OX40 transmembrane domain, a DAP10 transmembrane domain, a DAP12 transmembrane domain, a CD16a transmembrane domain, a DNAM-1 transmembrane domain, a KIR2DS1 transmembrane domain, a KIR3DS1 transmembrane domain, an NKp44 transmembrane domain, an NKp46 transmembrane domain, an FceRlg transmembrane domain, and an NKG2D transmembrane domain. In some aspects, the transmembrane domain is an OX40 transmembrane domain. In some aspects, the OX40 transmembrane domain comprises the amino acid sequence of SEQ ID NO: 244. In some aspects, the transmembrane domain is a CD8 transmembrane domain. In some aspects, the CD8 transmembrane domain comprises an amino acid sequence of SEQ ID NO: 236 or SEQ ID NO: 242.

In some aspects, the CAR comprises a spacer region between the antigen-binding domain and the transmembrane domain. In some aspects, the spacer region is derived from a protein selected from the group consisting of: CD8, CD28, IgG4, IgG1, LNGFR, PDGFR-beta, and MAG. In some aspects, the spacer region is a CD8 hinge. In some aspects, the CD8 hinge comprises the amino acid sequence of SEQ ID NO: 226 or SEQ ID NO: 228.

In some aspects, the ACP comprises a DNA binding domain and a transcriptional effector domain. In some aspects, the transcriptional effector domain comprises a transcriptional activator domain. In some aspects, the transcriptional activator domain is selected from the group consisting of: a Herpes Simplex Virus Protein 16 (VP16) activation domain; an activation domain comprising four tandem copies of VP16, a VP64 activation domain; a p65 activation domain of NFκB; an Epstein-Barr virus R transactivator (Rta) activation domain; a tripartite activator comprising the VP64, the p65, and the Rta activation domains (VPR activation domain); a histone acetyltransferase (HAT) core domain of the human E1A-associated protein p300 (p300 HAT core activation domain). In some aspects, the transcriptional activator domain comprises a VPR activation domain. In some aspects, the VPR activation domain comprises the amino acid sequence of SEQ ID NO: 325. In some aspects, the transcriptional effector domain comprises a transcriptional repressor domain. In some aspects, the transcriptional repressor domain is selected from the group consisting of: a Krüppel associated box (KRAB) repression domain; a truncated Krüppel associated box (KRAB) repression domain; a Repressor Element Silencing Transcription Factor (REST) repression domain; a WRPW motif of the hairy-related basic helix-loop-helix repressor proteins, the motif is known as a WRPW repression domain; a DNA (cytosine-5)-methyltransferase 3B (DNMT3B) repression domain; and an HP1 alpha chromoshadow repression domain.

In some aspects, the DNA binding domain comprises a zinc finger (ZF) protein domain. In some aspects, the ZF protein domain is modular in design and comprises an array of zinc finger motifs. In some aspects, the ZF protein domain comprises an array of one to ten zinc finger motifs. In some aspect,s the ZF protein domain comprises the amino acid sequence of SEQ ID NO: 320.

In some aspects, the ACP further comprises a repressible protease and one or more cognate cleavage sites of the repressible protease. In some aspects, the repressible protease is hepatitis C virus (HCV) nonstructural protein 3 (NS3). In some aspects, the NS3 protease comprises the amino acid sequence of SEQ ID NO: 321.In some aspects, the cognate cleavage site of the repressible protease comprises an NS3 protease cleavage site. In some aspects, the NS3 protease cleavage site comprises a NS3/NS4A, a NS4A/NS4B, a NS4B/NS5A, or a NS5A/NS5B junction cleavage site. In some aspects, the NS3 protease is repressible by a protease inhibitor. In some aspects, the protease inhibitor is selected from the group consisting of: simeprevir, danoprevir, asunaprevir, ciluprevir, boceprevir, sovaprevir, paritaprevir, telaprevir, grazoprevir, glecaprevir, and voxiloprevir. In some aspects, the protease inhibitor is grazoprevir (GRZ). In some aspects, the ACP further comprises a nuclear localization signal (NLS). In some aspects, the NLS comprises the amino acid sequence of SEQ ID NO: 296.In

some aspects, the one or more cognate cleavage sites of the repressible protease are localized between the DNA binding domain and the transcriptional effector domain.

In some aspects, the ACP further comprises a hormone binding domain of estrogen receptor variant ERT2.

In some aspects, the ACP-responsive promoter is a synthetic promoter. In some aspects, the ACP-responsive promoter comprises an ACP binding domain sequence and a minimal promoter sequence. In some aspects, the ACP binding domain sequence comprises one or more zinc finger binding sites.

In some aspects, the first engineered nucleic acid comprises a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 309. In some aspects, the first engineered nucleic acid comprises a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 326. In some aspects, the first engineered nucleic acid comprises a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 310. In some aspects, the first engineered nucleic acid comprises a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 327. In some aspects, the first engineered nucleic acid comprises a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 314. In some aspects, the first the engineered nucleic acid comprises a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 315. In some aspects, the second engineered nucleic acid comprises a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 317. In some aspects, the second engineered nucleic acid comprises a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 318.

In another aspect, provided herein is an immunoresponsive cell comprising: (a) a first engineered nucleic acid comprising the nucleotide sequence of SEQ ID NO: 310; and (b) a second engineered nucleic acid comprising the nucleotide sequence of SEQ ID NO: 317.

In another aspect, provided herein is an immunoresponsive cell comprising: (a) a first engineered nucleic acid comprising the nucleotide sequence of SEQ ID NO: 327; and (b) a

second engineered nucleic acid comprising the nucleotide sequence of SEQ ID NO: 317.In some aspects, the cell is selected from the group consisting of: a T cell, a CD8+ T cell, a CD4+ T cell, a gamma-delta T cell, a cytotoxic T lymphocyte (CTL), a regulatory T cell, a viral-specific T cell, a Natural Killer T (NKT) cell, a Natural Killer (NK) cell, a B cell, a tumor-infiltrating lymphocyte (TIL), an innate lymphoid cell, a mast cell, an eosinophil, a basophil, a neutrophil, a myeloid cell, a macrophage, a monocyte, a dendritic cell, an erythrocyte, a platelet cell, a human embryonic stem cell (ESC), an ESC-derived cell, a pluripotent stem cell, a mesenchymal stromal cell (MSC), an induced pluripotent stem cell (iPSC), and an iPSC-derived cell. In some aspects, the cell is a Natural Killer (NK) cell. In some aspects, the cell is autologous. In some aspects, the cell is allogeneic.

In some aspects, provided herein is an engineered nucleic acid comprising: a first expression cassette comprising a first promoter operably linked to a first, exogenous polynucleotide sequence encoding IL15, and a second expression cassette comprising a second promoter operably linked to a second exogenous polynucleotide sequence encoding a chimeric antigen receptor (CAR) that binds to GPC3, wherein the first exogenous polynucleotide sequence encodes a membrane-cleavable chimeric protein, oriented from N-terminal to C-terminal, having the formula: S – C – MT or MT – C – S wherein S comprises a secretable effector molecule comprising the IL15, C comprises a protease cleavage site, and MT comprises a cell membrane tethering domain, and wherein S – C – MT or MT – C – S is configured to be expressed as a single polypeptide.

In some aspects,

a. the first expression cassette and the second expression cassette are oriented within the first engineered nucleic acid in a head-to-tail directionality,

b. the first exogenous polynucleotide sequence and the second exogenous polynucleotide sequence are separated by a linker polynucleotide sequence comprising an E2A/T2A ribosome skipping element, and

c. the CAR that binds to GPC3 comprises a CD28 intracellular signaling domain or an OX40 intracellular signaling domain.

In another aspect, provided herein is engineered nucleic acid comprising: a first expression cassette comprising a first promoter operably linked to a first, exogenous polynucleotide sequence encoding a chimeric antigen receptor (CAR) that binds to GPC3 and a second exogenous polynucleotide sequence encoding IL15, wherein the first exogenous polynucleotide sequence encodes a membrane-cleavable chimeric protein, oriented from N-terminal to C-terminal, having the formula: S – C – MT or MT – C – S wherein S comprises a secretable effector molecule comprising the IL15, C comprises a protease cleavage site, and MT

comprises a cell membrane tethering domain, and wherein S – C – MT or MT – C – S is configured to be expressed as a single polypeptide. In some aspects, a. the first exogenous polynucleotide sequence and the second exogenous polynucleotide sequence are separated by a linker polynucleotide sequence comprising an E2A/T2A ribosome skipping element, and b. the CAR that binds to GPC3 comprises a CD28 intracellular signaling domain or an OX40 intracellular signaling domain.

In some aspects, the engineered nucleic acid comprises a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 309. In some aspects, the engineered nucleic acid comprises a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 326. In some aspects, the engineered nucleic acid comprises a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 310. In some aspects, the engineered nucleic acid comprises a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 327. In some aspects, the engineered nucleic acid comprises a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 314. In some aspects, the engineered nucleic acid comprises a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 315.

In another aspect, provided herein is an engineered nucleic acid comprising the nucleotide sequence of SEQ ID NO: 310.

In another aspect, provided herein is an engineered nucleic acid comprising the nucleotide sequence of SEQ ID NO: 327.

In another aspect, provided herein is an engineered nucleic acid comprising: a first expression cassette comprising a synthetic transcription factor-responsive promoter operably linked to a first exogenous polynucleotide sequence encoding an IL12p70 fusion protein, and a second expression cassette comprising a second promoter operably linked to a second exogenous polynucleotide sequence encoding an activation-conditional control polypeptide (ACP), wherein the ACP comprises a synthetic transcription factor comprising a DNA-binding domain and a transcriptional effector domain, wherein the ACP is capable of inducing expression of the first exogenous polynucleotide sequence by binding to the ACP-responsive promoter, wherein the first exogenous polynucleotide sequence encodes a membrane-cleavable

chimeric protein, oriented from N-terminal to C-terminal, having the formula: S – C – MT or MT – C – S wherein

S comprises a secretable effector molecule comprising the IL12p70 fusion protein, C comprises a protease cleavage site, and MT comprises a cell membrane tethering domain, and wherein S – C – MT or MT – C – S is configured to be expressed as a single polypeptide.

In some aspects,

a. the first expression cassette and the second expression cassette are oriented within the first engineered nucleic acid in a head-to-head directionality, and

b. the ACP comprises a DNA binding domain and a transcriptional effector domain, wherein the transcriptional activator domain comprises a VPR activation domain.

In some aspects, the engineered nucleic acid comprises a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 317. In some aspects, the engineered nucleic acid comprises a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 318.

In another aspect, provided herein is engineered nucleic acid comprising the nucleotide sequence of SEQ ID NO: 317.

In another aspect, provided herein is an expression vector comprising any one of the engineered nucleic acids described herein.

In some aspects, provided herein is an immunoresponsive cell comprising the engineered nucleic acid or expression vector of any one of the above aspects.

Also provided herein is a pharmaceutical composition comprising any of the immunoresponsive cells described herein, any one of the engineered nucleic acids described herein, and/or any one of the expression vectors described herein and a pharmaceutically acceptable carrier, pharmaceutically acceptable excipient, or a combination thereof.

Also provided herein is a method of treating a subject in need thereof, the method comprising administering a therapeutically effective dose of any of the immunoresponsive cells described herein, any one of the engineered nucleic acids described herein, any one of the expression vectors described herein, and/or pharmaceutical compositions described herein.

Also provided herein is a method of stimulating a cell-mediated immune response to a tumor cell in a subject, the method comprising administering to a subject having a tumor a therapeutically effective dose of any of the immunoresponsive cells described herein, any one of the engineered nucleic acids described herein, any one of the expression vectors described herein, and/or pharmaceutical compositions described herein.

Also provided herein is a method of reducing tumor volume in a subject, the method comprising administering to a subject having a tumor a composition comprising any of the immunoresponsive cells described herein, any one of the engineered nucleic acids described herein, any one of the expression vectors described herein, and/or pharmaceutical compositions described herein.

Also provided herein is a method of providing an anti-tumor immunity in a subject, the method comprising administering to a subject in need thereof a therapeutically effective dose of any of the immunoresponsive cells described herein, any one of the engineered nucleic acids described herein, any one of the expression vectors described herein, and/or pharmaceutical compositions described herein.

In some aspects, the tumor comprises a GPC3-expressing tumor. In some aspects, the tumor is selected from the group consisting of: hepatocellular carcinoma (HCC), ovarian clear cell carcinoma, melanoma, squamous cell carcinoma of the lung, hepatoblastoma, nephroblastoma (Wilms tumor), and yolk sac tumor.

A method of treating a subject having cancer, the method comprising administering a therapeutically effective dose of any of the immunoresponsive cells described herein, any one of the engineered nucleic acids described herein, any one of the expression vectors described herein, and/or pharmaceutical compositions described herein. In some aspects, the cancer comprises a GPC3-expressing cancer. In some aspects, the cancer is selected from the group consisting of: hepatocellular carcinoma (HCC), ovarian clear cell carcinoma, melanoma, squamous cell carcinoma of the lung, hepatoblastoma, nephroblastoma (Wilms tumor), and yolk sac tumor.

In some aspects, the administering comprises systemic administration. In some aspects, the administering comprises intratumoral administration. In some aspects, the immunoresponsive cell is derived from the subject. In some aspects, the immunoresponsive cell is allogeneic with reference to the subject.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 illustrates a schematic of a cytokine-CAR bidirectional construct in head-to-head directionality (**FIG. 1A**), head-to-tail directionality (**FIG. 1B**), tail-to-tail directionality (**FIG. 1C**), and.an exemplary anti-GPC3 CAR + IL15 bidirectional construct (**FIG. 1D**).

FIG. 2 provides CAR expression plots assessed by flow cytometry for cells transduced with lentivirus encoding a CAR + IL15 bidirectional construct and cells transduced with a lentivirus encoding the CAR-only (day 7).

FIG. 3 provides CAR expression plots assessed by flow cytometry for cells transduced with retrovirus encoding a CAR + IL15 bidirectional construct and cells transduced with a retrovirus encoding the CAR-only (day 7).

FIG. 4 provides CAR expression plots assessed by flow cytometry for cells transduced with lentivirus encoding a CAR + IL15 bidirectional construct and cells transduced with a lentivirus encoding the CAR-only (day 15).

FIG. 5 provides CAR expression plots assessed by flow cytometry for cells transduced with retrovirus encoding a CAR + IL15 bidirectional construct and cells transduced with a retrovirus encoding the CAR-only (day 15).

FIG. 6 provides IL15 levels assessed by immunoassay for NK cells transduced with lentiviruses encoding CAR + IL15 bidirectional construct ("Lenti") or γ-retroviruses encoding CAR + IL15 bidirectional constructs ("SinVec").

FIG. 7 provides killing by NK cells transduced with lentiviruses encoding CAR-only or CAR + IL15 bidirectional constructs, as assessed by a co-culture killing assay.

FIG. 8 provides killing by NK cells transduced with γ-retroviruses encoding CAR-only or CAR + IL15 bidirectional constructs, as assessed by a co-culture killing assay.

FIG. 9 illustrates schematics for bidirectionally orientated constructs, including IL12 expression cassettes having mRNA destabilization elements in the 3' untranslated region.

FIG. 10 provides IL12 levels assessed by immunoassay for NK cells transduced with bidirectional constructs including an inducible IL12 expression cassette and an expression cassette encoding a synthetic transcription factor.

FIG. 11 illustrates a schematic of bidirectional construct encoding a cleavable release IL15.

FIG. 12 provides a summary of IL15 bicistronic constructs tested and performance in functional assays.

FIG. 13A and **FIG. 13B** provide expression plots as assessed by flow cytometry for NK cells transduced with SB06251, SB06257, and SB06254, for GPC3 CAR and IL15. Two independent replicates are shown (**FIG. 13A** and **FIG. 13B**).

FIG. 14A and **FIG. 14B** provides secreted IL15 levels as assessed by immunoassay for NK cells tranduced with SB06251, SB06257, and SB06254. Two independent replicates are shown (**FIG. 14A** and **FIG. 14B**).

FIG. 15A and **FIG. 15B** provide cell growth of target cell population following co-culture with NK cells tranduced with SB06251, SB06257, and SB06254. Two independent replicates are shown (**FIG. 15A** and **FIG. 15B**).

FIG. 16 provides target cell counts in a serial-killing assay when co-cultured with NK cells tranduced with SB06251, SB06257, and SB06254.

FIG. 17A and **FIG. 17B** provide expression plots as assessed by flow cytometry for NK cells transduced with SB06252, SB06258, and SB06255, for GPC3 CAR and IL15. Two independent replicates are shown (**FIG. 17A** and **FIG. 17B**).

FIG. 18A and **FIG. 18B** provide secreted IL15 levels as assessed by immunoassay for NK cells tranduced with SB06252, SB06258, and SB06255. Two independent replicates are shown (**FIG. 18A** and **FIG. 18B**).

FIG. 19A and **FIG. 19B** provide cell growth of target cell population following co-culture with NK cells tranduced with SB06252, SB06258, and SB06255. Two independent replicates are shown (**FIG. 19A** and **FIG. 19B**).

FIG. 20 provides target cell counts in a serial-killing assay when co-cultured with NK cells transduced with SB06252, SB06258, and SB06255.

FIG. 21A and **FIG. 21B** provide expression plots as assessed by flow cytometry for NK cells transduced with bicistronic constructs SB06261, SB6294, and SB6298, for GPC3 CAR and IL15. Two independent replicates are shown (**FIG. 21A** and **FIG. 21B**).

FIG. 22A and **FIG. 22B** provide secreted IL15 levels as assessed by immunoassay for NK cells tranduced with SB06261, SB6294, and SB6298. Two independent replicates are shown (**FIG. 22A** and **FIG. 22B**).

FIG. 23A and **FIG. 23B** provide cell growth of target cell population following co-culture with NK cells tranduced with SB06252, SB06258, and SB06255. Two independent replicates are shown (**FIG. 23A** and **FIG. 23B**).

FIG. 24A and **FIG. 24B** provide characterization of cleavable release IL15 bicstronic constructs SB06691, SB06692, and SB06693. Expression plots as assessed by flow cytometry for NK cells transduced with SB06691, SB06692, and SB06693, for GPC3 CAR and IL15, are shown in **FIG. 24A.** Secreted IL15 levels as assessed by immunoassay for NK cells tranduced with SB06691, SB06692, and SB06693 are shown in **FIG. 24B.**

FIG. 25 illustrates a schematic of a bidirectional construct encoding a cleavable release IL12.

FIG. 26 provides a dose-response curve of IL12 secretion for NK cells following treatment with grazoprevir (GRZ).

FIG. 27A and **FIG. 27B** provide *in vivo* mouse data demonstrating IL12 levels in mouse blood following injectetion with NK cells tranduced with SB04599, SB05042, and SB05058. IL12 levels are shown in **FIG. 27A** and IL12 fold change is shown in **FIG. 27B**.

FIGs. 28A - C provide characterization of cells transduced with different constructs expressing the GPC3 CAR and IL15. **FIG. 28A** shows flow cytometry plots demonstrating expression of GPC3 CAR, membrane bound IL15, and respective copy numbers on NK cells transduced with different GPC3 CAR/IL15 expression constructs. **FIG. 28B** shows measurement of secreted IL-15. **FIG. 28C** shows cell killing of HepG2 as assessed by a serial killing assay.

FIG. 29A and **FIG. 29B** provide additional data of serial killing using transduced NK Cells. **FIG. 29A** shows serial killing of HepG2 cells. **FIG. 29B** shows serial killing of HuH-7 cells.

FIG. 30A and **FIG. 30B** provide data assessing transduced NK cell function using rapid expansion (G-Rex). **FIG. 30A** shows expression of GPC3 CAR, membrane bound IL 15(mIL15), and secreted IL15 (sIL15). **FIG. 30B** shows serial killing of the transduced NK cells.

FIG. 31 provides results from a xenograft tumor model as measured by bioluminescence imaging, in which mice are injected with NK cells.

FIG. 32A and **FIG. 32B** provide the results of a xenograft tumor model in mice that are injected with NK cells and summary. **FIG. 32A** provides a survival curve of mice treated with NK cells. **FIG. 32B** provides a summary of the median survival of mice treated with the NK cells.

FIG. 33 provides results of a BLI experiment to assess tumor reduction in mice injected with NK cells.

FIG. 34 provides a quantification of each condition in terms of BLI measurements that were normalized to day 10.

FIG. 35A and **FIG. 35B** provide results from a xenograft tumor (HepG2) mouse model in which mice were injected three times with NK cells over the course of the study. **FIG. 35A** provides results of mice that were imaged using BLI. **FIG. 35B** provides a time course of fold change of BLI over the course of the study.

FIG. 36A and **FIG. 36B** provide the fold change BLI in mice injected with transduced NK cells. **FIG. 36A** provides results corresponding to measurements performed 13 days after tumor implantation. **FIG. 36B** provides results corresponding to measurements performed 20 days after tumor implantation.

FIG. 37A and **FIG. 37B** provide results of tumor reduction in a xenograft model. **FIG. 37A** shows a summary of the BLI Fold change in two different in vivo experiments. **FIG. 37B** shows a summary of the normalized mean BLI Fold change in two different in vivo experiments, but the treatment groups are separated, and animal are tracked individually.

FIG. 38A and **FIG. 38B** provide results from a xenograft tumor model in which NK cells are injected intratumorally. **FIG. 38A** provides measurements of tumor volume. **FIG. 38B** shows a survival curve.

FIG. 39A and **FIG. 39B** provide results for expression of IL-12 in the presence or absence of grazoprevir. **FIG. 39A** provides measurements of concentration and fold change 24 hours after induction with grazoprevir. **FIG. 39B** provides measurements of concentration and fold change 72 hours after induction.

FIG. 40 provides results from a mouse that was injected NK cells expressing regulated IL12 at different concentrations and throughout the experiment.

FIG. 41 provides expression (GPC3 CAR and IL15) results of co-transduction with the IL-12 and GPC3 CAR/IL15 constructs into NK cells.

FIG. 42A and **FIG. 42B** provide results of secreted IL15 and secreted IL12 expression in the presence or absence of grazoprevir. **FIG. 42A** provides measurements of secreted IL15 concentration. **FIG. 42B** provides measurements of secreted IL12 expression.

FIG. 43 provides measurements of secreted IL15 and secreted IL12 of NK cells during a serial killing assay.

FIGs. 44A-D provide results of a serial killing assay for different co-transductions in NK cells for cell killing of Huh-7 and HepG2 cells. **FIG. 44A** provides the serial killing results for NK cells co-transduced with SB05042 + SB06258. **FIG. 44B** provides the serial killing results for NK cells co-transduced with SB05042 + SB06257. **FIG. 44C** provides the serial killing results for NK cells co-transduced with SB05042 + SB06294. **FIG. 44D** provides a combination of the results in **FIGs. 44A-C**.

FIGs. 45A-C provide results from assessment of the clonal selection of NK cells expressing the GPC3 CAR. **FIG. 45A** provides results on copies per cell. **FIG. 45B** provides results of GCP3 CAR expression. **FIG. 45C** provides results for IL15 expression. **FIG. 45D** provides measurement of secreted IL15.

FIG. 46A and **FIG. 46B** provide flow cytometry data of GPC3 CAR and IL15 expression on selected clones transduced with SB06258. **FIG. 46A** provides results of selected clones. **FIG. 46B** provides results of selected clones further transduced with SB05042 (IL12).

DETAILED DESCRIPTION

Immunoresponsive cells are provided for herein.

In a first instance, immunoresponsive cells are engineered to have the following:

(a) a first engineered nucleic acid comprising a first expression cassette comprising a first promoter operably linked to a first, exogenous polynucleotide sequence encoding a first

cytokine, and a second expression cassette comprising a second promoter operably linked to a second exogenous polynucleotide sequence encoding a chimeric antigen receptor (CAR) that binds to GPC3; and

(b) a second engineered nucleic acid comprising

a third expression cassette comprising a synthetic transcription factor-responsive promoter operably linked to a third exogenous polynucleotide sequence encoding a second cytokine, and a fourth expression cassette comprising a fourth promoter operably linked to a fourth exogenous polynucleotide sequence encoding an activation-conditional control polypeptide (ACP), wherein the ACP comprises a synthetic transcription factor comprising a DNA-binding domain and a transcriptional effector domain,

wherein the ACP is capable of inducing expression of the third exogenous polynucleotide sequence by binding to the ACP-responsive promoter,

wherein at least one of the first exogenous polynucleotide sequence and the third exogenous polynucleotide sequence encodes a membrane-cleavable chimeric protein, oriented from N-terminal to C-terminal, having the formula: **S – C – MT** or **MT – C – S** configured to be expressed as a single polypeptide.

In a second instance, immunoresponsive cells are engineered to have the following:

(a) a first engineered nucleic acid comprising

a first expression cassette comprising a first promoter operably linked to a first exogenous polynucleotide sequence encoding a first cytokine and a second exogenous polynucleotide sequence encoding a chimeric antigen receptor (CAR) that binds to GPC3, and

a second expression cassette comprising a synthetic transcription factor-responsive promoter operably linked to a third exogenous polynucleotide sequence encoding a second cytokine; and

(b) a second engineered nucleic acid comprising

a third expression cassette comprising a third promoter operably linked to fourth exogenous polynucleotide sequence encoding an activation-conditional control polypeptide (ACP), wherein the ACP comprises a synthetic transcription factor comprising a DNA-binding domain and a transcriptional effector domain,

wherein the ACP is capable of inducing expression of the third exogenous polynucleotide sequence by binding to the ACP-responsive promoter, wherein the ACP comprises a synthetic transcription factor,

wherein at least one of the first exogenous polynucleotide sequence and the third exogenous polynucleotide sequence encodes a membrane-cleavable chimeric protein, oriented from N-terminal to C-terminal, having the formula: **S – C – MT** or **MT – C – S** configured to be expressed as a single polypeptide.

S refers to a secretable effector molecule. C refers to a protease cleavage site. MT refers to a cell membrane tethering domain.

The ACP of the immunoresponsive cells includes a synthetic transcription factor. A synthetic transcription factor is a non-naturally occurring protein that includes a DNA-binding domain and a transcriptional effector domain and is capable of modulating (*i.e.*, activating or repressing) transcription through binding to a cognate promoter recognized by the DNA-binding domain (an ACP-responsive promoter). In some embodiments, the ACP is a transcriptional repressor. In some embodiments, the ACP is a transcriptional activator.

The membrane-cleavable chimeric protein is engineered such that secretion of the effector molecule can be regulated in a protease-dependent manner. Specifically, the membrane-cleavable chimeric protein is engineered such that secretion of the effector molecule can be regulated as part of a "Membrane-Cleavable" system, where incorporation of a protease cleavage site ("C") and a cell membrane tethering domain ("MT") allow for regulated secretion of an effector molecule in a protease-dependent manner. Without wishing to be bound by theory, the components of the Membrane-Cleavable system present in the membrane-cleavable chimeric protein generally regulate secretion through the below cellular processes:

- MT: The cell membrane tethering domain contains a transmembrane domain (or a transmembrane-intracellular domain) that directs cellular-trafficking of the chimeric protein such that the protein is inserted into, or otherwise associated with, a cell membrane ("tethered")
- C: Following expression and localization of the chimeric protein into the cell membrane, the protease cleavage site directs cleavage of the chimeric protein such that the effector molecule is released ("secreted") into the extracellular space. Generally, the protease cleavage site is protease-specific, including sites engineered to be protease-specific. The protease cleavage site can be selected or engineered to achieve optimal protein expression, cell-type specific cleavage, cell-state specific cleavage, and/or cleavage and release of the payload at desired kinetics (*e.g.*, ratio of membrane-bound to secreted chimeric protein levels)

In some aspects, membrane-cleavable chimeric proteins (or engineered nucleic acids encoding the membrane-cleavable chimeric proteins) are provided for herein having a protein of interest (*e.g.*, any of the effector molecules described herein), a protease cleavage site, and a cell membrane tethering domain.

An "effector molecule," refers to a molecule (*e.g.*, a nucleic acid such as DNA or RNA, or a protein (polypeptide) or peptide) that binds to another molecule and modulates the biological activity of that molecule to which it binds. For example, an effector molecule may act

as a ligand to increase or decrease enzymatic activity, gene expression, or cell signaling. Thus, in some embodiments, an effector molecule modulates (activates or inhibits) different immunomodulatory mechanisms. By directly binding to and modulating a molecule, an effector molecule may also indirectly modulate a second, downstream molecule.

In general, for all membrane-cleavable chimeric proteins described herein, an effector molecule is a cytokine or active fragment thereof (the secretable effector molecule referred to as "S" in the formula **S – C – MT** or **MT – C – S**) that includes a cytokine or active fragments thereof.

The term "modulate" encompasses maintenance of a biological activity, inhibition (partial or complete) of a biological activity, and stimulation/activation (partial or complete) of a biological activity. The term also encompasses decreasing or increasing (*e.g.*, enhancing) a biological activity. Two different effector molecules are considered to "modulate different tumor-mediated immunosuppressive mechanisms" when one effector molecule modulates a tumor-mediated immunosuppressive mechanism (*e.g.*, stimulates T cell signaling) that is different from the tumor-mediated immunosuppressive mechanism modulated by the other effector molecule (*e.g.*, stimulates antigen presentation and/or processing).

Modulation by an effector molecule may be direct or indirect. Direct modulation occurs when an effector molecule binds to another molecule and modulates activity of that molecule. Indirect modulation occurs when an effector molecule binds to another molecule, modulates activity of that molecule, and as a result of that modulation, the activity of yet another molecule (to which the effector molecule is not bound) is modulated.

In some embodiments, modulation of a tumor-mediated immunosuppressive mechanism by at least one effector molecule results in an increase in an immunostimulatory and/or anti-tumor immune response (*e.g.*, systemically or in the tumor microenvironment) by at least 10% (*e.g.*, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, or 200%). For example, modulation of a tumor-mediated immunosuppressive mechanism may result in an increase in an immunostimulatory and/or anti-tumor immune response by at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 100%. In some embodiments, modulation of a tumor-mediated immunosuppressive mechanism results in an increase in an immunostimulatory and/or anti-tumor immune response 10-20%, 10-30%, 10-40%, 10-50%, 10-60%, 10-70%, 10-80%, 10-90%, 10-100%, 10-200%, 20-30%, 20-40%, 20-50%, 20-60%, 20-70%, 20-80%, 20-90%, 20-100%, 20-200%, 50-60%, 50-70%, 50-80%, 50-90%, 50-100%, or 50-200%. It should be understood that "an increase" in an immunostimulatory and/or anti-tumor immune response, for example, systemically or in a tumor

microenvironment, is relative to the immunostimulatory and/or anti-tumor immune response that would otherwise occur, in the absence of the effector molecule(s).

In some embodiments, modulation of a tumor-mediated immunosuppressive mechanism by at least one effector molecule results in an increase in an immunostimulatory and/or anti-tumor immune response (*e.g.*, systemically or in the tumor microenvironment) by at least 2 fold (*e.g.*, 2, 3, 4, 5, 10, 25, 20, 25, 50, or 100 fold). For example, modulation of a tumor-mediated immunosuppressive mechanism may result in an increase in an immunostimulatory and/or anti-tumor immune response by at least 3 fold, at least 5 fold, at least 10 fold, at least 20 fold, at least 50 fold, or at least 100 fold. In some embodiments, modulation of a tumor-mediated immunosuppressive mechanism results in an increase in an immunostimulatory and/or anti-tumor immune response by 2-10, 2-20, 2-30, 2-40, 2-50, 2-60, 2-70, 2-80, 2-90, or 2-100 fold.

Non-limiting examples of immunostimulatory and/or anti-tumor immune mechanisms include T cell signaling, activity and/or recruitment, antigen presentation and/or processing, natural killer cell-mediated cytotoxic signaling, activity and/or recruitment, dendritic cell differentiation and/or maturation, immune cell recruitment, pro-inflammatory macrophage signaling, activity and/or recruitment, stroma degradation, immunostimulatory metabolite production, stimulator of interferon genes (STING) signaling (which increases the secretion of IFN and Th1 polarization, promoting an anti-tumor immune response), and/or Type I interferon signaling. An effector molecule may stimulate at least one (one or more) of the foregoing immunostimulatory mechanisms, thus resulting in an increase in an immunostimulatory response. Changes in the foregoing immunostimulatory and/or anti-tumor immune mechanisms may be assessed, for example, using *in vitro* assays for T cell proliferation or cytotoxicity, *in vitro* antigen presentation assays, expression assays (*e.g.*, of particular markers), and/or cell secretion assays (*e.g.*, of cytokines).

In some embodiments, modulation of a tumor-mediated immunosuppressive mechanism by at least one effector molecule results in a decrease in an immunosuppressive response (*e.g.*, systemically or in the tumor microenvironment) by at least 10% (*e.g.*, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 100%, or 200%). For example, modulation of a tumor-mediated immunosuppressive mechanism may result in a decrease in an immunosuppressive response by at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 100%. In some embodiments, modulation of a tumor-mediated immunosuppressive mechanism results in a decrease in an immunosuppressive response 10-20%, 10-30%, 10-40%, 10-50%, 10-60%, 10-70%, 10-80%, 10-90%, 10-100%, 10-200%, 20-30%, 20-40%, 20-50%, 20-60%, 20-70%, 20-80%, 20-90%, 20-100%, 20-200%, 50-60%, 50-70%, 50-80%, 50-90%, 50-100%, or 50-200%. It should be understood that “a decrease” in an

immunosuppressive response, for example, systemically or in a tumor microenvironment, is relative to the immunosuppressive response that would otherwise occur, in the absence of the effector molecule(s).

In some embodiments, modulation of a tumor-mediated immunosuppressive mechanism by at least one effector molecule results in a decrease in an immunosuppressive response (*e.g.*, systemically or in the tumor microenvironment) by at least 2 fold (*e.g.*, 2, 3, 4, 5, 10, 25, 20, 25, 50, or 100 fold). For example, modulation of a tumor-mediated immunosuppressive mechanism may result in a decrease in an immunosuppressive response by at least 3 fold, at least 5 fold, at least 10 fold, at least 20 fold, at least 50 fold, or at least 100 fold. In some embodiments, modulation of a tumor-mediated immunosuppressive mechanism results in a decrease in an immunosuppressive response by 2-10, 2-20, 2-30, 2-40, 2-50, 2-60, 2-70, 2-80, 2-90, or 2-100 fold.

Non-limiting examples of immunosuppressive mechanisms include negative costimulatory signaling, pro-apoptotic signaling of cytotoxic cells (*e.g.*, T cells and/or NK cells), T regulatory (Treg) cell signaling, tumor checkpoint molecule production/maintenance, myeloid-derived suppressor cell signaling, activity and/or recruitment, immunosuppressive factor/metabolite production, and/or vascular endothelial growth factor signaling. An effector molecule may inhibit at least one (one or more) of the foregoing immunosuppressive mechanisms, thus resulting in a decrease in an immunosuppressive response. Changes in the foregoing immunosuppressive mechanisms may be assessed, for example, by assaying for an increase in T cell proliferation and/or an increase in IFNγ production (negative co-stimulatory signaling, T_{reg} cell signaling and/or MDSC); Annexin V/PI flow staining (pro-apoptotic signaling); flow staining for expression, *e.g.*, PDL1 expression (tumor checkpoint molecule production/maintenance); ELISA, LUMINEX®, RNA via qPCR, enzymatic assays, *e.g.*, IDO tryptophan catabolism (immunosuppressive factor/metabolite production); and phosphorylation of PI3K, Akt, p38 (VEGF signaling).

In some embodiments, effector molecules function additively: the effect of two effector molecules, for example, may be equal to the sum of the effect of the two effector molecules functioning separately. In other embodiments, effector molecules function synergistically: the effect of two effector molecules, for example, may be greater than the combined function of the two effector molecules.

Effector molecules that modulate tumor-mediated immunosuppressive mechanisms and/or modify tumor microenvironments may be any of the cytokines described herein.

In some embodiments, at least one of the effector molecules stimulates an immunostimulatory mechanism in the tumor microenvironment and/or inhibits an immunosuppressive mechanism in the tumor microenvironment.

In some embodiments, at least one of the effector molecules (a) stimulates T cell signaling, activity and/or recruitment, (b) stimulates antigen presentation and/or processing, (c) stimulates natural killer cell-mediated cytotoxic signaling, activity and/or recruitment, (d) stimulates dendritic cell differentiation and/or maturation, (e) stimulates immune cell recruitment, (f) stimulates pro-inflammatory macrophage signaling, activity and/or recruitment or inhibits anti-inflammatory macrophage signaling, activity and/or recruitment, (g) stimulates stroma degradation, (h) stimulates immunostimulatory metabolite production, (i) stimulates Type I interferon signaling, (j) inhibits negative costimulatory signaling, (k) inhibits pro-apoptotic signaling of anti-tumor immune cells, (l) inhibits T regulatory (T_{reg}) cell signaling, activity and/or recruitment, (m) inhibits tumor checkpoint molecules, (n) stimulates stimulator of interferon genes (STING) signaling, (o) inhibits myeloid-derived suppressor cell signaling, activity and/or recruitment, (p) degrades immunosuppressive factors/metabolites, (q) inhibits vascular endothelial growth factor signaling, and/or (r) directly kills tumor cells.

Non-limiting examples of cytokines are listed in Table 1 and specific sequences encoding exemplary effector molecules are listed in Table 2. Effector molecules can be human, such as those listed in Table 1 or Table 2 or human equivalents of murine effector molecules listed in Table 1 or Table 2. Effector molecules can be human-derived, such as the endogenous human effector molecule or an effector molecule modified and/or optimized for function, *e.g.*, codon optimized to improve expression, modified to improve stability, or modified at its signal sequence (*see* below). Various programs and algorithms for optimizing function are known to those skilled in the art and can be selected based on the improvement desired, such as codon optimization for a specific species (*e.g.*, human, mouse, bacteria, etc.).

Table 1. Exemplary Effector Molecules

Effector name	Category	Function
IFNbeta	Cytokine	T cell response, tumor cell killing
IFNgamma	Cytokine	T cell response, tumor cell killing
IL-12 (*e.g.*, IL12p70 fusion)	Cytokine	T cells, NK cells
IL-1beta	Cytokine	T cells, NK cells
IL-15	Cytokine	Stimulates T-cells and NK
IL-2	Cytokine	Stimulates T-cells and NK
IL-21	Cytokine	Stimulates T-cells
IL-24	Cytokine	Stimulates T-cells

Effector name	Category	Function
IL36-gamma	Cytokine	Stimulates T-cells
IL-7	Cytokine	Stimulates T-cells
IL-22	Cytokine	Stimulates T-cells
IL-18	Cytokine	Stimulates T-cells

Table 2: Sequences encoding exemplary effector molecules

IL-12 (Human) (SEQ ID NO: 56)
ATGTGCCATCAGCAGCTTGTCATATCTTGGTTTTCACTTGTATTCCTGGCCAGCCCTTTGGTTGCGAT CTGGGAGCTCAAGAAGGATGTGTACGTTGTAGAGCTGGACTGGTACCCCGATGCTCCCGGTGAGAT GGTCGTTTTGACATGTGACACTCCAGAAGAGGACGGTATTACGTGGACTCTGGACCAGTCCTCCGA AGTTCTTGGTTCTGGTAAGACTCTGACTATCCAGGTGAAAGAATTTGGGGATGCGGGACAATACAC ATGCCACAAGGGAGGCGAGGTGTTGTCTCATAGTTTGCTGCTTCTCCACAAGAAAGAGGATGGAAT CTGGAGCACCGACATACTCAAGGATCAAAAGGAACCCAAAAATAAGACATTTCTGCGATGTGAGGC TAAGAACTATAGTGGCCGCTTCACTTGTTGGTGGCTGACTACCATCAGCACAGATCTCACGTTTTCA GTAAAAAGTAGTAGAGGTTCAAGTGATCCTCAAGGGGTAACGTGCGGTGCTGCAACACTGTCTGCT GAACGCGTAAGAGGAGATAATAAGGAGTACGAGTATTCCGTAGAATGCCAAGAGGACAGTGCTTGT CCTGCGGCCGAGGAGTCTCTCCCAATAGAAGTGATGGTGGACGCGGTGCATAAACTGAAATATGAG AACTACACAAGCAGTTTTTTTATAAGAGATATCATCAAGCCCGATCCGCCGAAGAATTTGCAACTTA AACCGCTTAAAAACTCACGCCAGGTTGAAGTATCCTGGGAGTATCCGGATACATGGTCAACACCAC ACAGCTATTTTTCCCTTACCTTCTGTGTGCAGGTCCAAGGGAAGAGCAAAAGGGAGAAGAAGGACA GGGTATTCACTGATAAAACTTCCGCGACGGTCATCTGCCGAAAAAACGCTAGTATATCTGTACGGG CGCAGGATAGGTACTATAGTTCTTCTTGGTCTGAGTGGGCCTCAGTTCCGTGCTCTGGGGGAGGAAG TGGAGGAGGGTCCGGCGGTGGAAGCGGGGGAGGGAGTCGCAACTTGCCAGTGGCTACACCAGATC CAGGCATGTTTCCATGTCTGCATCATTCCCAGAATCTCCTGAGAGCGGTGTCAAATATGCTCCAAAA AGCGAGACAAACACTGGAATTTTACCCGTGTACCAGTGAGGAGATTGATCACGAGGACATAACCAA GGACAAGACCTCAACTGTAGAAGCGTGTTTGCCGCTGGAGTTGACTAAGAATGAGTCCTGCCTCAA TTCCAGAGAAACTTCATTCATTACTAACGGCAGTTGTCTTGCATCCCGGAAAACGTCCTTTATGATG GCCCTTTGCCTTAGTTCAATTTACGAGGATCTTAAAATGTATCAAGTGGAGTTTAAAACCATGAATG CTAAACTTCTTATGGACCCCAAACGACAAATTTTTCTGGATCAGAATATGCTTGCCGTGATAGACGA ACTCATGCAGGCGCTTAATTTTAACTCCGAAACAGTTCCACAAAAATCTAGCCTTGAAGAACCTGAT TTTTATAAAACGAAGATTAAACTGTGTATCCTGCTGCATGCCTTTCGCATCCGAGCTGTCACAATCG ATAGGGTTATGTCCTACCTTAACGCGAGCtaG
IL-12p70 (Human; codon optimized; bold denotes signal sequence) (SEQ ID NO: 57)
ATGTGCCATCAGCAACTCGTCATCTCCTGGTTCTCCCTTGTGTTCCTCGCTTCCCCTCTGGTCG CCATTTGGGAACTGAAGAAGGACGTCTACGTGGTCGAGCTGGATTGGTACCCGGACGCCCCTGGAG AAATGGTCGTGCTGACTTGCGATACGCCAGAAGAGGACGGCATAACCTGGACCCTGGATCAGAGCT CCGAGGTGCTCGGAAGCGGAAAGACCCTGACCATTCAAGTCAAGGAGTTCGGCGACGCGGGCCAGT ACACTTGCCACAAGGGTGGCGAAGTGCTGTCCCACTCCCTGCTGCTGCTGCACAAGAAAGAGGATG GAATCTGGTCCACTGACATCCTCAAGGACCAAAAAGAACCGAAGAACAAGACCTTCCTCCGCTGCG AAGCCAAGAACTACAGCGGTCGGTTCACCTGTTGGTGGCTGACGACAATCTCCACCGACCTGACTTT CTCCGTGAAGTCGTCACGGGGATCAAGCGATCCTCAGGGCGTGACCTGTGGAGCCGCCACTCTGTC CGCCGAGAGAGTCAGGGGAGACAACAAGGAATATGAGTACTCCGTGGAATGCCAGGAGGACAGCG CCTGCCCTGCCGCGGAAGAGTCCCTGCCTATCGAGGTCATGGTCGATGCCGTGCATAAGCTGAAAT ACGAGAACTACACTTCCTCCTTCTTTATCCGCGACATCATCAAGCCTGACCCCCCCAAGAACTTGCA GCTGAAGCCACTCAAGAACTCCCGCCAAGTGGAAGTGTCTTGGGAATATCCAGACACTTGGAGCAC CCCGCACTCATACTTCTCGCTCACTTTCTGTGTGCAAGTGCAGGGAAAGTCCAAACGGGAGAAGAA AGACCGGGTGTTCACCGACAAAACCTCCGCCACTGTGATTTGTCGGAAGAACGCGTCAATCAGCGT CCGGGCGCAGGATAGATACTACTCGTCCTCCTGGAGCGAATGGGCCAGCGTGCCTTGTTCCGGTGG CGGATCAGGCGGAGGTTCAGGAGGAGGCTCCGGAGGAGGTTCCCGGAACCTCCCTGTGGCAACCCC CGACCCTGGAATGTTCCCGTGCCTACACCACTCCCAAAACCTCCTGAGGGCTGTGTCGAACATGTTG CAGAAGGCCCGCCAGACCCTTGAGTTCTACCCCTGCACCTCGGAAGAAATTGATCACGAGGACATC ACCAAGGACAAGACCTCGACCGTGGAAGCCTGCCTGCCGCTGGAACTGACCAAGAACGAATCGTGT CTGAACTCCCGCGAGACAAGCTTTATCACTAACGGCAGCTGCCTGGCGTCGAGAAAGACCTCATTC

ATGATGGCGCTCTGTCTTTCCTCGATCTACGAAGATCTGAAGATGTATCAGGTCGAGTTCAAGACCA TGAACGCCAAGCTGCTCATGGACCCGAAGCGGCAGATCTTCCTGGACCAGAATATGCTCGCCGTGA TTGATGAACTGATGCAGGCCCTGAATTTCAACTCCGAGACTGTGCCTCAAAAGTCCAGCCTGGAAG AACCGGACTTCTACAAGACCAAGATCAAGCTGTGCATCCTGTTGCACGCTTTCCGCATTCGAGCCGT GACCATTGACCGCGTGATGTCCTACCTGAACGCCAGT
IL-12 (Mouse) (SEQ ID NO: 58)
ATGTGTCCACAGAAGCTGACAATAAGTTGGTTTGCCATTGTCCTCCTGGTGAGCCCACTCATGGCAA TGTGGGAACTCGAAAAGGATGTCTACGTGGTAGAAGTAGATTGGACTCCAGACGCGCCAGGGGAG ACAGTGAATTTGACATGTGACACACCAGAAGAAGATGACATTACATGGACATCTGACCAACGCCAT GGCGTAATAGGGAGTGGGAAAACACTCACGATCACAGTTAAAGAGTTCTTGGATGCTGGTCAATAT ACTTGCCATAAAGGCGGCGAGACACTCAGCCACTCACATTTGCTTTTGCATAAAAAAGAGAATGGC ATTTGGAGCACTGAAATACTTAAGAACTTTAAGAACAAGACATTTCTCAAGTGTGAGGCCCCTAATT ACAGCGGCAGGTTCACGTGCTCATGGCTGGTCCAGCGCAACATGGACCTCAAGTTTAACATAAAAT CTTCTTCCTCTTCACCTGACTCCAGAGCTGTTACTTGCGGCATGGCTTCTCTGAGCGCAGAAAAAGT AACGTTGGATCAAAGAGACTACGAAAAGTACTCTGTTTCTTGTCAAGAGGATGTTACGTGCCCGAC GGCCGAAGAAACGCTTCCAATTGAACTCGCGTTGGAAGCTCGCCAACAAAACAAGTATGAAAACTA CAGTACAAGCTTCTTTATACGGGATATAATTAAACCCGATCCCCCCAAGAACTTGCAAATGAAACC ACTTAAGAACAGCCAGGTGGAAGTTTCCTGGGAGTATCCAGACTCATGGAGTACTCCTCACAGCTA TTTTTCTCTGAAATTCTTTGTAAGGATACAACGGAAGAAAGAGAAGATGAAAGAGACCGAGGAGGG TTGTAATCAGAAGGGAGCGTTTCTCGTGGAGAAAACGTCTACCGAAGTCCAATGTAAAGGTGGCAA TGTGTGCGTCCAAGCTCAGGATAGATACTATAATTCAAGTTGCTCCAAGTGGGCCTGTGTTCCATGC CGCGTTCGGAGCGGGGGAGGTAGCGGAGGAGGTAGTGGGGGTGGGTCAGGAGGAGGGAGTCGAGT TATCCCGGTGTCAGGCCCCGCACGCTGCTTGAGCCAGAGTCGCAACCTCCTTAAGACAACAGATGA CATGGTGAAAACAGCACGCGAAAAGCTTAAACACTACTCTTGTACGGCGGAGGATATTGATCACGA GGATATTACCCGAGACCAAACTAGCACTTTGAAAACCTGTCTGCCCCTTGAACTTCATAAAAATGAG AGCTGTCTGGCTACACGAGAGACGTCAAGTACGACTAGGGGCAGCTGTCTCCCGCCGCAAAAGACA AGCCTCATGATGACGCTCTGTTTGGGTTCCATTTACGAGGACTTGAAAATGTATCAAACGGAGTTCC AGGCTATAAATGCGGCGTTGCAGAACCATAACCATCAACAAATTATACTTGATAAAGGCATGTTGG TGGCGATTGATGAACTCATGCAGAGTCTCAATCACAACGGGGAAACGTTGAGACAGAAACCCCCAG TCGGTGAAGCGGACCCATATCGAGTAAAAATGAAGCTCTGCATTCTGCTTCACGCATTCAGCACTAG AGTTGTTACCATCAACCGGGTAATGGGATATCTCTCCAGTGCGtaG
IL21 (Human; codon optimized; bold denotes signal sequence) (SEQ ID NO: 59)
ATGGAACGCATTGTGATCTGCCTGATGGTCATCTTCCTGGGCACCTTAGTGCACAAGTCGAGC AGCCAGGGACAGGACAGGCACATGATTAGAATGCGCCAGCTCATCGATATCGTGGACCAGTTGAA GAACTACGTGAACGACCTGGTGCCCGAGTTCCTGCCGGCCCCCGAAGATGTGGAAACCAATTGCGA ATGGTCGGCATTTTCCTGCTTTCAAAAGGCACAGCTCAAGTCCGCTAACACCGGGAACAACGAACG GATCATCAACGTGTCCATCAAAAAGCTGAAGCGGAAGCCTCCCTCCACCAACGCCGGACGGAGGCA GAAGCATAGGCTGACTTGCCCGTCATGCGACTCCTACGAGAAGAAGCCGCCGAAGGAGTTCCTGGA GCGGTTCAAGTCGCTCCTGCAAAAGATGATTCATCAGCACCTGTCCTCCCGGACTCATGGGTCTGAG GATTCA
IL-12p70_T2A_IL21 (Human; codon optimized; bold denotes signal sequences) (SEQ ID NO: 60)
ATGTGCCATCAGCAACTCGTCATCTCCTGGTTCTCCCTTGTGTTCCTCGCTTCCCCTCTGGTCG CCATTTGGGAACTGAAGAAGGACGTCTACGTGGTCGAGCTGGATTGGTACCCGGACGCCCCTGGAG AAATGGTCGTGCTGACTTGCGATACGCCAGAAGAGGACGGCATAACCTGGACCCTGGATCAGAGCT CCGAGGTGCTCGGAAGCGGAAAGACCCTGACCATTCAAGTCAAGGAGTTCGGCGACGCGGGCCAGT ACACTTGCCACAAGGGTGGCGAAGTGCTGTCCCACTCCCTGCTGCTGCTGCACAAGAAAGAGGATG GAATCTGGTCCACTGACATCCTCAAGGACCAAAAAGAACCGAAGAACAAGACCTTCCTCCGCTGCG AAGCCAAGAACTACAGCGGTCGGTTCACCTGTTGGTGGCTGACGACAATCTCCACCGACCTGACTTT CTCCGTGAAGTCGTCACGGGGATCAAGCGATCCTCAGGGCGTGACCTGTGGAGCCGCCACTCTGTC CGCCGAGAGAGTCAGGGGAGACAACAAGGAATATGAGTACTCCGTGGAATGCCAGGAGGACAGCG CCTGCCCTGCCGCGGAAGAGTCCCTGCCTATCGAGGTCATGGTCGATGCCGTGCATAAGCTGAAAT ACGAGAACTACACTTCCTCCTTCTTTATCCGCGACATCATCAAGCCTGACCCCCCCAAGAACTTGCA GCTGAAGCCACTCAAGAACTCCCGCCAAGTGGAAGTGTCTTGGGAATATCCAGACACTTGGAGCAC CCCGCACTCATACTTCTCGCTCACTTTCTGTGTGCAAGTGCAGGGAAAGTCCAAACGGGAGAAGAA AGACCGGGTGTTCACCGACAAAACCTCCGCCACTGTGATTTGTCGGAAGAACGCGTCAATCAGCGT CCGGGCGCAGGATAGATACTACTCGTCCTCCTGGAGCGAATGGGCCAGCGTGCCTTGTTCCGGTGG

CGGATCAGGCGGAGGTTCAGGAGGAGGCTCCGGAGGAGGTTCCCGGAACCTCCCTGTGGCAACCCC
CGACCCTGGAATGTTCCCGTGCCTACACCACTCCCAAAACCTCCTGAGGGCTGTGTCGAACATGTTG
CAGAAGGCCCGCCAGACCCTTGAGTTCTACCCCTGCACCTCGGAAGAAATTGATCACGAGGACATC
ACCAAGGACAAGACCTCGACCGTGGAAGCCTGCCTGCCGCTGGAACTGACCAAGAACGAATCGTGT
CTGAACTCCCGCGAGACAAGCTTTATCACTAACGGCAGCTGCCTGGCGTCGAGAAAGACCTCATTC
ATGATGGCGCTCTGTCTTTCCTCGATCTACGAAGATCTGAAGATGTATCAGGTCGAGTTCAAGACCA
TGAACGCCAAGCTGCTCATGGACCCGAAGCGGCAGATCTTCCTGGACCAGAATATGCTCGCCGTGA
TTGATGAACTGATGCAGGCCCTGAATTTCAACTCCGAGACTGTGCCTCAAAAGTCCAGCCTGGAAG
AACCGGACTTCTACAAGACCAAGATCAAGCTGTGCATCCTGTTGCACGCTTTCCGCATTCGAGCCGT
GACCATTGACCGCGTGATGTCCTACCTGAACGCCAGTAGACGGAAACGCGGAAGCGGAGAGGGCA
GAGGCTCGCTGCTTACATGCGGGGACGTGGAAGAGAACCCCGGTCCG**ATGGAACGCATTGTGATC**
TGCCTGATGGTCATCTTCCTGGGCACCTTAGTGCACAAGTCGAGCAGCCAGGGACAGGACAGG
CACATGATTAGAATGCGCCAGCTCATCGATATCGTGGACCAGTTGAAGAACTACGTGAACGACCTG
GTGCCCGAGTTCCTGCCGGCCCCCGAAGATGTGGAAACCAATTGCGAATGGTCGGCATTTTCCTGCT
TTCAAAAGGCACAGCTCAAGTCCGCTAACACCGGGAACAACGAACGGATCATCAACGTGTCCATCA
AAAAGCTGAAGCGGAAGCCTCCCTCCACCAACGCCGGACGGAGGCAGAAGCATAGGCTGACTTGC
CCGTCATGCGACTCCTACGAGAAGAAGCCGCCGAAGGAGTTCCTGGAGCGGTTCAAGTCGCTCCTG
CAAAAGATGATTCATCAGCACCTGTCCTCCCGGACTCATGGGTCTGAGGATTCA

IL-12_2A_CCL21a (Human) (SEQ ID NO: 61)

ATGTGCCATCAGCAGCTTGTCATATCTTGGTTTTCACTTGTATTCCTGGCCAGCCCTTTGGTTGCGAT
CTGGGAGCTCAAGAAGGATGTGTACGTTGTAGAGCTGGACTGGTACCCCGATGCTCCCGGTGAGAT
GGTCGTTTTGACATGTGACACTCCAGAAGAGGACGGTATTACGTGGACTCTGGACCAGTCCTCCGA
AGTTCTTGGTTCTGGTAAGACTCTGACTATCCAGGTGAAAGAATTTGGGGATGCGGGACAATACAC
ATGCCACAAGGGAGGCGAGGTGTTGTCTCATAGTTTGCTGCTTCTCCACAAGAAAGAGGATGGAAT
CTGGAGCACCGACATACTCAAGGATCAAAAGGAACCCAAAAATAAGACATTTCTGCGATGTGAGGC
TAAGAACTATAGTGGCCGCTTCACTTGTTGGTGGCTGACTACCATCAGCACAGATCTCACGTTTTCA
GTAAAAAGTAGTAGAGGTTCAAGTGATCCTCAAGGGGTAACGTGCGGTGCTGCAACACTGTCTGCT
GAACGCGTAAGAGGAGATAATAAGGAGTACGAGTATTCCGTAGAATGCCAAGAGGACAGTGCTTGT
CCTGCGGCCGAGGAGTCTCTCCCAATAGAAGTGATGGTGGACGCGGTGCATAAACTGAAATATGAG
AACTACACAAGCAGTTTTTTTATAAGAGATATCATCAAGCCCGATCCGCCGAAGAATTTGCAACTTA
AACCGCTTAAAAACTCACGCCAGGTTGAAGTATCCTGGGAGTATCCGGATACATGGTCAACACCAC
ACAGCTATTTTTCCCTTACCTTCTGTGTGCAGGTCCAAGGGAAGAGCAAAAGGGAGAAGAAGGACA
GGGTATTCACTGATAAAACTTCCGCGACGGTCATCTGCCGAAAAAACGCTAGTATATCTGTACGGG
CGCAGGATAGGTACTATAGTTCTTCTTGGTCTGAGTGGGCCTCAGTTCCGTGCTCTGGGGGAGGAAG
TGGAGGAGGGTCCGGCGGTGGAAGCGGGGGAGGGAGTCGCAACTTGCCAGTGGCTACACCAGATC
CAGGCATGTTTCCATGTCTGCATCATTCCCAGAATCTCCTGAGAGCGGTGTCAAATATGCTCCAAAA
AGCGAGACAAACACTGGAATTTTACCCGTGTACCAGTGAGGAGATTGATCACGAGGACATAACCAA
GGACAAGACCTCAACTGTAGAAGCGTGTTTGCCGCTGGAGTTGACTAAGAATGAGTCCTGCCTCAA
TTCCAGAGAAACTTCATTCATTACTAACGGCAGTTGTCTTGCATCCCGGAAAACGTCCTTTATGATG
GCCCTTTGCCTTAGTTCAATTTACGAGGATCTTAAAATGTATCAAGTGGAGTTTAAAACCATGAATG
CTAAACTTCTTATGGACCCCAAACGACAAATTTTTCTGGATCAGAATATGCTTGCCGTGATAGACGA
ACTCATGCAGGCGCTTAATTTTAACTCCGAAACAGTTCCACAAAAATCTAGCCTTGAAGAACCTGAT
TTTTATAAAACGAAGATTAAACTGTGTATCCTGCTGCATGCCTTTCGCATCCGAGCTGTCACAATCG
ATAGGGTTATGTCCTACCTTAACGCGAGCCGGCGCAAGAGGGGTTCCGGAGAGGGAAGGGGTAGTC
TGCTCACCTGCGGCGATGTTGAAGAAAATCCTGGTCCCATGGCGCAAAGTCTGGCTCTTTCACTCCT
GATCCTGGTCTTGGCCTTCGGGATTCCGAGGACCCAAGGAAGTGATGGTGGCGCCCAAGATTGTTG
CCTTAAATACAGCCAGCGGAAAATACCCGCGAAAGTGGTCAGGAGTTATAGAAAACAGGAGCCTTC
CCTGGGTTGTAGTATCCCCGCCATACTTTTCCTCCCGAGAAAACGGAGCCAGGCCGAACTGTGCGCT
GACCCTAAGGAACTTTGGGTGCAACAACTTATGCAACACCTGGATAAGACACCTTCTCCTCAAAAG
CCAGCTCAGGGCTGCCGAAAAGATAGAGGCGCCTCAAAAACCGGAAAAAAGGGCAAAGGTTCTAA
AGGATGTAAGCGGACTGAACGCTCTCAAACGCCTAAAGGGCCGtaG

IL-12_2A_CCL21a (Mouse) (SEQ ID NO: 62)

ATGTGTCCACAGAAGCTGACAATAAGTTGGTTTGCCATTGTCCTCCTGGTGAGCCCACTCATGGCAA
TGTGGGAACTCGAAAAGGATGTCTACGTGGTAGAAGTAGATTGGACTCCAGACGCGCCAGGGGAG
ACAGTGAATTTGACATGTGACACACCAGAAGAAGATGACATTACATGGACATCTGACCAACGCCAT
GGCGTAATAGGGAGTGGGAAAACACTCACGATCACAGTTAAAGAGTTCTTGGATGCTGGTCAATAT
ACTTGCCATAAAGGCGGCGAGACACTCAGCCACTCACATTTGCTTTTGCATAAAAAAGAGAATGGC
ATTTGGAGCACTGAAATACTTAAGAACTTTAAGAACAAGACATTTCTCAAGTGTGAGGCCCCTAATT

ACAGCGGCAGGTTCACGTGCTCATGGCTGGTCCAGCGCAACATGGACCTCAAGTTTAACATAAAAT
CTTCTTCCTCTTCACCTGACTCCAGAGCTGTTACTTGCGGCATGGCTTCTCTGAGCGCAGAAAAAGT
AACGTTGGATCAAAGAGACTACGAAAAGTACTCTGTTTCTTGTCAAGAGGATGTTACGTGCCCGAC
GGCCGAAGAAACGCTTCCAATTGAACTCGCGTTGGAAGCTCGCCAACAAAACAAGTATGAAAACTA
CAGTACAAGCTTCTTTATACGGGATATAATTAAACCCGATCCCCCCAAGAACTTGCAAATGAAACC
ACTTAAGAACAGCCAGGTGGAAGTTTCCTGGGAGTATCCAGACTCATGGAGTACTCCTCACAGCTA
TTTTTCTCTGAAATTCTTTGTAAGGATACAACGGAAGAAAGAGAAGATGAAAGAGACCGAGGAGGG
TTGTAATCAGAAGGGAGCGTTTCTCGTGGAGAAAACGTCTACCGAAGTCCAATGTAAAGGTGGCAA
TGTGTGCGTCCAAGCTCAGGATAGATACTATAATTCAAGTTGCTCCAAGTGGGCCTGTGTTCCATGC
CGCGTTCGGAGCGGGGGAGGTAGCGGAGGAGGTAGTGGGGGTGGGTCAGGAGGAGGGAGTCGAGT
TATCCCGGTGTCAGGCCCCGCACGCTGCTTGAGCCAGAGTCGCAACCTCCTTAAGACAACAGATGA
CATGGTGAAAACAGCACGCGAAAAGCTTAAACACTACTCTTGTACGGCGGAGGATATTGATCACGA
GGATATTACCCGAGACCAAACTAGCACTTTGAAAACCTGTCTGCCCCTTGAACTTCATAAAAATGAG
AGCTGTCTGGCTACACGAGAGACGTCAAGTACGACTAGGGGCAGCTGTCTCCCGCCGCAAAAGACA
AGCCTCATGATGACGCTCTGTTTGGGTTCCATTTACGAGGACTTGAAAATGTATCAAACGGAGTTCC
AGGCTATAAATGCGGCGTTGCAGAACCATAACCATCAACAAATTATACTTGATAAAGGCATGTTGG
TGGCGATTGATGAACTCATGCAGAGTCTCAATCACAACGGGGAAACGTTGAGACAGAAACCCCCAG
TCGGTGAAGCGGACCCATATCGAGTAAAAATGAAGCTCTGCATTCTGCTTCACGCATTCAGCACTAG
AGTTGTTACCATCAACCGGGTAATGGGATATCTCTCCAGTGCGCGGCGCAAGAGGGGTTCCGGAGA
GGGAAGGGGTAGTCTGCTCACCTGCGGCGATGTTGAAGAAAATCCTGGTCCCATGGCGCAAATGAT
GACCCTTTCCCTGCTGAGTCTTGTCCTCGCGCTCTGCATCCCGTGGACGCAGGGGTCTGATGGGGGG
GGCCAAGACTGTTGCCTGAAGTATTCACAAAAAAAGATACCGTACTCTATTGTCAGAGGGTACAGG
AAGCAAGAACCCTCCTTGGGTTGCCCTATACCAGCAATTCTTTTCTCCCCACGCAAGCATTCCAAAC
CAGAACTGTGTGCGAACCCCGAGGAGGGTTGGGTACAGAACTTGATGCGAAGGCTTGACCAGCCCC
CAGCCCCTGGCAAGCAGTCACCTGGGTGCAGAAAAAACAGAGGTACTTCAAAGAGCGGCAAGAAA
GGCAAAGGGAGTAAAGGATGTAAAAGAACGGAGCAGACCCAGCCTTCACGAGGCtaG

IL7 (Mouse) (SEQ ID NO: 64)

ATGTTTCATGTGTCCTTCAGGTACATATTTGGTATCCCACCACTTATATTGGTGCTCTTGCCTGTAAC
CAGCTCTGAATGTCATATAAAAGACAAGGAGGGCAAAGCATACGAGTCCGTATTGATGATCTCAAT
CGATGAACTTGACAAGATGACAGGGACCGATTCTAATTGTCCAAATAACGAGCCAAACTTCTTTCG
GAAACACGTGTGTGATGATACAAAAGAAGCTGCTTTTCTTAACAGAGCTGCCAGAAAACTCAAGCA
GTTCCTCAAGATGAATATATCCGAGGAATTTAACGTGCATCTCCTCACAGTATCTCAGGGAACTCAA
ACCCTTGTAAACTGCACTTCTAAGGAGGAGAAGAATGTCAAAGAGCAGAAGAAAAATGATGCATGT
TTTTTGAAACGGCTGTTGAGGGAGATCAAAACATGCTGGAATAAAATCCTCAAGGGCTCAATTtaG

IL-15 (Human) (SEQ ID NO: 65)

ATGGAAACAGACACATTGCTGCTTTGGGTATTGTTGCTCTGGGTGCCTGGATCAACAGGAAACTGGG
TAAACGTAATTTCAGATCTGAAGAAGATCGAGGACCTTATTCAATCCATGCACATCGATGCCACTCT
CTACACCGAAAGCGACGTTCACCCATCTTGCAAGGTGACCGCTATGAAATGTGAATTGTTGGAACTT
CAGGTAATTTCTCTGGAGAGCGGCGATGCCTCAATACATGACACCGTTGAAAATCTTATCATCCTTG
CTAATGATTCACTCTCTAGTAATGGGAACGTAACAGAGAGCGGGTGTAAGGAGTGTGAAGAACTGG
AGGAGAAAAACATTAAGGAATTTTTGCAGTCATTCGTCCATATAGTGCAAATGTTCATAAACACTTC
CAGAAGAAAGCGAGGCTCTGGGGAGGGGCGAGGCTCTCTGCTGACCTGTGGGGATGTAGAAGAGA
ATCCAGGTCCCATGGACCGGCTGACCAGCTCATTCCTGCTTCTGATTGTGCCAGCCTACGTGCTCTC
CATCACATGTCCTCCCCCAATGAGCGTCGAGCATGCTGACATCTGGGTGAAGTCATACTCCTTGTAC
AGCAGAGAGAGATACATTTGTAATTCCGGATTCAAGCGCAAGGCCGGCACCTCCTCTCTGACAGAG
TGCGTCCTTAACAAAGCAACCAACGTAGCACATTGGACCACACCATCCTTGAAGTGCATACGAGAA
CCTAAATCTTGCGATAAGACTCATACTTGTCCACCTTGTCCAGCCCCAGAACTGCTTGGCGGACCCT
CAGTATTTTTGTTCCCACCAAAGCCAAAAGACACACTCATGATATCCAGAACTCCTGAGGTGACCTG
TGTCGTTGTAGACGTTTCCCACGAAGATCCTGAAGTAAAATTCAACTGGTACGTGGATGGGGTCGAA
GTCCATAACGCCAAGACTAAACCAAGGGAGGAACAGTATAACTCTACTTACCGAGTAGTTTCTGTG
TTGACCGTGCTGCACCAGGACTGGTTGAACGGGAAGGAGTACAAATGCAAGGTGAGCAATAAAGCT
CTGCCCGCACCAATCGAAAAGACAATATCTAAGGCCAAGGGGCAGCCACGAGAGCCCCAGGTATA
CACACTGCCACCCTCACGCGATGAATTGACTAAGAACCAGGTTTCCCTGACCTGTCTTGTAAAAGGT
TTCTACCCTTCCGACATAGCTGTTGAGTGGGAAAGTAACGGGCAGCCAGAGAACAATTACAAGACA
ACTCCACCCGTTCTTGATAGCGATGGATCATTTTTTCTGTATTCCAAACTCACTGTCGATAAAAGTCG
CTGGCAGCAAGGCAATGTTTTTAGCTGCTCAGTCATGCACGAAGCACTGCATAATCACTACACACA
AAAAAGTTTGTCCCTTAGCCCTGGTAAGtaG

IL-15 (Human) (SEQ ID NO: 66)
ATGTACTCAATGCAGTTGGCCTCCTGTGTAACATTGACCTTGGTCCTCTTGGTCAACAGCAATTGGA TCGATGTACGCTACGACTTGGAGAAGATTGAGTCCCTTATACAGAGTATACACATAGATACAACCTT GTATACTGACAGTGACTTCCATCCCAGCTGTAAAGTGACTGCAATGAACTGTTTTTTGTTGGAGTTG CAAGTAATTCTGCATGAATACAGCAACATGACCCTCAATGAAACCGTTAGGAATGTCCTTTATCTCG CAAATTCTACTCTGAGTAGCAATAAGAATGTTGCCGAAAGCGGCTGCAAGGAGTGCGAAGAACTGG AGGAAAAAACTTTCACCGAGTTTCTCCAGAGTTTCATCAGAATTGTCCAAATGTTCATTAATACAAG TAGTGGTGGTGGGAGCGGGGGTGGAGGCAGTGGGGGAGGTGGGAGCGGAGGTGGAGGGTCCGGAG GGGGGAGCCTTCAAGGCACTACTTGTCCTCCACCCGTATCCATCGAGCACGCCGATATTCGAGTTAA AAATTATAGTGTTAATAGCAGAGAACGATACGTCTGCAACTCAGGGTTTAAGAGAAAGGCCGGAAC TTCAACTCTCATAGAATGCGTGATTAATAAGAATACTAACGTCGCACATTGGACTACTCCCAGTCTC AAGTGCATACGCGATCCATCTCTCGCTCATTACTCACCAGTACCTACAGTGGTTACTCCTAAGGTGA CCTCTCAGCCCGAATCACCATCTCCCAGCGCAAAAGAGCCTGAGGCCTTTTCTCCTAAATCAGACAC TGCTATGACTACAGAAACAGCCATAATGCCAGGAAGCCGGCTGACACCATCTCAAACTACCAGCGC AGGCACAACTGGGACTGGCTCCCACAAAAGCTCACGCGCACCAAGTCTCGCCGCAACAATGACATT GGAGCCTACAGCCAGCACATCTCTTAGAATCACAGAAATTTCTCCCCACAGTAGCAAGATGACCAA GGTGGCAATTAGTACCAGCGTCCTTCTTGTAGGAGCTGGAGTTGTGATGGCATTTTTGGCATGGTAT ATCAAAAGCAGGtaG
IL-15 (Mouse) (SEQ ID NO: 67)
ATGAAGATCCTCAAGCCATACATGCGAAACACTAGTATTAGCTGTTACTTGTGTTTTCTGCTGAATA GTCATTTTTTGACTGAAGCAGGAATCCATGTATTTATACTCGGTTGTGTGTCTGTAGGTCTGCCAAAG ACTGAGGCTAATTGGATTGACGTGCGCTATGATCTTGAAAAAATAGAGTCCTTGATTCAATCAATAC ACATCGATACCACTCTCTACACCGACAGTGATTTCCATCCTTCCTGCAAGGTAACAGCTATGAATTG CTTCCTCCTGGAGCTCCAAGTCATTCTCCATGAGTACTCCAACATGACTTTGAACGAAACTGTAAGA AACGTATTGTATCTGGCTAATAGCACCTTGTCTAGTAACAAAAATGTGGCAGAGAGCGGCTGCAAA GAATGTGAAGAATTGGAAGAGAAAACATTTACAGAGTTCCTGCAATCCTTTATTCGCATCGTCCAAA TGTTTATCAATACCTCTtaG
IL-15 (Mouse) (SEQ ID NO: 68)
ATGTATTCCATGCAACTTGCCAGTTGTGTAACCCTTACTCTCGTCCTGCTCGTTAATTCCGCTGGTGC TAACTGGATAGATGTTCGATACGATCTGGAAAAGATTGAGTCCCTTATCCAATCCATTCATATAGAT ACCACCCTTTATACTGACAGCGACTTCCATCCTTCTTGCAAGGTGACCGCTATGAATTGTTTCCTGCT GGAACTCCAAGTTATTCTGCATGAATACTCTAATATGACACTTAACGAGACCGTAAGAAATGTTCTC TATCTCGCTAATAGTACTTTGAGCTCAAATAAGAACGTGGCCGAGTCTGGGTGTAAGGAATGCGAA GAGCTGGAAGAAAAGACATTCACCGAGTTTCTCCAGTCTTTCATACGGATTGTGCAGATGTTTATCA ACACATCAGATTACAAAGACGACGATGATAAGtaG
IL-18 (Mouse) (SEQ ID NO: 69)
ATGGCAGCCATGTCTGAGGACTCTTGTGTGAACTTTAAAGAAATGATGTTCATAGACAATACACTCT ACTTTATACCTGAGGAGAATGGAGATTTGGAATCTGACAACTTTGGCAGGCTGCATTGCACTACCGC AGTTATCCGAAACATCAACGATCAGGTACTGTTTGTTGATAAAAGACAACCTGTATTCGAGGACATG ACCGACATAGATCAGTCTGCCTCAGAGCCCCAGACTAGGCTTATCATCTATATGTACAAGGACAGC GAAGTACGAGGCCTGGCTGTTACACTCTCAGTCAAAGACTCTAAGATGAGCACCCTGTCATGCAAG AACAAAATTATCAGTTTTGAGGAGATGGACCCACCTGAAAACATAGATGACATTCAGTCAGACCTC ATTTTTTTTCAAAAGCGGGTACCAGGACACAACAAAATGGAATTTGAATCATCACTCTACGAAGGA CATTTCCTTGCATGCCAGAAAGAGGATGACGCATTCAAATTGATCCTGAAAAAAAAGGACGAAAAT GGTGATAAATCAGTCATGTTTACATTGACCAATCTTCACCAAAGTtaG
IL-18 (Mouse) (SEQ ID NO: 70)
ATGGCTGCAATGTCTGAAGATAGCTGTGTCAACTTTAAGGAGATGATGTTCATTGATAATACTTTGT ACTTTATACCTGAAGAAAATGGAGACCTTGAGTCAGACAACTTCGGGAGACTGCACTGCACAACTG CCGTTATCCGAAACATAAATGATCAAGTATTGTTCGTGGACAAAAGACAACCAGTCTTTGAGGATAT GACAGACATCGACCAATCCGCATCTGAACCTCAGACTAGGCTGATCATCTATATGTACGCCGACTCC GAAGTAAGAGGCCTTGCTGTGACACTTAGTGTTAAGGATAGTAAGATGAGCACACTGTCCTGTAAG AATAAGATTATATCTTTTGAAGAGATGGACCCTCCCGAGAACATAGATGACATCCAGAGCGACTTG

ATCTTCTTTCAGAAGCGAGTGCCAGGCCATAACAAGATGGAATTTGAATCATCTCTTTATGAAGGCC ATTTCCTCGCATGTCAAAAGGAGGACGATGCCTTCAAGCTCATTCTGAAAAAAAAAGACGAGAACG GTGATAAGAGCGTGATGTTCACTCTGACAAATCTGCACCAGTCAtaG
IL-18 (Human) (SEQ ID NO: 71)
ATGTATCGCATGCAACTCCTGTCCTGCATTGCTCTGAGCTTGGCTTTGGTAACCAACTCATACTTCGG GAAACTGGAGAGTAAACTCTCCGTAATCAGGAATCTTAATGACCAAGTATTGTTTATTGACCAGGGC AACCGCCCGTTGTTCGAGGATATGACTGATTCTGACTGTCGGGATAACGCTCCGAGAACTATCTTTA TCATTTCAATGTACAAGGACAGCCAACCGCGGGGTATGGCTGTGACAATCAGTGTCAAATGTGAGA AGATTTCCACGCTGTCCTGCGAAAACAAGATAATTTCTTTCAAAGAAATGAACCCCCCTGACAATAT AAAGGATACAAAGAGTGATATCATCTTCTTTCAGAGGTCCGTGCCCGGCCACGATAATAAGATGCA ATTTGAAAGTTCATCTTATGAGGGGTACTTTTTGGCATGCGAGAAAGAAAGGGATCTCTTCAAGTTG ATCCTGAAGAAGGAGGACGAATTGGGCGACCGCTCCATCATGTTCACAGTCCAGAACGAGGACtaG
IL-18 (Human) (SEQ ID NO: 72)
ATGTACCGCATGCAGCTCCTGAGTTGTATTGCCCTTTCCCTCGCTCTCGTTACCAATTCTTACTTCGG TAAGCTTGCCTCTAAACTCTCTGTTATTAGGAACTTGAACGACCAAGTCCTTTTCATAGACCAAGGG AACAGACCACTGTTTGAAGATATGACGGATAGCGATTGCCGAGATAATGCCCCTAGGACGATTTTT ATCATTAGTATGTATGCGGACTCTCAACCGAGGGGGATGGCCGTTACTATAAGTGTGAAATGCGAG AAAATATCAACGCTCAGTTGTGAGAACAAAATCATAAGTTTCAAGGAGATGAATCCACCTGATAAC ATCAAAGACACTAAGTCTGATATTATATTTTTCCAACGAAGTGTTCCGGGACACGATAACAAAATGC AATTTGAGAGCTCCTCATACGAGGGCTACTTCCTCGCGTGTGAGAAAGAAAGGGATTTGTTTAAGCT TATCCTCAAGAAAGAGGACGAGTTGGGGGATCGGAGCATAATGTTTACCGTACAGAATGAGGACtaG
IL-21 (Mouse) (SEQ ID NO: 73)
ATGGAGCGGACACTCGTGTGTCTTGTCGTAATTTTTCTCGGGACAGTCGCACACAAGTCCTCACCCC AGGGTCCTGATCGCCTTCTCATACGCCTCCGACATTTGATCGACATTGTAGAGCAGCTCAAAATTTA CGAGAATGACCTCGATCCCGAGCTTTTGAGTGCTCCCCAAGACGTTAAGGGTCATTGCGAGCACGC AGCTTTTGCTTGCTTCCAGAAGGCCAAGTTGAAACCAAGCAACCCTGGTAATAATAAGACTTTCATC ATCGACTTGGTCGCCCAACTCCGAAGGAGGCTGCCTGCCCGGCGCGGAGGAAAAAAACAAAAGCA TATTGCAAAGTGTCCTTCATGTGATTCATACGAAAAGCGGACTCCCAAAGAGTTCTTGGAAAGGTTG AAATGGCTTCTTCAGAAGATGATTCATCAACATTTGTCAtaG
IFN-beta (Human) (SEQ ID NO: 74)
ATGACCAACAAATGCCTTTTGCAAATTGCCCTGCTTTTGTGTTTTAGCACTACCGCATTGAGCATGTC ATATAACCTCCTCGGCTTCCTTCAGAGATCATCAAACTTTCAGTGTCAGAAACTGCTTTGGCAACTT AATGGCAGGCTCGAATATTGTCTGAAAGATCGGATGAATTTCGACATTCCAGAAGAAATAAAACAG CTTCAACAATTCCAGAAAGAGGACGCCGCCCTGACTATTTACGAGATGCTCCAGAATATCTTCGCCA TTTTCCGGCAGGACAGCTCATCCACGGGGTGGAATGAGACTATTGTAGAAAATCTTCTGGCTAATGT GTACCATCAAATTAATCACCTCAAAACGGTGCTTGAGGAAAAACTTGAAAAGGAAGATTTCACACG GGGCAAGTTGATGTCCTCCCTGCACCTTAAACGATACTACGGCAGGATTCTTCATTACTTGAAGGCT AAGGAGTATAGCCATTGCGCGTGGACAATTGTACGGGTAGAAATACTGCGAAACTTTTATTTCATCA ACCGGCTCACTGGATACCTTAGAAATtaG
IFN-beta (Mouse) (SEQ ID NO: 75)
ATGAACAATCGGTGGATACTCCACGCCGCATTTCTCCTCTGCTTTAGCACGACGGCCCTGTCCATCA ACTACAAACAGCTTCAGTTGCAGGAGCGGACTAACATAAGGAAGTGCCAGGAACTGCTGGAACAG CTTAATGGTAAAATTAATCTTACATACCGAGCTGACTTCAAAATTCCTATGGAAATGACCGAGAAGA TGCAGAAATCCTACACGGCATTCGCCATCCAGGAAATGCTCCAGAACGTATTTCTCGTGTTCCGCAA TAATTTCTCTTCTACGGGTTGGAACGAAACCATTGTTGTTAGACTGCTTGACGAACTGCATCAGCAA ACCGTGTTCCTTAAAACCGTGCTTGAGGAGAAGCAGGAGGAGCGCCTGACTTGGGAGATGTCTAGT ACCGCACTTCACTTGAAATCCTACTACTGGCGCGTTCAGCGGTATCTGAAGCTGATGAAGTATAACT CATACGCCTGGATGGTAGTGCGCGCAGAGATCTTCAGAAACTTTCTTATCATCCGGCGACTGACCCG AAACTTTCAGAATtaG
IFN-gamma (Human) (SEQ ID NO: 76)

ATGAAGTACACTAGCTATATATTGGCCTTCCAGCTTTGCATCGTATTGGGTAGCCTCGGATGCTATT GCCAAGACCCGTATGTCAAAGAAGCCGAAAATCTCAAAAAGTATTTCAATGCCGGACACTCAGACG TCGCGGATAACGGTACACTGTTTCTTGGCATCCTGAAAAATTGGAAGGAAGAGAGTGACAGAAAAA TAATGCAGTCACAAATAGTGTCCTTTTACTTTAAGCTGTTCAAAAATTTCAAGGATGACCAAAGTAT CCAGAAGAGTGTTGAAACTATCAAAGAGGACATGAATGTGAAATTCTTTAACAGTAATAAGAAGAA GCGCGATGACTTCGAGAAACTCACTAATTACAGCGTAACGGATCTTAACGTCCAACGCAAGGCAAT CCACGAGCTTATACAGGTAATGGCTGAGCTTAGTCCCGCAGCCAAGACAGGGAAGAGAAAAAGGT CTCAAATGCTTTTTCGGGGCCGGCGAGCTTCACAAtaG
IFN-gamma (Mouse) (SEQ ID NO: 77)
ATGAACGCTACGCATTGCATCCTCGCACTCCAATTGTTCCTCATGGCTGTGTCAGGGTGTTACTGTC ACGGTACTGTCATAGAAAGCCTCGAATCCCTGAATAACTATTTTAACAGTAGCGGTATAGATGTAGA AGAAAAGTCTCTCTTTCTTGACATCTGGAGGAATTGGCAAAAGGATGGAGACATGAAGATTCTCCA ATCTCAGATTATATCATTTTACTTGAGGCTTTTTGAGGTTCTGAAGGATAACCAGGCGATCAGCAAT AATATCAGCGTAATTGAATCTCACCTTATTACAACATTTTTCTCAAATTCCAAGGCAAAGAAAGATG CTTTCATGTCTATCGCGAAATTTGAGGTGAACAATCCTCAGGTACAAAGGCAAGCCTTTAACGAGCT GATTAGAGTTGTACATCAGTTGTTGCCCGAAAGTAGTCTTAGAAAACGCAAACGGAGCCGATGCtaG
IFN-alpha (Mouse) (SEQ ID NO: 78)
ATGGCAAGGTTGTGCGCTTTTCTCATGGTACTGGCTGTGCTCTCCTATTGGCCTACTTGTTCTCTGGG ATGCGACTTGCCACAGACCCACAATCTGCGGAATAAGAGGGCTCTGACTCTGCTGGTGCAAATGAG ACGGCTCTCTCCACTTAGCTGTTTGAAAGATAGAAAGGATTTCGGGTTCCCCCAGGAGAAGGTGGA TGCCCAGCAGATCAAGAAGGCACAGGCTATCCCCGTCCTTTCCGAGCTGACCCAGCAAATTTTGAA CATCTTTACAAGTAAGGATAGTTCAGCTGCATGGAATACCACACTTTTGGATTCTTTTTGTAACGATC TGCATCAGCAGCTGAACGATCTCCAGGGATGCCTGATGCAGCAAGTCGGCGTGCAAGAATTTCCAC TCACCCAGGAGGACGCTCTGCTCGCAGTGCGAAAGTATTTTCACCGAATTACCGTGTACCTCCGGGA GAAAAAGCATTCACCCTGCGCTTGGGAAGTAGTCAGGGCCGAAGTATGGAGAGCCCTTAGTAGCTC CGCTAATGTACTGGGCCGGTTGCGGGAAGAGAAAtaG

The first engineered nucleic acid can include a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 309. The first engineered nucleic acid can include a nucleotide sequence having the sequence shown in SEQ ID NO: 309.

The first engineered nucleic acid can include a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 326. The first engineered nucleic acid can include a nucleotide sequence having the sequence shown in SEQ ID NO: 326.

The first engineered nucleic acid can include a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 310. The first engineered nucleic acid can include a nucleotide sequence having the sequence shown in SEQ ID NO: 310.

The first engineered nucleic acid can include a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 327. The first engineered nucleic acid can include a nucleotide sequence having the sequence shown in SEQ ID NO: 327.

The first engineered nucleic acid can include a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 314. The first engineered nucleic acid can include a nucleotide sequence having the sequence shown in SEQ ID NO: 314.

The first engineered nucleic acid can include a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 315. The first engineered nucleic acid can include a nucleotide sequence having the sequence shown in SEQ ID NO: 315.

The second engineered nucleic acid can include a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 317. The second engineered nucleic acid can include a nucleotide sequence having the sequence shown in SEQ ID NO: 317.

The second engineered nucleic acid can include a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 318. The second engineered nucleic acid can include a nucleotide sequence having the sequence shown in SEQ ID NO: 318.

The first engineered nucleic acid can include a nucleotide sequence having the sequence shown in SEQ ID NO: 310; and (b) the second engineered nucleic acid can include a nucleotide sequence having the sequence shown in SEQ ID NO: 317.

The first engineered nucleic acid can include a nucleotide sequence having the sequence shown in SEQ ID NO: 327; and (b) the second engineered nucleic acid can include a nucleotide sequence having the sequence shown in SEQ ID NO: 317.

The first engineered nucleic acid can include a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 310; and (b) the second engineered nucleic acid can include a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 317.

The first engineered nucleic acid can include a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 327; and (b) the second engineered nucleic acid can include a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 317.

Immunoresponsive cells provided for herein can include any one of the engineered nucleic acids described herein. Immunoresponsive cells provided for herein can include combinations of any one of the engineered nucleic acids described herein. Immunoresponsive cells provided for herein can include two or more of any one of the engineered nucleic acids described herein.

Immunoresponsive cells provided for herein can include a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 309. Immunoresponsive cells provided for herein can include a nucleotide sequence having the sequence shown in SEQ ID NO: 309.

Immunoresponsive cells provided for herein can include a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 326. Immunoresponsive cells provided for herein can include a nucleotide sequence having the sequence shown in SEQ ID NO: 326.

Immunoresponsive cells provided for herein can include a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 310. Immunoresponsive cells provided for herein can include a nucleotide sequence having the sequence shown in SEQ ID NO: 310.

Immunoresponsive cells provided for herein can include a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 327. Immunoresponsive cells provided for herein can include a nucleotide sequence having the sequence shown in SEQ ID NO: 327.

Immunoresponsive cells provided for herein can include a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 314. Immunoresponsive cells provided for herein can include a nucleotide sequence having the sequence shown in SEQ ID NO: 314.

Immunoresponsive cells provided for herein can include a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 315. Immunoresponsive cells provided for herein can include a nucleotide sequence having the sequence shown in SEQ ID NO: 315.

Immunoresponsive cells provided for herein can include a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 317. Immunoresponsive cells provided for herein can include a nucleotide sequence having the sequence shown in SEQ ID NO: 317.

Immunoresponsive cells provided for herein can include a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 318. Immunoresponsive cells provided for herein can include a nucleotide sequence having the sequence shown in SEQ ID NO: 318.

Immunoresponsive cells provided for herein can include a first engineered nucleic acid including a nucleotide sequence having the sequence shown in SEQ ID NO: 310; and (b) a second engineered nucleic acid including a nucleotide sequence having the sequence shown in SEQ ID NO: 317.

Immunoresponsive cells provided for herein can include a first engineered nucleic acid including a nucleotide sequence having the sequence shown in SEQ ID NO: 327; and (b) a second engineered nucleic acid including a nucleotide sequence having the sequence shown in SEQ ID NO: 317.

Immunoresponsive cells provided for herein can include a first engineered nucleic acid including a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 310; and (b) a second engineered nucleic acid including a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 317.

Immunoresponsive cells provided for herein can include a first engineered nucleic acid including a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 327; and (b) a second engineered nucleic acid including a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 317.

Expression vectors provided for herein can include any one of the engineered nucleic acids described herein. Expression vectors provided for herein can include combinations of any one of the engineered nucleic acids described herein. Expression vectors provided for herein can include two or more of any one of the engineered nucleic acids described herein.

Expression vectors provided for herein can include a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 309. Expression vectors provided for herein can include a nucleotide sequence having the sequence shown in SEQ ID NO: 309.

Expression vectors provided for herein can include a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 326. Expression vectors provided for herein can include a nucleotide sequence having the sequence shown in SEQ ID NO: 326.

Expression vectors provided for herein can include a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 310. Expression vectors provided for herein can include a nucleotide sequence having the sequence shown in SEQ ID NO: 310.

Expression vectors provided for herein can include a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 327. Expression vectors provided for herein can include a nucleotide sequence having the sequence shown in SEQ ID NO: 327.

Expression vectors provided for herein can include a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 314. Expression vectors provided for herein can include a nucleotide sequence having the sequence shown in SEQ ID NO: 314.

Expression vectors provided for herein can include a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 315. Expression vectors provided for herein can include a nucleotide sequence having the sequence shown in SEQ ID NO: 315.

Expression vectors provided for herein can include a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 317. Expression vectors provided for herein can include a nucleotide sequence having the sequence shown in SEQ ID NO: 317.

Expression vectors provided for herein can include a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 318. Expression vectors provided for herein can include a nucleotide sequence having the sequence shown in SEQ ID NO: 318.

Expression vectors provided for herein can include a first engineered nucleic acid including a nucleotide sequence having the sequence shown in SEQ ID NO: 310; and (b) a

second engineered nucleic acid including a nucleotide sequence having the sequence shown in SEQ ID NO: 317.

Expression vectors provided for herein can include a first engineered nucleic acid including a nucleotide sequence having the sequence shown in SEQ ID NO: 327; and (b) a second engineered nucleic acid including a nucleotide sequence having the sequence shown in SEQ ID NO: 317.

Expression vectors provided for herein can include a first engineered nucleic acid including a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 310; and (b) a second engineered nucleic acid including a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 317.

Expression vectors provided for herein can include a first engineered nucleic acid including a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 327; and (b) a second engineered nucleic acid including a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 317.

Secretion Signals and Signal-Anchors

The one or more effector molecules (*e.g.*, any of the cytokines described herein) of the membrane-cleavable chimeric proteins provided for herein are in general secretable effector molecules having a secretion signal peptide (also referred to as a signal peptide or signal sequence) at the chimeric protein's N-terminus (*e.g.*, an effector molecule's N-terminus for **S – C – MT**) that direct newly synthesized proteins destined for secretion or membrane localization (also referred to as membrane insertion) to the proper protein processing pathways. For chimeric proteins having the formula **MT – C – S**, a membrane tethering domain generally has a signal-anchor sequence (*e.g.*, signal-anchor sequences of a Type II transmembrane protein) that direct newly synthesized proteins destined for membrane localization to the proper protein processing pathways. For chimeric proteins having the formula **S – C – MT**, a membrane tethering domain having a reverse signal-anchor sequence (*e.g.*, signal-anchor sequences of certain Type III transmembrane proteins) can be used, generally without a separate secretion signal peptide, that direct newly synthesized proteins destined for membrane localization to the proper protein processing pathways.

In general, for all membrane-cleavable chimeric proteins described herein, the one or more effector molecules are secretable effector molecules (referred to as "S" in the formula **S – C – MT** or **MT – C – S**). In embodiments with two or more chimeric proteins, each chimeric protein can comprise a secretion signal. In embodiments with two or more chimeric proteins, each chimeric protein can comprise a secretion signal such that each effector molecule is capable of secretion from an engineered cell following cleavage of the protease cleavage site.

The secretion signal peptide operably associated with an effector molecule can be a native secretion signal peptide (*e.g.*, the secretion signal peptide generally endogenously associated with the given effector molecule, such as a cytokine's endogenous secretion signal peptide). The secretion signal peptide operably associated with an effector molecule can be a non-native secretion signal peptide native secretion signal peptide. Non-native secretion signal peptides can promote improved expression and function, such as maintained secretion, in particular environments, such as tumor microenvironments. Non-limiting examples of non-native secretion signal peptide are shown in Table 3.

Table 3. Exemplary Signal Secretion Peptides

Name	Protein SEQUENCE	Source (Uniprot)	DNA SEQUENCE
IL-12	MCHQQLVISWFSL VFLASPLVA (SEQ ID NO: 112)	P29460	ATGTGTCACCAGCAGCTCGTTATATC CTGGTTTAGTTTGGTGTTTCTCGCTTC ACCCCTGGTGGCA (SEQ ID NO: 31)
IL-12 (Codon Optimized)	MCHQQLVISWFSL VFLASPLVA (SEQ ID NO: 112)	-	ATGTGCCATCAGCAACTCGTCATCTC CTGGTTCTCCCTTGTGTTCCTCGCTTC CCCTCTGGTCGCC (SEQ ID NO: 32)
IL-2 (Optimized)	MQLLSCIALILALV (SEQ ID NO: 113)	-	ATGCAACTGCTGTCATGTATCGCACT CATCCTGGCGCTGGTA (SEQ ID NO: 33)
IL-2 (Native)	MYRMQLLSCIALSL ALVTNS (SEQ ID NO: 114)	P60568	ATGTATCGGATGCAACTTTTGAGCTG CATCGCATTGTCTCTGGCGCTGGTGA CAAATTCC (SEQ ID NO: 34)
Trypsinogen-2	MNLLLILTFVAAAV A (SEQ ID NO: 115)	P07478	ATGAATCTCTTGCTCATACTTACGTTT GTCGCTGCTGCCGTTGCG (SEQ ID NO: 35)
Gaussia Luciferase	MGVKVLFALICIAV AEA (SEQ ID NO: 116)	-	ATGGGCGTGAAGGTCTTGTTTGCCCT TATCTGCATAGCTGTTGCGGAGGCG (SEQ ID NO: 36)
CD5	MPMGSLQPLATLY LLGMLVASCLG (SEQ ID NO: 117)	P06127	ATGCCGATGGGGAGCCTTCAACCTTT GGCAACGCTTTATCTTCTGGGGATGT TGGTTGCTAGTTGCCTTGGG (SEQ ID NO: 37)
IgKVII (mouse)	METDTLLLWVLLL WVPGSTGD (SEQ ID NO: 118)		ATGGAAACTGACACGTTGTTGCTGTG GGTATTGCTCTTGTGGGTCCCAGGAT CTACGGGCGAC (SEQ ID NO: 38)
IgKVII (human)	MDMRVPAQLLGLL LLWLRGARC (SEQ ID NO: 119)	P01597	ATGGATATGAGGGTTCCCGCCCAGCT TTTGGGGCTGCTTTTGTTGTGGCTTCG AGGGGCTCGGTGT (SEQ ID NO: 39)
VSV-G	MKCLLYLAFLFIGV NC (SEQ ID NO: 120)	-	ATGAAGTGTCTGTTGTACCTGGCGTT TCTGTTCATTGGTGTAAACTGT (SEQ ID NO: 40)

Name	Protein SEQUENCE	Source (Uniprot)	DNA SEQUENCE
Prolactin	MNIKGSPWKGSLLLLVSNLLLCQSVAP (SEQ ID NO: 121)	P01236	ATGAATATCAAAGGAAGTCCGTGGAAGGGTAGTCTCCTGCTGCTCCTCGTATCTAACCTTCTCCTTTGTCAATCCGTGGCACCC (SEQ ID NO: 41)
Serum albumin preproprotein	MKWVTFISLLFLFSSAYS (SEQ ID NO: 122)	P02768	ATGAAATGGGTAACATTCATATCACTTCTCTTTCTGTTCAGCTCTGCGTATTCT (SEQ ID NO: 42)
Azurocidin preproprotein	MTRLTVLALLAGLLASSRA (SEQ ID NO: 123)	20160	ATGACAAGGCTTACTGTTTTGGCTCTCCTCGCTGGACTCTTGGCTTCCTCCCGAGCA (SEQ ID NO: 43)
Osteonectin (BM40)	MRAWIFFLLCLAGRALA (SEQ ID NO: 124)	P09486	ATGAGGGCTTGGATTTTTTTTCTGCTCTGCCTTGCCGGTCGAGCCCTGGCG (SEQ ID NO: 44)
CD33	MPLLLLLPLLWAGALA (SEQ ID NO: 125)	P20138	ATGCCTCTTCTGCTTTTGCTTCCTCTTTTGTGGGCAGGTGCCCTCGCA (SEQ ID NO: 45)
IL-6	MNSFSTSAFGPVAFSLGLLLVLPAAFPAP (SEQ ID NO: 126)	P05231	ATGAACTCTTTCTCAACCTCTGCGTTTGGTCCGGTCGCTTTCTCCCTTGGGCTCCTGCTTGTCTTGCCAGCAGCGTTTCCTGCGCCA (SEQ ID NO: 46)
IL-8	MTSKLAVALLAAFLISAALC (SEQ ID NO: 127)	P10145	ATGACAAGTAAACTGGCGGTAGCCTTGCTCGCGGCCTTTTTGATTTCCGCAGCCCTTTGT (SEQ ID NO: 47)
CCL2	MKVSAALLCLLLIAATFIPQGLA (SEQ ID NO: 128)	P13500	ATGAAGGTAAGTGCAGCGTTGCTTTGCCTTCTCCTCATTGCAGCGACCTTTATTCCTCAAGGGCTGGCC (SEQ ID NO: 48)
TIMP2	MGAAARTLRLALGLLLLATLLRPADA (SEQ ID NO: 129)	P16035	ATGGGAGCGGCAGCTAGAACACTTCGACTTGCCCTTGGGCTCTTGCTCCTTGCAACCCTCCTTAGACCTGCCGACGCA (SEQ ID NO: 49)
VEGFB	MSPLLRRLLLAALLQLAPAQA (SEQ ID NO: 130)	P49765	ATGTCACCGTTGTTGCGGAGATTGCTGTTGGCCGCACTTTTGCAACTGGCTCCTGCTCAAGCC (SEQ ID NO: 50)
Osteoprotegerin	MNNLLCCALVFLDISIKWTTQ (SEQ ID NO: 131)	O00300	ATGAATAACCTGCTCTGTTGTGCGCTCGTGTTCCTGGACATTTCTATAAAATGGACAACGCAA (SEQ ID NO: 51)
Serpin E1	MQMSPALTCLVLGLALVFGEGSA (SEQ ID NO: 132)	P05121	ATGCAAATGTCTCCTGCCCTTACCTGTCTCGTACTTGGTCTTGCGCTCGTATTTGGAGAGGGATCAGCC (SEQ ID NO: 52)
GROalpha	MARAALSAAPSNPRLLRVALLLLLLVAAGRRAAG (SEQ ID NO: 133)	P09341	ATGGCAAGGGCTGCACTCAGTGCTGCCCCGTCTAATCCCAGATTGCTTCGAGTTGCATTGCTTCTTCTGTTGCTGGTTGCAGCTGGTAGGAGAGCAGCGGGT (SEQ ID NO: 53)
CXCL12	MNAKVVVVLVLVLTALCLSDG (SEQ ID NO: 134)	P48061	ATGAATGCAAAAGTCGTGGTCGTGCTGGTTTTGGTTCTGACGGCGTTGTGTCTTAGTGATGGG (SEQ ID NO: 54)
IL-21 (Codon Optimized)	MERIVICLMVIFLGTLVHKSSS (SEQ ID NO: 135)	Q9HBE4	ATGGAACGCATTGTGATCTGCCTGATGGTCATCTTCCTGGGCACCTTAGTGCACAAGTCGAGCAGC (SEQ ID NO: 55)
CD8	MALPVTALLLPLALLLHAARP (SEQ ID NO: 136)	-	ATGGCCTTACCAGTGACCGCCTTGCTCCTGCCGCTGGCCTTGCTGCTCCACGCCGCCAGGCCG (SEQ ID NO: 139)
CD8 (Codon Optimized)	MALPVTALLLPLALLLHAARP (SEQ ID NO: 137)	-	ATGGCGCTCCCGGTGACAGCACTTCTCTTGCCTCTTGCCCTGCTGTTGCATGCCGCGCGCCCA (SEQ ID NO: 140)
GMCSFRa	MLLVTSLLLCELPHPAFLLIP (SEQ ID NO: 138)	-	ATGTTGCTCGTGACATCCCTCTTGCTTTGTGAGTTGCCTCATCCCGCATTCCTGCTCATCCCA (SEQ ID NO: 141)

Name	Protein SEQUENCE	Source (Uniprot)	DNA SEQUENCE
GM-CSFRa	MLLLVTSLLLCELP HPAFLLIP (SEQ ID NO: 216)		ATGCTGCTGCTGGTCACATCTCTGCT GCTGTGCGAGCTGCCCCATCCTGCCT TTCTGCTGATCCCT (SEQ ID NO: 217)
NKG2D	PFFFCCFIAVAMGIR FIIMVA (SEQ ID NO: 192)		CCCTTCTTCTTCTGTTGCTTTATCGCC GTGGCCATGGGCATCCGCTTCATCAT TATGGTGGCC (SEQ ID NO: 193)
IgE	MDWTWILFLVAAA TRVHS (SEQ ID NO: 218)	-	ATGGACTGGACCTGGATCCTGTTTCT GGTGGCCGCTGCCACAAGAGTGCAC AGC (SEQ ID NO: 214)

Protease Cleavage Site

In general, all membrane-cleavable chimeric proteins described herein contain a protease cleavage site (referred to as "C" in the formula **S – C – MT** or **MT – C – S**). In general, the protease cleavage site can be any amino acid sequence motif capable of being cleaved by a protease. Examples of protease cleavage sites include, but are not limited to, a Type 1 transmembrane protease cleavage site, a Type II transmembrane protease cleavage site, a GPI anchored protease cleavage site, an ADAM8 protease cleavage site, an ADAM9 protease cleavage site, an ADAM10 protease cleavage site, an ADAM12 protease cleavage site, an ADAM15 protease cleavage site, an ADAM17 protease cleavage site, an ADAM19 protease cleavage site, an ADAM20 protease cleavage site, an ADAM21 protease cleavage site, an ADAM28 protease cleavage site, an ADAM30 protease cleavage site, an ADAM33 protease cleavage site, a BACE1 protease cleavage site, a BACE2 protease cleavage site, a SIP protease cleavage site, an MT1-MMP protease cleavage site, an MT3-MMP protease cleavage site, an MT5-MMP protease cleavage site, a furin protease cleavage site, a PCSK7 protease cleavage site, a matriptase protease cleavage site, a matriptase-2 protease cleavage site, an MMP9 protease cleavage site, or an NS3 protease cleavage site.

One example of a protease cleavage site is a hepatitis C virus (HCV) nonstructural protein 3 (NS3) protease cleavage site, including, but not limited to, a NS3/NS4A, a NS4A/NS4B, a NS4B/NS5A, or a NS5A/NS5B cleavage site. For a description of NS3 protease and representative sequences of its cleavage sites for various strains of HCV, see, e.g., Hepatitis C Viruses: Genomes and Molecular Biology (S.L. Tan ed., Taylor & Francis, 2006), Chapter 6, pp. 163-206; herein incorporated by reference in its entirety. For example, the sequences of HCV NS4A/4B protease cleavage site; HCV NS5A/5B protease cleavage site; C-terminal degron with NS4A/4B protease cleavage site; N-terminal degron with HCV NS5A/5B protease cleavage site are provided. Representative NS3 sequences are listed in the National Center for Biotechnology Information (NCBI) database. See, for example, NCBI entries: Accession Nos. YP_001491553, YP_001469631, YP_001469632, NP_803144, NP_671491, YP_001469634, YP_001469630, YP_001469633, ADA68311, ADA68307, AFP99000, AFP98987, ADA68322,

AFP99033, ADA68330, AFP99056, AFP99041, CBF60982, CBF60817, AHH29575, AIZ00747, AIZ00744, ABI36969, ABN05226, KF516075, KF516074, KF516056, AB826684, AB826683, JX171009, JX171008, JX171000, EU847455, EF154714, GU085487, JX171065, JX171063; all of which sequences (as entered by the date of filing of this application) are herein incorporated by reference.

Another example of a protease cleavage site is an ADAM17-specific protease (also referred to as Tumor Necrosis Factor-α Converting Enzyme [TACE]) cleavage site. An ADAM17-specific protease cleavage site can be an endogenous sequence of a substrate naturally cleaved by ADAM17. An ADAM17-specific protease cleavage site can be an engineered sequence capable of being cleaved by ADAM17. An engineered ADAM17-specific protease cleavage site can be an engineered for specific desired properties including, but not limited to, optimal expression of the chimeric proteins, specificity for ADAM17, rate-of-cleavage by ADAM17, ratio of secreted and membrane-bound chimeric protein levels, and cleavage in different cell states. A protease cleavage site can be selected for specific cleavage by ADAM17. For example, certain protease cleavage sites capable of being cleaved by ADAM17 are also capable of cleavage by additional ADAM family proteases, such as ADAM10. Accordingly, an ADAM17-specific protease cleavage site can be selected and/or engineered such that cleavage by other proteases, such as ADAM10, is reduced or eliminated. A protease cleavage site can be selected for rate-of-cleavage by ADAM17. For example, it can be desirable to select a protease cleavage site demonstrating a specific rate-of-cleavage by ADAM17, such as reduced cleavage kinetics relative to an endogenous sequence of a substrate naturally cleaved by ADAM17. In such cases, in general, a specific rate-of-cleavage can be selected to regulate the rate of processing of the chimeric protein, which in turn regulates the rate of release/secretion of the payload effector molecule. Accordingly, an ADAM17-specific protease cleavage site can be selected and/or engineered such that the sequence demonstrates a desired rate-of-cleavage by ADAM17. A protease cleavage site can be selected for both specific cleavage by ADAM17 and rate-of-cleavage by ADAM17. Exemplary ADAM17-specific protease cleavage sites, including those demonstrating particular specificity and rate-of-cleavage kinetics, are shown in **Table 4A** below with reference to the site of cleavage (P5-P1: N-terminal; P1'-P5': C-terminal). Further details of ADAM17 and ADAM10, including expression and protease cleavage sites, are described in Sharma, *et al.* (J Immunol October 15, 2017, 199 (8) 2865-2872), Pham *et al.* (Anticancer Res. 2017 Oct;37(10):5507-5513), Caescu *et al.* (Biochem J. 2009 Oct 23; 424(1): 79–88), and Tucher *et al.* (J. Proteome Res. 2014, 13, 4, 2205–2214), each herein incorporated by reference for purposes.

Table 4A – Potential ADAM17 Protease Cleavage Site Sequences

P5	P4	P3	P2	P1	P1'	P2'	P3'	P4'	P5'		FULL SEQ	SEQ ID NO
P	R	A	E	A	V	K	G	G			PRAEAVKGG	179
P	R	A	E	A	L	K	G	G			PRAEALKGG	180
P	R	A	E	Y	S	K	G	G			PRAEYSKGG	181
P	R	A	E	P	I	K	G	G			PRAEPIKGG	182
P	R	A	E	A	Y	K	G	G			PRAEAYKGG	183
P	R	A	E	S	S	K	G	G			PRAESSKGG	184
P	R	A	E	F	T	K	G	G			PRAEFTKGG	185
D	E	P	H	Y	S	Q	R	R			DEPHYSQRR	187
P	P	L	G	P	I	F	N	P	G		PPLGPIFNPG	188
P	L	A	Q	A	Y	R	S	S			PLAQAYRSS	189
T	P	I	D	S	S	F	N	P	D		TPIDSSFNPD	190
V	T	P	E	P	I	F	S	L	I		VTPEPIFSLI	191
P	R	A	E	A	A	K	G	G			PRAEAAKGG	186

In some embodiments, the protease cleavage site comprises a first region having the amino acid sequence of PRAE (SEQ ID NO: 176). In some embodiments, the protease cleavage site comprises a second region having the amino acid sequence of KGG (SEQ ID NO: 177). In some embodiments, the first region is located N-terminal to the second region. In some embodiments, the protease cleavage site comprises the amino acid sequence of PRAEX_1X_2KGG (SEQ ID NO: 178), wherein X_1 is A, Y, P, S, or F, and wherein X_2 is V, L, S, I, Y, T, or A. In some embodiments, the protease cleavage site comprises the amino acid sequence of PRAEAVKGG (SEQ ID NO: 179). In some embodiments, the protease cleavage site comprises the amino acid sequence of PRAEALKGG (SEQ ID NO: 180). In some embodiments, the protease cleavage site comprises the amino acid sequence of PRAEYSKGG (SEQ ID NO: 181). In some embodiments, the protease cleavage site comprises the amino acid sequence of PRAEPIKGG (SEQ ID NO: 182). In some embodiments, the protease cleavage site comprises the amino acid sequence of PRAEAYKGG (SEQ ID NO: 183). In some embodiments, the protease cleavage site comprises the amino acid sequence of PRAESSKGG (SEQ ID NO: 184). In some embodiments, the protease cleavage site comprises the amino acid sequence of PRAEFTKGG (SEQ ID NO: 185). In some embodiments, the protease cleavage site comprises the amino acid sequence of PRAEAAKGG (SEQ ID NO: 186). In some embodiments, the protease cleavage site comprises the amino acid sequence of DEPHYSQRR (SEQ ID NO: 187). In some embodiments, the protease cleavage site comprises the amino acid sequence of PPLGPIFNPG (SEQ ID NO: 188). In some embodiments, the protease cleavage site comprises

the amino acid sequence of PLAQAYRSS (SEQ ID NO: 189). In some embodiments, the protease cleavage site comprises the amino acid sequence of TPIDSSFNPD (SEQ ID NO: 190). In some embodiments, the protease cleavage site comprises the amino acid sequence of VTPEPIFSLI (SEQ ID NO: 191).

In certain embodiments, a cleavage site comprises a linker sequence. A cleavage site may be flanked on the N terminal and/or C terminal sides by a linker sequence. For example and without limitation, the cleavage site may be flanked on both the N terminal and C terminal sides by a partial glycine-serine (GS) linker sequence. Upon cleavage, the N terminal partial GS linker, and C terminal partial GS linker, join to form a GS linker sequence, such as SEQ ID NO: 215.

In certain embodiments, the cleavage site and linker comprise the amino acid sequence of SGGGGSGGGGSGVTPEPIFSLIGGGSGGGGSGGGSLQ (SEQ ID NO: 287). An exemplary nucleic acid sequence encoding SEQ ID NO: 287 is TCTGGCGGCGGAGGATCTGGCGGAGGTGGAAGCGGAGTTACACCCGAGCCTATCTT CAGCCTGATCGGAGGCGGTAGCGGAGGCGGAGGAAGTGGTGGCGGATCTCTGCAA (SEQ ID NO: 288). In some embodiments, nucleic acids encoding SEQ ID NO: 287 may comprise SEQ ID NO: 288, or a nucleic acid sequence that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 288.

In certain embodiments, the protease cleavage site is N-terminal to a linker. In certain embodiments, the protease cleavage site and linker comprise the amino acid sequence of PRAEALKGGSGGGGSGGGGSGGGGSGGGGSGGGSLQ (SEQ ID NO: 289). An exemplary nucleic acid sequence encoding SEQ ID NO: 289 is CCCAGAGCCGAGGCTCTGAAAGGCGGATCAGGCGGCGGTGGTAGTGGAGGCGGAG GCTCAGGCGGCGGAGGTTCCGGAGGTGGCGGTTCCGGCGGAGGATCTCTTCAAT (SEQ ID NO: 292). In some embodiments, nucleic acids encoding SEQ ID NO: 289 may comprise SEQ ID NO: 292, or a nucleic acid sequence that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 292.

In some embodiments, the protease cleavage site comprises the amino acid sequence of ITQGLAVSTISSFF (SEQ ID NO: 198), which is a cleavage site that is native to CD16 and is cleavable by ADAM17. In certain embodiments, SEQ ID NO: 198 is comprised within a linker. In certain embodiments, the linker comprises the amino acid sequence of SGGGGSGGGGSGITQGLAVSTISSFFGGGSGGGGSGGGSLQ (SEQ ID NO: 290). An exemplary nucleic acid sequence encoding SEQ ID NO: 290 is

AGCGGCGGAGGTGGTAGCGGAGGCGGAGGATCTGGAATTACACAGGGACTCGCCG TGTCTACAATCTCCAGCTTCTTTGGTGGCGGTAGTGGCGGCGGTGGCAGTGGCGGTG GATCTCTTCAA (SEQ ID NO: 291). In some embodiments, nucleic acids encoding SEQ ID NO: 290 may comprise SEQ ID NO: 291, or a nucleic acid sequence that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 291.

The protease cleavage site can be C-terminal of the secretable effector molecule. The protease cleavage site can be N-terminal of the secretable effector molecule. In general, for all membrane-cleavable chimeric proteins described herein, the protease cleavage site is either: (1) C-terminal of the secretable effector molecule and N-terminal of the cell membrane tethering domain (in other words, the protease cleavage site is in between the secretable effector molecule and the cell membrane tethering domain); or (2) N-terminal of the secretable effector molecule and C-terminal of the cell membrane tethering domain (also between the secretable effector molecule and the cell membrane tethering domain with domain orientation inverted). The protease cleavage site can be connected to the secretable effector molecule by a polypeptide linker, *i.e.*, a polypeptide sequence not generally considered to be part of the effector molecule or protease cleavage site. The protease cleavage site can be connected to the cell membrane tethering domain by a polypeptide linker, *i.e.*, a polypeptide sequence not generally considered to be part of the cell membrane tethering domain or protease cleavage site. A polypeptide linker can be any amino acid sequence that connects a first polypeptide sequence and a second polypeptide sequence. A polypeptide linker can be a flexible linker (*e.g.*, a Gly-Ser-Gly sequence). Examples of polypeptide linkers include, but are not limited to, GSG linkers (*e.g.*, $[GS]_4GG$ [SEQ ID NO: 182]), $A(EAAAK)_3A$ (SEQ ID NO: 183), and Whitlow linkers (*e.g.*, a "KEGS" linker such as the amino acid sequence KESGSVSSEQLAQFRSLD (SEQ ID NO: 184), an eGK linker such as the amino acid sequence EGKSSGSGSESKST (SEQ ID NO: 185), an LR1 linker such as the amino acid sequence SGGGGSGGGGSGGGGSGGGGSGGGSLQ (SEQ ID NO: 215), and linkers described in more detail in Issued U.S. Pat. No. 5,990,275 herein incorporated by reference). Additional exemplary polypeptide linkers include SGGGGSGGGGSG (SEQ ID NO: 194), TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD (SEQ ID NO: 196), and GGGSGGGGSGGGSLQ (SEQ ID NO: 197). Other polypeptide linkers may be selected based on desired properties (*e.g.*, length, flexibility, amino acid composition, *etc.*) and are known to those skilled in the art. An exemplary nucleic acid sequence encoding SEQ ID NO: 196 is ACCACCACACCAGCTCCTCGGCCACCAACTCCAGCTCCAACAATTGCCAGCCAGCC TCTGTCTCTGAGGCCCGAAGCTTGTAGACCTGCTGCAGGCGGAGCCGTGCATACAA

GAGGACTGGATTTCGCCTGCGAC (SEQ ID NO: 337). In certain embodiments, a nucleic acid encoding SEQ ID NO: 196 comprises a sequence that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 337.

In the Membrane-Cleavable system, following expression and localization of the chimeric protein into the cell membrane, the protease cleavage site directs cleavage of the chimeric protein such that the effector molecule is released ("secreted") into the extracellular space of a cell.

In general, a protease that cleaves the protease cleavage site is a protease specific for that specific protease cleavage site. For example, in the case of a disintegrin and metalloproteinase ("ADAM") family protease, the protease that cleaves a specific ADAM protease cleavage site is generally limited to the ADAM protease(s) that specifically recognize the specific ADAM protease cleavage site motif. A protease cleavage site can be selected and/or engineered such that cleavage by undesired proteases is reduced or eliminated. Proteases can be membrane-bound or membrane-associated. Proteases can be secreted, *e.g.*, secreted in a specific cellular environment, such as a tumor microenvironment ("TME").

A protease that cleaves the protease cleavage site of the chimeric protein can be expressed in the same cell that expresses the chimeric protein. A protease that cleaves the protease cleavage site of the chimeric protein can be endogenous to a cell expressing the chimeric protein. In other words, a cell engineered to express the chimeric protein can endogenously express the protease specific for the protease cleavage site present in the chimeric protein. Endogenous expression of the protease refers to both expression under generally homeostatic conditions (*e.g.*, a cell generally considered to be healthy), and also to differential expression under non-homeostatic conditions (*e.g.*, upregulated expression in a tumor cell). The protease cleavage site can be selected based on the known proteases endogenously expressed by a desired cell population. In such cases, in general, the cleavage of the protease cleavage site (and thus release/secretion of a payload) can be restricted to only those cells of interest due to the cell-restricted protease needing to come in contact with the protease cleavage site of chimeric protein expressed in the same cell. For example, and without wishing to be bound by theory, ADAM17 is believed to be restricted in its endogenous expression to NK cell and T cells. Thus, selection of an ADAM17-specific protease cleavage site may restrict the cleavage of the protease cleavage site to NK cell and T cells co-expressing the chimeric protein. In other examples, a protease cleavage site can be selected for a specific tumor-associated protease known to be expressed in a particular tumor population of interest (*e.g.*, in a specific tumor cell engineered to express the chimeric protein). Protease and/or expression databases can be used to

select an appropriate protease cleavage site, such as selecting a protease cleavage site cleaved by a tumor-associated proteases through consulting Oncomine (www.oncomine.org), the European Bioinformatic Institute (www.ebi.ac.uk) in particular (www.ebi.ac.uk/gxa), PMAP (www.proteolysis.org), ExPASy Peptide Cutter (ca.expasy.org/tools/peptide cutter) and PMAP.Cut DB (cutdb.burnham.org), each of which is incorporated by reference for all purposes.

A protease that cleaves the protease cleavage site of the chimeric protein can be heterologous to a cell expressing the chimeric protein. For example, a cell engineered to express the chimeric protein can also be engineered to express a protease not generally expressed by the cell that is specific for the protease cleavage site present in the chimeric protein. A cell engineered to express both the chimeric protein and the protease can be engineered to express each from separate engineered nucleic acids or from a multicistronic systems (multicistronic and multi-promoter systems are described in greater detail in the Section herein titled "Multicistronic and Multiple Promoter Systems"). Heterologous proteases and their corresponding protease cleavage site can be selected as described above with reference to endogenous proteases.

A protease that cleaves the protease cleavage site of the chimeric protein can be expressed on a separate distinct cell than the cell that expresses the chimeric protein. For example, the protease can be generally expressed in a specific cellular environment, such as a tumor microenvironment. In such cases, in general, the cleavage of the protease cleavage site can be restricted to only those cellular environments of interest (*e.g.*, a tumor microenvironment) due to the environment-restricted protease needing to come in contact with the protease cleavage site. In embodiments having membrane-cleavable chimeric proteins, in general, the secretion of the effector molecule can be restricted to only those cellular environments of interest (*e.g.*, a tumor microenvironment) due to the environment-restricted protease needing to come in contact with the protease cleavage site. A protease that cleaves the protease cleavage site of the chimeric protein can be endogenous to the separate distinct cell. A protease that cleaves the protease cleavage site of the chimeric protein can be heterologous to the separate distinct cell. For example, the separate distinct cell can be engineered to express a protease not generally expressed by the separate distinct cell.

Proteases include, but are not limited to, a Type 1 transmembrane protease, a Type II transmembrane protease, a GPI anchored protease, an ADAM8 protease, an ADAM9 protease, an ADAM10 protease, an ADAM12 protease, an ADAM15 protease, an ADAM17 protease, an ADAM19 protease, an ADAM20 protease, an ADAM21 protease, an ADAM28 protease, an ADAM30 protease, an ADAM33 protease, a BACE1 protease, a BACE2 protease, a SIP protease, an MT1-MMP protease, an MT3-MMP protease, an MT5-MMP protease, a furin

protease, a PCSK7 protease, a matriptase protease, a matriptase-2 protease, and an MMP9 protease. A protease can be an NS3 protease. A protease can be an ADAM17 protease.

Proteases can be tumor associated proteases, such as, a cathepsin, a cysteine protease, an aspartyl protease, a serine protease, or a metalloprotease. Specific examples of tumor associated proteases include Cathepsin B, Cathepsin L, Cathepsin S, Cathepsin D, Cathepsin E, Cathepsin A, Cathepsin G, Thrombin, Plasmin, Urokinase, Tissue Plasminogen Activator, Metalloproteinase 1 (MMP1), MMP2, MMP3, MMP4, MMP7, MMP8, MMP9, MMP10, MMP11, MMP12, MMP13, MMP14, MMP15, MMP16, MMP17, MMP20, MMP21, MMP23, MMP24, MMP25, MMP26, MMP28, ADAM, ADAMTS, CD10 (CALLA), or prostate specific antigen. Proteases can also include, but are not limited to, proteases listed in Table 4B below. Exemplary cognate protease cleavage sites for certain proteases are also listed in Table 4B.

Table 4B: Exemplary Proteases with Cognate Cleavage Sites and Inhibitors

Protease (UniProt Accession No.)	Cognate cleavage site	Protease inhibitors
HCV NS4A/4B	DEMEECSQHL (SEQ ID NO: 142) EDVVPCSMG (SEQ ID NO: 143)	Simeprevir, Danoprevir, Asunaprevir, Ciluprevir, Boceprevir, Sovaprevir, Paritaprevir, Telaprevir, Grazoprevir
HCV NS5A/5B	DEMEECSQHL (SEQ ID NO: 142) EDVVPCSMG (SEQ ID NO: 143)	Simeprevir, Danoprevir, Asunaprevir, Ciluprevir, Boceprevir, Sovaprevir, Paritaprevir, Telaprevir, Grazoprevir
HCV NS3	DEMEECSQHL (SEQ ID NO: 142) EDVVPCSMG (SEQ ID NO: 143)	Simeprevir, Danoprevir, Asunaprevir, Ciluprevir, Boceprevir, Sovaprevir, Paritaprevir, Telaprevir, Grazoprevir
HCV NS2-3	DEMEECSQHL (SEQ ID NO: 142) EDVVPCSMG (SEQ ID NO: 143)	Simeprevir, Danoprevir, Asunaprevir, Ciluprevir, Boceprevir, Sovaprevir, Paritaprevir, Telaprevir, Grazoprevir
HIV-1 protease (SEQ ID NO: 144)		Amprenavir, Atazanavir, Darunavir, Fosamprenavir, Indinavir, Lopinavir, Nelfinavir, Ritonavir, Saquinavir, Tipranavir
Signal peptidase (P67812, P15367, P00804, P0803)	preference of eukaryotic signal peptidase for cleavage after residue 20 (Xaa^{20}) of pre(⊗pro)apoA-II: Ala, Cys > Gly	

Protease (UniProt Accession No.)	Cognate cleavage site	Protease inhibitors
	> Ser, Thr > Pro > Asn, Val, Ile, Leu, Tyr, His, Arg, Asp.	
proprotein convertases cleaving at hydrophobic residues (e.g., Leu, Phe, Val, or Met) (Q16549, Q8NBP7, Q92824, P29120, Q6UW60, P29122, Q9QXV0)	(R/K)-X-(hydrophobic)-X↓, where X is any amino acid	
proprotein convertases cleaving at small amino acid residues such as Ala or Thr (Q16549, Q8NBP7, Q92824, P29120, Q6UW60, P29122)	(K/R)-(X)n-(K/R)↓, where n is 0, 2, 4 or 6 and X is any amino acid	
proopiomelanocortin converting enzyme (PCE) (Q9UO77615, O776133)	Cleavage at paired basic residues in certain prohormones, either between them, or on the carboxyl side	
chromaffin granule aspartic protease (CGAP)	tends to cleave dipeptide bonds that have hydrophobic residues as well as a beta-methylene group	
prohormone thiol protease (cathepsin L1) (P07154, P07711, P06797, P25975, Q28944)		
carboxypeptidases (e.g., carboxypeptidase E/H, carboxypeptidase D and carboxypeptidase Z) (Q9M099, P15169, Q04609, P08819, P08818, O77564, P70627, O35409, P07519, Q8VZU3, P22792, P15087, P16870, Q9JHH6, Q96IY4, Q7L8A9)	cleaves a peptide bond at the carboxy-terminal (C-terminal) end of a protein or peptide	
aminopeptidases (e.g., arginine aminopeptidase, lysine aminopeptidase, aminopeptidase B)	cleaves a peptide bond at the amino-terminal (N-terminal) end of a protein or peptide	
prolyl endopeptidase (Q12884, P48147, P97321, Q4J6C6)	Hydrolysis of Pro-|-Xaa >> Ala-|-Xaa in oligopeptides. Release of an N-terminal dipeptide, Xaa-Yaa-|-Zaa-, from a polypeptide, preferentially when	

Protease (UniProt Accession No.)	Cognate cleavage site	Protease inhibitors
	Yaa is Pro, provided Zaa is neither Pro nor hydroxyproline	
aminopeptidase N (P97449, P15144, P15145, P15684)	Release of an N-terminal amino acid, Xaa-|-Yaa- from a peptide, amide or arylamide. Xaa is preferably Ala, but may be most amino acids including Pro (slow action). When a terminal hydrophobic residue is followed by a prolyl residue, the two may be released as an intact Xaa-Pro dipeptide	
insulin degrading enzyme (P14735, P35559, Q9JHR7, P22817, Q24K02)	Degradation of insulin, glucagon and other polypeptides. No action on proteins. Cleaves multiple short polypeptides that vary considerably in sequence	
Calpain (O08529, P17655, Q07009, Q27971, P20807, P07384, O35350, O14815, P04632, Q9Y6Q1, O15484, Q9HC96, A6NHC0, Q9UMQ6)	No specific amino acid sequence is uniquely recognized by calpains. Amongst protein substrates, tertiary structure elements rather than primary amino acid sequences appear to be responsible for directing cleavage to a specific substrate. Amongst peptide and small-molecule substrates, the most consistently reported specificity is for small, hydrophobic amino acids (e.g., leucine, valine and isoleucine) at the P2 position, and large hydrophobic amino acids (e.g., phenylalanine and tyrosine) at the P1 position. One fluorogenic calpain substrate is (EDANS)-Glu-Pro-Leu-Phe=Ala-Glu-Arg-Lys-(DABCYL), (EDANSEPLFAERKDABCYL (SEQ ID NO: 145)) with cleavage occurring at the Phe=Ala bond.	
caspase 1 (P29466, P29452)	Strict requirement for an Asp residue at position P1 and has a preferred cleavage sequence of Tyr-Val-Ala-Asp-|- (YVAD; SEQ ID NO: 146).	
caspase 2 (P42575, P29594)	Strict requirement for an Asp residue at P1, with 316-asp being essential for proteolytic activity	

Protease (UniProt Accession No.)	Cognate cleavage site	Protease inhibitors
	and has a preferred cleavage sequence of Val-Asp-Val-Ala-Asp-|- (VDVAD; SEQ ID NO: 147).	
caspase 3 (P42574, P70677)	Strict requirement for an Asp residue at positions P1 and P4. It has a preferred cleavage sequence of Asp-Xaa-Xaa-Asp-|- with a hydrophobic amino-acid residue at P2 and a hydrophilic amino-acid residue at P3, although Val or Ala are also accepted at this position.	
caspase 4 (P70343, P49662)	Strict requirement for Asp at the P1 position. It has a preferred cleavage sequence of Tyr-Val-Ala-Asp-|- (YVAD; SEQ ID NO: 146) but also cleaves at Asp-Glu-Val-Asp-|-(DEVD; SEQ ID NO: 148).	
caspase 5 (P51878)	Strict requirement for Asp at the P1 position. It has a preferred cleavage sequence of Tyr-Val-Ala-Asp-|-(YVAD; SEQ ID NO: 146) but also cleaves at Asp-Glu-Val-Asp-|- -|-(DEVD; SEQ ID NO: 148).	
caspase 6 (P55212)	Strict requirement for Asp at position P1 and has a preferred cleavage sequence of Val-Glu-His-Asp-|-(VEHD; SEQ ID NO: 149).	
caspase 7 (P97864, P55210)	Strict requirement for an Asp residue at position P1 and has a preferred cleavage sequence of Asp-Glu-Val-Asp-|- (DEVD; SEQ ID NO: 148).	
caspase 8 (Q8IRY7, O89110, Q14790)	Strict requirement for Asp at position P1 and has a preferred cleavage sequence of (Leu/Asp/Val)-Glu-Thr-Asp-|-(Gly/Ser/Ala).	
caspase 9 (P55211, Q8C3Q9, Q5IS54)	Strict requirement for an Asp residue at position P1 and with a marked preference for His at position P2. It has a preferred cleavage sequence of Leu-Gly-His-Asp-|-Xaa (LGHD; SEQ ID NO: 150).	
caspase 10 (Q92851)	Strict requirement for Asp at position P1 and has a preferred cleavage sequence of Leu-Gln-	

Protease (UniProt Accession No.)	Cognate cleavage site	Protease inhibitors
	Thr-Asp-|-Gly (LQTDG; SEQ ID NO: 151).	
puromycin sensitive aminopeptidase (P55786, Q11011)	Release of an N-terminal amino acid, preferentially alanine, from a wide range of peptides, amides and arylamides.	
angiotensin converting enzyme (ACE) (P12821, P09470, Q9BYF1) SEQ ID NO: 156	Release of a C-terminal dipeptide, oligopeptide-|-Xaa-Yaa, when Xaa is not Pro, and Yaa is neither Asp nor Glu.	Benazepril (Lotensin), Captopril, Enalapril (Vasotec), Fosinopril, Lisinopril (Prinivil, Zestril), Moexipril, Perindopril (Aceon), Quinapril (Accupril), Ramipril (Altace), Trandolapril (Mavik), Zofenopril
pyroglutamyl peptidase II (Q9NXJ5)	Release of the N-terminal pyroglutamyl group from pGlu--His-Xaa tripeptides and pGlu--His-Xaa-Gly tetrapeptides	
dipeptidyl peptidase IV (P27487, P14740, P28843)	Release of an N-terminal dipeptide, Xaa-Yaa-|-Zaa-, from a polypeptide, preferentially when Yaa is Pro, provided Zaa is neither Pro nor hydroxyproline	
N-arginine dibasic convertase (O43847, Q8BHG1)	Hydrolysis of polypeptides, preferably at -Xaa-|-Arg-Lys-, and less commonly at -Arg-|-Arg-Xaa-, in which Xaa is not Arg or Lys	
endopeptidase 24.15 (thimet oligopeptidase) (P52888, P24155)	Preferential cleavage of bonds with hydrophobic residues at P1, P2 and P3' and a small residue at P1' in substrates of 5 to 15 residues	
endopeptidase 24.16 (neurolysin) (Q9BYT8, Q91YP2)	Preferential cleavage in neurotensin: 10-Pro-|-Tyr-11	
amyloid precursor protein secretase alpha (P05067, P12023, Q9Y5Z0, P56817)	Endopeptidase of broad specificity.	
amyloid precursor protein secretase beta (P05067, P12023, Q9Y5Z0, P56817)	Broad endopeptidase specificity. Cleaves Glu-Val-Asn-Leu-|-Asp-Ala-Glu-Phe (EVNLDAEF; SEQ ID NO: 152) in the Swedish variant of Alzheimer's amyloid precursor protein	
amyloid precursor protein secretase gamma (P05067, P12023, Q9Y5Z0, P56817)	intramembrane cleavage of integral membrane proteins	

Protease (UniProt Accession No.)	Cognate cleavage site	Protease inhibitors
MMP 1 (P03956, Q9EPL5uy)	Cleavage of the triple helix of collagen at about three-quarters of the length of the molecule from the N-terminus, at 775-Gly-|-Ile-776 in the alpha-1(I) chain. Cleaves synthetic substrates and alpha-macroglobulins at bonds where P1' is a hydrophobic residue.	SB-3CT *p*-OH SB-3CT *O*-phosphate SB-3CT RXP470.1
MMP 2 (P08253, P33434)	Cleavage of gelatin type I and collagen types IV, V, VII, X. Cleaves the collagen-like sequence Pro-Gln-Gly-|-Ile-Ala-Gly-Gln (PQGIAGQ; SEQ ID NO: 153).	SB-3CT *p*-OH SB-3CT *O*-phosphate SB-3CT RXP470.1
MMP 3 (P08254, P28862)	Preferential cleavage where P1', P2' and P3' are hydrophobic residues.	SB-3CT *p*-OH SB-3CT *O*-phosphate SB-3CT RXP470.1
MMP 7 (P09237, Q10738)	Cleavage of 14-Ala-|-Leu-15 and 16-Tyr-|-Leu-17 in B chain of insulin. No action on collagen types I, II, IV, V. Cleaves gelatin chain alpha-2(I) > alpha-1(I).	SB-3CT *p*-OH SB-3CT *O*-phosphate SB-3CT RXP470.1
MMP 8 (P22894, O70138)	Can degrade fibrillar type I, II, and III collagens. Cleavage of interstitial collagens in the triple helical domain. Unlike EC 3.4.24.7, this enzyme cleaves type III collagen more slowly than type I.	SB-3CT *p*-OH SB-3CT *O*-phosphate SB-3CT RXP470.1
MMP 9 (P14780, P41245)	Cleavage of gelatin types I and V and collagen types IV and V. Cleaves KiSS1 at a Gly-|-Leu bond. Cleaves type IV and type V collagen into large C-terminal three quarter fragments and shorter N-terminal one quarter fragments. Degrades fibronectin but not laminin or Pz-peptide.	SB-3CT *p*-OH SB-3CT *O*-phosphate SB-3CT RXP470.1
MMP 10 (P09238, O55123)	Can degrade fibronectin, gelatins of type I, III, IV, and V; weakly collagens III, IV, and V.	SB-3CT *p*-OH SB-3CT *O*-phosphate SB-3CT RXP470.1
MMP 11 (P24347, Q02853)	A(A/Q)(N/A)↓(L/Y)(T/V/M/R)(R/K)	SB-3CT *p*-OH SB-3CT *O*-phosphate SB-3CT

Protease (UniProt Accession No.)	Cognate cleavage site	Protease inhibitors
	G(G/A)E↓LR ↓ denotes the cleavage site	RXP470.1
MMP 12 (P39900, P34960)	Hydrolysis of soluble and insoluble elastin. Specific cleavages are also produced at 14-Ala-|-Leu-15 and 16-Tyr-|-Leu-17 in the B chain of insulin Has significant elastolytic activity. Can accept large and small amino acids at the P1' site, but has a preference for leucine. Aromatic or hydrophobic residues are preferred at the P1 site, with small hydrophobic residues (preferably alanine) occupying P3	SB-3CT *p*-OH SB-3CT *O*-phosphate SB-3CT RXP470.1
MMP 13 (P45452, P33435)	Cleaves triple helical collagens, including type I, type II and type III collagen, but has the highest activity with soluble type II collagen. Can also degrade collagen type IV, type XIV and type X	SB-3CT *p*-OH SB-3CT *O*-phosphate SB-3CT RXP470.1
MMP 14 (P50281, P53690)	Activates progelatinase A by cleavage of the propeptide at 37-Asn-|-Leu-38. Other bonds hydrolyzed include 35-Gly-|-Ile-36 in the propeptide of collagenase 3, and 341-Asn-|-Phe-342, 441-Asp-|-Leu-442 and 354-Gln-|-Thr-355 in the aggrecan interglobular domain.	SB-3CT *p*-OH SB-3CT *O*-phosphate SB-3CT RXP470.1
urokinase plasminogen activator (uPA) (P00749, P06869)	Specific cleavage of Arg-|-Val bond in plasminogen to form plasmin.	Plasminogen activator inhibitors (PAI)
tissue plasminogen activator (tPA) (P00750, P11214)	Specific cleavage of Arg-|-Val bond in plasminogen to form plasmin.	Plasminogen activator inhibitors (PAI)
tissue plasminogen activator (tPA) (P00750, P11214)	Specific cleavage of Arg-|-Val bond in plasminogen to form plasmin.	Plasminogen activator inhibitors (PAI)
Plasmin (P00747, P20918)	Preferential cleavage: Lys-|-Xaa > Arg-|-Xaa, higher selectivity than trypsin. Converts fibrin into soluble products.	α-2-antiplasmin (AP)
Thrombin (P00734, P19221)	Cleaves bonds after Arg and Lys Converts fibrinogen to fibrin and activates factors V, VII, VIII, XIII,	

Protease (UniProt Accession No.)	Cognate cleavage site	Protease inhibitors
	and, in complex with thrombomodulin, protein C.	
BMP-1 (procollagen C-peptidase) (P13497, P98063)	Cleavage of the C-terminal propeptide at Ala-|-Asp in type I and II procollagens and at Arg-|-Asp in type III.	
ADAM (Q9P0K1, Q9UKQ2, Q9JLN6, O14672, Q13444, P78536, Q13443, O43184, P78325, Q9UKF5, Q9BZ11, Q9H2U9, Q99965, O75077, Q9H013, O43506)		SB-3CT *p*-OH SB-3CT *O*-phosphate SB-3CT RXP470.1
granzyme A (P12544, P11032)	Preferential cleavage: -Arg-|-Xaa-, -Lys-|-Xaa- >> -Phe-|-Xaa- in small molecule substrates.	
granzyme B (P10144, P04187)	Preference for bulky and aromatic residues at the P1 position and acidic residues at the P3' and P4' sites.	
granzyme M (P51124, Q03238)	Cleaves peptide substrates after methionine, leucine, and norleucine.	
tobacco Etch virus (TEV) protease (P04517, P0CK09)	E-Xaa-Xaa-Y -Xaa-Q-(G/S), with cleavage occurring between Q and G/S. The most common sequence is ENLYFQS (SEQ ID NO: 154)	
chymotrypsin-like serine protease (P08217, Q9UNI1, Q91X79, P08861, P09093, P08218)		-*Thermobifida fusca* Thermopin -*Pyrobaculum aerophilum* Aeropin -*Thermococcus kodakaraensis* Tk-serpin -*Alteromonas sp.* Marinostatin -*Streptomyces misionensis* SMTI -*Streptomyces sp.* chymostatin
alphavirus proteases (P08411, P03317, P13886, Q8JUX6, Q86924, Q4QXJ8, Q8QL53, P27282, Q5XXP4)		
chymotrypsin-like cysteine proteases		-*Thermobifida fusca* Thermopin

Protease (UniProt Accession No.)	Cognate cleavage site	Protease inhibitors
(Q86TL0, Q14790, Q99538, O15553)		-*Pyrobaculum aerophilum* Aeropin -*Thermococcus kodakaraensis* Tk-serpin -*Alteromonas sp.* Marinostatin -*Streptomyces misionensis* SMTI -*Streptomyces sp.* chymostatin
papain-like cysteine proteases (P25774, P53634, Q96K76)		
picornavirus leader proteases (P03305, P03311, P13899)		
HIV proteases (P04585, P03367, P04584, P03369, P12497, P03366, P04587)		
Herpesvirus proteases (P10220, Q2HRB6, O40922, Q69527)		
adenovirus proteases (P03252, P24937, Q83906, P68985, P09569, P11825, P10381)		
Streptomyces griseus protease A (SGPA) (P00776)		
Streptomyces griseus protease B (SGPB) (P00777)		
alpha-lytic protease (P85142, P00778)		
serine proteases (P48740, P98064, Q9UL52, P05981, O60235)		
cysteine proteases (Q86TL0, Q14790, Q8WYN0, Q96DT6, P55211)		
aspartic proteases (Q9Y5Z0, P56817, Q00663, Q53RT3, P0CY27)		
threonine proteases (Q9UI38, Q16512, Q9H6P5, Q8IWU2)		
Mast cell (MC) chymase (CMA1) (NM_001836)	Abz-HPFHL(SEQ ID NO: 155)-Lys(Dnp)-NH2	BAY 1142524 SUN13834

Protease (UniProt Accession No.)	Cognate cleavage site	Protease inhibitors
Rat mast cell protease-1, -2, -3, -4, -5 (NM_017145, NM_172044, NM_001170466, NM_019321, NM_013092)	Abz-HPFHL(SEQ ID NO: 155)-Lys(Dnp)-NH2	TY-51469
Rat vascular chymase (RVCH) (O70500)	Abz-HPFHL(SEQ ID NO: 155)-Lys(Dnp)-NH2	
DENV NS3pro (NS2B/NS3) SEQ ID NOs: 157, 158, 159, 160	A strong preference for basic amino acid residues (Arg/Lys) at the P1 positions was observed, whereas the preferences for the P2– 4 sites were in the order of Arg > Thr > Gln/Asn/Lys for P2, Lys > Arg > Asn for P3, and Nle > Leu > Lys > Xaa for P4. The prime site substrate specificity was for small and polar amino acids in P1 and P3.	Anthraquinone BP13944 ZINC04321905 MB21 Policresulen SK-12 NSC135618 Biliverdin

A protease can be any of the following human proteases (MEROPS peptidase database number provided in parentheses; Rawlings N. D., Morton F. R., Kok, C. Y., Kong, J. & Barrett A. J. (2008) MEROPS: the peptidase database. Nucleic Acids Res. 36 Database issue, D320-325; herein incorporated by reference for all purposes): pepsin A (MER000885), gastricsin (MER000894), memapsin-2 (MER005870), renin (MER000917), cathepsin D (MER000911), cathepsin E (MER000944), memapsin-1 (MER005534), napsin A (MER004981), Mername-AA034 peptidase (MER014038), pepsin A4 (MER037290), pepsin A5 (Homo sapiens) (MER037291), hCG1733572 (Homo sapiens)-type putative peptidase (MER107386), napsin B pseudogene (MER004982), CYMP g.p. (Homo sapiens) (MER002929), subfamily A1A unassigned peptidases (MER181559), mouse mammary tumor virus retropepsin (MER048030), rabbit endogenous retrovirus endopeptidase (MER043650), S71-related human endogenous retropepsin (MER001812), RTVL-H-type putative peptidase (MER047117), RTVL-H-type putative peptidase (MER047133), RTVL-H-type putative peptidase (MER047160), RTVL-H-type putative peptidase (MER047206), RTVL-H-type putative peptidase (MER047253), RTVL-H-type putative peptidase (MER047260), RTVL-H-type putative peptidase (MER047291), RTVL-H-type putative peptidase (MER047418), RTVL-H-type putative peptidase (MER047440), RTVL-H-type putative peptidase (MER047479), RTVL-H-type putative peptidase (MER047559), RTVL-H-type putative peptidase (MER047583), RTVL-H-type putative peptidase (MER015446), human endogenous retrovirus retropepsin homologue 1 (MER015479), human endogenous retrovirus retropepsin homologue 2 (MER015481),

endogenous retrovirus retropepsin pseudogene 1 (Homo sapiens chromosome 14) (MER029977), endogenous retrovirus retropepsin pseudogene 2 (Homo sapiens chromosome 8) (MER029665), endogenous retrovirus retropepsin pseudogene 3 (Homo sapiens chromosome 17) (MER002660), endogenous retrovirus retropepsin pseudogene 3 (Homo sapiens chromosome 17) (MER030286), endogenous retrovirus retropepsin pseudogene 3 (Homo sapiens chromosome 17) (MER047144), endogenous retrovirus retropepsin pseudogene 5 (Homo sapiens chromosome 12) (MER029664), endogenous retrovirus retropepsin pseudogene 6 (Homo sapiens chromosome 7) (MER002094), endogenous retrovirus retropepsin pseudogene 7 (Homo sapiens chromosome 6) (MER029776), endogenous retrovirus retropepsin pseudogene 8 (Homo sapiens chromosome Y) (MER030291), endogenous retrovirus retropepsin pseudogene 9 (Homo sapiens chromosome 19) (MER029680), endogenous retrovirus retropepsin pseudogene 10 (Homo sapiens chromosome 12) (MER002848), endogenous retrovirus retropepsin pseudogene 11 (Homo sapiens chromosome 17) (MER004378), endogenous retrovirus retropepsin pseudogene 12 (Homo sapiens chromosome 11) (MER003344), endogenous retrovirus retropepsin pseudogene 13 (Homo sapiens chromosome 2 and similar) (MER029779), endogenous retrovirus retropepsin pseudogene 14 (Homo sapiens chromosome 2) (MER029778), endogenous retrovirus retropepsin pseudogene 15 (Homo sapiens chromosome 4) (MER047158), endogenous retrovirus retropepsin pseudogene 15 (Homo sapiens chromosome 4) (MER047332), endogenous retrovirus retropepsin pseudogene 15 (Homo sapiens chromosome 4) (MER003182), endogenous retrovirus retropepsin pseudogene 16 (MER047165), endogenous retrovirus retropepsin pseudogene 16 (MER047178), endogenous retrovirus retropepsin pseudogene 16 (MER047200), endogenous retrovirus retropepsin pseudogene 16 (MER047315), endogenous retrovirus retropepsin pseudogene 16 (MER047405), endogenous retrovirus retropepsin pseudogene 16 (MER030292), endogenous retrovirus retropepsin pseudogene 17 (Homo sapiens chromosome 8) (MER005305), endogenous retrovirus retropepsin pseudogene 18 (Homo sapiens chromosome 4) (MER030288), endogenous retrovirus retropepsin pseudogene 19 (Homo sapiens chromosome 16) (MER001740), endogenous retrovirus retropepsin pseudogene 21 (Homo sapiens) (MER047222), endogenous retrovirus retropepsin pseudogene 21 (Homo sapiens) (MER047454), endogenous retrovirus retropepsin pseudogene 21 (Homo sapiens) (MER047477), endogenous retrovirus retropepsin pseudogene 21 (Homo sapiens) (MER004403), endogenous retrovirus retropepsin pseudogene 22 (Homo sapiens chromosome X) (MER030287), subfamily A2A non-peptidase homologues (MER047046), subfamily A2A non-peptidase homologues (MER047052), subfamily A2A non-peptidase homologues (MER047076), subfamily A2A non-peptidase homologues (MER047080), subfamily A2A non-

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(MER003645), epitheliasin (MER003736), kallikrein-related peptidase 4 (MER005266), prosemin (MER004214), chymopasin (MER001503), kallikrein-related peptidase 11 (MER004861), kallikrein-related peptidase 11 (MER216142), trypsin-2 type A (MER000021), HtrA1 peptidase (Homo sapiens-type) (MER002577), HtrA2 peptidase (MER208413), HtrA2 peptidase (MER004093), HtrA3 peptidase (MER014795), HtrA4 peptidase (MER016351), Tysnd1 peptidase (MER050461), TMPRSS12 peptidase (MER017085), HAT-like putative peptidase 2 (MER021884), trypsin C (MER021898), kallikrein-related peptidase 7 (MER002001), matriptase (MER003735), kallikrein-related peptidase 13 (MER005269), kallikrein-related peptidase 9 (MER005270), matriptase-2 (MER005278), umbilical vein peptidase (MER005421), LCLP peptidase (MER001900), spinesin (MER014385), marapsin-2 (MER021929), complement factor D-like putative peptidase (MER056164), ovochymase-2 (MER022410), HAT-like 4 peptidase (MER044589), ovochymase 1 domain 1 (MER022412), epidermis-specific SP-like putative peptidase (MER029900), testis serine peptidase 5 (MER029901), Mername-AA258 peptidase (MER000285), polyserase-IA unit 1 (MER030879), polyserase-IA unit 2 (MER030880), testis serine peptidase 2 (human-type) (MER033187), hypothetical acrosin-like peptidase (Homo sapiens) (MER033253), HAT-like 5 peptidase (MER028215), polyserase-3 unit 1 (MER061763), polyserase-3 unit 2 (MER061748), peptidase similar to tryptophan/serine protease (MER056263), polyserase-2 unit 1 (MER061777), Mername-AA123 peptidase (MER021930), HAT-like 2 peptidase (MER099184), hCG2041452-like protein (MER099172), hCG22067 (Homo sapiens) (MER099169), brain-rescue-factor-1 (Homo sapiens) (MER098873), hCG2041108 (Homo sapiens) (MER099173), polyserase-2 unit 2 (MER061760), polyserase-2 unit 3 (MER065694), Mername-AA201 (peptidase homologue) MER099175, secreted trypsin-like serine peptidase homologue (MER030000), polyserase-1A unit 3 (MER029880), azurocidin (MER000119), haptoglobin-1 (MER000233), haptoglobin-related protein (MER000235), macrophage-stimulating protein (MER001546), hepatocyte growth factor (MER000185), protein Z (MER000227), TESP1 protein (MER047214), LOC136242 protein (MER016132), plasma kallikrein-like protein 4 (MER016346), PRSS35 protein (MER016350), DKFZp586H2123-like protein (MER066474), apolipoprotein (MER000183), psi-KLK1 pseudogene (Homo sapiens) (MER033287), tryptase pseudogene I (MER015077), tryptase pseudogene II (MER015078), tryptase pseudogene III (MER015079), subfamily S1A unassigned peptidases (MER216982), subfamily S1A unassigned peptidases (MER216148), amidophosphoribosyltransferase precursor (MER003314), glutamine-fructose-6-phosphate transaminase 1 (MER003322), glutamine:fructose-6-phosphate amidotransferase (MER012158), Mername-AA144 protein (MER021319), asparagine synthetase (MER033254), family C44 non-peptidase homologues (MER159286), family C44 unassigned peptidases

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containing mucin-like hormone receptor-like 3 (MER037288), EGF-like module containing mucin-like hormone receptor-like 1 (MER037278), EGF-like module containing mucin-like hormone receptor-like 4 (MER037294), cadherin EGF LAG seven-pass G-type receptor 2 precursor (Homo sapiens) (MER045397), Gpr64 (Mus musculus)-type protein (MER123205), GPR56 (Homo sapiens)-type protein (MER122057), latrophilin 2 (MER122199), latrophilin-1 (MER126380), latrophilin 3 (MER124612), protocadherin Flamingo 2 (MER124239), ETL protein (MER126267), G protein-coupled receptor 112 (MER126114), seven transmembrane helix receptor (MER125448), Gpr114 protein (MER159320), GPR126 vascular inducible G protein-coupled receptor (MER140015), GPR125 (Homo sapiens)-type protein (MER159279), GPR116 (Homo sapiens)-type G-protein coupled receptor (MER159280), GPR128 (Homo sapiens)-type G-protein coupled receptor (MER162015), GPR133 (Homo sapiens)-type protein (MER159334), GPR110 G-protein coupled receptor (MER159277), GPR97 protein (MER159322), KPG_006 protein (MER161773), KPG_008 protein (MER161835), KPG_009 protein (MER159335), unassigned homologue (MER166269), GPR113 protein (MER159352), brain-specific angiogenesis inhibitor 2 (MER159746), PIDD auto-processing protein unit 1 (MER020001), PIDD auto-processing protein unit 2 (MER063690), MUC1 self-cleaving mucin (MER074260), dystroglycan (MER054741), proprotein convertase 9 (MER022416), site-1 peptidase (MER001948), furin (MER000375), proprotein convertase 1 (MER000376), proprotein convertase 2 (MER000377), proprotein convertase 4 (MER028255), PACE4 proprotein convertase (MER000383), proprotein convertase 5 (MER002578), proprotein convertase 7 (MER002984), tripeptidyl-peptidase II (MER000355), subfamily S8A non-peptidase homologues (MER201339), subfamily S8A non-peptidase homologues (MER191613), subfamily S8A unassigned peptidases (MER191611), subfamily S8A unassigned peptidases (MER191612), subfamily S8A unassigned peptidases (MER191614), tripeptidyl-peptidase I (MER003575), prolyl oligopeptidase (MER000393), dipeptidyl-peptidase IV (eukaryote) (MER000401), acylaminoacyl-peptidase (MER000408), fibroblast activation protein alpha subunit (MER000399), PREPL A protein (MER004227), dipeptidyl-peptidase 8 (MER013484), dipeptidyl-peptidase 9 (MER004923), FLJ1 putative peptidase (MER017240), Mername-AA194 putative peptidase (MER017353), Mername-AA195 putative peptidase (MER017367), Mername-AA196 putative peptidase (MER017368), Mername-AA197 putative peptidase (MER017371), C14orf29 protein (MER033244), hypothetical protein (MER033245), hypothetical esterase/lipase/thioesterase (MER047309), protein bat5 (MER037840), hypothetical protein flj40219 (MER033212), hypothetical protein flj37464 (MER033240), hypothetical protein flj33678 (MER033241), dipeptidylpeptidase homologue DPP6 (MER000403), dipeptidylpeptidase homologue DPP10 (MER005988), protein similar to Mus

musculus chromosome 20 open reading frame 135 (MER037845), kynurenine formamidase (MER046020), thyroglobulin precursor (MER011604), acetylcholinesterase (MER033188), cholinesterase (MER033198), carboxylesterase D1 (MER033213), liver carboxylesterase (MER033220), carboxylesterase 3 (MER033224), carboxylesterase 2 (MER033226), bile salt-dependent lipase (MER033227), carboxylesterase-related protein (MER033231), neuroligin 3 (MER033232), neuroligin 4, X-linked (MER033235), neuroligin 4, Y-linked (MER033236), esterase D (MER043126), arylacetamide deacetylase (MER033237), KIAA1363-like protein (MER033242), hormone-sensitive lipase (MER033274), neuroligin 1 (MER033280), neuroligin 2 (MER033283), family S9 non-peptidase homologues (MER212939), family S9 non-peptidase homologues (MER211490), subfamily S9C unassigned peptidases (MER192341), family S9 unassigned peptidases (MER209181), family S9 unassigned peptidases (MER200434), family S9 unassigned peptidases (MER209507), family S9 unassigned peptidases (MER209142), serine carboxypeptidase A (MER000430), vitellogenic carboxypeptidase-like protein (MER005492), RISC peptidase (MER010960), family S15 unassigned peptidases (MER199442), family S15 unassigned peptidases (MER200437), family S15 unassigned peptidases (MER212825), lysosomal Pro-Xaa carboxypeptidase (MER000446), dipeptidyl-peptidase II (MER004952), thymus-specific serine peptidase (MER005538), epoxide hydrolase-like putative peptidase (MER031614), Loc328574-like protein (MER033246), abhydrolase domain-containing protein 4 (MER031616), epoxide hydrolase (MER000432), mesoderm specific transcript protein (MER199890), mesoderm specific transcript protein (MER017123), cytosolic epoxide hydrolase (MER029997), cytosolic epoxide hydrolase (MER213866), similar to hypothetical protein FLJ22408 (MER031608), CGI-58 putative peptidase (MER030163), Williams-Beuren syndrome critical region protein 21 epoxide hydrolase (MER031610), epoxide hydrolase (MER031612), hypothetical protein 922408 (epoxide hydrolase) (MER031617), monoglyceride lipase (MER033247), hypothetical protein (MER033249), valacyclovir hydrolase (MER033259), Ccg1-interacting factor b (MER210738), glycosylasparaginase precursor (MER003299), isoaspartyl dipeptidase (threonine type) (MER031622). taspase-1 (MER016969), gamma-glutamyltransferase 5 (mammalian-type) (MER001977), gamma-glutamyltransferase 1 (mammalian-type) (MER001629), gamma-glutamyltransferase 2 (Homo sapiens) (MER001976), gamma-glutamyltransferase-like protein 4 (MER002721). gamma-glutamyltransferase-like protein 3 (MER016970). similar to gamma-glutamyltransferase 1 precursor (Homo sapiens) (MER026204). similar to gamma-glutamyltransferase 1 precursor (Homo sapiens) (MER026205). Mername-AA211 putative peptidase (MER026207). gamma-glutamyltransferase 6 (MER159283). gamma-glutamyl transpeptidase homologue (chromosome 2, Homo sapiens) (MER037241). polycystin-1 (MER126824), KIAA1879 protein

(MER159329). polycystic kidney disease 1-like 3 (MER172554). gamma-glutamyl hydrolase (MER002963). guanine 5″-monophosphate synthetase (MER043387). carbamoyl-phosphate synthase (Homo sapiens-type) (MER078640). dihydro-orotase (N-terminal unit) (Homo sapiens-type) (MER060647). DJ-1 putative peptidase (MER003390). Mername-AA100 putative peptidase (MER014802). Mername-AA101 non-peptidase homologue (MER014803). KIAA0361 protein (Homo sapiens-type) (MER042827). F1134283 protein (Homo sapiens) (MER044553). non-peptidase homologue chromosome 21 open reading frame 33 (Homo sapiens) (MER160094). family C56 non-peptidase homologues (MER177016), family C56 non-peptidase homologues (MER176613). family C56 non-peptidase homologues (MER176918). EGF-like module containing mucin-like hormone receptor-like 2 (MER037230). CD97 antigen (human type) (MER037286). EGF-like module containing mucin-like hormone receptor-like 3 (MER037288). EGF-like module containing mucin-like hormone receptor-like 1 (MER037278). EGF-like module containing mucin-like hormone receptor-like 4 (MER037294). cadherin EGF LAG seven-pass G-type receptor 2 precursor (Homo sapiens) (MER045397), Gpr64 (Mus musculus)-type protein (MER123205). GPR56 (Homo sapiens)-type protein (MER122057). latrophilin 2 (MER122199). latrophilin-1 (MER126380). latrophilin 3 (MER124612). protocadherin Flamingo 2 (MER124239). ETL protein (MER126267). G protein-coupled receptor 112 (MER126114). seven transmembrane helix receptor (MER125448). Gpr114 protein (MER159320). GPR126 vascular inducible G protein-coupled receptor (MER140015). GPR125 (Homo sapiens)-type protein (MER159279). GPR116 (Homo sapiens)-type G-protein coupled receptor (MER159280). GPR128 (Homo sapiens)-type G-protein coupled receptor (MER162015). GPR133 (Homo sapiens)-type protein (MER159334) GPR110 G-protein coupled receptor (MER159277), GPR97 protein (MER159322), KPG_006 protein (MER161773) KPG_008 protein (MER161835), KPG_009 protein (MER159335), unassigned homologue (MER166269), GPR113 protein (MER159352), brain-specific angiogenesis inhibitor 2 (MER159746), PIDD auto-processing protein unit 1 (MER020001), PIDD auto-processing protein unit 2 (MER063690), MUC1 self-cleaving mucin (MER074260), dystroglycan (MER054741), proprotein convertase 9 (MER022416), site-1 peptidase (MER001948), furin (MER000375), proprotein convertase 1 (MER000376), proprotein convertase 2 (MER000377), proprotein convertase 4 (MER028255), PACE4 proprotein convertase (MER000383), proprotein convertase 5 (MER002578), proprotein convertase 7 (MER002984), tripeptidyl-peptidase II (MER000355), subfamily S8A non-peptidase homologues (MER201339), subfamily S8A non-peptidase homologues (MER191613), subfamily S8A unassigned peptidases (MER191611), subfamily S8A unassigned peptidases (MER191612), subfamily S8A unassigned peptidases (MER191614), tripeptidyl-peptidase I (MER003575), prolyl oligopeptidase (MER000393),

dipeptidyl-peptidase IV (eukaryote) (MER000401), acylaminoacyl-peptidase (MER000408), fibroblast activation protein alpha subunit (MER000399), PREPL A protein (MER004227), dipeptidyl-peptidase 8 (MER013484), dipeptidyl-peptidase 9 (MER004923), FLJ1 putative peptidase (MER017240), Mername-AA194 putative peptidase (MER017353), Mername-AA195 putative peptidase (MER017367), Mername-AA196 putative peptidase (MER017368), Mername-AA197 putative peptidase (MER017371), C14orf29 protein (MER033244), hypothetical protein (MER033245), hypothetical esterase/lipase/thioesterase (MER047309), protein bat5 (MER037840), hypothetical protein flj40219 (MER033212), hypothetical protein flj37464 (MER033240), hypothetical protein flj33678 (MER033241), dipeptidylpeptidase homologue DPP6 (MER000403), dipeptidylpeptidase homologue DPP10 (MER005988), protein similar to Mus musculus chromosome 20 open reading frame 135 (MER037845), kynurenine formamidase (MER046020), thyroglobulin precursor (MER011604), acetylcholinesterase (MER033188), cholinesterase (MER033198), carboxylesterase D1 (MER033213), liver carboxylesterase (MER033220), carboxylesterase 3 (MER033224), carboxylesterase 2 (MER033226), bile salt-dependent lipase (MER033227), carboxylesterase-related protein (MER033231), neuroligin 3 (MER033232), neuroligin 4, X-linked (MER033235), neuroligin 4, Y-linked (MER033236), esterase D (MER043126), arylacetamide deacetylase (MER033237), KIAA1363-like protein (MER033242), hormone-sensitive lipase (MER033274), neuroligin 1 (MER033280), neuroligin 2 (MER033283), family S9 non-peptidase homologues (MER212939), family S9 non-peptidase homologues (MER211490), subfamily S9C unassigned peptidases (MER192341), family S9 unassigned peptidases (MER209181), family S9 unassigned peptidases (MER200434), family S9 unassigned peptidases (MER209507), family S9 unassigned peptidases (MER209142), serine carboxypeptidase A (MER000430), vitellogenic carboxypeptidase-like protein (MER005492), RISC peptidase (MER010960), family S15 unassigned peptidases (MER199442), family S15 unassigned peptidases (MER200437), family S15 unassigned peptidases (MER212825), lysosomal Pro-Xaa carboxypeptidase (MER000446), dipeptidyl-peptidase II (MER004952), thymus-specific serine peptidase (MER005538), epoxide hydrolase-like putative peptidase (MER031614), Loc328574-like protein (MER033246), abhydrolase domain-containing protein 4 (MER031616), epoxide hydrolase (MER000432), mesoderm specific transcript protein (MER199890), mesoderm specific transcript protein (MER017123), cytosolic epoxide hydrolase (MER029997), cytosolic epoxide hydrolase (MER213866), similar to hypothetical protein FLJ22408 (MER031608), CGI-58 putative peptidase (MER030163), Williams-Beuren syndrome critical region protein 21 epoxide hydrolase (MER031610), epoxide hydrolase (MER031612), hypothetical protein flj22408

(epoxide hydrolase) (MER031617), monoglyceride lipase (MER033247), hypothetical protein (MER033249), valacyclovir hydrolase (MER033259), Ccg1-interacting factor b (MER210738).

Protease enzymatic activity can be regulated. For example, certain proteases can be inactivated by the presence or absence of a specific agent (e.g., that binds to the protease, such as specific small molecule inhibitors). Such proteases can be referred to as a "repressible protease." Exemplary inhibitors for certain proteases are listed in Table 4B. For example, an NS3 protease can be repressed by a protease inhibitor including, but not limited to, simeprevir, danoprevir, asunaprevir, ciluprevir, boceprevir, sovaprevir, paritaprevir, telaprevir, grazoprevir, glecaprevir, and voxiloprevir. In another example, protease activity can be regulated through regulating expression of the protease itself, such as engineering a cell to express a protease using an inducible promoter system (*e.g.*, Tet On/Off systems) or cell-specific promoters (promoters that can be used to express a heterologous protease are described in more detail in the Section herein titled "Promoters"). A protease can also contain a degron, such as any of the degrons described herein, and can be regulated using any of the degron systems described herein.

Protease enzymatic activity can also be regulated through selection of a specific protease cleavage site. For example, a protease cleavage site can be selected and/or engineered such that the sequence demonstrates a desired rate-of-cleavage by a desired protease, such as reduced cleavage kinetics relative to an endogenous sequence of a substrate naturally cleaved by the desired protease. As another example, a protease cleavage site can be selected and/or engineered such that the sequence demonstrates a desired rate-of-cleavage in a cell-state specific manner. For example, various cell states (*e.g.*, following cellular signaling, such as immune cell activation) can influence the expression and/or localization of certain proteases. As an illustrative example, ADAM17 protein levels and localization is known to be influenced by signaling, such as through Protein kinase C (PKC) signaling pathways (*e.g.*, activation by the PKC activator Phorbol-12-myristat-13-acetat [PMA]). Accordingly, a protease cleavage site can be selected and/or engineered such that cleavage of the protease cleavage site and subsequent release of an effector molecule is increased or decreased, as desired, depending on the protease properties (*e.g.*, expression and/or localization) in a specific cell state. As another example, a protease cleavage site (particularly in combination with a specific membrane tethering domain) can be selected and/or engineered for optimal protein expression of the chimeric protein.

Cell Membrane Tethering Domain

The membrane-cleavable chimeric proteins provided for herein include a cell-membrane tethering domain (referred to as "MT" in the formula **S – C – MT** or **MT – C – S**). In general, the cell-membrane tethering domain can be any amino acid sequence motif capable of directing

the chimeric protein to be localized to (*e.g.*, inserted into), or otherwise associated with, the cell membrane of the cell expressing the chimeric protein. The cell-membrane tethering domain can be a transmembrane-intracellular domain. The cell-membrane tethering domain can be a transmembrane domain. The cell-membrane tethering domain can be an integral membrane protein domain (*e.g.*, a transmembrane domain). The cell-membrane tethering domain can be derived from a Type I, Type II, or Type III transmembrane protein. The cell-membrane tethering domain can include post-translational modification tag, or motif capable of post-translational modification to modify the chimeric protein to include a post-translational modification tag, where the post-translational modification tag allows association with a cell membrane. Examples of post-translational modification tags include, but are not limited to, lipid-anchor domains (*e.g.*, a GPI lipid-anchor, a myristoylation tag, or palmitoylation tag). Examples of cell-membrane tethering domains include, but are not limited to, a transmembrane-intracellular domain and/or transmembrane domain derived from PDGFR-beta, CD8, CD28, CD3zeta-chain, CD4, 4-1BB, OX40, ICOS, CTLA-4, PD-1, LAG-3, 2B4, LNGFR, NKG2D, EpoR, TNFR2, B7-1, or BTLA. The cell membrane tethering domain can be a cell surface receptor or a cell membrane-bound portion thereof. Sequences of exemplary cell membrane tethering domains are provided in **Table 4C**.

Table 4C.

Source	Amino Acid Sequence	DNA Sequence
B7-1	LLPSWAITLISVNGIFVICCLTYCF APRCRERRRNERLRRESVRPV (SEQ ID NO: 219)	CTGCTGCCAAGCTGGGCCATCACACTGATC TCCGTGAACGGCATCTTCGTGATCTGTTGC CTGACCTACTGCTTCGCCCCTCGGTGCAGA GAGCGGAGAAGAAACGAACGGCTGCGGA GAGAATCTGTGCGGCCTGTG (SEQ ID NO: 220) OR CTGCTGCCTAGCTGGGCCATCACACTGATC TCCGTGAACGGCATCTTCGTGATCTGCTGC CTGACCTACTGCTTCGCCCCTAGATGCAGA GAGCGGCGGAGAAACGAACGGCTGAGAA GAGAATCTGTGCGGCCCGTT (SEQ ID NO: 331)

In general, for all membrane-cleavable chimeric proteins described herein, the cell membrane tethering domain is either: (1) C-terminal of the protease cleavage site and N-terminal of any intracellular domain, if present (in other words, the cell membrane tethering domain is in between the protease cleavage site and, if present, an intracellular domain); or (2)

N-terminal of the protease cleavage site and C-terminal of any intracellular domain, if present (also between the protease cleavage site and, if present, an intracellular domain with domain orientation inverted). In embodiments featuring a degron associated with the chimeric protein, the degron domain is the terminal cytoplasmic-oriented domain, specifically relative to the cell membrane tethering (in other words, the cell membrane tethering domain is in between the protease cleavage site and the degron). The cell membrane tethering domain can be connected to the protease cleavage site by a polypeptide linker, *i.e.*, a polypeptide sequence not generally considered to be part of cell membrane tethering domain or protease cleavage site. The cell membrane tethering domain can be connected to an intracellular domain, if present, by a polypeptide linker, *i.e.*, a polypeptide sequence not generally considered to be part of the cell membrane tethering domain or the intracellular domain. The cell membrane tethering domain can be connected to the degron, if present, by a polypeptide linker, *i.e.*, a polypeptide sequence not generally considered to be part of the cell membrane tethering domain or degron. A polypeptide linker can be any amino acid sequence that connects a first polypeptide sequence and a second polypeptide sequence. A polypeptide linker can be a flexible linker (*e.g.*, a Gly-Ser-Gly sequence). Examples of polypeptide linkers include, but are not limited to, GSG linkers (*e.g.*, $[GS]_4GG$ [SEQ ID NO: 182]), $A(EAAAK)_3A$ (SEQ ID NO: 183), and Whitlow linkers (*e.g.*, a "KEGS" linker such as the amino acid sequence KESGSVSSEQLAQFRSLD (SEQ ID NO: 184), an eGK linker such as the amino acid sequence EGKSSGSGSESKST (SEQ ID NO: 185), an LR1 linker such as the amino acid sequence SGGGGSGGGGSGGGGSGGGGSGGGSLQ (SEQ ID NO: 215), and linkers described in more detail in Issued U.S. Pat. No. 5,990,275 herein incorporated by reference). Additional polypeptide linkers include SEQ ID NO: 194, SEQ ID NO: 196, and SEQ ID NO: 197. Other polypeptide linkers may be selected based on desired properties (*e.g.*, length, flexibility, amino acid composition etc.) and are known to those skilled in the art.

In general, the cell-membrane tethering domain is oriented such that the secreted effector molecule and the protease cleavage site are extracellularly exposed following insertion into, or association with, the cell membrane, such that the protease cleavage site is capable of being cleaved by its respective protease and releasing ("secreting") the effector molecule into the extracellular space.

Degron Systems and Domains

In some embodiments, any of the proteins described herein can include a degron domain including, but not limited to, a cytokine, a CAR, a protease, a transcription factor, a promoter or constituent of a promoter system (*e.g.*, an ACP), and/or any of the membrane-cleavable chimeric

protein described herein. In general, the degron domain can be any amino acid sequence motif capable of directing regulated degradation, such as regulated degradation through a ubiquitin-mediated pathway. In the presence of an immunomodulatory drug (IMiD), the degron domain directs ubiquitin-mediated degradation of a degron-fusion protein.

The degron domain can be a cereblon (CRBN) polypeptide substrate domain capable of binding CRBN in response to an immunomodulatory drug (IMiD) including, but not limited to, IKZF1, IKZF3, CKla, ZFP91, GSPT1, MEIS2, GSS E4F1, ZN276, ZN517, ZN582, ZN653, ZN654, ZN692, ZN787, and ZN827, or a fragment thereof that is capable of drug-inducible binding of CRBN. The CRBN polypeptide substrate domain can be a chimeric fusion product of native CRBN polypeptide sequences, such as a IKZF3/ZFP91/IKZF3 chimeric fusion product having the amino acid sequence of FNVLMVHKRSHTGERPLQCEICGFTCRQKGNLLRHIKLHTGEKPFKCHLCNYACQRRD AL (SEQ ID NO: 175). Degron domains, and in particular CRBN degron systems, are described in more detail in International Application Pub. No. WO2019/089592Al, herein incorporated by reference for all purposes. Other examples of degron domains include, but are not limited to HCV NS4 degron, PEST (two copies of residues 277-307 of human IκBα; SEQ ID NO: 161), GRR (residues 352-408 of human p105; SEQ ID NO: 162), DRR (residues 210-295 of yeast Cdc34; SEQ ID NO: 163), SNS (tandem repeat of SP2 and NB (SP2-NB-SP2 of influenza A or influenza B; *e.g.*, SEQ ID NO: 164), RPB (four copies of residues 1688-1702 of yeast RPB; SEQ ID NO: 165), SPmix (tandem repeat of SP1 and SP2 (SP2-SP1-SP2-SP1-SP2 of influenza A virus M2 protein; SEQ ID NO: 166), NS2 (three copies of residues 79-93 of influenza A virus NS protein; SEQ ID NO: 167), ODC (residues 106-142 of ornithine decarboxylase; SEQ ID NO: 168), Nek2A, mouse ODC (residues 422–461; SEQ ID NO: 169), mouse ODC_DA (residues 422-461 of mODC including D433A and D434A point mutations), an APC/C degron, a COP1 E3 ligase binding degron motif, a CRL4-Cdt2 binding PIP degron, an actinfilin-binding degron, a KEAP1 binding degron, a KLHL2 and KLHL3 binding degron, an MDM2 binding motif, an N-degron, a hydroxyproline modification in hypoxia signaling, a phytohormone-dependent SCF-LRR-binding degron, an SCF ubiquitin ligase binding phosphodegron, a phytohormone-dependent SCF-LRR-binding degron, a DSGxxS phospho-dependent degron, an Siah binding motif, an SPOP SBC docking motif, or a PCNA binding PIP box.

Regulated degradation can be drug-inducible. Drugs capable of mediating/regulating degradation can be small-molecule compounds. Drugs capable of mediating/regulating degradation can include an "immunomodulatory drug" (IMiD). In general, as used herein, IMiDs refer to a class of small-molecule immunomodulatory drugs containing an imide group. Cereblon (CRBN) is known target of IMiDs and binding of an IMiD to CRBN or a CRBN

polypeptide substrate domain alters the substrate specificity of the CRBN E3 ubiquitin ligase complex leading to degradation of proteins having a CRBN polypeptide substrate domain (*e.g.*, any of secretable effector molecules or other proteins of interest described herein). For degron domains having a CRBN polypeptide substrate domain, examples of imide-containing IMiDs include, but are not limited to, a thalidomide, a lenalidomide, or a pomalidomide. The IMiD can be an FDA-approved drug.

Proteins described herein can contain a degron domain (*e.g.*, referred to as "D" in the formula **S – C – MT – D** or **D – MT – C – S** for membrane-cleavable chimeric proteins described herein). In the absence of an IMiD, degron/ubiquitin-mediated degradation of the chimeric protein does not occur. Following expression and localization of the chimeric protein into the cell membrane, the protease cleavage site directs cleavage of the chimeric protein such that the effector molecule is released ("secreted") into the extracellular space. In the presence of an immunomodulatory drug (IMiD), the degron domain directs ubiquitin-mediated degradation of the chimeric protein such that secretion of the effector molecule is reduced or eliminated. In general, for membrane-cleavable chimeric proteins fused to a degron domain, the degron domain is the terminal cytoplasmic-oriented domain, specifically relative to the cell membrane tethering domain, *e.g.*, the most C-terminal domain in the formula **S – C – MT – D** or the most N-terminal domain in the formula **D – MT – C – S** . The degron domain can be connected to the cell membrane tethering domain by a polypeptide linker, *i.e.*, a polypeptide sequence not generally considered to be part of the cell membrane tethering domain or the degron domain. A polypeptide linker can be any amino acid sequence that connects a first polypeptide sequence and a second polypeptide sequence. A polypeptide linker can be a flexible linker (*e.g.*, a Gly-Ser-Gly sequence). Examples of polypeptide linkers include, but are not limited to, GSG linkers (*e.g.*, $[GS]_4GG$ [SEQ ID NO: 182]), $A(EAAAK)_3A$ (SEQ ID NO: 183), and Whitlow linkers (*e.g.*, a "KEGS" linker such as the amino acid sequence KESGSVSSEQLAQFRSLD (SEQ ID NO: 184), an eGK linker such as the amino acid sequence EGKSSGSGSESKST (SEQ ID NO: 185), an LR1 linker such as the amino acid sequence SGGGGSGGGGSGGGGSGGGGSGGGSLQ (SEQ ID NO: 215), and linkers described in more detail in Issued U.S. Pat. No. 5,990,275 herein incorporated by reference). Additional polypeptide linkers include SEQ ID NO: 194, SEQ ID NO: 196, and SEQ ID NO: 197. Other polypeptide linkers may be selected based on desired properties (*e.g.*, length, flexibility, amino acid composition etc.) and are known to those skilled in the art. In general, the degron is oriented in relation to the cell membrane tethering domain such that the degron is exposed to the cytosol following localization to the cell membrane such that the degron domain is capable of

mediating degradation (*e.g.*, exposure to the cytosol and cytosol) and is capable of mediating ubiquitin-mediated degradation.

For degron-fusion proteins, the degron domain can be N-terminal or C-terminal of the protein of interest, *e.g.*, the effector molecule. The degron domain can be connected to the protein of interest by a polypeptide linker, *i.e.*, a polypeptide sequence not generally considered to be part of the protein of interest or the degron domain. A polypeptide linker can be any amino acid sequence that connects a first polypeptide sequence and a second polypeptide sequence. A polypeptide linker can be a flexible linker (*e.g.*, a Gly-Ser-Gly sequence). Examples of polypeptide linkers include, but are not limited to, GSG linkers (*e.g.*, $[GS]_4GG$ [SEQ ID NO: 182]), $A(EAAAK)_3A$ (SEQ ID NO: 183), and Whitlow linkers (*e.g.*, a "KEGS" linker such as the amino acid sequence KESGSVSSEQLAQFRSLD (SEQ ID NO: 184), an eGK linker such as the amino acid sequence EGKSSGSGSESKST (SEQ ID NO: 185), an LR1 linker such as the amino acid sequence SGGGGSGGGGSGGGGSGGGGSGGGSLQ (SEQ ID NO: 215), and linkers described in more detail in Issued U.S. Pat. No. 5,990,275 herein incorporated by reference). Additional polypeptide linkers include SEQ ID NO: 194, SEQ ID NO: 196, and SEQ ID NO: 197. Other polypeptide linkers may be selected based on desired properties (*e.g.*, length, flexibility, amino acid composition etc.) and are known to those skilled in the art. A polypeptide linker can be cleavable, *e.g.*, any of the protease cleavage sites described herein.

Engineered Nucleic Acids

Provided herein are engineered nucleic acids (*e.g.*, an expression cassette) encoding at least one protein of the present disclosure, such as the cytokines, CARs, ACPs, and/or membrane-cleavable chimeric proteins having the formula **S – C – MT** or **MT – C – S** described herein. Provided herein are engineered nucleic acids (*e.g.*, an expression cassette) encoding two or more proteins, such as two or more of the cytokines, CARs, ACPs, and/or membrane-cleavable chimeric proteins having the formula **S – C – MT** or **MT – C – S** described herein.

In certain embodiments described herein, the engineered nucleic acids encode an expression cassette containing a promoter and an exogenous polynucleotide sequence encoding the cytokines, CARs, ACPs, and/or membrane-cleavable chimeric protein, oriented from N-terminal to C-terminal, having the formula: **S – C – MT** or **MT – C – S**. S refers to a secretable effector molecule. C refers to a protease cleavage site. MT refers to a cell membrane tethering domain. The promoter is operably linked to the exogenous polynucleotide sequence and **S – C – MT** or **MT – C – S** is configured to be expressed as a single polypeptide.

In certain embodiments described herein, the engineered nucleic acids encode an expression cassette containing a promoter and an exogenous polynucleotide sequence encoding a cytokine. In certain embodiments described herein, the engineered nucleic acids encode an expression cassette containing a promoter and an exogenous polynucleotide sequence encoding a CAR. In certain embodiments described herein, the engineered nucleic acids encode an expression cassette containing a promoter and an exogenous polynucleotide sequence encoding a membrane-cleavable chimeric protein having a protein of interest (*e.g.*, any of the effector molecules described herein). The promoter is operably linked to the exogenous polynucleotide sequence and the membrane-cleavable chimeric protein is configured to be expressed as a single polypeptide.

In certain embodiments described herein, the engineered nucleic acids encode an expression cassette containing a promoter and an exogenous polynucleotide sequence encoding a combination of the cytokines, CARs, ACPs, and/or membrane-cleavable chimeric proteins described herein. In certain embodiments described herein, the engineered nucleic acids encode an expression cassette containing a promoter and an exogenous polynucleotide sequence encoding a cytokine and CAR. In certain embodiments described herein, the engineered nucleic acids encode an expression cassette containing a promoter and an exogenous polynucleotide sequence encoding a cytokine and an ACP.

In certain embodiments described herein, the engineered nucleic acids encode two or more expression cassettes each containing a promoter and an exogenous polynucleotide sequence encoding a cytokine, CAR, ACP, and/or membrane-cleavable chimeric protein described herein. In certain embodiments described herein, the engineered nucleic acids encode two or more expression cassettes each containing a promoter and each separately encoding an exogenous polynucleotide sequence encoding a cytokine and CAR, respectively. In certain embodiments described herein, the engineered nucleic acids encode two or more expression cassettes each containing a promoter and each separately encoding an exogenous polynucleotide sequence encoding a cytokine and an ACP, respectively. In certain embodiments, the two or more expression cassettes are oriented in a head-to-tail orientation. In certain embodiments, the two or more expression cassettes are oriented in a head-to-head orientation. In certain embodiments, the two or more expression cassettes are oriented in a tail-to-tail orientation. In some cases, each expression cassette contains its own promoter to drive expression of the polynucleotide sequence encoding a cytokine and/or CAR. In certain embodiments, the cytokine and CAR are organized as such: 5'-cytokine-CAR-3' or 5'-CAR-cytokine-3'.

An "engineered nucleic acid" is a nucleic acid that does not occur in nature. It should be understood, however, that while an engineered nucleic acid as a whole is not naturally-

occurring, it may include nucleotide sequences that occur in nature. In some embodiments, an engineered nucleic acid comprises nucleotide sequences from different organisms (*e.g.,* from different species). For example, in some embodiments, an engineered nucleic acid includes a murine nucleotide sequence, a bacterial nucleotide sequence, a human nucleotide sequence, and/or a viral nucleotide sequence. The term "engineered nucleic acids" includes recombinant nucleic acids and synthetic nucleic acids. A "recombinant nucleic acid" refers to a molecule that is constructed by joining nucleic acid molecules and, in some embodiments, can replicate in a live cell. A "synthetic nucleic acid" refers to a molecule that is amplified or chemically, or by other means, synthesized. Synthetic nucleic acids include those that are chemically modified, or otherwise modified, but can base pair with naturally- occurring nucleic acid molecules. Modifications include, but are not limited to, one or more modified internucleotide linkages and non-natural nucleic acids. Modifications are described in further detail in U.S. Pat. No. 6,673,611 and U.S. Application Publication 2004/0019001 and, each of which is incorporated by reference in their entirety. Modified internucleotide linkages can be a phosphorodithioate or phosphorothioate linkage. Non-natural nucleic acids can be a locked nucleic acid (LNA), a peptide nucleic acid (PNA), glycol nucleic acid (GNA), a phosphorodiamidate morpholino oligomer (PMO or "morpholino"), and threose nucleic acid (TNA). Non-natural nucleic acids are described in further detail in International Application WO 1998/039352, U.S. Application Pub. No. 2013/0156849, and U.S. Pat. Nos. 6,670,461; 5,539,082; 5,185,444, each herein incorporated by reference in their entirety. Recombinant nucleic acids and synthetic nucleic acids also include those molecules that result from the replication of either of the foregoing. Engineered nucleic acid of the present disclosure may be encoded by a single molecule (*e.g.*, included in the same plasmid or other vector) or by multiple different molecules (*e.g.*, multiple different independently-replicating molecules). Engineered nucleic acids can be an isolated nucleic acid. Isolated nucleic acids include, but are not limited to a cDNA polynucleotide, an RNA polynucleotide, an RNAi oligonucleotide (*e.g.*, siRNAs, miRNAs, antisense oligonucleotides, shRNAs, etc.), an mRNA polynucleotide, a circular plasmid, a linear DNA fragment, a vector, a minicircle, a ssDNA, a bacterial artificial chromosome (BAC), and yeast artificial chromosome (YAC), and an oligonucleotide.

Engineered nucleic acid of the present disclosure may be produced using standard molecular biology methods (*see*, *e.g.*, Green and Sambrook, Molecular Cloning, A Laboratory Manual, 2012, Cold Spring Harbor Press). In some embodiments, engineered nucleic acid constructs are produced using GIBSON ASSEMBLY® Cloning (see, *e.g.*, Gibson, D.G. *et al.* Nature Methods, 343-345, 2009; and Gibson, D.G. *et al.* Nature Methods, 901-903, 2010, each of which is incorporated by reference herein). GIBSON ASSEMBLY® typically uses three

enzymatic activities in a single-tube reaction: 5' exonuclease, the 3' extension activity of a DNA polymerase and DNA ligase activity. The 5 ' exonuclease activity chews back the 5 ' end sequences and exposes the complementary sequence for annealing. The polymerase activity then fills in the gaps on the annealed regions. A DNA ligase then seals the nick and covalently links the DNA fragments together. The overlapping sequence of adjoining fragments is much longer than those used in Golden Gate Assembly, and therefore results in a higher percentage of correct assemblies. In some embodiments, engineered nucleic acid constructs are produced using IN-FUSION® cloning (Clontech).

Promoters

In general, in all embodiments described herein, the engineered nucleic acids encoding the proteins herein (*e.g.*, a cytokine, CAR, ACP, and/or membrane-cleavable chimeric protein described herein) encode an expression cassette containing a promoter and an exogenous polynucleotide sequence encoding the protein. In some embodiments, an engineered nucleic acid (*e.g.*, an engineered nucleic acid comprising an expression cassette) comprises a promoter operably linked to a nucleotide sequence (*e.g.*, an exogenous polynucleotide sequence) encoding at least 2 distinct proteins. For example, the engineered nucleic acid may comprise a promoter operably linked to a nucleotide sequence encoding at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 8, at least 9, or at least 10 distinct proteins. In some embodiments, an engineered nucleic acid comprises a promoter operably linked to a nucleotide sequence encoding 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more distinct proteins. In some embodiments, an engineered nucleic acid (*e.g.*, an engineered nucleic acid comprising an expression cassette) comprises a promoter operably linked to a nucleotide sequence (*e.g.*, an exogenous polynucleotide sequence) encoding at least 2 cytokines. For example, the engineered nucleic acid may comprise a promoter operably linked to a nucleotide sequence encoding at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 8, at least 9, or at least 10 cytokines. In some embodiments, an engineered nucleic acid comprises a promoter operably linked to a nucleotide sequence encoding 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more cytokines. In some embodiments, an engineered nucleic acid (*e.g.*, an engineered nucleic acid comprising an expression cassette) comprises a promoter operably linked to a nucleotide sequence (*e.g.*, an exogenous polynucleotide sequence) encoding at least 2 membrane-cleavable chimeric proteins. For example, the engineered nucleic acid may comprise a promoter operably linked to a nucleotide sequence encoding at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 8, at least 9, or at least 10 membrane-cleavable chimeric proteins. In some embodiments,

an engineered nucleic acid comprises a promoter operably linked to a nucleotide sequence encoding 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more membrane-cleavable chimeric proteins.

A "promoter" refers to a control region of a nucleic acid sequence at which initiation and rate of transcription of the remainder of a nucleic acid sequence are controlled. A promoter may also contain sub-regions at which regulatory proteins and molecules may bind, such as RNA polymerase and other transcription factors. Promoters may be constitutive, inducible, repressible, tissue-specific or any combination thereof. A promoter drives expression or drives transcription of the nucleic acid sequence that it regulates. Herein, a promoter is considered to be "operably linked" when it is in a correct functional location and orientation in relation to a nucleic acid sequence it regulates to control ("drive") transcriptional initiation and/or expression of that sequence.

A promoter may be one naturally associated with a gene or sequence, as may be obtained by isolating the 5' non-coding sequences located upstream of the coding segment of a given gene or sequence. Such a promoter can be referred to as "endogenous." In some embodiments, a coding nucleic acid sequence may be positioned under the control of a recombinant or heterologous promoter, which refers to a promoter that is not normally associated with the encoded sequence in its natural environment. Such promoters may include promoters of other genes; promoters isolated from any other cell; and synthetic promoters or enhancers that are not "naturally occurring" such as, for example, those that contain different elements of different transcriptional regulatory regions and/or mutations that alter expression through methods of genetic engineering that are known in the art. In addition to producing nucleic acid sequences of promoters and enhancers synthetically, sequences may be produced using recombinant cloning and/or nucleic acid amplification technology, including polymerase chain reaction (PCR) (see, *e.g.*, U.S. Pat. No. 4,683,202 and U.S. Pat. No. 5,928,906).

Promoters of an engineered nucleic acid may be "inducible promoters," which refer to promoters that are characterized by regulating (*e.g.*, initiating or activating) transcriptional activity when in the presence of, influenced by or contacted by a signal. The signal may be endogenous or a normally exogenous condition (*e.g.*, light), compound (*e.g.*, chemical or non-chemical compound) or protein (*e.g.*, cytokine) that contacts an inducible promoter in such a way as to be active in regulating transcriptional activity from the inducible promoter. Activation of transcription may involve directly acting on a promoter to drive transcription or indirectly acting on a promoter by inactivation a repressor that is preventing the promoter from driving transcription. Conversely, deactivation of transcription may involve directly acting on a promoter to prevent transcription or indirectly acting on a promoter by activating a repressor that then acts on the promoter.

A promoter is "responsive to" or "modulated by" a local tumor state (*e.g.*, inflammation or hypoxia) or signal if in the presence of that state or signal, transcription from the promoter is activated, deactivated, increased, or decreased. In some embodiments, the promoter comprises a response element. A "response element" is a short sequence of DNA within a promoter region that binds specific molecules (*e.g.*, transcription factors) that modulate (regulate) gene expression from the promoter. Response elements that may be used in accordance with the present disclosure include, without limitation, a phloretin-adjustable control element (PEACE), a zinc-finger DNA-binding domain (DBD), an interferon-gamma-activated sequence (GAS) (Decker, T. *et al. J Interferon Cytokine Res.* 1997 Mar;17(3):121-34, incorporated herein by reference), an interferon-stimulated response element (ISRE) (Han, K. J. *et al. J Biol Chem.* 2004 Apr 9;279(15):15652-61, incorporated herein by reference), a NF-kappaB response element (Wang, V. *et al.* Cell Reports. 2012; 2(4): 824-839, incorporated herein by reference), and a STAT3 response element (Zhang, D. *et al. J of Biol Chem.* 1996; 271: 9503-9509, incorporated herein by reference). Other response elements are encompassed herein. Response elements can also contain tandem repeats (*e.g.*, consecutive repeats of the same nucleotide sequence encoding the response element) to generally increase sensitivity of the response element to its cognate binding molecule. Tandem repeats can be labeled 2X, 3X, 4X, 5X, *etc.* to denote the number of repeats present.

Non-limiting examples of responsive promoters (also referred to as "inducible promoters") (*e.g.*, TGF-beta responsive promoters) are listed in Table 5A, which shows the design of the promoter and transcription factor, as well as the effect of the inducer molecule towards the transcription factor (TF) and transgene transcription (T) is shown (B, binding; D, dissociation; n.d., not determined) (A, activation; DA, deactivation; DR, derepression) (see Horner, M. & Weber, W. *FEBS Letters* 586 (2012) 20784-2096m, and references cited therein). Non-limiting examples of components of inducible promoters include those presented in **Table 5B**.

Table 5A. Examples of Responsive Promoters

System	Promoter and operator	Transcription factor (TF)	Inducer molecule	Response to inducer	
				TF	T
Transcriptional activator-responsive promoters					
AIR	PAIR (OalcA-PhCMVmin)	AlcR	Acetaldehyde	n.d.	A
ART	PART (OARG-PhCMVmin)	ArgR-VP16	l-Arginine	B	A
BIT	PBIT3 (OBirA3-PhCMVmin)	BIT (BirA-VP16)	Biotin	B	A

System	Promoter and operator	Transcription factor (TF)	Inducer molecule	Response to inducer	
Cumate – activator	PCR5 (OCuO6-PhCMVmin)	cTA (CymR-VP16)	Cumate	D	DA
Cumate – reverse activator	PCR5 (OCuO6-PhCMVmin)	rcTA (rCymR-VP16)	Cumate	B	A
E-OFF	PETR (OETR-PhCMVmin)	ET (E-VP16)	Erythromycin	D	DA
NICE-OFF	PNIC (ONIC-PhCMVmin)	NT (HdnoR-VP16)	6-Hydroxy-nicotine	D	DA
PEACE	PTtgR1 (OTtgR-PhCMVmin)	TtgA1 (TtgR-VP16)	Phloretin	D	DA
PIP-OFF	PPIR (OPIR-Phsp70min)	PIT (PIP-VP16)	Pristinamycin I	D	DA
QuoRex	PSCA (OscbR-PhCMVmin)PSPA (OpapRI-PhCMVmin)	SCA (ScbR-VP16)	SCB1	D	DA
Redox	PROP (OROP-PhCMVmin)	REDOX (REX-VP16)	NADH	D	DA
TET-OFF	PhCMV∗-1 (OtetO7-PhCMVmin)	tTA (TetR-VP16)	Tetracycline	D	DA
TET-ON	PhCMV∗-1 (OtetO7-PhCMVmin)	rtTA (rTetR-VP16)	Doxycycline	B	A
TIGR	PCTA (OrheO-PhCMVmin)	CTA (RheA-VP16)	Heat	D	DA
TraR	O7x(tra box)-PhCMVmin	p65-TraR	3-Oxo-C8-HSL	B	A
VAC-OFF	P1VanO2 (OVanO2-PhCMVmin)	VanA1 (VanR-VP16)	Vanillic acid	D	DA
Transcriptional repressor-responsive promoters					
Cumate - repressor	PCuO (PCMV5-OCuO)	CymR	Cumate	D	DR
E-ON	PETRON8 (PSV40-OETR8)	E-KRAB	Erythromycin	D	DR
NICE-ON	PNIC (PSV40-ONIC8)	NS (HdnoR-KRAB)	6-Hydroxy-nicotine	D	DR
PIP-ON	PPIRON (PSV40-OPIR3)	PIT3 (PIP-KRAB)	Pristinamycin I	D	DR
Q-ON	PSCAON8 (PSV40-OscbR8)	SCS (ScbR-KRAB)	SCB1	D	DR
TET-ON repressor-based	OtetO-PHPRT	tTS-H4 (TetR-HDAC4)	Doxycycline	D	DR
T-REX	PTetO (PhCMV-OtetO2)	TetR	Tetracycline	D	DR
UREX	PUREX8 (PSV40-OhucO8)	mUTS (KRAB-HucR)	Uric acid	D	DR
VAC-ON	PVanON8 (PhCMV-OVanO8)	VanA4 (VanR-KRAB)	Vanillic acid	D	DR
Hybrid promoters					
QuoRexPIP-ON(NOT IF gate)	OscbR8-OPIR3-PhCMVmin	SCAPIT3	SCB1Pristinamycin I	DD	DADR
QuoRexE-ON(NOT IF gate)	OscbR-OETR8-PhCMVmin	SCAE-KRAB	SCB1Erythromycin	DD	DADR
TET-OFFE-ON(NOT IF gate)	OtetO7-OETR8-PhCMVmin	tTAE-KRAB	TetracyclineErythromycin	DD	DADR

System	Promoter and operator	Transcription factor (TF)	Inducer molecule	Response to inducer	
TET-OFFPIP-ONE-ON	OtetO7-OPIR3-OETR8-PhCMVmin	tTAPIT3E-KRAB	TetracyclinePristinamycin IErythromycin	DDD	DADRDR

Table 5B. Exemplary Components of Inducible Promoters

Name	DNA SEQUENCE
minimal promoter; minP	AGAGGGTATATAATGGAAGCTCGACTTCCAG (SEQ ID NO: 1)
NFkB response element protein promoter; 5x NFkB-RE	GGGAATTTCCGGGGACTTTCCGGGAATTTCCGGGGACTTTCCGGGAATTTCC (SEQ ID NO: 2)
CREB response element protein promoter; 4x CRE	CACCAGACAGTGACGTCAGCTGCCAGATCCCATGGCCGTCATACTGTGACGTCTTTCAGACACCCCATTGACGTCAATGGGAGAA (SEQ ID NO: 3)
NFAT response element protein promoter; 3x NFAT binding sites	GGAGGAAAAACTGTTTCATACAGAAGGCGTGGAGGAAAAACTGTTTCATACAGAAGGCGTGGAGGAAAAACTGTTTCATACAGAAGGCGT (SEQ ID NO: 4)
SRF response element protein promoter; 5x SRE	AGGATGTCCATATTAGGACATCTAGGATGTCCATATTAGGACATCTAGGATGTCCATATTAGGACATCTAGGATGTCCATATTAGGACATCTAGGATGTCCATATTAGGACATCT (SEQ ID NO: 5)
SRF response element protein promoter 2; 5x SRF-RE	AGTATGTCCATATTAGGACATCTACCATGTCCATATTAGGACATCTACTATGTCCATATTAGGACATCTTGTATGTCCATATTAGGACATCTAAAATGTCCATATTAGGACATCT (SEQ ID NO: 6)
AP1 response element protein promoter; 6x AP1-RE	TGAGTCAGTGACTCAGTGAGTCAGTGACTCAGTGAGTCAGTGACTCAG (SEQ ID NO: 7)
TCF-LEF response element promoter; 8x TCF-LEF-RE	AGATCAAAGGGTTTAAGATCAAAGGGCTTAAGATCAAAGGGTATAAGATCAAAGGGCCTAAGATCAAAGGGACTAAGATCAAAGGGTTTAAGATCAAAGGGCTTAAGATCAAAGGGCCTA (SEQ ID NO: 8)
SBEx4	GTCTAGACGTCTAGACGTCTAGACGTCTAGAC (SEQ ID NO: 9)
SMAD2/3 - CAGACA x4	CAGACACAGACACAGACACAGACA (SEQ ID NO: 10)
STAT3 binding site	Ggatccggtactcgagatctgcgatctaagtaagcttggcattccggtactgttggtaaagccac (SEQ ID NO: 11)
minCMV	taggcgtgtacggtgggaggcctatataagcagagctcgtttagtgaaccgtcagatcgcctgga (SEQ ID NO: 170)
YB_TATA	TCTAGAGGGTATATAATGGGGGCCA (SEQ ID NO: 171)
minTK	Ttcgcatattaaggtgacgcgtgtggcctcgaacaccgagcgaccctgcagcgacccgcttaa (SEQ ID NO: 172)

Non-limiting examples of promoters include the cytomegalovirus (CMV) promoter, the elongation factor 1-alpha (EF1a) promoter, the elongation factor (EFS) promoter, the MND promoter (a synthetic promoter that contains the U3 region of a modified MoMuLV LTR with myeloproliferative sarcoma virus enhancer), the phosphoglycerate kinase (PGK) promoter, the spleen focus-forming virus (SFFV) promoter, the simian virus 40 (SV40) promoter, and the ubiquitin C (UbC) promoter (see Table 5C).

Table 5C. Exemplary Constitutive Promoters

Name	DNA SEQUENCE
CMV	GTTGACATTGATTATTGACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATATGGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCAACGACCCCCGCCCATTGACGTCAATAATGACGTATG

Name	DNA SEQUENCE
	TTCCCATAGTAACGCCAATAGGGACTTTCCATTGACGTCAATGGGTGGAGTAT TTACGGTAAACTGCCCACTTGGCAGTACATCAAGTGTATCATATGCCAAGTAC GCCCCCTATTGACGTCAATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGT ACATGACCTTATGGGACTTTCCTACTTGGCAGTACATCTACGTATTAGTCATCG CTATTACCATGGTGATGCGGTTTTGGCAGTACATCAATGGGCGTGGATAGCGG TTTGACTCACGGGGATTTCCAAGTCTCCACCCCATTGACGTCAATGGGAGTTT GTTTTGGCACCAAAATCAACGGGACTTTCCAAAATGTCGTAACAACTCCGCCC CATTGACGCAAATGGGCGGTAGGCGTGTACGGTGGGAGGTCTATATAAGCAG AGCTC (SEQ ID NO: 12)
EF1a	GGCTCCGGTGCCCGTCAGTGGGCAGAGCGCACATCGCCCACAGTCCCCGAGA AGTTGGGGGGAGGGGTCGGCAATTGAACCGGTGCCTAGAGAAGGTGGCGCGG GGTAAACTGGGAAAGTGATGCCGTGTACTGGCTCCGCCTTTTTCCCGAGGGTG GGGGAGAACCGTATATAAGTGCAGTAGTCGCCGTGAACGTTCTTTTTCGCAAC GGGTTTGCCGCCAGAACACAGGTAAGTGCCGTGTGTGGTTCCCGCGGGCCTGG CCTCTTTACGGGTTATGGCCCTTGCGTGCCTTGAATTACTTCCACCTGGCTGCA GTACGTGATTCTTGATCCCGAGCTTCGGGTTGGAAGTGGGTGGGAGAGTTCGA GGCCTTGCGCTTAAGGAGCCCCTTCGCCTCGTGCTTGAGTTGAGGCCTGGCCT GGGCGCTGGGGCCGCCGCGTGCGAATCTGGTGGCACCTTCGCGCCTGTCTCGC TGCTTTCGATAAGTCTCTAGCCATTTAAAATTTTTGATGACCTGCTGCGACGCT TTTTTTCTGGCAAGATAGTCTTGTAAATGCGGGCCAAGATCTGCACACTGGTA TTTCGGTTTTTGGGGCCGCGGGCGGCGACGGGGCCCGTGCGTCCCAGCGCACA TGTTCGGCGAGGCGGGGCCTGCGAGCGCGACCACCGAGAATCGGACGGGGGT AGTCTCAAGCTGGCCGGCCTGCTCTGGTGCCTGTCCTCGCGCCGCCGTGTATC GCCCCGCCCCGGGCGGCAAGGCTGGCCCGGTCGGCACCAGTTGCGTGAGCGG AAAGATGGCCGCTTCCCGGTCCTGCTGCAGGGAGCTCAAAATGGAGGACGCG GCGCTCGGGAGAGCGGGCGGGTGAGTCACCCACACAAAGGAAAAGGGCCTTT CCGTCCTCAGCCGTCGCTTCATGTGACTCCACGGAGTACCGGGCGCCGTCCAG GCACCTCGATTAGTTCTCGAGCTTTTGGAGTACGTCGTCTTTAGGTTGGGGGG AGGGGTTTTATGCGATGGAGTTTCCCCACACTGAGTGGGTGGAGACTGAAGTT AGGCCAGCTTGGCACTTGATGTAATTCTCCTTGGAATTTGCCCTTTTTGAGTTT GGATCTTGGTTCATTCTCAAGCCTCAGACAGTGGTTCAAAGTTTTTTTCTTCCA TTTCAGGTGTCGTGA (SEQ ID NO: 13)
EFS	GGATCTGCGATCGCTCCGGTGCCCGTCAGTGGGCAGAGCGCACATCGCCCAC AGTCCCCGAGAAGTTGGGGGGAGGGGTCGGCAATTGAACCGGTGCCTAGAGA AGGTGGCGCGGGGTAAACTGGGAAAGTGATGTCGTGTACTGGCTCCGCCTTTT TCCCGAGGGTGGGGGAGAACCGTATATAAGTGCAGTAGTCGCCGTGAACGTT CTTTTTCGCAACGGGTTTGCCGCCAGAACACAGCTGAAGCTTCGAGGGGCTCG CATCTCTCCTTCACGCGCCCGCCGCCCTACCTGAGGCCGCCATCCACGCCGGT TGAGTCGCGTTCTGCCGCCTCCCGCCTGTGGTGCCTCCTGAACTGCGTCCGCC GTCTAGGTAAGTTTAAAGCTCAGGTCGAGACCGGGCCTTTGTCCGGCGCTCCC TTGGAGCCTACCTAGACTCAGCCGGCTCTCCACGCTTTGCCTGACCCTGCTTGC TCAACTCTACGTCTTTGTTTCGTTTTCTGTTCTGCGCCGTTACAGATCCAAGCT GTGACCGGCGCCTAC (SEQ ID NO: 14)
MND	TTTATTTAGTCTCCAGAAAAAGGGGGGAATGAAAGACCCCACCTGTAGGTTTG GCAAGCTAGGATCAAGGTTAGGAACAGAGAGACAGCAGAATATGGGCCAAA CAGGATATCTGTGGTAAGCAGTTCCTGCCCCGGCTCAGGGCCAAGAACAGTTG GAACAGCAGAATATGGGCCAAACAGGATATCTGTGGTAAGCAGTTCCTGCCC CGGCTCAGGGCCAAGAACAGATGGTCCCCAGATGCGGTCCCGCCCTCAGCAG TTTCTAGAGAACCATCAGATGTTTCCAGGGTGCCCCAAGGACCTGAAATGACC CTGTGCCTTATTTGAACTAACCAATCAGTTCGCTTCTCGCTTCTGTTCGCGCGC TTCTGCTCCCCGAGCTCAATAAAAGAGCCCA (SEQ ID NO: 15)
PGK	GGGGTTGGGGTTGCGCCTTTTCCAAGGCAGCCCTGGGTTTGCGCAGGGACGCG GCTGCTCTGGGCGTGGTTCCGGGAAACGCAGCGGCGCCGACCCTGGGTCTCGC ACATTCTTCACGTCCGTTCGCAGCGTCACCCGGATCTTCGCCGCTACCCTTGTG GGCCCCCCGGCGACGCTTCCTGCTCCGCCCCTAAGTCGGGAAGGTTCCTTGCG GTTCGCGGCGTGCCGGACGTGACAAACGGAAGCCGCACGTCTCACTAGTACC CTCGCAGACGGACAGCGCCAGGGAGCAATGGCAGCGCGCCGACCGCGATGGG CTGTGGCCAATAGCGGCTGCTCAGCGGGGCGCGCCGAGAGCAGCGGCCGGGA AGGGGCGGTGCGGGAGGCGGGGTGTGGGGCGGTAGTGTGGGCCCTGTTCCTG CCCGCGCGGTGTTCCGCATTCTGCAAGCCTCCGGAGCGCACGTCGGCAGTCGG CTCCCTCGTTGACCGAATCACCGACCTCTCTCCCCAG (SEQ ID NO: 16)

Name	DNA SEQUENCE
SFFV	GTAACGCCATTTTGCAAGGCATGGAAAAATACCAAACCAAGAATAGAGAAGT TCAGATCAAGGGCGGGTACATGAAAATAGCTAACGTTGGGCCAAACAGGATA TCTGCGGTGAGCAGTTTCGGCCCCGGCCCGGGGCCAAGAACAGATGGTCACC GCAGTTTCGGCCCCGGCCCGAGGCCAAGAACAGATGGTCCCCAGATATGGCC CAACCCTCAGCAGTTTCTTAAGACCCATCAGATGTTTCCAGGCTCCCCCAAGG ACCTGAAATGACCCTGCGCCTTATTTGAATTAACCAATCAGCCTGCTTCTCGCT TCTGTTCGCGCGCTTCTGCTTCCCGAGCTCTATAAAAGAGCTCACAACCCCTC ACTCGGCGCGCCAGTCCTCCGACAGACTGAGTCGCCCGGG (SEQ ID NO: 17)
SV40	CTGTGGAATGTGTGTCAGTTAGGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGG CAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCAGGTGTGGAAAG TCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTC AGCAACCATAGTCCCGCCCCTAACTCCGCCCATCCCGCCCCTAACTCCGCCCA GTTCCGCCCATTCTCCGCCCCATGGCTGACTAATTTTTTTTATTTATGCAGAGG CCGAGGCCGCCTCTGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTTT GGAGGCCTAGGCTTTTGCAAAAAGCT (SEQ ID NO: 18)
SV40 alt	GTGTGTCAGTTAGGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTA TGCAAAGCATGCATCTCAATTAGTCAGCAACCAGGTGTGGAAAGTCCCCAGG CTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATCTCAATTAGTCAGCAACC ATAGTCCCGCCCCTAACTCCGCCCATCCCGCCCCTAACTCCGCCCAGTTCCGC CCATTCTCCGCCCCATGGCTGACTAATTTTTTTTATTTATGCAGAGGCCGAGGC CGCCTCTGCCTCTGAGCTATTCCAGAAGTAGTGAGGAGGCTTTTTTGGAGGCC TAGGCTTTTGCAAA (SEQ ID NO: 295)
UbC	GCGCCGGGTTTTGGCGCCTCCCGCGGGCGCCCCCCTCCTCACGGCGAGCGCTG CCACGTCAGACGAAGGGCGCAGGAGCGTTCCTGATCCTTCCGCCCGGACGCTC AGGACAGCGGCCCGCTGCTCATAAGACTCGGCCTTAGAACCCCAGTATCAGC AGAAGGACATTTTAGGACGGGACTTGGGTGACTCTAGGGCACTGGTTTTCTTT CCAGAGAGCGGAACAGGCGAGGAAAAGTAGTCCCTTCTCGGCGATTCTGCGG AGGGATCTCCGTGGGGCGGTGAACGCCGATGATTATATAAGGACGCGCCGGG TGTGGCACAGCTAGTTCCGTCGCAGCCGGGATTTGGGTCGCGGTTCTTGTTTG TGGATCGCTGTGATCGTCACTTGGTGAGTTGCGGGCTGCTGGGCTGGCCGGGG CTTTCGTGGCCGCCGGGCCGCTCGGTGGGACGGAAGCGTGTGGAGAGACCGC CAAGGGCTGTAGTCTGGGTCCGCGAGCAAGGTTGCCCTGAACTGGGGGTTGG GGGGAGCGCACAAAATGGCGGCTGTTCCCGAGTCTTGAATGGAAGACGCTTG TAAGGCGGGCTGTGAGGTCGTTGAAACAAGGTGGGGGGCATGGTGGGCGGCA AGAACCCAAGGTCTTGAGGCCTTCGCTAATGCGGGAAAGCTCTTATTCGGGTG AGATGGGCTGGGGCACCATCTGGGGACCCTGACGTGAAGTTTGTCACTGACTG GAGAACTCGGGTTTGTCGTCTGGTTGCGGGGGCGGCAGTTATGCGGTGCCGTT GGGCAGTGCACCCGTACCTTTGGGAGCGCGCGCCTCGTCGTGTCGTGACGTCA CCCGTTCTGTTGGCTTATAATGCAGGGTGGGGCCACCTGCCGGTAGGTGTGCG GTAGGCTTTTCTCCGTCGCAGGACGCAGGGTTCGGGCCTAGGGTAGGCTCTCC TGAATCGACAGGCGCCGGACCTCTGGTGAGGGGAGGGATAAGTGAGGCGTCA GTTTCTTTGGTCGGTTTTATGTACCTATCTTCTTAAGTAGCTGAAGCTCCGGTT TTGAACTATGCGCTCGGGGTTGGCGAGTGTGTTTTGTGAAGTTTTTTAGGCAC CTTTTGAAATGTAATCATTTGGGTCAATATGTAATTTTCAGTGTTAGACTAGTA AAGCTTCTGCAGGTCGACTCTAGAAAATTGTCCGCTAAATTCTGGCCGTTTTT GGCTTTTTTGTTAGAC (SEQ ID NO: 19)
hEF1aV1	GGCTCCGGTGCCCGTCAGTGGGCAGAGCGCACATCGCCCACAGTCCCCGAGA AGTTGGGGGGAGGGGTCGGCAATTGAACCGGTGCCTAGAGAAGGTGGCGCGG GGTAAACTGGGAAAGTGATGTCGTGTACTGGCTCCGCCTTTTTCCCGAGGGTG GGGGAGAACCGTATATAAGTGCAGTAGTCGCCGTGAACGTTCTTTTTCGCAAC GGGTTTGCCGCCAGAACACAGGTAAGTGCCGTGTGTGGTTCCCGCGGGCCTGG CCTCTTTACGGGTTATGGCCCTTGCGTGCCTTGAATTACTTCCACCTGGCTGCA GTACGTGATTCTTGATCCCGAGCTTCGGGTTGGAAGTGGGTGGGAGAGTTCGA GGCCTTGCGCTTAAGGAGCCCCTTCGCCTCGTGCTTGAGTTGAGGCCTGGCCT GGGCGCTGGGGCCGCCGCGTGCGAATCTGGTGGCACCTTCGCGCCTGTCTCGC TGCTTTCGATAAGTCTCTAGCCATTTAAAATTTTTGATGACCTGCTGCGACGCT TTTTTTCTGGCAAGATAGTCTTGTAAATGCGGGCCAAGATCTGCACACTGGTA TTTCGGTTTTTGGGGCCGCGGGCGGCGACGGGGCCCGTGCGTCCCAGCGCACA TGTTCGGCGAGGCGGGGCCTGCGAGCGCGGCCACCGAGAATCGGACGGGGGT AGTCTCAAGCTGGCCGGCCTGCTCTGGTGCCTGGTCTCGCGCCGCCGTGTATC GCCCCGCCCTGGGCGGCAAGGCTGGCCCGGTCGGCACCAGTTGCGTGAGCGG

Name	DNA SEQUENCE
	AAAGATGGCCGCTTCCCGGCCCTGCTGCAGGGAGCTCAAAATGGAGGACGCG GCGCTCGGGAGAGCGGGCGGGTGAGTCACCCACACAAAGGAAAAGGGCCTTT CCGTCCTCAGCCGTCGCTTCATGTGACTCCACGGAGTACCGGGCGCCGTCCAG GCACCTCGATTAGTTCTCGAGCTTTTGGAGTACGTCGTCTTTAGGTTGGGGGG AGGGGTTTTATGCGATGGAGTTTCCCCACACTGAGTGGGTGGAGACTGAAGTT AGGCCAGCTTGGCACTTGATGTAATTCTCCTTGGAATTTGCCCTTTTTGAGTTT GGATCTTGGTTCATTCTCAAGCCTCAGACAGTGGTTCAAAGTTTTTTTCTTCCA TTTCAGGTGTCGTGA (SEQ ID NO: 20)
hCAGG	ACTAGTTATTAATAGTAATCAATTACGGGGTCATTAGTTCATAGCCCATATAT GGAGTTCCGCGTTACATAACTTACGGTAAATGGCCCGCCTGGCTGACCGCCCA ACGACCCCCGCCCATTGACGTCAATAATGACGTATGTTCCCATAGTAACGCCA ATAGGGACTTTCCATTGACGTCAATGGGTGGAGTATTTACGGTAAACTGCCCA CTTGGCAGTACATCAAGTGTATCATATGCCAAGTACGCCCCCTATTGACGTCA ATGACGGTAAATGGCCCGCCTGGCATTATGCCCAGTACATGACCTTATGGGAC TTTCCTACTTGGCAGTACATCTACGTATTAGTCATCGCTATTACCATGGTCGAG GTGAGCCCCACGTTCTGCTTCACTCTCCCCATCTCCCCCCCCTCCCCACCCCCA ATTTTGTATTTATTTATTTTTTAATTATTTTGTGCAGCGATGGGGGCGGGGGGG GGGGGGGGGCGCGCGCCAGGCGGGGCGGGGCGGGGCGAGGGGCGGGGCGGG GCGAGGCGGAGAGGTGCGGCGGCAGCCAATCAGAGCGGCGCGCTCCGAAAG TTTCCTTTTATGGCGAGGCGGCGGCGGCGGCGGCCCTATAAAAAGCGAAGCG CGCGGCGGGCGGGGAGTCGCTGCGACGCTGCCTTCGCCCCGTGCCCCGCTCCG CCGCCGCCTCGCGCCGCCCGCCCCGGCTCTGACTGACCGCGTTACTCCCACAG GTGAGCGGGCGGGACGGCCCTTCTCCTCCGGGCTGTAATTAGCGCTTGGTTTA ATGACGGCTTGTTTCTTTTCTGTGGCTGCGTGAAAGCCTTGAGGGGCTCCGGG AGGGCCCTTTGTGCGGGGGGAGCGGCTCGGGGGGTGCGTGCGTGTGTGTGTG CGTGGGGAGCGCCGCGTGCGGCTCCGCGCTGCCCGGCGGCTGTGAGCGCTGC GGGCGCGGCGCGGGGCTTTGTGCGCTCCGCAGTGTGCGCGAGGGGAGCGCGG CCGGGGGCGGTGCCCCGCGGTGCGGGGGGGGCTGCGAGGGGAACAAAGGCT GCGTGCGGGGTGTGTGCGTGGGGGGGTGAGCAGGGGGTGTGGGCGCGTCGGT CGGGCTGCAACCCCCCCTGCACCCCCCTCCCCGAGTTGCTGAGCACGGCCCGG CTTCGGGTGCGGGGCTCCGTACGGGGCGTGGCGCGGGGCTCGCCGTGCCGGG CGGGGGGTGGCGGCAGGTGGGGGTGCCGGGCGGGGCGGGGCCGCCTCGGGC CGGGGAGGGCTCGGGGGAGGGGCGCGGCGGCCCCCGGAGCGCCGGCGGCTG TCGAGGCGCGGCGAGCCGCAGCCATTGCCTTTTATGGTAATCGTGCGAGAGG GCGCAGGGACTTCCTTTGTCCCAAATCTGTGCGGAGCCGAAATCTGGGAGGCG CCGCCGCACCCCCTCTAGCGGGCGCGGGGCGAAGCGGTGCGGCGCCGGCAGG AAGGAAATGGGCGGGGAGGGCCTTCGTGCGTCGCCGCGCCGCCGTCCCCTTCT CCCTCTCCAGCCTCGGGGCTGTCCGCGGGGGGACGGCTGCCTTCGGGGGGGA CGGGGCAGGGCGGGGTTCGGCTTCTGGCGTGTGACCGGCGGCTCTAGAGCCT CTGCTAACCATGTTCATGCCTTCTTCTTTTTCCTACAGCTCCTGGGCAACGTGC TGGTTATTGTGCTGTCTCATCATTTTGGCAAAGAATTC (SEQ ID NO: 21)
hEF1aV2	Gggcagagcgcacatcgcccacagtccccgagaagttgggggggaggggtcggcaattgaaccggtgcctagagaaggtgg cgcggggtaaactgggaaagtgatgtcgtgtactggctccgccttttcccgagggtgggggagaaccgtatataagtgcagtag tcgccgtgaacgttcttttcgcaacgggtttgccgccagaacacag (SEQ ID NO: 22)
hACTb	CCACTAGTTCCATGTCCTTATATGGACTCATCTTTGCCTATTGCGACACACACT CAATGAACACCTACTACGCGCTGCAAAGAGCCCCGCAGGCCTGAGGTGCCCC CACCTCACCACTCTTCCTATTTTTGTGTAAAAATCCAGCTTCTTGTCACCACCT CCAAGGAGGGGGAGGAGGAGGAAGGCAGGTTCCTCTAGGCTGAGCCGAATG CCCCTCTGTGGTCCCACGCCACTGATCGCTGCATGCCCACCACCTGGGTACAC ACAGTCTGTGATTCCCGGAGCAGAACGGACCCTGCCCACCCGGTCTTGTGTGC TACTCAGTGGACAGACCCAAGGCAAGAAAGGGTGACAAGGACAGGGTCTTCC CAGGCTGGCTTTGAGTTCCTAGCACCGCCCCGCCCCCAATCCTCTGTGGCACA TGGAGTCTTGGTCCCCAGAGTCCCCCAGCGGCCTCCAGATGGTCTGGGAGGGC AGTTCAGCTGTGGCTGCGCATAGCAGACATACAACGGACGGTGGGCCCAGAC CCAGGCTGTGTAGACCCAGCCCCCCCGCCCCGCAGTGCCTAGGTCACCCACTA ACGCCCCAGGCCTGGTCTTGGCTGGGCGTGACTGTTACCCTCAAAAGCAGGCA GCTCCAGGGTAAAAGGTGCCCTGCCCTGTAGAGCCCACCTTCCTTCCCAGGGC TGCGGCTGGGTAGGTTTGTAGCCTTCATCACGGGCCACCTCCAGCCACTGGAC CGCTGGCCCCTGCCCTGTCCTGGGGAGTGTGGTCCTGCGACTTCTAAGTGGCC GCAAGCCACCTGACTCCCCCAACACCACACTCTACCTCTCAAGCCCAGGTCTC TCCCTAGTGACCCACCCAGCACATTTAGCTAGCTGAGCCCCACAGCCAGAGGT

Name	DNA SEQUENCE
	CCTCAGGCCCTGCTTTCAGGGCAGTTGCTCTGAAGTCGGCAAGGGGGAGTGAC TGCCTGGCCACTCCATGCCCTCCAAGAGCTCCTTCTGCAGGAGCGTACAGAAC CCAGGGCCCTGGCACCCGTGCAGACCCTGGCCCACCCCACCTGGGCGCTCAGT GCCCAAGAGATGTCCACACCTAGGATGTCCCGCGGTGGGTGGGGGGCCCGAG AGACGGGCAGGCCGGGGGCAGGCCTGGCCATGCGGGGCCGAACCGGGCACT GCCCAGCGTGGGGCGCGGGGGCCACGGCGCGCGCCCCCAGCCCCCGGGCCCA GCACCCCAAGGCGGCCAACGCCAAAACTCTCCCTCCTCCTCTTCCTCAATCTC GCTCTCGCTCTTTTTTTTTTCGCAAAAGGAGGGGAGAGGGGGTAAAAAAATG CTGCACTGTGCGGCGAAGCCGGTGAGTGAGCGGCGCGGGGCCAATCAGCGTG CGCCGTTCCGAAAGTTGCCTTTTATGGCTCGAGCGGCCGCGGCGGCGCCCTAT AAAACCCAGCGGCGCGACGCGCCACCACCGCCGAGACCGCGTCCGCCCCGCG AGCACAGAGCCTCGCCTTTGCCGATCCGCCGCCCGTCCACACCCGCCGCCAGgt aagcccggccagccgaccggggcaggcggctcacggcccggccgcaggcggccgcggcccccttcgcccgtgcagagccg ccgtctgggccgcagcgggggggcgcatgggggggggaaccggaccgccgtgggggggcgcgggagaagccccctgggcctcc ggagatgggggacaccccacgccagttcggaggcgcgaggccgcgctcgggaggcgcgctccgggggtgccgctctcggg gcgggggcaaccggcggggtctttgtctgagccgggctcttgccaatggggatcgcagggtgggcgcggcggagcccccgc caggcccggtgggggctgggggcgccattgcgcgtgcgcgctggtcctttgggcgctaactgcgtgcgcgctgggaattggcgc taattgcgcgtgcgcgctgggactcaaggcgctaactgcgcgtgcgttctggggcccggggtgccgcggcctgggctggggc gaaggcgggctcggccggaaggggtggggtcgccgcggctcccgggcgcttgcgcgcacttcctgcccgagccgctggccg cccgagggtgtggccgctgcgtgcgcgcgcgccgacccggcgctgtttgaaccgggcggaggcggggctggcgcccggttg ggaggggggttggggcctggcttcctgccgcgcgccgcggggacgcctccgaccagtgtttgccttttatggtaataacgcggcc ggcccggcttcctttgtccccaatctgggcgcgcgccggcgcccccctggcggcctaaggactcggcgcgccggaagtggcca gggcgggggcgacctcggctcacagcgcgcccggctat (SEQ ID NO: 23)
heIF4A1	GTTGATTTCCTTCATCCCTGGCACACGTCCAGGCAGTGTCGAATCCATCTCTGC TACAGGGGAAAACAAATAACATTTGAGTCCAGTGGAGACCGGGAGCAGAAGT AAAGGGAAGTGATAACCCCCAGAGCCCGGAAGCCTCTGGAGGCTGAGACCTC GCCCCCCTTGCGTGATAGGGCCTACGGAGCCACATGACCAAGGCACTGTCGCC TCCGCACGTGTGAGAGTGCAGGGCCCCAAGATGGCTGCCAGGCCTCGAGGCC TGACTCTTCTATGTCACTTCCGTACCGGCGAGAAAGGCGGGCCCTCCAGCCAA TGAGGCTGCGGGGCGGGCCTTCACCTTGATAGGCACTCGAGTTATCCAATGGT GCCTGCGGGCCGGAGCGACTAGGAACTAACGTCATGCCGAGTTGCTGAGCGC CGGCAGGCGGGGCCGGGGCGGCCAAACCAATGCGATGGCCGGGGCGGAGTC GGGCGCTCTATAAGTTGTCGATAGGCGGGCACTCCGCCCTAGTTTCTAAGGAC CATG (SEQ ID NO: 24)
hGAPDH	AGTTCCCCAACTTTCCCGCCTCTCAGCCTTTGAAAGAAAGAAAGGGGAGGGG GCAGGCCGCGTGCAGTCGCGAGCGGTGCTGGGCTCCGGCTCCAATTCCCCATC TCAGTCGCTCCCAAAGTCCTTCTGTTTCATCCAAGCGTGTAAGGGTCCCCGTCC TTGACTCCCTAGTGTCCTGCTGCCCACAGTCCAGTCCTGGGAACCAGCACCGA TCACCTCCCATCGGGCCAATCTCAGTCCCTTCCCCCCTACGTCGGGGCCCACA CGCTCGGTGCGTGCCCAGTTGAACCAGGCGGCTGCGGAAAAAAAAAAGCGGG GAGAAAGTAGGGCCCGGCTACTAGCGGTTTTACGGGCGCACGTAGCTCAGGC CTCAAGACCTTGGGCTGGGACTGGCTGAGCCTGGCGGGAGGCGGGGTCCGAG TCACCGCCTGCCGCCGCGCCCCCGGTTTCTATAAATTGAGCCCGCAGCCTCCC GCTTCGCTCTCTGCTCCTCCTGTTCGACAGTCAGCCGCATCTTCTTTTGCGTCG CCAGgtgaagacgggcggagagaaacccgggaggctagggacggcctgaaggcggcaggggcgggcgcaggccgga tgtgttcgcgccgctgcggggtgggcccgggcggcctccgcattgcaggggcgggcggaggacgtgatgcggcgcgggctg ggcatggaggcctggtgggggagggggaggggaggcgtgggtgcggccggggccactaggcgctcactgttctctccctccg cgcagCCGAGCCACATCGCTGAGACAC (SEQ ID NO: 25)
hGRP78	AGTGCGGTTACCAGCGGAAATGCCTCGGGGTCAGAAGTCGCAGGAGAGATAG ACAGCTGCTGAACCAATGGGACCAGCGGATGGGGCGGATGTTATCTACCATT GGTGAACGTTAGAAACGAATAGCAGCCAATGAATCAGCTGGGGGGGCGGAGC AGTGACGTTTATTGCGGAGGGGGCCGCTTCGAATCGGCGGCGGCCAGCTTGGT GGCCTGGGCCAATGAACGGCCTCCAACGAGCAGGGCCTTCACCAATCGGCGG CCTCCACGACGGGGCTGGGGGAGGGTATATAAGCCGAGTAGGCGACGGTGAG GTCGACGCCGGCCAAGACAGCACAGACAGATTGACCTATTGGGGTGTTTCGC GAGTGTGAGAGGGAAGCGCCGCGGCCTGTATTTCTAGACCTGCCCTTCGCCTG GTTCGTGGCGCCTTGTGACCCCGGGCCCCTGCCGCCTGCAAGTCGGAAATTGC GCTGTGCTCCTGTGCTACGGCCTGTGGCTGGACTGCCTGCTGCTGCCCAACTG GCTGGCAC (SEQ ID NO: 26)
hGRP94	TAGTTTCATCACCACCGCCACCCCCCCGCCCCCCCCGCCATCTGAAAGGGTTCT AGGGGATTTGCAACCTCTCTCGTGTGTTTCTTCTTTCCGAGAAGCGCCGCCAC

Name	DNA SEQUENCE
	ACGAGAAAGCTGGCCGCGAAAGTCGTGCTGGAATCACTTCCAACGAAACCCC AGGCATAGATGGGAAAGGGTGAAGAACACGTTGCCATGGCTACCGTTTCCCC GGTCACGGAATAAACGCTCTCTAGGATCCGGAAGTAGTTCCGCCGCGACCTCT CTAAAAGGATGGATGTGTTCTCTGCTTACATTCATTGGACGTTTTCCCTTAGAG GCCAAGGCCGCCCAGGCAAAGGGGCGGTCCCACGCGTGAGGGGCCCGCGGA GCCATTTGATTGGAGAAAAGCTGCAAACCCTGACCAATCGGAAGGAGCCACG CTTCGGGCATCGGTCACCGCACCTGGACAGCTCCGATTGGTGGACTTCCGCCC CCCCTCACGAATCCTCATTGGGTGCCGTGGGTGCGTGGTGCGGCGCGATTGGT GGGTTCATGTTTCCCGTCCCCCGCCCGCGAGAAGTGGGGGTGAAAAGCGGCC CGACCTGCTTGGGGTGTAGTGGGCGGACCGCGCGGCTGGAGGTGTGAGGATC CGAACCCAGGGGTGGGGGGTGGAGGCGGCTCCTGCGATCGAAGGGGACTTGA GACTCACCGGCCGCACGTC (SEQ ID NO: 27)
hHSP70	GGGCCGCCCACTCCCCCTTCCTCTCAGGGTCCCTGTCCCCTCCAGTGAATCCCA GAAGACTCTGGAGAGTTCTGAGCAGGGGGCGGCACTCTGGCCTCTGATTGGTC CAAGGAAGGCTGGGGGGCAGGACGGGAGGCGAAAACCCTGGAATATTCCCG ACCTGGCAGCCTCATCGAGCTCGGTGATTGGCTCAGAAGGGAAAAGGCGGGT CTCCGTGACGACTTATAAAAGCCCAGGGGCAAGCGGTCCGGATAACGGCTAG CCTGAGGAGCTGCTGCGACAGTCCACTACCTTTTTCGAGAGTGACTCCCGTTG TCCCAAGGCTTCCCAGAGCGAACCTGTGCGGCTGCAGGCACCGGCGCGTCGA GTTTCCGGCGTCCGGAAGGACCGAGCTCTTCTCGCGGATCCAGTGTTCCGTTT CCAGCCCCCAATCTCAGAGCGGAGCCGACAGAGAGCAGGGAACCC (SEQ ID NO: 28)
hKINb	GCCCCACCCCCGTCCGCGTTACAACCGGGAGGCCCGCTGGGTCCTGCACCGTC ACCCTCCTCCCTGTGACCGCCCACCTGATACCCAAACAACTTTCTCGCCCCTCC AGTCCCCAGCTCGCCGAGCGCTTGCGGGGAGCCACCCAGCCTCAGTTTCCCCA GCCCCGGGCGGGGCGAGGGGCGATGACGTCATGCCGGCGCGCGGCATTGTGG GGCGGGGCGAGGCGGGGCGCCGGGGGGAGCAACACTGAGACGCCATTTTCGG CGGCGGGAGCGGCGCAGGCGGCCGAGCGGGACTGGCTGGGTCGGCTGGGCTG CTGGTGCGAGGAGCCGCGGGGCTGTGCTCGGCGGCCAAGGGGACAGCGCGTG GGTGGCCGAGGATGCTGCGGGGCGGTAGCTCCGGCGCCCCTCGCTGGTGACT GCTGCGCCGTGCCTCACACAGCCGAGGCGGGCTCGGCGCACAGTCGCTGCTCC GCGCTCGCGCCCGGCGGCGCTCCAGGTGCTGACAGCGCGAGAGAGCGCGGCC TCAGGAGCAACAC (SEQ ID NO: 29)
hUBIb	TTCCAGAGCTTTCGAGGAAGGTTTCTTCAACTCAAATTCATCCGCCTGATAATT TTCTTATATTTTCCTAAAGAAGGAAGAGAAGCGCATAGAGGAGAAGGGAAAT AATTTTTTAGGAGCCTTTCTTACGGCTATGAGGAATTTGGGGCTCAGTTGAAA AGCCTAAACTGCCTCTCGGGAGGTTGGGCGCGGCGAACTACTTTCAGCGGCGC ACGGAGACGGCGTCTACGTGAGGGGTGATAAGTGACGCAACACTCGTTGCAT AAATTTGCGCTCCGCCAGCCCGGAGCATTTAGGGGCGGTTGGCTTTGTTGGGT GAGCTTGTTTGTGTCCCTGTGGGTGGACGTGGTTGGTGATTGGCAGGATCCTG GTATCCGCTAACAGgtactggcccacagccgtaaagacctgcgggggcgtgagagggggaatgggtgaggtc aagctggaggcttcttggggttgggtgggccgctgaggggaggggagggcgaggtgacgcgacacccggcctttctgggag agtgggccttgttgacctaaggggggcgagggcagttggcacgcgcacgcgccgacagaaactaacagacattaaccaacag cgattccgtcgcgtttacttgggaggaaggcggaaaagaggtagtttgtgtggcttctggaaaccctaaatttggaatcccagtatg agaatggtgtcccttcttgtgtttcaatgggatttttacttcgcgagtcttgtgggtttggttttgttttcagtttgcctaacaccgtgcttag gtttgaggcagattggagttcggtcgggggagtttgaatatccggaacagttagtggggaaagctgtggacgcttggtaagagag cgctctggattttccgctgttgacgttgaaaccttgaatgacgaatttcgtattaagtgacttagccttgtaaaattgaggggaggcttg cggaatattaacgtatttaaggcattttgaaggaatagttgctaattttgaagaatattaggtgtaaaagcaagaaatacaatgatcct gaggtgacacgcttatgttttacttttaaactagGTCACC (SEQ ID NO: 30)
CAG	gacattgattattgactagttattaatagtaatcaattacggggtcattagttcatagcccatatatggagttccg cgttacataacttacggtaaatggcccgcctggctgaccgcccaacgacccccgcccattgacgtcaataa tgacgtatgttcccatagtaacgccaatagggactttccattgacgtcaatgggtggagtatttacggtaaact gcccacttggcagtacatcaagtgtatcatatgccaagtacgccccctattgacgtcaatgacggtaaatgg cccgcctggcattatgcccagtacatgaccttatgggactttcctacttggcagtacatctacgtattagtcatc gctattaccatggtcgaggtgagccccacgttctgcttcactctccccatctccccccccctccccacccccca attttgtatttatttatttttttaattattttgtgcagcgatgggggcgggggggggggggggcgcgcgccag gcggggcggggcggggcgaggggcggggcggggcgaggcggagaggtgcggcggcagccaatc agagcggcgcgctccgaaagtttccttttatggcgaggcggcggcggcggccctataaaaagcgaa gcgcgcggcgggcg (SEQ ID NO: 173)

Name	DNA SEQUENCE
HLP	Tgtttgctgcttgcaatgtttgcccattttagggtggacacaggacgctgtggtttctgagccaggggggcga ctcagatcccagccagtggacttagcccctgtttgctcctccgataactggggtgaccttggttaatattcacc agcagcctcccccgttgcccctctggatccactgcttaaatacggacgaggacagggccctgtctcctcag cttcaggcaccaccactgacctgggacagtgaat (SEQ ID NO: 174)

The promoter can be a tissue-specific promoter. In general, a tissue-specific promoter directs transcription of a nucleic acid, (*e.g.*, the engineered nucleic acids encoding the proteins herein (*e.g.*, a cytokine, CAR, ACP, and/or membrane-cleavable chimeric protein described herein) such that expression is limited to a specific cell type, organelle, or tissue. Tissue-specific promoters include, but are not limited to, albumin (liver specific, Pinkert et al., (1987)), lymphoid specific promoters (Calame and Eaton, 1988), particular promoters of T-cell receptors (Winoto and Baltimore, (1989)) and immunoglobulins; Banerji et al., (1983); Queen and Baltimore, 1983), neuron specific promoters (e.g. the neurofilament promoter; Byrne and Ruddle, 1989), pancreas specific promoters (Edlund et al., (1985)) or mammary gland specific promoters (milk whey promoter, U.S. Pat. No. 4,873,316 and European Application Publication No. 264,166) as well as developmentally regulated promoters such as the murine hox promoters (Kessel and Gruss, Science 249:374-379 (1990)) or the α-fetoprotein promoter (Campes and Tilghman, Genes Dev. 3:537-546 (1989)), the contents of each of which are fully incorporated by reference herein. The promoter can be constitutive in the respective specific cell type, organelle, or tissue. Tissue-specific promoters and/or regulatory elements can also include promoters from the liver fatty acid binding (FAB) protein gene, specific for colon epithelial cells; the insulin gene, specific for pancreatic cells; the transphyretin, .alpha.1- antitrypsin, plasminogen activator inhibitor type 1 (PAI-I), apolipoprotein AI and LDL receptor genes, specific for liver cells; the myelin basic protein (MBP) gene, specific for oligodendrocytes; the glial fibrillary acidic protein (GFAP) gene, specific for glial cells; OPSIN, specific for targeting to the eye; and the neural-specific enolase (NSE) promoter that is specific for nerve cells. Examples of tissue-specific promoters include, but are not limited to, the promoter for creatine kinase, which has been used to direct expression in muscle and cardiac tissue and immunoglobulin heavy or light chain promoters for expression in B cells. Other tissue specific promoters include the human smooth muscle alpha-actin promoter. Exemplary tissue-specific expression elements for the liver include but are not limited to HMG-COA reductase promoter, sterol regulatory element 1, phosphoenol pyruvate carboxy kinase (PEPCK) promoter, human C-reactive protein (CRP) promoter, human glucokinase promoter, cholesterol L 7-alpha hydroylase (CYP-7) promoter, beta- galactosidase alpha-2,6 sialylkansferase promoter, insulin-like growth factor binding protein (IGFBP-I) promoter, aldolase B promoter, human transferrin promoter, and collagen type I promoter. Exemplary tissue-specific expression elements for the prostate

include but are not limited to the prostatic acid phosphatase (PAP) promoter, prostatic secretory protein of 94 (PSP 94) promoter, prostate specific antigen complex promoter, and human glandular kallikrein gene promoter (hgt-1). Exemplary tissue- specific expression elements for gastric tissue include but are not limited to the human H+/K+-ATPase alpha subunit promoter. Exemplary tissue-specific expression elements for the pancreas include but are not limited to pancreatitis associated protein promoter (PAP), elastase 1 transcriptional enhancer, pancreas specific amylase and elastase enhancer promoter, and pancreatic cholesterol esterase gene promoter. Exemplary tissue-specific expression elements for the endometrium include, but are not limited to, the uteroglobin promoter. Exemplary tissue-specific expression elements for adrenal cells include, but are not limited to, cholesterol side-chain cleavage (SCC) promoter. Exemplary tissue-specific expression elements for the general nervous system include, but are not limited to, gamma-gamman enolase (neuron- specific enolase, NSE) promoter. Exemplary tissue-specific expression elements for the brain include, but are not limited to, the neurofilament heavy chain (NF-H) promoter. Exemplary tissue-specific expression elements for lymphocytes include, but are not limited to, the human CGL-1/granzyme B promoter, the terminal deoxy transferase (TdT), lambda 5, VpreB, and lck (lymphocyte specific tyrosine protein kinase p561ck) promoter, the humans CD2 promoter and its 3 ' transcriptional enhancer, and the human NK and T cell specific activation (NKG5) promoter. Exemplary tissue-specific expression elements for the colon include, but are not limited to, pp60c-src tyrosine kinase promoter, organ-specific neoantigens (OSNs) promoter, and colon specific antigen-P promoter. Tissue-specific expression elements for breast cells are for example, but are not limited to, the human alpha-lactalbumin promoter. Exemplary tissue-specific expression elements for the lung include, but are not limited to, the cystic fibrosis transmembrane conductance regulator (CFTR) gene promoter.

In some embodiments, a promoter of the present disclosure is modulated by signals within a tumor microenvironment. A tumor microenvironment is considered to modulate a promoter if, in the presence of the tumor microenvironment, the activity of the promoter is increased or decreased by at least 10%, relative to activity of the promoter in the absence of the tumor microenvironment. In some embodiments, the activity of the promoter is increased or decreased by at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 100%, relative to activity of the promoter in the absence of the tumor microenvironment. For example, the activity of the promoter is increased or decreased by 10-20%, 10-30%, 10-40%, 10-50%, 10-60%, 10-70%, 10-80%, 10-90%, 10-100%, 10-200%, 20-30%, 20-40%, 20-50%, 20-60%, 20-70%, 20-80%, 20-90%, 20-100%, 20-200%, 50-60%,

50-70%, 50-80%, 50-90%, 50-100%, or 50-200%, relative to activity of the promoter in the absence of the tumor microenvironment.

In some embodiments, the activity of the promoter is increased or decreased by at least 2 fold (*e.g.*, 2, 3, 4, 5, 10, 25, 20, 25, 50, or 100 fold), relative to activity of the promoter in the absence of the tumor microenvironment. For example, the activity of the promoter is increased or decreased by at least 3 fold, at least 5 fold, at least 10 fold, at least 20 fold, at least 50 fold, or at least 100 fold, relative to activity of the promoter in the absence of the tumor microenvironment. In some embodiments, the activity of the promoter is increased or decreased by 2-10, 2-20, 2-30, 2-40, 2-50, 2-60, 2-70, 2-80, 2-90, or 2-100 fold, relative to activity of the promoter in the absence of the tumor microenvironment.

In some embodiments, a promoter of the present disclosure is activated under a hypoxic condition. A "hypoxic condition" is a condition where the body or a region of the body is deprived of adequate oxygen supply at the tissue level. Hypoxic conditions can cause inflammation (*e.g.*, the level of inflammatory cytokines increase under hypoxic conditions). In some embodiments, the promoter that is activated under hypoxic condition is operably linked to a nucleotide encoding a protein that decreases the expression of activity of inflammatory cytokines, thus reducing the inflammation caused by the hypoxic condition. In some embodiments, the promoter that is activated under hypoxic conditions comprises a hypoxia responsive element (HRE). A "hypoxia responsive element (HRE)" is a response element that responds to hypoxia-inducible factor (HIF). The HRE, in some embodiments, comprises a consensus motif NCGTG (where N is either A or G).

Activation-Conditional Control Polypeptide (ACP) Promoter Systems

In some embodiments, a synthetic promoter is a promoter system including an activation-conditional control polypeptide- (ACP-) binding domain sequence and a promoter sequence. Such a system is also referred to herein as an "ACP-responsive promoter." In general, an ACP promoter system includes a first expression cassette encoding an activation-conditional control polypeptide (ACP) and a second expression cassette encoding an ACP-responsive promoter operably linked to an exogenous polynucleotide sequence, such as the exogenous polynucleotide sequence encoding the cytokines, including membrane-cleavable chimeric proteins versions of cytokines, described herein or any other protein of interest (*e.g.*, a protease or CAR). In some embodiments, the first expression cassette and second expression cassette are each encoded by a separate engineered nucleic acid. In other embodiments, the first expression cassette and the second expression cassette are encoded by the same engineered nucleic acid. The ACP-responsive promoter can be operably linked to a nucleotide sequence encoding a

single protein of interest or multiple proteins of interest. In some embodiments, a synthetic promoter comprises the nucleic acid sequence of AATTAACGGGTTTCGTAACAATCGCATGAGGATTCGCAACGCCTTTGAAGCAGTCG ACGCCGAAGTCCCGTCTCAGTAAAGGTTGAAGCAGTCGACGCCGAAGAATCGGACT GCCTTCGTATGAAGCAGTCGACGCCGAAGGTATCAGTCGCCTCGGAATGAAGCAGT CGACGCCGAAGATTCGTAAGAGGCTCACTCTCCCTTACACGGAGTGGATAACTAGT TCTAGAGGGTATATAATGGGGGCCAACGCGTACCGGTGTC (SEQ ID NO: 298). In some embodiments, a synthetic promoter comprises a sequence that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 298. In some embodiments, a synthetic promoter comprises the nucleic acid sequence of CGGGTTTCGTAACAATCGCATGAGGATTCGCAACGCCTTCGGCGTAGCCGATGTCG CGCTCCCGTCTCAGTAAAGGTCGGCGTAGCCGATGTCGCGCAATCGGACTGCCTTCG TACGGCGTAGCCGATGTCGCGCGTATCAGTCGCCTCGGAACGGCGTAGCCGATGTC GCGCATTCGTAAGAGGCTCACTCTCCCTTACACGGAGTGGATAACTAGTTCTAGAG GGTATATAATGGGGGCCA (SEQ ID NO: 299). In some embodiments, a synthetic promoter comprises a sequence that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 299.

The promoters of the ACP promoter system, *e.g.*, either a promoter driving expression of the ACP or the promoter sequence of the ACP-responsive promoter, can include any of the promoter sequences described herein (*see* "Promoters" above). The ACP-responsive promoter can be derived from minP, NFkB response element, CREB response element, NFAT response element, SRF response element 1, SRF response element 2, AP1 response element, TCF-LEF response element promoter fusion, Hypoxia responsive element, SMAD binding element, STAT3 binding site, minCMV, YB_TATA, minTK, inducer molecule responsive promoters, and tandem repeats thereof. In some embodiments, the ACP-responsive promoter includes a minimal promoter.

In some embodiments, the ACP-binding domain includes one or more zinc finger binding sites. In some embodiments, the ACP-responsive promoter includes a minimal promoter and the ACP-binding domain includes one or more zinc finger binding sites. The ACP-binding domain can include 1, 2, 3, 4,5 ,6 7, 8, 9, 10, or more zinc finger binding sites. In some embodiments, the transcription factor is a zinc-finger-containing transcription factor. In some embodiments, the zinc-finger-containing transcription factor is a synthetic transcription factor. In some embodiments, the ACP-binding domain includes one or more zinc finger binding sites

and the ACP has a DNA-binding zinc finger protein domain (ZF protein domain). In some embodiments, the ACP has a DNA-binding zinc finger protein domain (ZF protein domain) and an effector domain. In some embodiments, the ACP-binding domain includes one or more zinc finger binding sites and the ACP has a DNA-binding zinc finger protein domain (ZF protein domain) and an effector domain. In some embodiments, the ZF protein domain is modular in design and is composed of zinc finger arrays (ZFA). A zinc finger array comprises multiple zinc finger protein motifs that are linked together. Each zinc finger motif binds to a different nucleic acid motif. This results in a ZFA with specificity to any desired nucleic acid sequence, *e.g.*, a ZFA with desired specificity to an ACP-binding domain having a specific zinc finger binding site composition and/or configuration. The ZF motifs can be directly adjacent to each other, or separated by a flexible linker sequence. In some embodiments, a ZFA is an array, string, or chain of ZF motifs arranged in tandem. A ZFA can have 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12,1 3, 14, or 15 zinc finger motifs. The ZFA can have from 1-10, 1-15, 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 2-3, 2-4, 2-5, 2-6, 2-7, 2-8, 2-9, 2-10, 3-4, 3-5 3-6, 3-7, 3-8, 3-9, 3-10, 4-5, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, or 5-15 zinc finger motifs. The ZF protein domain can have 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or more ZFAs. The ZF domain can have from 1-10, 1-15, 1-2, 1-3, 1-4, 1-5, 1-6, 1-7, 1-8, 1-9, 2-3, 2-4, 2-5, 2-6, 2-7, 2-8, 2-9, 2-10, 3-4, 3-5 3-6, 3-7, 3-8, 3-9, 3-10, 4-5, 4-6, 4-7, 4-8, 4-9, 4-10, 5-6, 5-7, 5-8, 5-9, 5-10, or 5-15 ZFAs. In some embodiments, the ZF protein domain comprises one to ten ZFA(s). In some embodiments, the ZF protein domain comprises at least one ZFA. In some embodiments, the ZF protein domain comprises at least two ZFAs. In some embodiments, the ZF protein domain comprises at least three ZFAs. In some embodiments, the ZF protein domain comprises at least four ZFAs. In some embodiments, the ZF protein domain comprises at least five ZFAs. In some embodiments, the ZF protein domain comprises at least ten ZFAs.

In some embodiments, the DNA-binding domain comprises a tetracycline (or derivative thereof) repressor (TetR) domain.

The ACP can also further include an effector domain, such as a transcriptional effector domain. For instance, a transcriptional effector domain can be the effector or activator domain of a transcription factor. Transcription factor activation domains are also known as transactivation domains, and act as scaffold domains for proteins such as transcription coregulators that act to activate or repress transcription of genes. Any suitable transcriptional effector domains can be used in the ACP including, but not limited to, a Herpes Simplex Virus Protein 16 (VP16) activation domain; an activation domain consisting of four tandem copies of VP16, a VP64 activation domain; a p65 activation domain of NFκB; an Epstein-Barr virus R transactivator (Rta) activation domain; a tripartite activator comprising the VP64, the p65, and

the Rta activation domains, the tripartite activator is known as a VPR activation domain; a histone acetyltransferase (HAT) core domain of the human E1A-associated protein p300, known as a p300 HAT core activation domain; a Krüppel associated box (KRAB) repression domain; a Repressor Element Silencing Transcription Factor (REST) repression domain; a WRPW motif of the hairy-related basic helix-loop-helix repressor proteins, the motif is known as a WRPW repression domain; a DNA (cytosine-5)-methyltransferase 3B (DNMT3B) repression domain; and an HP1 alpha chromoshadow repression domain, or any combination thereof.

In some embodiments, the effector domain is s transcription effector domain selected from: a Herpes Simplex Virus Protein 16 (VP16) activation domain; an activation domain consisting of four tandem copies of VP16, a VP64 activation domain; a p65 activation domain of NFκB; an Epstein-Barr virus R transactivator (Rta) activation domain; a tripartite activator comprising the VP64, the p65, and the Rta activation domains, the tripartite activator is known as a VPR activation domain; a histone acetyltransferase (HAT) core domain of the human E1A-associated protein p300, known as a p300 HAT core activation domain; a Krüppel associated box (KRAB) repression domain; a Repressor Element Silencing Transcription Factor (REST) repression domain; a WRPW motif of the hairy-related basic helix-loop-helix repressor proteins, the motif is known as a WRPW repression domain; a DNA (cytosine-5)-methyltransferase 3B (DNMT3B) repression domain; and an HP1 alpha chromoshadow repression domain.

In some embodiments, the ACP is a small molecule (*e.g.*, drug) inducible polypeptide. For example, in some embodiments, the ACP may be induced by tetracycline (or derivative thereof), and comprises a TetR domain and a VP16 effector domain. In some embodiments, the ACP includes an estrogen receptor variant, such as ERT2, and may be regulated by tamoxifen, or a metabolite thereof (such as 4-hydroxy-tamoxifen [4-OHT], N-desmethyltamoxifen, tamoxifen-N-oxide, or endoxifen), through tamoxifen-controlled nuclear localization. In some embodiments, the ACP comprises a nuclear-localization signal (NLS). In certain embodiments, the NLS comprises the amino acid sequence of MPKKKRKV (SEQ ID NO: 296). An exemplary nucleic acid sequence encoding SEQ ID NO: 296 is ATGCCCAAGAAGAAGCGGAAGGTT (SEQ ID NO: 297) or ATGCCCAAGAAAAAGCGGAAGGTG (SEQ ID NO: 340). In some embodiments, a nucleic acid sequence encoding SEQ ID NO: 296 may comprise SEQ ID NO: 297 or SEQ ID NO: 340, or comprises a sequence that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 297 or SEQ ID NO: 340.

In some embodiments, the ACP is a small molecule (e.g., drug) inducible polypeptide that includes a repressible protease and one or more cognate cleavage sites of the repressible

protease. In some embodiments, a repressible protease is active (cleaves a cognate cleavage site) in the absence of the specific agent and is inactive (does not cleave a cognate cleavage site) in the presence of the specific agent. In some embodiments, the specific agent is a protease inhibitor. In some embodiments, the protease inhibitor specifically inhibits a given repressible protease of the present disclosure. The repressible protease can be any of the proteases described herein that is capable of inactivation by the presence or absence of a specific agent (*see* "Protease Cleavage Site" above for exemplary repressible proteases, cognate cleavage sites, and protease inhibitors).

In some embodiments, the ACP has a degron domain (*see* "Degron Systems and Domains" above for exemplary degron sequences). The degron domain can be in any order or position relative to the individual domains of the ACP. For example, the degron domain can be N-terminal of the repressible protease, C-terminal of the repressible protease, N-terminal of the ZF protein domain, C-terminal of the ZF protein domain, N-terminal of the effector domain, or C-terminal of the effector domain.

Exemplary sequences of components of ACPs and exemplary ACPs of the present disclosure are provided in **Table 5D**. In some embodiments, nucleic acids may comprise a sequence in Table 5D, or a nucleic acid sequence that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to a sequence in Table 5D.

Table 5D.

Design	Amino Acid Sequence	Nucleic Acid Sequence
NLS + miniVPR activation domain + NS3 protease + ZFBD DNA binding domain	MPKKKRKVDALDDFDLDMLG SDALDDFDLDMLGSDALDDF DLDMLGSDALDDFDLDMLINS RSSGSPKKKRKVGSGGGSGGS GSVLPQAPAPAPAPAMVSALA QAPAPVPVLAPGPPQAVAPPA PKPTQAGEGTLSEALLQLQFD DEDLGALLGNSTDPAVFTDLA SVDNSEFQQLLNQGIPVAPHTT EPMLMEYPEAITRLVTGAQRP PDPAPAPLGAPGLPNGLLSGDE DFSSIADMDFSALLSGGGSGGS GSDLSHPPPRGHLDELTTTLES MTEDLNLDSPLTPELNEILDTF LNDECLLHAMHISTGLSIFDTS LFEDVVCCHSIYGKKKGDIDT YRYIGSSGTGCVVIVGRIVLSG	ATGCCCAAGAAAAAGCGGAAGGTGGACGCC CTGGACGACTTCGATCTGGATATGCTGGGCA GCGACGCTCTGGATGATTTTGACCTGGACAT GCTCGGCTCTGATGCACTCGACGATTTCGAC CTCGATATGTTGGGATCTGATGCCCTTGATG ACTTTGATCTCGACATGTTGATCAATAGCCG GTCCAGCGGCAGCCCCAAGAAGAAGAGAAA AGTCGGCTCTGGCGGCGGATCTGGCGGTTCT GGATCTGTTTTGCCCCAAGCTCCTGCTCCTGC ACCAGCTCCAGCTATGGTTTCTGCTCTGGCTC AGGCTCCAGCTCCTGTGCCTGTTCTTGCTCCT GGACCTCCTCAGGCTGTTGCTCCACCAGCAC CTAAACCTACACAGGCCGGCGAGGGAACAC TGTCTGAAGCTCTGCTGCAGCTCCAGTTCGA CGACGAAGATCTGGGAGCCCTGCTGGGCAAT AGCACAGATCCTGCCGTGTTCACCGATCTGG CCAGCGTGGACAATAGCGAGTTCCAGCAGCT

	SGTSAPITAYAQQTRGLLGCIIT SLTGRDKNQVEGEVQIVSTAT QTFLATCINGVCWAVYHGAG TRTIASPKGPVIQMYTNVDQD LVGWPAPQGSRSLTPCTCGSS DLYLVTRHADVIPVRRRGDSR GSLLSPRPISYLKGSSGGPLLCP AGHAVGLFRAAVCTRGVAKA VDFIPVENLETTMRSPVFTDNS SPPAVTLTHPITKIDREVLYQEF DEMEECSQHMSRPGERPFQCR ICMRNFSNMSNLTRHTRTHTG EKPFQCRICMRNFSDRSVLRR HLRTHTGSQKPFQCRICMRNF SDPSNLARHTRTHTGEKPFQC RICMRNFSDRSSLRRHLRTHTG SQKPFQCRICMRNFSQSGTLHR HTRTHTGEKPFQCRICMRNFS QRPNLTRHLRTHLRGS (SEQ ID NO: 301)	CCTGAACCAGGGCATTCCTGTGGCTCCTCAC ACCACCGAGCCTATGCTGATGGAATACCCCG AGGCCATCACCAGACTGGTCACCGGTGCTCA AAGACCACCTGATCCGGCTCCAGCACCTCTT GGAGCACCTGGACTGCCTAATGGACTGCTGT CTGGCGACGAGGACTTCAGCTCTATCGCCGA CATGGATTTCAGCGCCCTGCTCAGTGGCGGT GGAAGCGGAGGAAGTGGCAGCGATCTTTCTC ACCCTCCACCTAGAGGCCACCTGGACGAGCT GACAACCACACTGGAATCCATGACCGAGGA CCTGAACCTGGACAGCCCTCTGACACCCGAG CTGAACGAGATCCTGGACACCTTCCTGAACG ACGAGTGTCTGCTGCACGCCATGCACATCTC TACCGGCCTGAGCATCTTCGACACCAGCCTG TTTGAGGATGTCGTGTGCTGCCACAGCATCT ACGGCAAGAAGAAGGGCGACATCGACACCT ACCGGTACATCGGCAGCTCTGGCACAGGCTG TGTGGTCATCGTGGGCAGAATCGTGCTGTCT GGCAGCGGAACAAGCGCCCCTATCACAGCCT ATGCTCAGCAGACAAGAGGCCTGCTGGGCTG CATCATCACAAGCCTGACCGGCAGAGACAA GAACCAGGTGGAAGGCGAGGTGCAGATCGT GTCTACAGCTACCCAGACCTTCCTGGCCACC TGTATCAATGGCGTGTGCTGGGCCGTGTATC ACGGCGCTGGAACCAGAACAATCGCCTCTCC TAAGGGCCCCGTGATCCAGATGTACACCAAC GTGGACCAGGACCTCGTTGGCTGGCCTGCTC CTCAAGGCAGCAGAAGCCTGACACCTTGCAC CTGTGGCTCCAGCGATCTGTACCTGGTCACC AGACACGCCGACGTGATCCCTGTCAGAAGA AGAGGGGATTCCAGAGGCAGCCTGCTGAGC CCTAGACCTATCAGCTACCTGAAGGGCTCTA GCGGCGGACCTCTGCTTTGTCCTGCTGGACA TGCCGTGGGCCTGTTTAGAGCCGCCGTGTGT ACAAGAGGCGTGGCCAAAGCCGTGGACTTC ATCCCCGTGGAAAACCTGGAAACCACCATGC GGAGCCCCGTGTTCACCGACAATTCTAGCCC TCCAGCCGTGACACTGACACACCCCATCACC AAGATCGACAGAGAGGTGCTGTACCAAGAG TTCGACGAGATGGAAGAGTGCAGCCAGCAC ATGTCTAGACCTGGCGAGAGGCCCTTCCAGT GCCGGATCTGCATGCGGAACTTCAGCAACAT

		GAGCAACCTGACCAGACACACCCGGACACA CACAGGCGAGAAGCCTTTTCAGTGCAGAATC TGTATGCGCAATTTCTCCGACAGAAGCGTGC TGCGGAGACACCTGAGAACCCACACCGGCA GCCAGAAACCATTCCAGTGTCGCATCTGTAT GAGAAACTTTAGCGACCCCTCCAATCTGGCC CGGCACACCAGAACACATACCGGGGAAAAA CCCTTTCAGTGTAGGATATGCATGAGGAATT TTTCCGACCGGTCCAGCCTGAGGCGGCACCT GAGGACACATACTGGCTCCCAAAAGCCGTTC CAATGTCGGATATGTATGCGCAACTTTAGCC AGAGCGGCACCCTGCACAGACACACAAGAA CCCATACTGGCGAGAAACCTTTCCAATGTAG AATCTGCATGCGAAATTTTTCCCAGCGGCCT AATCTGACCAGGCATCTGAGGACCCACCTGA GAGGATCT (SEQ ID NO: 306)
NLS + ZFBD DNA binding domain + NS3 protease + miniVPR activation domain	MPKKKRKVMSRPGERPFQCRI CMRNFSNMSNLTRHTRTHTGE KPFQCRICMRNFSDRSVLRRH LRTHTGSQKPFQCRICMRNFS DPSNLARHTRTHTGEKPFQCRI CMRNFSDRSSLRRHLRTHTGS QKPFQCRICMRNFSQSGTLHR HTRTHTGEKPFQCRICMRNFS QRPNLTRHLRTHLRGSEDVVC CHSIYGKKKGDIDTYRYIGSSG TGCVVIVGRIVLSGSGTSAPIT AYAQQTRGLLGCIITSLTGRDK NQVEGEVQIVSTATQTFLATCI NGVCWAVYHGAGTRTIASPK GPVIQMYTNVDQDLVGWPAP QGSRSLTPCTCGSSDLYLVTRH ADVIPVRRRGDSRGSLLSPRPIS YLKGSSGGPLLCPAGHAVGLF RAAVCTRGVAKAVDFIPVENL ETTMRSPVFTDNSSPPAVTLTH PITKIDREVLYQEFDEMEECSQ HDALDDFDLDMLGSDALDDF DLDMLGSDALDDFDLDMLGS DALDDFDLDMLINSRSSGSPK KKRKVGSGGGSGGSGSVLPQA PAPAPAPAMVSALAQAPAPVP	ATGCCCAAGAAAAAGCGGAAGGTGATGTCT AGACCTGGCGAGAGGCCCTTCCAGTGCCGGA TCTGCATGCGGAACTTCAGCAACATGAGCAA CCTGACCAGACACACCCGGACACACACAGG CGAGAAGCCTTTTCAGTGCAGAATCTGTATG CGCAATTTCTCCGACAGAAGCGTGCTGCGGA GACACCTGAGAACCCACACCGGCAGCCAGA AACCATTCCAGTGTCGCATCTGTATGAGAAA CTTTAGCGACCCCTCCAATCTGGCCCGGCAC ACCAGAACACATACCGGGGAAAAACCCTTTC AGTGTAGGATATGCATGAGGAATTTTTCCGA CCGGTCCAGCCTGAGGCGGCACCTGAGGAC ACATACTGGCTCCCAAAAGCCGTTCCAATGT CGGATATGTATGCGCAACTTTAGCCAGAGCG GCACCCTGCACAGACACACAAGAACCCATA CTGGCGAGAAACCTTTCCAATGTAGAATCTG CATGCGAAATTTTTCCCAGCGGCCTAATCTG ACCAGGCATCTGAGGACCCACCTGAGAGGA TCTGAGGATGTCGTGTGCTGCCACAGCATCT ACGGCAAGAAGAAGGGCGACATCGACACCT ACCGGTACATCGGCAGCTCTGGCACAGGCTG TGTGGTCATCGTGGGCAGAATCGTGCTGTCT GGCAGCGGAACAAGCGCCCCTATCACAGCCT ATGCTCAGCAGACAAGAGGCCTGCTGGGCTG CATCATCACAAGCCTGACCGGCAGAGACAA GAACCAGGTGGAAGGCGAGGTGCAGATCGT

	VLAPGPPQAVAPPAPKPTQAG EGTLSEALLQLQFDDEDLGAL LGNSTDPAVFTDLASVDNSEF QQLLNQGIPVAPHTTEPMLME YPEAITRLVTGAQRPPDPAPAP LGAPGLPNGLLSGDEDFSSIAD MDFSALLSGGGSGGSGSDLSH PPPRGHLDELTTTLESMTEDLN LDSPLTPELNEILDTFLNDECLL HAMHISTGLSIFDTSLF (SEQ ID NO: 302)	GTCTACAGCTACCCAGACCTTCCTGGCCACC TGTATCAATGGCGTGTGCTGGGCCGTGTATC ACGGCGCTGGAACCAGAACAATCGCCTCTCC TAAGGGCCCCGTGATCCAGATGTACACCAAC GTGGACCAGGACCTCGTTGGCTGGCCTGCTC CTCAAGGCAGCAGAAGCCTGACACCTTGCAC CTGTGGCTCCAGCGATCTGTACCTGGTCACC AGACACGCCGACGTGATCCCTGTCAGAAGA AGAGGGGATTCCAGAGGCAGCCTGCTGAGC CCTAGACCTATCAGCTACCTGAAGGGCTCTA GCGGCGGACCTCTGCTTTGTCCTGCTGGACA TGCCGTGGGCCTGTTTAGAGCCGCCGTGTGT ACAAGAGGCGTGGCCAAAGCCGTGGACTTC ATCCCCGTGGAAAACCTGGAAACCACCATGC GGAGCCCCGTGTTCACCGACAATTCTAGCCC TCCAGCCGTGACACTGACACACCCCATCACC AAGATCGACAGAGAGGTGCTGTACCAAGAG TTCGACGAGATGGAAGAGTGCAGCCAGCAC GACGCCCTGGACGACTTCGATCTGGATATGC TGGGCAGCGACGCTCTGGATGATTTTGACCT GGACATGCTCGGCTCTGATGCACTCGACGAT TTCGACCTCGATATGTTGGGATCTGATGCCC TTGATGACTTTGATCTCGACATGTTGATCAAT AGCCGGTCCAGCGGCAGCCCCAAGAAGAAG AGAAAAGTCGGCTCTGGCGGCGGATCTGGC GGTTCTGGATCTGTTTTGCCCCAAGCTCCTGC TCCTGCACCAGCTCCAGCTATGGTTTCTGCTC TGGCTCAGGCTCCAGCTCCTGTGCCTGTTCTT GCTCCTGGACCTCCTCAGGCTGTTGCTCCAC CAGCACCTAAACCTACACAGGCCGGCGAGG GAACACTGTCTGAAGCTCTGCTGCAGCTCCA GTTCGACGACGAAGATCTGGGAGCCCTGCTG GGCAATAGCACAGATCCTGCCGTGTTCACCG ATCTGGCCAGCGTGGACAATAGCGAGTTCCA GCAGCTCCTGAACCAGGGCATTCCTGTGGCT CCTCACACCACCGAGCCTATGCTGATGGAAT ACCCCGAGGCCATCACCAGACTGGTCACCGG TGCTCAAAGACCACCTGATCCGGCTCCAGCA CCTCTTGGAGCACCTGGACTGCCTAATGGAC TGCTGTCTGGCGACGAGGACTTCAGCTCTAT CGCCGACATGGATTTCAGCGCCCTGCTCAGT GGCGGTGGAAGCGGAGGAAGTGGCAGCGAT

		CTTTCTCACCCTCCACCTAGAGGCCACCTGG ACGAGCTGACAACCACACTGGAATCCATGAC CGAGGACCTGAACCTGGACAGCCCTCTGACA CCCGAGCTGAACGAGATCCTGGACACCTTCC TGAACGACGAGTGTCTGCTGCACGCCATGCA CATCTCTACCGGCCTGAGCATCTTCGACACC AGCCTGTTT (SEQ ID NO: 305)
NLS + ZFBD DNA binding domain + NS3 protease + miniVPR activation domain	MPKKKRKVSRPGERPFQCRIC MRNFSRRHGLDRHTRTHTGEK PFQCRICMRNFSDHSSLKRHLR THTGSQKPFQCRICMRNFSVR HNLTRHLRTHTGEKPFQCRIC MRNFSDHSNLSRHLKTHTGSQ KPFQCRICMRNFSQRSSLVRHL RTHTGEKPFQCRICMRNFSESG HLKRHLRTHLRGSEDVVCCHS IYGKKKGDIDTYRYIGSSGTGC VVIVGRIVLSGSGTSAPITAYA QQTRGLLGCIITSLTGRDKNQV EGEVQIVSTATQTFLATCINGV CWAVYHGAGTRTIASPKGPVI QMYTNVDQDLVGWPAPQGSR SLTPCTCGSSDLYLVTRHADVI PVRRRGDSRGSLLSPRPISYLK GSSGGPLLCPAGHAVGLFRAA VCTRGVAKAVDFIPVENLETT MRSPVFTDNSSPPAVTLTHPIT KIDREVLYQEFDEMEECSQHD ALDDFDLDMLGSDALDDFDL DMLGSDALDDFDLDMLGSDA LDDFDLDMLINSRSSGSPKKK RKVGSGGGSGGSGSVLPQAPA PAPAPAMVSALAQAPAPVPVL APGPPQAVAPPAPKPTQAGEG TLSEALLQLQFDDEDLGALLG NSTDPAVFTDLASVDNSEFQQ LLNQGIPVAPHTTEPMLMEYP EAITRLVTGAQRPPDPAPAPLG APGLPNGLLSGDEDFSSIADM DFSALLSGGGSGGSGSDLSHPP PRGHLDELTTTLESMTEDLNL DSPLTPELNEILDTFLNDECLL	ATGCCCAAGAAGAAGCGGAAGGTTTCCCGG CCTGGCGAGAGGCCTTTCCAGTGCAGAATCT GCATGCGGAACTTCAGCAGACGGCACGGCCT GGACAGACACACCAGAACACACACAGGCGA GAAACCCTTCCAGTGCCGGATCTGTATGAGA AATTTCAGCGACCACAGCAGCCTGAAGCGGC ACCTGAGAACCCATACCGGCAGCCAGAAAC CATTTCAGTGTAGGATATGCATGCGCAATTT CTCCGTGCGGCACAACCTGACCAGACACCTG AGGACACACACCGGGGAGAAGCCTTTTCAAT GTCGCATATGCATGAGAAACTTCTCTGACCA CTCCAACCTGAGCCGCCACCTCAAAACCCAC ACCGGCTCTCAAAAGCCCTTCCAATGTAGAA TATGTATGAGGAACTTTAGCCAGCGGAGCAG CCTCGTGCGCCATCTGAGAACTCACACTGGC GAAAAGCCGTTTCAATGCCGTATCTGTATGC GCAACTTTAGCGAGAGCGGCCACCTGAAGA GACATCTGCGCACACACCTGAGAGGCAGCG AGGATGTCGTGTGCTGCCACAGCATCTACGG AAAGAAGAAGGGCGACATCGACACCTATCG GTACATCGGCAGCAGCGGCACAGGCTGTGTT GTGATCGTGGGCAGAATCGTGCTGAGCGGCT CTGGAACAAGCGCCCCTATCACAGCCTACGC TCAGCAGACAAGAGGCCTGCTGGGCTGCATC ATCACAAGCCTGACCGGCAGAGACAAGAAC CAGGTGGAAGGCGAGGTGCAGATCGTGTCT ACAGCTACCCAGACCTTCCTGGCCACCTGTA TCAATGGCGTGTGCTGGGCCGTGTATCACGG CGCTGGCACAAGAACAATCGCCTCTCCAAAG GGCCCCGTGATCCAGATGTACACCAACGTGG ACCAGGACCTCGTTGGCTGGCCTGCTCCTCA AGGCAGCAGAAGCCTGACACCTTGCACCTGT GGCTCCAGCGATCTGTACCTGGTCACCAGAC ACGCCGACGTGATCCCTGTCAGAAGAAGAG GGGATTCCAGAGGCAGCCTGCTGAGCCCTAG

	HAMHISTGLSIFDTSLF (SEQ ID NO: 303)	ACCTATCAGCTACCTGAAGGGCAGCTCTGGC GGACCTCTGCTTTGTCCTGCTGGACATGCCG TGGGCCTGTTTAGAGCCGCCGTGTGTACAAG AGGCGTGGCCAAAGCCGTGGACTTCATCCCC GTGGAAAACCTGGAAACCACCATGCGGAGC CCCGTGTTCACCGACAATTCTAGCCCTCCAG CCGTGACACTGACACACCCCATCACCAAGAT CGACAGAGAGGTGCTGTACCAAGAGTTCGA CGAGATGGAAGAGTGCAGCCAGCACGACGC TCTTGATGACTTTGACCTGGATATGCTCGGA TCAGATGCCCTGGACGATTTCGATCTGGACA TGTTGGGGTCTGATGCTCTCGACGACTTCGA TCTGGATATGCTTGGAAGTGACGCGCTGGAT GATTTCGACCTTGACATGCTCATCAATTCTCG ATCCAGTGGAAGCCCGAAAAAGAAACGCAA GGTGGGAAGTGGGGGCGGCTCCGGTGGGAG CGGTAGTGTATTGCCTCAAGCTCCCGCGCCC GCTCCTGCTCCGGCAATGGTTTCAGCTCTGG CACAAGCTCCAGCTCCAGTGCCTGTGCTCGC CCCTGGCCCTCCGCAGGCCGTAGCACCTCCC GCCCCCAAACCGACGCAAGCCGGTGAGGGG ACTCTCTCTGAAGCCTTGCTGCAGCTTCAGTT CGATGATGAAGATCTGGGCGCGCTCTTGGGG AACAGCACGGATCCGGCAGTATTTACGGACC TCGCATCAGTTGACAATAGTGAATTTCAACA ACTTCTTAACCAGGGAATACCGGTTGCGCCC CATACGACGGAACCTATGCTGATGGAGTACC CTGAAGCTATAACCAGACTCGTAACTGGCGC CCAACGCCCGCCCGACCCGGCTCCTGCGCCG CTGGGTGCGCCGGGTCTTCCGAATGGTCTTC TCTCAGGGGACGAAGATTTCAGTTCCATTGC GGATATGGACTTTTCCGCGCTCCTGAGTGGG GGTGGCTCTGGAGGCTCTGGTTCCGACCTCA GCCATCCTCCACCGAGAGGACACCTCGACGA GCTGACAACCACCCTCGAAAGTATGACGGA AGATCTGAACTTGGATTCCCCCCTTACCCCA GAACTGAATGAAATCCTCGATACGTTCTTGA ACGATGAGTGCCTTTTGCACGCCATGCATAT ATCAACAGGTTTGTCTATCTTCGACACGTCC CTCTTTTGA (SEQ ID NO: 304)
miniVPR activator domain	DALDDFDLDMLGSDALDDFD LDMLGSDALDDFDLDMLGSD	GACGCCCTGGACGACTTCGATCTGGATATGC TGGGCAGCGACGCTCTGGATGATTTTGACCT

	ALDDFDLDMLINSRSSGSPKK KRKVGSGGGSGGSGSVLPQAP APAPAPAMVSALAQAPAPVPV LAPGPPQAVAPPAPKPTQAGE GTLSEALLQLQFDDEDLGALL GNSTDPAVFTDLASVDNSEFQ QLLNQGIPVAPHTTEPMLMEY PEAITRLVTGAQRPPDPAPAPL GAPGLPNGLLSGDEDFSSIAD MDFSALLSGGGSGGSGSDLSH PPPRGHLDELTTTLESMTEDLN LDSPLTPELNEILDTFLNDECLL HAMHISTGLSIFDTSLF (SEQ ID NO: 325)	GGACATGCTCGGCTCTGATGCACTCGACGAT TTCGACCTCGATATGTTGGGATCTGATGCCC TTGATGACTTTGATCTCGACATGTTGATCAAT AGCCGGTCCAGCGGCAGCCCCAAGAAGAAG AGAAAAGTCGGCTCTGGCGGCGGATCTGGC GGTTCTGGATCTGTTTTGCCCCAAGCTCCTGC TCCTGCACCAGCTCCAGCTATGGTTTCTGCTC TGGCTCAGGCTCCAGCTCCTGTGCCTGTTCTT GCTCCTGGACCTCCTCAGGCTGTTGCTCCAC CAGCACCTAAACCTACACAGGCCGGCGAGG GAACACTGTCTGAAGCTCTGCTGCAGCTCCA GTTCGACGACGAAGATCTGGGAGCCCTGCTG GGCAATAGCACAGATCCTGCCGTGTTCACCG ATCTGGCCAGCGTGGACAATAGCGAGTTCCA GCAGCTCCTGAACCAGGGCATTCCTGTGGCT CCTCACACCACCGAGCCTATGCTGATGGAAT ACCCCGAGGCCATCACCAGACTGGTCACCGG TGCTCAAAGACCACCTGATCCGGCTCCAGCA CCTCTTGGAGCACCTGGACTGCCTAATGGAC TGCTGTCTGGCGACGAGGACTTCAGCTCTAT CGCCGACATGGATTTCAGCGCCCTGCTCAGT GGCGGTGGAAGCGGAGGAAGTGGCAGCGAT CTTTCTCACCCTCCACCTAGAGGCCACCTGG ACGAGCTGACAACCACACTGGAATCCATGAC CGAGGACCTGAACCTGGACAGCCCTCTGACA CCCGAGCTGAACGAGATCCTGGACACCTTCC TGAACGACGAGTGTCTGCTGCACGCCATGCA CATCTCTACCGGCCTGAGCATCTTCGACACC AGCCTGTTT (SEQ ID NO: 322) OR GACGCTCTTGATGACTTTGACCTGGATATGC TCGGATCAGATGCCCTGGACGATTTCGATCT GGACATGTTGGGGTCTGATGCTCTCGACGAC TTCGATCTGGATATGCTTGGAAGTGACGCGC TGGATGATTTCGACCTTGACATGCTCATCAA TTCTCGATCCAGTGGAAGCCCGAAAAAGAA ACGCAAGGTGGGAAGTGGGGGCGGCTCCGG TGGGAGCGGTAGTGTATTGCCTCAAGCTCCC GCGCCCGCTCCTGCTCCGGCAATGGTTTCAG CTCTGGCACAAGCTCCAGCTCCAGTGCCTGT GCTCGCCCCTGGCCCTCCGCAGGCCGTAGCA CCTCCCGCCCCCAAACCGACGCAAGCCGGTG

		AGGGGACTCTCTCTGAAGCCTTGCTGCAGCT TCAGTTCGATGATGAAGATCTGGGCGCGCTC TTGGGGAACAGCACGGATCCGGCAGTATTTA CGGACCTCGCATCAGTTGACAATAGTGAATT TCAACAACTTCTTAACCAGGGAATACCGGTT GCGCCCCATACGACGGAACCTATGCTGATGG AGTACCCTGAAGCTATAACCAGACTCGTAAC TGGCGCCCAACGCCCGCCCGACCCGGCTCCT GCGCCGCTGGGTGCGCCGGGTCTTCCGAATG GTCTTCTCTCAGGGGACGAAGATTTCAGTTC CATTGCGGATATGGACTTTTCCGCGCTCCTG AGTGGGGGTGGCTCTGGAGGCTCTGGTTCCG ACCTCAGCCATCCTCCACCGAGAGGACACCT CGACGAGCTGACAACCACCCTCGAAAGTATG ACGGAAGATCTGAACTTGGATTCCCCCCTTA CCCCAGAACTGAATGAAATCCTCGATACGTT CTTGAACGATGAGTGCCTTTTGCACGCCATG CATATATCAACAGGTTTGTCTATCTTCGACA CGTCCCTCTTTTGA (SEQ ID NO: 343)
ZF5-7 Zinc finger domain	MSRPGERPFQCRICMRNFSNM SNLTRHTRTHTGEKPFQCRIC MRNFSDRSVLRRHLRTHTGSQ KPFQCRICMRNFSDPSNLARHT RTHTGEKPFQCRICMRNFSDRS SLRRHLRTHTGSQKPFQCRIC MRNFSQSGTLHRHTRTHTGEK PFQCRICMRNFSQRPNLTRHLR THLRGS (SEQ ID NO: 320)	ATGTCTAGACCTGGCGAGAGGCCCTTCCAGT GCCGGATCTGCATGCGGAACTTCAGCAACAT GAGCAACCTGACCAGACACACCCGGACACA CACAGGCGAGAAGCCTTTTCAGTGCAGAATC TGTATGCGCAATTTCTCCGACAGAAGCGTGC TGCGGAGACACCTGAGAACCCACACCGGCA GCCAGAAACCATTCCAGTGTCGCATCTGTAT GAGAAACTTTAGCGACCCCTCCAATCTGGCC CGGCACACCAGAACACATACCGGGGAAAAA CCCTTTCAGTGTAGGATATGCATGAGGAATT TTTCCGACCGGTCCAGCCTGAGGCGGCACCT GAGGACACATACTGGCTCCCAAAAGCCGTTC CAATGTCGGATATGTATGCGCAACTTTAGCC AGAGCGGCACCCTGCACAGACACACAAGAA CCCATACTGGCGAGAAACCTTTCCAATGTAG AATCTGCATGCGAAATTTTTCCCAGCGGCCT AATCTGACCAGGCATCTGAGGACCCACCTGA GAGGATCT (SEQ ID NO: 323)
NS3 protease	EDVVCCHSIYGKKKGDIDTYR YIGSSGTGCVVIVGRIVLSGSG TSAPITAYAQQTRGLLGCIITSL TGRDKNQVEGEVQIVSTATQT FLATCINGVCWAVYHGAGTR	GAGGATGTCGTGTGCTGCCACAGCATCTACG GCAAGAAGAAGGGCGACATCGACACCTACC GGTACATCGGCAGCTCTGGCACAGGCTGTGT GGTCATCGTGGGCAGAATCGTGCTGTCTGGC AGCGGAACAAGCGCCCCTATCACAGCCTATG

	TIASPKGPVIQMYTNVDQDLV GWPAPQGSRSLTPCTCGSSDL YLVTRHADVIPVRRRGDSRGS LLSPRPISYLKGSSGGPLLCPA GHAVGLFRAAVCTRGVAKAV DFIPVENLETTMRSPVFTDNSS PPAVTLTHPITKIDREVLYQEF DEMEECSQH (SEQ ID NO: 321)	CTCAGCAGACAAGAGGCCTGCTGGGCTGCAT CATCACAAGCCTGACCGGCAGAGACAAGAA CCAGGTGGAAGGCGAGGTGCAGATCGTGTCT ACAGCTACCCAGACCTTCCTGGCCACCTGTA TCAATGGCGTGTGCTGGGCCGTGTATCACGG CGCTGGAACCAGAACAATCGCCTCTCCTAAG GGCCCCGTGATCCAGATGTACACCAACGTGG ACCAGGACCTCGTTGGCTGGCCTGCTCCTCA AGGCAGCAGAAGCCTGACACCTTGCACCTGT GGCTCCAGCGATCTGTACCTGGTCACCAGAC ACGCCGACGTGATCCCTGTCAGAAGAAGAG GGGATTCCAGAGGCAGCCTGCTGAGCCCTAG ACCTATCAGCTACCTGAAGGGCTCTAGCGGC GGACCTCTGCTTTGTCCTGCTGGACATGCCG TGGGCCTGTTTAGAGCCGCCGTGTGTACAAG AGGCGTGGCCAAAGCCGTGGACTTCATCCCC GTGGAAAACCTGGAAACCACCATGCGGAGC CCCGTGTTCACCGACAATTCTAGCCCTCCAG CCGTGACACTGACACACCCCATCACCAAGAT CGACAGAGAGGTGCTGTACCAAGAGTTCGA CGAGATGGAAGAGTGCAGCCAGCAC (SEQ ID NO: 195) OR GAGGATGTCGTGTGCTGCCACAGCATCTACG GAAAGAAGAAGGGCGACATCGACACCTATC GGTACATCGGCAGCAGCGGCACAGGCTGTGT TGTGATCGTGGGCAGAATCGTGCTGAGCGGC TCTGGAACAAGCGCCCCTATCACAGCCTACG CTCAGCAGACAAGAGGCCTGCTGGGCTGCAT CATCACAAGCCTGACCGGCAGAGACAAGAA CCAGGTGGAAGGCGAGGTGCAGATCGTGTCT ACAGCTACCCAGACCTTCCTGGCCACCTGTA TCAATGGCGTGTGCTGGGCCGTGTATCACGG CGCTGGCACAAGAACAATCGCCTCTCCAAAG GGCCCCGTGATCCAGATGTACACCAACGTGG ACCAGGACCTCGTTGGCTGGCCTGCTCCTCA AGGCAGCAGAAGCCTGACACCTTGCACCTGT GGCTCCAGCGATCTGTACCTGGTCACCAGAC ACGCCGACGTGATCCCTGTCAGAAGAAGAG GGGATTCCAGAGGCAGCCTGCTGAGCCCTAG ACCTATCAGCTACCTGAAGGGCAGCTCTGGC GGACCTCTGCTTTGTCCTGCTGGACATGCCG

		TGGGCCTGTTTAGAGCCGCCGTGTGTACAAG AGGCGTGGCCAAAGCCGTGGACTTCATCCCC GTGGAAAACCTGGAAACCACCATGCGGAGC CCCGTGTTCACCGACAATTCTAGCCCTCCAG CCGTGACACTGACACACCCCATCACCAAGAT CGACAGAGAGGTGCTGTACCAAGAGTTCGA CGAGATGGAAGAGTGCAGCCAGCAC (SEQ ID NO: 342)

Multicistronic and Multiple Promoter Systems

In some embodiments, engineered nucleic acids (*e.g.*, an engineered nucleic acid comprising an expression cassette) are configured to produce multiple proteins (*e.g.*, a cytokine, CAR, ACP, membrane-cleavable chimeric protein, and/or combinations thereof). For example, nucleic acids may be configured to produce 2-20 different proteins. In some embodiments, nucleic acids are configured to produce 2-20, 2-19, 2-18, 2-17, 2-16, 2-15, 2-14, 2-13, 2-12, 2-11, 2-10, 2-9, 2-8, 2-7, 2-6, 2-5, 2-4, 2-3, 3-20, 3-19, 3-18, 3-17, 3-16, 3-15, 3-14, 3-13, 3-12, 3-11, 3-10, 3-9, 3-8, 3-7, 3-6, 3-5, 3-4, 4-20, 4-19, 4-18, 4-17, 4-16, 4-15, 4-14, 4-13, 4-12, 4-11, 4-10, 4-9, 4-8, 4-7, 4-6, 4-5, 5-20, 5-19, 5-18, 5-17, 5-16, 5-15, 5-14, 5-13, 5-12, 5-11, 5-10, 5-9, 5-8, 5-7, 5-6, 6-20, 6-19, 6-18, 6-17, 6-16, 6-15, 6-14, 6-13, 6-12, 6-11, 6-10, 6-9, 6-8, 6-7, 7-20, 7-19, 7-18, 7-17, 7-16, 7-15, 7-14, 7-13, 7-12, 7-11, 7-10, 7-9, 7-8, 8-20, 8-19, 8-18, 8-17, 8-16, 8-15, 8-14, 8-13, 8-12, 8-11, 8-10, 8-9, 9-20, 9-19, 9-18, 9-17, 9-16, 9-15, 9-14, 9-13, 9-12, 9-11, 9-10, 10-20, 10-19, 10-18, 10-17, 10-16, 10-15, 10-14, 10-13, 10-12, 10-11, 11-20, 11-19, 11-18, 11-17, 11-16, 11-15, 11-14, 11-13, 11-12, 12-20, 12-19, 12-18, 12-17, 12-16, 12-15, 12-14, 12-13, 13-20, 13-19, 13-18, 13-17, 13-16, 13-15, 13-14, 14-20, 14-19, 14-18, 14-17, 14-16, 14-15, 15-20, 15-19, 15-18, 15-17, 15-16, 16-20, 16-19, 16-18, 16-17, 17-20, 17-19, 17-18, 18-20, 18-19, or 19-20 proteins. In some embodiments, nucleic acids are configured to produce 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 proteins.

In some embodiments, engineered nucleic acids can be multicistronic, *i.e.*, more than one separate polypeptide (*e.g.*, multiple proteins, such as a cytokine, CAR, ACP, and/or membrane-cleavable chimeric protein described herein) can be produced from a single mRNA transcript. Engineered nucleic acids can be multicistronic through the use of various linkers, *e.g.*, a polynucleotide sequence encoding a first protein can be linked to a nucleotide sequence encoding a second protein, such as in a first gene:linker:second gene 5' to 3' orientation. A linker can encode a 2A ribosome skipping element, such as T2A. Other 2A ribosome skipping elements include, but are not limited to, E2A, P2A, and F2A. 2A ribosome skipping elements allow production of separate polypeptides encoded by the first and second genes are produced

during translation. A linker can encode a cleavable linker polypeptide sequence, such as a Furin cleavage site or a TEV cleavage site, wherein following expression the cleavable linker polypeptide is cleaved such that separate polypeptides encoded by the first and second genes are produced. A cleavable linker can include a polypeptide sequence, such as such a flexible linker (*e.g.*, a Gly-Ser-Gly sequence), that further promotes cleavage. In some embodiments, an engineered nucleic acid disclosed herein comprises an E2A/T2A ribosome skipping element. In certain embodiments, the E2A/T2A ribosome skipping element comprises the amino acid sequence of GSGQCTNYALLKLAGDVESNPGPGSGEGRGSLLTCGDVEENPGP (SEQ ID NO: 281). An exemplary nucleic acid encoding SEQ ID NO: 281 is GGTAGCGGCCAGTGTACCAACTACGCCCTGCTGAAACTGGCCGGCGACGTGGAATC TAATCCTGGACCTGGATCTGGCGAGGGACGCGGGAGTCTACTGACGTGTGGAGACG TGGAGGAAAACCCTGGACCT (SEQ ID NO: 282). In certain embodiments, a nucleic acid encoding SEQ ID NO: 281 comprises a sequence that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 282. In some embodiments, an engineered nucleic acid disclosed herein comprises an E2A/T2A ribosome skipping element. In certain embodiments, the E2A/T2A ribosome skipping element comprises the amino acid sequence of QCTNYALLKLAGDVESNPGPGSGEGRGSLLTCGDVEENPGP (SEQ ID NO: 283). An exemplary nucleic acid encoding SEQ ID NO: 283 is CAGTGTACCAACTACGCCCTGCTGAAACTGGCCGGCGACGTGGAATCTAATCCTGG ACCTGGATCTGGCGAGGGACGCGGGAGTCTACTGACGTGTGGAGACGTGGAGGAA AACCCTGGACCT (SEQ ID NO: 284). In certain embodiments, a nucleic acid encoding SEQ ID NO: 283 comprises a sequence that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 284.

A linker can encode an Internal Ribosome Entry Site (IRES), such that separate polypeptides encoded by the first and second genes are produced during translation. A linker can encode a splice acceptor, such as a viral splice acceptor.

A linker can be a combination of linkers, such as a Furin-2A linker that can produce separate polypeptides through 2A ribosome skipping followed by further cleavage of the Furin site to allow for complete removal of 2A residues. In some embodiments, a combination of linkers can include a Furin sequence, a flexible linker, and 2A linker. Accordingly, in some embodiments, the linker is a Furin-Gly-Ser-Gly-2A fusion polypeptide. In some embodiments, a linker of the present disclosure is a Furin-Gly-Ser-Gly-T2A fusion polypeptide.

In general, a multicistronic system can use any number or combination of linkers, to express any number of genes or portions thereof (*e.g.*, an engineered nucleic acid can encode a first, a second, and a third protein, each separated by linkers such that separate polypeptides encoded by the first, second, and third proteins are produced).

Engineered nucleic acids can use multiple promoters to express genes from multiple ORFs, *i.e.*, more than one separate mRNA transcript can be produced from a single engineered nucleic acid. For example, a first promoter can be operably linked to a polynucleotide sequence encoding a first protein, and a second promoter can be operably linked to a polynucleotide sequence encoding a second protein. In general, any number of promoters can be used to express any number of proteins. In some embodiments, at least one of the ORFs expressed from the multiple promoters can be multicistronic.

Expression cassettes encoded on the same engineered nucleic acid can be oriented in any manner suitable for expression of the encoded exogenous polynucleotide sequences. Expression cassettes encoded on the same engineered nucleic acid can be oriented in the same direction, *i.e.*, transcription of separate cassettes proceeds in the same direction. Constructs oriented in the same direction can be organized in a head-to-tail format referring to the 5′ end (head) of the first gene being adjacent to the 3′ end (tail) of the upstream gene. Expression cassettes encoded on the same engineered nucleic acid can be oriented in an opposite direction, *i.e.*, transcription of separate cassettes proceeds in the opposite direction (also referred to herein as "bidirectional"). Expression cassettes encoded on the same engineered nucleic acid oriented in opposite directions can be oriented in a "head-to-head" directionality. As used herein, head-to-head refers to the 5′ end (head) of a first gene of a bidirectional construct being adjacent to the 5′ end (head) of an upstream gene of the bidirectional construct. Expression cassettes encoded on the same engineered nucleic acid oriented in opposite directions can be oriented in a "tail-to-tail" directionality. As used herein, tail-to-tail refers to the 3′ end (tail) of a first gene of a bidirectional construct being adjacent to the 3′ end (tail) of an upstream gene of the bidirectional construct. For example, and without limitation, **FIG. 1** schematically depicts a cytokine-CAR bidirectional construct in head-to-head directionality (**FIG. 1A**), head-to-tail directionality (**FIG. 1B**), and tail-to-tail directionality (**FIG. 1C**).

"Linkers," as used herein can refer to polypeptides that link a first polypeptide sequence and a second polypeptide sequence, the multicistronic linkers described above, or the additional promoters that are operably linked to additional ORFs described above.

Exogenous polynucleotide sequences encoded by the expression cassette can include a 3'untranslated region (UTR) comprising an mRNA-destabilizing element that is operably linked to the exogenous polynucleotide sequence, such as exogenous polynucleotide sequences

encoding a cytokine (e.g., IL12 or IL12p70). In some embodiments, the mRNA-destabilizing element comprises an AU-rich element and/or a stem-loop destabilizing element (SLDE). In some embodiments, the mRNA-destabilizing element comprises an AU-rich element. In some embodiments, the AU-rich element includes at least two overlapping motifs of the sequence ATTTA (SEQ ID NO: 209). In some embodiments, the AU-rich element comprises ATTTATTTATTTATTTATTTA (SEQ ID NO: 210). In some embodiments, the mRNA-destabilizing element comprises a stem-loop destabilizing element (SLDE). In some embodiments, the SLDE comprises CTGTTTAATATTTAAACAG (SEQ ID NO: 211). In some embodiments, the mRNA-destabilizing element comprises at least one AU-rich element and at least one SLDE. "AuSLDE" as used herein refers to an AU-rich element operably linked to a stem-loop destabilizing element (SLDE). An exemplary AuSLDE sequence comprises ATTTATTTATTTATTTATTTAacatcggttccCTGTTTAATATTTAAACAG (SEQ ID NO: 212). In some embodiments, the mRNA-destabilizing element comprises a 2X AuSLDE. An exemplary AuSLDE sequence is provided as ATTTATTTATTTATTTATTTAacatcggttccCTGTTTAATATTTAAACAGtgcggtaagcATTTATTTATTTATTTATTTAacatcggttccCTGTTTAATATTTAAACAG (SEQ ID NO: 213).

In certain embodiments, an engineered nucleic acid described herein comprises an insulator sequence. Such insulator sequences function to prevent inappropriate interactions between adjacent regions of a construct. In certain embodiments, an insulator sequence comprises the nucleic acid sequence of ACAATGGCTGGCCCATAGTAAATGCCGTGTTAGTGTGTTAGTTGCTGTTCTTCCACGTCAGAAGAGGCACAGACAAATTACCACCAGGTGGCGCTCAGAGTCTGCGGAGGCATCACAACAGCCCTGAATTTGAATCCTGCTCTGCCACTGCCTAGTTGAGACCTTTTACTACCTGACTAGCTGAGACATTTACGACATTTACTGGCTCTAGGACTCATTTTATTCATTTCATTACTTTTTTTTCTTTGAGACGGAATCTCGCTCT (SEQ ID NO: 300). In certain embodiments, an insulator sequence comprises a sequence that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 300.

Engineered Cells

Provided herein are engineered immunoresponsive cells, and methods of producing the engineered immunoresponsive cells, that produce a protein described herein (*e.g.*, a cytokine, CAR, ACP, and/or membrane-cleavable chimeric protein described herein). In general, engineered immunoresponsive cells of the present disclosure may be engineered to express the proteins provided for herein, such as a cytokine, CAR, ACP, and/or the membrane-cleavable

chimeric proteins having the formula **S – C – MT** or **MT – C – S** described herein. These cells are referred to herein as "engineered cells." These cells, which typically contain engineered nucleic acid, do not occur in nature. In some embodiments, the cells are engineered to include a nucleic acid comprising a promoter operably linked to a nucleotide sequence encoding a protein, for example, a cytokine, CAR, ACP, and/or a membrane-cleavable chimeric protein. An engineered cell can comprise an engineered nucleic acid integrated into the cell's genome. An engineered cell can comprise an engineered nucleic acid capable of expression without integrating into the cell's genome, for example, engineered with a transient expression system such as a plasmid or mRNA.

The present disclosure also encompasses additivity and synergy between a protein(s) and the engineered cell from which they are produced. In some embodiments, cells are engineered to produce at least two (*e.g.*, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) proteins, for example at least each of a cytokine, CAR, ACP, and membrane-cleavable chimeric protein. In general, immunoresponsive cells provide herein are engineered to produce at least one membrane-cleavable chimeric protein having a cytokine effector molecule that is not natively produced by the cells, a CAR, and an ACP. In general, immunoresponsive cells provide herein are engineered to produce at least two cytokines, at least one of which is a membrane-cleavable chimeric protein having a cytokine effector molecule, a CAR, and an ACP. Such an effector molecule may, for example, complement the function of effector molecules natively produced by the cells.

In some embodiments, a cell (e.g., an immune cell) is engineered to produce multiple proteins. For example, cells may be engineered to produce 2-20 different proteins, such as 2-20 different membrane-cleavable proteins. In some embodiments, a cell (e.g., an immunoresponsive cell) is engineered to produce at least 4 distinct proteins exogenous to the cell. In some embodiments, a cell (e.g., an immunoresponsive cell) is engineered to produce 4 distinct proteins exogenous to the cell. In some embodiments, cells engineered to produce 2-20, 2-19, 2-18, 2-17, 2-16, 2-15, 2-14, 2-13, 2-12, 2-11, 2-10, 2-9, 2-8, 2-7, 2-6, 2-5, 2-4, 2-3, 3-20, 3-19, 3-18, 3-17, 3-16, 3-15, 3-14, 3-13, 3-12, 3-11, 3-10, 3-9, 3-8, 3-7, 3-6, 3-5, 3-4, 4-20, 4-19, 4-18, 4-17, 4-16, 4-15, 4-14, 4-13, 4-12, 4-11, 4-10, 4-9, 4-8, 4-7, 4-6, 4-5, 5-20, 5-19, 5-18, 5-17, 5-16, 5-15, 5-14, 5-13, 5-12, 5-11, 5-10, 5-9, 5-8, 5-7, 5-6, 6-20, 6-19, 6-18, 6-17, 6-16, 6-15, 6-14, 6-13, 6-12, 6-11, 6-10, 6-9, 6-8, 6-7, 7-20, 7-19, 7-18, 7-17, 7-16, 7-15, 7-14, 7-13, 7-12, 7-11, 7-10, 7-9, 7-8, 8-20, 8-19, 8-18, 8-17, 8-16, 8-15, 8-14, 8-13, 8-12, 8-11, 8-10, 8-9, 9-20, 9-19, 9-18, 9-17, 9-16, 9-15, 9-14, 9-13, 9-12, 9-11, 9-10, 10-20, 10-19, 10-18, 10-17, 10-16, 10-15, 10-14, 10-13, 10-12, 10-11, 11-20, 11-19, 11-18, 11-17, 11-16, 11-15, 11-14, 11-13, 11-12, 12-20, 12-19, 12-18, 12-17, 12-16, 12-15, 12-14, 12-13, 13-20, 13-19, 13-18, 13-17, 13-16, 13-15, 13-14, 14-20, 14-19, 14-18, 14-17, 14-16, 14-15, 15-20, 15-19, 15-18, 15-17, 15-16, 16-20,

16-19, 16-18, 16-17, 17-20, 17-19, 17-18, 18-20, 18-19, or 19-20 proteins. In some embodiments, cells are engineered to produce 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, or 20 proteins.

In some embodiments, engineered cells comprise one or more engineered nucleic acids encoding a promoter operably linked to a nucleotide sequence encoding a protein (*e.g.*, an expression cassette). In some embodiments, cells are engineered to include a plurality of engineered nucleic acids, *e.g.*, at least two engineered nucleic acids, each encoding a promoter operably linked to a nucleotide sequence encoding at least one (*e.g.*, 1, 2 or 3) protein. For example, cells may be engineered to comprise at least 2, at least 3, at least 4, at least 5, at least 6, at least 7, at least 8, at least 8, at least 9, or at least 10, engineered nucleic acids, each encoding a promoter operably linked to a nucleotide sequence encoding at least one (*e.g.*, 1, 2 or 3) protein. In some embodiments, the cells are engineered to comprise 2, 3, 4, 5, 6, 7, 8, 9, 10, or more engineered nucleic acids, each encoding a promoter operably linked to a nucleotide sequence encoding at least one (*e.g.*, 1, 2 or 3) protein. Engineered cells can comprise an engineered nucleic acid encoding at least one of the linkers described above, such as polypeptides that link a first polypeptide sequence and a second polypeptide sequence, one or more multicistronic linker described above, one or more additional promoters operably linked to additional ORFs, or a combination thereof.

In some embodiments, a cell (e.g., an immune cell) is engineered to express a protease. In some embodiments, a cell is engineered to express a protease heterologous to a cell. In some embodiments, a cell is engineered to express a protease heterologous to a cell expressing a protein, such as a heterologous protease that cleaves the protease cleavage site of a membrane-cleavable chimeric protein. In some embodiments, engineered cells comprise one or more engineered nucleic acids encoding a promoter operably linked to a nucleotide sequence encoding a protease, such as a heterologous protease. Protease and protease cleavage sites are described in greater detail in the Section herein titled "Protease Cleavage site."

Also provided herein are engineered cells that are engineered to produce multiple proteins, at least two of which include effector molecules that modulate different tumor-mediated immunosuppressive mechanisms. In some embodiments, at least one (*e.g.*, 1, 2, 3, 4, 5, or more) protein includes an effector molecule that stimulates at least one immunostimulatory mechanism in the tumor microenvironment, or inhibits at least one immunosuppressive mechanism in the tumor microenvironment. In some embodiments, at least one (*e.g.*, 1, 2, 3, 4, 5, or more) protein includes an effector molecule that inhibits at least one immunosuppressive mechanism in the tumor microenvironment, and at least one protein (*e.g.*, 1, 2, 3, 4, 5, or more) inhibits at least one immunosuppressive mechanism in the tumor microenvironment. In yet other

embodiments, at least two (*e.g.*, 2, 3, 4, 5, or more) of the proteins are effector molecules that each stimulate at least one immunostimulatory mechanism in the tumor microenvironment. In still other embodiments, at least two (*e.g.*, 1, 2, 3, 4, 5, or more) of the proteins are effector molecules that each inhibit at least one immunosuppressive mechanism in the tumor microenvironment.

In some embodiments, a cell (e.g., an immune cell) is engineered to produce at least one protein including an effector molecule that stimulates T cell or NK cell signaling, activity and/or recruitment. In some embodiments, a cell is engineered to produce at least one protein that includes an effector molecule that stimulates antigen presentation and/or processing. In some embodiments, a cell is engineered to produce at least one protein that includes an effector molecule that stimulates natural killer cell-mediated cytotoxic signaling, activity and/or recruitment. In some embodiments, a cell is engineered to produce at least one protein that includes an effector molecule that stimulates dendritic cell differentiation and/or maturation. In some embodiments, a cell is engineered to produce at least one protein that includes an effector molecule that stimulates immune cell recruitment. In some embodiments, a cell is engineered to produce at least one protein includes an effector molecule that that stimulates M1 macrophage signaling, activity and/or recruitment. In some embodiments, a cell is engineered to produce at least one protein that includes an effector molecule that stimulates Th1 polarization. In some embodiments, a cell is engineered to produce at least one protein that includes an effector molecule that stimulates stroma degradation. In some embodiments, a cell is engineered to produce at least one protein that includes an effector molecule that stimulates immunostimulatory metabolite production. In some embodiments, a cell is engineered to produce at least one protein that includes an effector molecule that stimulates Type I interferon signaling. In some embodiments, a cell is engineered to produce at least one protein that includes an effector molecule that inhibits negative costimulatory signaling. In some embodiments, a cell is engineered to produce at least one protein that includes an effector molecule that inhibits pro-apoptotic signaling (*e.g.*, via TRAIL) of anti-tumor immune cells. In some embodiments, a cell is engineered to produce at least one protein that includes an effector molecule that inhibits T regulatory (T_{reg}) cell signaling, activity and/or recruitment. In some embodiments, a cell is engineered to produce at least one protein that includes an effector molecule that inhibits tumor checkpoint molecules. In some embodiments, a cell is engineered to produce at least one protein that includes an effector molecule that activates stimulator of interferon genes (STING) signaling. In some embodiments, a cell is engineered to produce at least one protein that includes an effector molecule that inhibits myeloid-derived suppressor cell signaling, activity and/or recruitment. In some embodiments, a cell is engineered to produce at

least one protein that includes an effector molecule that degrades immunosuppressive factors/metabolites. In some embodiments, a cell is engineered to produce at least one protein that includes an effector molecule that inhibits vascular endothelial growth factor signaling. In some embodiments, a cell is engineered to produce at least one protein that includes an effector molecule that directly kills tumor cells (*e.g.*, granzyme, perforin, oncolytic viruses, cytolytic peptides and enzymes, anti-tumor antibodies, *e.g.*, that trigger ADCC).

In some embodiments, at least one protein including an effector molecule that: stimulates T cell signaling, activity and/or recruitment, stimulates antigen presentation and/or processing, stimulates natural killer cell-mediated cytotoxic signaling , activity and/or recruitment, stimulates dendritic cell differentiation and/or maturation, stimulates immune cell recruitment, stimulates macrophage signaling, stimulates stroma degradation, stimulates immunostimulatory metabolite production, or stimulates Type I interferon signaling; and at least one protein including an effector molecule that inhibits negative costimulatory signaling, inhibits pro-apoptotic signaling of anti-tumor immune cells, inhibits T regulatory (Treg) cell signaling, activity and/or recruitment, inhibits tumor checkpoint molecules, activates stimulator of interferon genes (STING) signaling, inhibits myeloid-derived suppressor cell signaling, activity and/or recruitment, degrades immunosuppressive factors/metabolites, inhibits vascular endothelial growth factor signaling, or directly kills tumor cells.

In some embodiments, an immunoresponsive cell is engineered to produce at least one effector molecule cytokine selected from IL15, IL12, an IL12p70 fusion protein, IL18, and IL21. In some embodiments, an immunoresponsive cell is engineered to produce at least two effector molecule cytokines selected from IL15, IL12, an IL12p70 fusion protein, IL18, and IL21. In some embodiments, an immunoresponsive cell is engineered to produce at least two effector molecule cytokines selected from IL15, IL12, an IL12p70 fusion protein, IL18, and IL21. In some embodiments, an immunoresponsive cell is engineered to produce at least the effector molecule cytokines IL15 and IL12p70 fusion protein. In some embodiments, an immunoresponsive cell is engineered to produce at least one membrane-cleavable chimeric protein including an effector molecule cytokine selected from IL15, IL12, an IL12p70 fusion protein, IL18, and IL21. In some embodiments, an immunoresponsive cell is engineered to produce at least two membrane-cleavable chimeric protein including effector molecule cytokines selected from IL15, IL12, an IL12p70 fusion protein, IL18, and IL21.

In certain embodiments, the IL15 comprises the amino acid sequence of NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLLELQVISLESGDASIH DTVENLIILANNSLSSNGNVTESGCKECEELEEKNIKEFLQSFVHIVQMFINTS (SEQ ID NO: 285). An exemplary nucleic acid sequence encoding SEQ ID NO: 285 is

AATTGGGTCAACGTGATCAGCGACCTGAAGAAGATCGAGGACCTGATCCAGAGCAT
GCACATCGACGCCACACTGTACACCGAGAGCGACGTGCACCCTAGCTGTAAAGTGA
CCGCCATGAAGTGCTTTCTGCTGGAACTGCAAGTGATCAGCCTGGAAAGCGGCGAC
GCCAGCATCCACGACACCGTGGAAAACCTGATCATCCTGGCCAACAACAGCCTGAG
CAGCAACGGCAATGTGACCGAGTCCGGCTGCAAAGAGTGCGAGGAACTGGAAGAG
AAGAATATCAAAGAGTTCCTGCAGAGCTTCGTGCACATCGTGCAGATGTTCATCAA
CACAAGC (SEQ ID NO: 286). In certain embodiments, a nucleic acid encoding SEQ ID NO: 285 comprises a sequence that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 286.

In certain embodiments, the IL12p70 comprises the amino acid sequence of
MCHQQLVISWFSLVFLASPLVAIWELKKDVYVVELDWYPDAPGEMVVLTCDTPEEDGI
TWTLDQSSEVLGSGKTLTIQVKEFGDAGQYTCHKGGEVLSHSLLLLHKKEDGIWSTDIL
KDQKEPKNKTFLRCEAKNYSGRFTCWWLTTISTDLTFSVKSSRGSSDPQGVTCGAATLS
AERVRGDNKEYEYSVECQEDSACPAAEESLPIEVMVDAVHKLKYENYTSSFFIRDIIKPD
PPKNLQLKPLKNSRQVEVSWEYPDTWSTPHSYFSLTFCVQVQGKSKREKKDRVFTDKT
SATVICRKNASISVRAQDRYYSSSWSEWASVPCSGGGSGGGSGGGSGGGSRNLPVATP
DPGMFPCLHHSQNLLRAVSNMLQKARQTLEFYPCTSEEIDHEDITKDKTSTVEACLPLE
LTKNESCLNSRETSFITNGSCLASRKTSFMMALCLSSIYEDLKMYQVEFKTMNAKLLMD
PKRQIFLDQNMLAVIDELMQALNFNSETVPQKSSLEEPDFYKTKIKLCILLHAFRIRAVTI
DRVMSYLNAS (SEQ ID NO: 293). An exemplary nucleic acid sequence encoding SEQ ID NO: 293 is
ATGTGTCACCAGCAGCTGGTCATCAGCTGGTTCAGCCTGGTGTTCCTGGCCTCTCCT
CTGGTGGCCATCTGGGAGCTGAAGAAAGACGTGTACGTGGTGGAACTGGACTGGTA
TCCCGATGCTCCTGGCGAGATGGTGGTGCTGACCTGCGATACCCCTGAAGAGGACG
GCATCACCTGGACACTGGATCAGTCTAGCGAGGTGCTCGGCAGCGGCAAGACCCTG
ACCATCCAAGTGAAAGAGTTTGGCGACGCCGGCCAGTACACCTGTCACAAAGGCGG
AGAAGTGCTGAGCCACAGCCTGCTGCTGCTCCACAAGAAAGAGGATGGCATTTGGA
GCACCGACATCCTGAAGGACCAGAAAGAGCCCAAGAACAAGACCTTCCTGAGATG
CGAGGCCAAGAACTACAGCGGCCGGTTCACATGTTGGTGGCTGACCACCATCAGCA
CCGACCTGACCTTCAGCGTGAAGTCCAGCAGAGGCAGCAGTGATCCTCAGGGCGTT
ACATGTGGCGCCGCTACACTGTCTGCCGAAAGAGTGCGGGGCGACAACAAAGAATA
CGAGTACAGCGTGGAATGCCAAGAGGACAGCGCCTGTCCAGCCGCCGAAGAGTCTC
TGCCTATCGAAGTGATGGTGGACGCCGTGCACAAGCTGAAGTACGAGAACTACACC
TCCAGCTTTTTCATCCGGGACATCATCAAGCCCGATCCTCCAAAGAACCTGCAGCTG

AAGCCTCTGAAGAACAGCAGACAGGTGGAAGTGTCCTGGGAGTACCCCGACACCTG
GTCTACACCCCACAGCTACTTCAGCCTGACCTTTTGCGTGCAAGTGCAGGGCAAGTC
CAAGCGCGAGAAAAAGGACCGGGTGTTCACCGACAAGACCAGCGCCACCGTGATC
TGCAGAAAGAACGCCAGCATCAGCGTCAGAGCCCAGGACCGGTACTACAGCAGCTC
TTGGAGCGAATGGGCCAGCGTGCCATGTTCTGGCGGAGGAAGCGGTGGCGGATCAG
GTGGTGGATCTGGCGGCGGATCTAGAAACCTGCCTGTGGCCACTCCTGATCCTGGC
ATGTTCCCTTGTCTGCACCACAGCCAGAACCTGCTGAGAGCCGTGTCCAACATGCTG
CAGAAGGCCAGACAGACCCTGGAATTCTACCCCTGCACCAGCGAGGAAATCGACCA
CGAGGACATCACCAAGGATAAGACCAGCACCGTGGAAGCCTGCCTGCCTCTGGAAC
TGACCAAGAACGAGAGCTGCCTGAACAGCCGGGAAACCAGCTTCATCACCAACGGC
TCTTGCCTGGCCAGCAGAAAGACCTCCTTCATGATGGCCCTGTGCCTGAGCAGCATC
TACGAGGACCTGAAGATGTACCAGGTGGAATTCAAGACCATGAACGCCAAGCTGCT
GATGGACCCCAAGCGGCAGATCTTCCTGGACCAGAATATGCTGGCCGTGATCGACG
AGCTGATGCAGGCCCTGAACTTCAACAGCGAGACAGTGCCCCAGAAGTCTAGCCTG
GAAGAACCCGACTTCTACAAGACCAAGATCAAGCTGTGCATCCTGCTGCACGCCTT
CCGGATCAGAGCCGTGACCATCGACAGAGTGATGAGCTACCTGAACGCCTCT (SEQ ID NO: 294). In certain embodiments, a nucleic acid encoding SEQ ID NO: 293 comprises a sequence that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 294.

In general, a cell (e.g., an immune cell or a stem cell) is engineered to produce two or more cytokines, including at least one of the cytokines being in a membrane-cleavable chimeric protein format (*e.g.*, "S" in the formula **S – C – MT** or **MT – C – S**).

In some embodiments, a cell is engineered to produce at least one membrane-cleavable chimeric protein where the secretable effector molecule (*e.g.*, "S" in the formula **S – C – MT** or **MT – C – S**) is IL15, IL12, an IL12p70 fusion protein, IL18, or IL21.

In some embodiments, a cell is engineered to produce at least one membrane-cleavable chimeric protein where the secretable effector molecule (*e.g.*, "S" in the formula **S – C – MT** or **MT – C – S**) is IL-15. In some embodiments, a cell is engineered to produce at least one membrane-cleavable chimeric protein where the secretable effector molecule is IL-15 and the cell is further engineered to produce one or more additional cytokines. In some embodiments, a cell is engineered to produce at least one membrane-cleavable chimeric protein where the secretable effector molecule is IL-15 and the cell is further engineered to produce IL12, an IL12p70 fusion protein, IL18, or IL21. In some embodiments, a cell is engineered to produce at least one membrane-cleavable chimeric protein where the secretable effector molecule is IL-15 and the cell is further engineered to produce IL-12. In some embodiments, a cell is engineered to

produce at least one membrane-cleavable chimeric protein where the secretable effector molecule is IL-15 and the cell is further engineered to produce an IL12p70 fusion protein.

In some embodiments, a cell is engineered to produce at least one membrane-cleavable chimeric protein where the secretable effector molecule (*e.g.*, "S" in the formula **S – C – MT** or **MT – C – S**) is IL-15 and the cell is further engineered to produce one or more additional membrane-cleavable chimeric proteins. In some embodiments, a cell is engineered to produce at least one membrane-cleavable chimeric protein where the secretable effector molecule (*e.g.*, "S" in the formula **S – C – MT** or **MT – C – S**) is IL-15 and the cell is further engineered to produce one or more additional membrane-cleavable chimeric proteins including IL12, an IL12p70 fusion protein, IL18, and IL21. In some embodiments, a cell is engineered to produce at least one membrane-cleavable chimeric protein where the secretable effector molecule (*e.g.*, "S" in the formula **S – C – MT** or **MT – C – S**) is IL15 and the cell is further engineered to produce an additional membrane-cleavable chimeric proteins including IL12p70.

In some embodiments, a cell is engineered to produce at least one membrane-cleavable chimeric protein where the secretable effector molecule (*e.g.*, "S" in the formula **S – C – MT** or **MT – C – S**) is an IL12p70. In some embodiments, a cell is engineered to produce at least one membrane-cleavable chimeric protein where the secretable effector molecule is IL12p70 and the cell is further engineered to produce one or more additional cytokines. In some embodiments, a cell is engineered to produce at least one membrane-cleavable chimeric protein where the secretable effector molecule is IL12p70 and the cell is further engineered to produce IL15, IL18, or IL21. In some embodiments, a cell is engineered to produce at least one membrane-cleavable chimeric protein where the secretable effector molecule is IL12p70 and the cell is further engineered to produce IL15.

In some embodiments, a cell is engineered to produce at least one membrane-cleavable chimeric protein where the secretable effector molecule (*e.g.*, "S" in the formula **S – C – MT** or **MT – C – S**) is IL12p70 and the cell is further engineered to produce one or more additional membrane-cleavable chimeric proteins. In some embodiments, a cell is engineered to produce at least one membrane-cleavable chimeric protein where the secretable effector molecule (*e.g.*, "S" in the formula **S – C – MT** or **MT – C – S**) is IL12p70 and the cell is further engineered to produce one or more additional membrane-cleavable chimeric proteins including IL15, IL18, and IL21. In some embodiments, a cell is engineered to produce at least one membrane-cleavable chimeric protein where the secretable effector molecule (*e.g.*, "S" in the formula **S – C – MT** or **MT – C – S**) is IL12p70 and the cell is further engineered to produce an additional membrane-cleavable chimeric proteins including IL15.

A cell can also be further engineered to express additional proteins in addition to the cytokines and/or the membrane-cleavable chimeric proteins having the formula **S – C – MT** or **MT – C – S** described herein. As provided herein, an immunoresponsive cell is engineered to express a chimeric antigen receptor (CAR) that binds to GPC3. Also as provided herein, an immunoresponsive cell is engineered to express an ACP that includes a synthetic transcription factor.

A CAR can include an antigen-binding domain, such as an antibody, an antigen-binding fragment of an antibody, a F(ab) fragment, a F(ab') fragment, a single chain variable fragment (scFv), or a single-domain antibody (sdAb). An antigen recognizing receptors can include an scFv. An scFv can include a heavy chain variable domain (VH) and a light chain variable domain (VL), which can be separated by a peptide linker. For example, an scFv can include the structure VH-L-VL or VL-L-VH, wherein VH is the heavy chain variable domain, L is the peptide linker, and VL is the light chain variable domain. In certain embodiments, the peptide linker is a gly-ser linker. In certain embodiments, the peptide linker is a (GGGGS)3 linker comprising the sequence of GGGGSGGGGSGGGGS (SEQ ID NO: 223). An exemplary nucleic acid sequence encoding SEQ ID NO: 223 is GGCGGCGGAGGATCTGGCGGAGGTGGAAGTGGCGGAGGCGGATCT (SEQ ID NO: 224) or GGCGGCGGAGGAAGCGGAGGCGGAGGATCCGGTGGTGGTGGATCT (SEQ ID NO: 332). In certain embodiments, a nucleic acid encoding SEQ ID NO: 223 comprises a sequence that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 224 or SEQ ID NO: 332.

A CAR can have one or more intracellular signaling domains, such as a CD3zeta-chain intracellular signaling domain, a CD97 intracellular signaling domain, a CD11a-CD18 intracellular signaling domain, a CD2 intracellular signaling domain, an ICOS intracellular signaling domain, a CD27 intracellular signaling domain, a CD154 intracellular signaling domain, a CD8 intracellular signaling domain, an OX40 intracellular signaling domain, a 4-1BB intracellular signaling domain, a CD28 intracellular signaling domain, a ZAP40 intracellular signaling domain, a CD30 intracellular signaling domain, a GITR intracellular signaling domain, an HVEM intracellular signaling domain, a DAP10 intracellular signaling domain, a DAP12 intracellular signaling domain, a MyD88 intracellular signaling domain, a 2B4 intracellular signaling domain, a CD16a intracellular signaling domain, a DNAM-1 intracellular signaling domain, a KIR2DS1 intracellular signaling domain, a KIR3DS1 intracellular signaling domain, a NKp44 intracellular signaling domain, a NKp46 intracellular signaling domain, a FceRIg intracellular signaling domain, a NKG2D intracellular signaling domain, an EAT-2 intracellular

signaling domain, fragments thereof, combinations thereof, or combinations of fragments thereof. In some embodiments, the intracellular signaling domain comprises a sequence from **Table 6A**.

Table 6A.

Amino Acid Sequence	Nucleotide Sequence	Description
KSRQTPPLASVEMEAMEALP VTWGTSSRDEDLENCSHHL (SEQ ID NO: 265)	AAGTCCAGACAGACACCTCCTCTGGCCAGCGTGGA AATGGAAGCCATGGAAGCTCTGCCTGTGACCTGGG GCACCAGCTCCAGAGATGAGGACCTGGAAAACTG CTCCCACCACCTG (SEQ ID NO: 266)	IL-15Rα ICD
RSKRSRLLHSDYMNMTPRR PGPTRKHYQPYAPPRDFAAY RS (SEQ ID NO: 267)	CGGAGCAAGAGAAGCAGACTGCTGCACAGCGACT ACATGAACATGACCCCTAGACGGCCCGGACCTACC AGAAAGCACTACCAGCCTTACGCTCCTCCTAGAGA CTTCGCCGCCTACCGGTCC (SEQ ID NO: 268)	CD28 ICD
ALYLLRRDQRLPPDAHKPPG GGSFRTPIQEEQADAHSTLA KI (SEQ ID NO: 269)	GCTCTGTATCTGCTGCGGAGGGACCAAAGACTGCC TCCTGATGCTCACAAGCCTCCAGGCGGAGGCAGCT TCAGAACCCCTATCCAAGAGGAACAGGCCGACGC TCACAGCACCCTGGCCAAGATT (SEQ ID NO: 270)	OX40 ICD
KRGRKKLLYIFKQPFMRPVQ TTQEEDGCSCRFPEEEEGGC EL (SEQ ID NO: 271)	AAGCGGGGCAGAAAGAAGCTGCTGTACATCTTCA AGCAGCCCTTCATGCGGCCCGTGCAGACCACACAA GAGGAAGATGGCTGCTCCTGCAGATTCCCCGAGGA AGAAGAAGGCGGCTGCGAGCTG (SEQ ID NO: 272) OR AAGCGGGGCAGAAAGAAGCTGCTGTACATCTTCA AGCAGCCCTTCATGCGGCCCGTGCAGACCACACAA GAGGAAGATGGCTGCAGCTGTCGGTTCCCCGAGG AAGAAGAAGGCGGCTGCGAGCTG (SEQ ID NO: 339)	4-1BB ICD
RKRTRERASRASTWEGRRR LNTQTL (SEQ ID NO: 273)	CGGAAGCGGACAAGAGAGAGAGCCAGCAGAGCCT CTACCTGGGAGGGAAGAAGAAGGCTGAACACCCA GACACTC (SEQ ID NO: 274)	Nkp46 ICD
WRRKRKEKQSETSPKEFLTI YEDVKDLKTRRNHEQEQTF PGGGSTIYSMIQSQSSAPTSQ EPAYTLYSLIQPSRKSGSRKR NHSPSFNSTIYEVIGKSQPKA QNPARLSRKELENFDVYS (SEQ ID NO: 275)	TGGCGCCGCAAGCGGAAAGAGAAGCAGTCTGAGA CAAGCCCCAAAGAGTTCCTGACCATCTACGAGGAC GTGAAGGACCTGAAAACCCGGCGGAACCACGAGC AAGAGCAGACCTTTCCTGGCGGCGGAAGCACCATC TACAGCATGATCCAGAGCCAGTCTAGCGCCCCTAC CAGCCAAGAGCCTGCCTACACACTGTACTCCCTGA TCCAGCCTAGCAGAAAGAGCGGCAGCCGGAAGAG AAATCACAGCCCCAGCTTCAACAGCACGATCTACG AAGTGATCGGCAAGAGCCAGCCAAAGGCTCAGAA CCCTGCCAGGCTGAGCCGGAAAGAGCTGGAAAAC TTCGACGTGTACAGC (SEQ ID NO: 276)	2B4 ICD
RVKFSRSADAPAYKQGQNQ LYNELNLGRREEYDVLDKR RGRDPEMGGKPRRKNPQEG LYNELQKDKMAEAYSEIGM KGERRRGKGHDGLYQGLST ATKDTYDALHMQALPPR (SEQ ID NO: 277)	AGAGTGAAGTTCAGCAGAAGCGCCGACGCACCCG CCTATAAGCAGGGACAGAACCAGCTGTACAACGA GCTGAACCTGGGGAGAAGAGAAGAGTACGACGTG CTGGACAAGCGGAGAGGCAGAGATCCTGAGATGG GCGGCAAGCCCAGACGGAAGAATCCTCAAGAGGG CCTGTATAATGAGCTGCAGAAAGACAAGATGGCC GAGGCCTACAGCGAGATCGGAATGAAGGGCGAGC GCAGAAGAGGCAAGGGACACGATGGACTGTACCA GGGCCTGAGCACCGCCACCAAGGATACCTATGATG CCCTGCACATGCAGGCCCTGCCTCCAAGA (SEQ ID NO: 278)	CD3z mut

	OR AGAGTGAAGTTCAGCAGGAGCGCAGACGCCCCCG CGTACAAGCAGGGCCAGAACCAGCTCTATAACGA GCTCAATCTAGGACGAAGAGAGGAGTACGATGTTT TGGACAAGAGACGTGGCCGGGACCCTGAGATGGG GGGAAAGCCGAGAAGGAAGAACCCTCAGGAAGGC CTGTACAATGAACTGCAGAAAGATAAGATGGCGG AGGCCTACAGTGAGATTGGGATGAAAGGCGAGCG CCGGAGGGGCAAGGGGCACGATGGCCTTTACCAG GGTCTCAGTACAGCCACCAAGGACACCTACGACGC CCTTCACATGCAGGCCCTGCCCCCTCGC (SEQ ID NO: 334)	
RVKFSRSADAPAYQQGQNQ LYNELNLGRREEYDVLDKR RGRDPEMGGKPRRKNPQEG LYNELQKDKMAEAYSEIGM KGERRRGKGHDGLYQGLST ATKDTYDALHMQALPPR (SEQ ID NO: 279)	AGAGTGAAGTTCAGCAGATCCGCCGATGCTCCCGC CTATCAGCAGGGACAGAACCAGCTGTACAACGAG CTGAACCTGGGGAGAAGAGAAGAGTACGACGTGC TGGACAAGCGGAGAGGCAGAGATCCTGAGATGGG CGGCAAGCCCAGACGGAAGAATCCTCAAGAGGGC CTGTATAATGAGCTGCAGAAAGACAAGATGGCCG AGGCCTACAGCGAGATCGGAATGAAGGGCGAGCG CAGAAGAGGCAAGGGACACGATGGACTGTACCAG GGCCTGAGCACCGCCACCAAGGATACCTATGATGC CCTGCACATGCAGGCCCTGCCTCCAAGA (SEQ ID NO: 280)	CD3z

In some embodiments, a CAR can also comprise a spacer region that links the extracellular antigen-binding domain to the transmembrane domain. The spacer region may be flexible enough to allow the antigen-binding domain to orient in different directions to facilitate antigen recognition. In some embodiments, the spacer region may be a hinge from a human protein. For example, the hinge may be a human Ig (immunoglobulin) hinge, including without limitation an IgG4 hinge, an IgG2 hinge, a CD8a hinge, or an IgD hinge. In some embodiments, the spacer region may comprise an IgG4 hinge, an IgG2 hinge, an IgD hinge, a CD28 hinge, a KIR2DS2 hinge, an LNGFR hinge, or a PDGFR-beta extracellular linker. In some embodiments, the spacer region comprises a sequence from **Table 6B**.

Table 6B.

Amino Acid Sequence	Nucleic Acid Sequence	Description
TTTPAPRPPTPAPTIALQPLSLRPE ACRPAAGGAVHTRGLDFACD (SEQ ID NO: 226)	ACAACAACCCCTGCTCCTAGACCT CCTACACCAGCTCCTACAATCGCC CTGCAGCCTCTGTCTCTGAGGCCA GAAGCTTGTAGACCAGCTGCTGGC GGAGCCGTGCATACAAGAGGACT GGACTTCGCCTGTGAT (SEQ ID NO: 227)	CD8 hinge (S2L)
GALSNSIMYFSHFVPVFLPAKPTT TPAPRPPTPAPTIASQPLSLRPEAC RPAAGGAVHTRGLDFACD (SEQ ID NO: 228) OR	GGCGCCCTGAGCAACAGCATCAT GTACTTCAGCCACTTCGTGCCCGT GTTTCTGCCCGCCAAGCCTACAAC AACCCCTGCTCCTAGACCTCCTAC ACCAGCTCCTACAATCGCCAGCCA	CD8 hinge (FA)

Amino Acid Sequence	Nucleic Acid Sequence	Description
ALSNSIMYFSHFVPVFLPAKPTTT PAPRPPTPAPTIASQPLSLRPEACR PAAGGAVHTRGLDFACD (SEQ ID NO: 336)	GCCTCTGTCTCTGAGGCCAGAAGC TTGTAGACCTGCTGCAGGCGGAGC CGTGCATACAAGAGGACTGGATTT CGCCTGCGAC (SEQ ID NO: 229) OR GCCCTGAGCAACAGCATCATGTAC TTCAGCCACTTCGTGCCCGTGTTT CTGCCCGCCAAGCCTACAACAACC CCTGCTCCTAGACCTCCTACACCA GCTCCTACAATCGCCAGCCAGCCT CTGTCTCTGAGGCCAGAAGCTTGT AGACCTGCTGCAGGCGGAGCCGT GCATACAAGAGGACTGGATTTCG CCTGCGAC (SEQ ID NO: 335)	
AAAIEVMYPPPYLDNEKSNGTIIH VKGKHLCPSPLFPGPSKP (SEQ ID NO: 246)	GCAGCAGCTATCGAGGTGATGTAT CCTCCGCCCTACCTGGATAATGAA AAGAGTAATGGGACTATCATTCAT GTAAAAGGGAAGCATCTTTGTCCT TCTCCCCTTTTCCCCGGTCCGTCTA AACCT (SEQ ID NO: 247)	CD28 hinge
ESKYGPPCPSCP (SEQ ID NO: 248)	GAAAGCAAGTACGGTCCACCTTG CCCTAGCTGTCCG (SEQ ID NO: 249)	IgG4 minimal hinge
ESKYGPPAPSAP (SEQ ID NO: 250)	GAATCCAAGTACGGCCCCCCAGC GCCTAGTGCCCCA (SEQ ID NO: 251)	IgG4 minimal hinge, no disulfides
ESKYGPPCPPCP (SEQ ID NO: 252)	GAATCTAAATATGGCCCGCCATGC CCGCCTTGCCCA (SEQ ID NO: 253)	IgG4 S228P minimal hinge, enhanced disulfide formation
EPKSCDKTHTCP (SEQ ID NO: 254)	GAACCGAAGTCTTGTGATAAAACT CATACGTGCCCG (SEQ ID NO: 255)	IgG1 minimal hinge
AAAFVPVFLPAKPTTTPAPRPPTP APTIASQPLSLRPEACRPAAGGAV HTRGLDFACDIYIWAPLAGTCGV LLLSLVITLYCNHRN (SEQ ID NO: 256)	GCTGCTGCTTTCGTACCCGTGTTC CTCCCTGCTAAGCCTACGACTACC CCCGCACCGAGACCACCCACGCC AGCACCCACGATTGCTAGCCAGCC CCTTAGTTTGCGACCAGAAGCTTG TCGGCCTGCTGCTGGTGGCGCGGT ACATACCCGCGGCCTTGATTTTGC TTGCGATATATATATCTGGGCGCC TCTGGCCGGAACATGCGGGGTCCT	Extended CD8a hinge

Amino Acid Sequence	Nucleic Acid Sequence	Description
	CCTCCTTTCTCTGGTTATTACTCTC TACTGTAATCACAGGAAT (SEQ ID NO: 257)	
ACPTGLYTHSGECCKACNLGEGV AQPCGANQTVCEPCLDSVTFSDV VSATEPCKPCTECVGLQSMSAPC VEADDAVCRCAYGYYQDETTGR CEACRVCEAGSGLVFSCQDKQNT VCEECPDGTYSDEADAEC (SEQ ID NO: 258)	GCCTGCCCGACCGGGCTCTACACT CATAGCGGGGAATGTTGTAAGGC ATGTAACTTGGGTGAGGGCGTCGC ACAGCCCTGCGGAGCTAACCAAA CAGTGTGCGAACCCTGCCTCGATA GTGTGACGTTCTCTGATGTTGTAT CAGCTACAGAGCCTTGCAAACCAT GTACTGAGTGCGTTGGACTTCAGT CAATGAGCGCTCCATGTGTGGAG GCAGATGATGCGGTCTGTCGATGT GCTTACGGATACTACCAAGACGA GACAACAGGGCGGTGCGAGGCCT GTAGAGTTTGTGAGGCGGGCTCCG GGCTGGTGTTTTCATGTCAAGACA AGCAAAATACGGTCTGTGAAGAG TGCCCTGATGGCACCTACTCAGAC GAAGCAGATGCAGAATGC (SEQ ID NO: 259)	LNGFR hinge
ACPTGLYTHSGECCKACNLGEGV AQPCGANQTVC (SEQ ID NO: 260)	GCCTGCCCTACAGGACTCTACACG CATAGCGGTGAGTGTTGTAAAGC ATGCAACCTCGGGGAAGGTGTAG CCCAGCCATGCGGGGCTAACCAA ACCGTTTGC (SEQ ID NO: 261)	Truncated LNGFR hinge (TNFR-Cys1)
AVGQDTQEVIVVPHSLPFKV (SEQ ID NO: 262)	GCTGTGGGCCAGGACACGCAGGA GGTCATCGTGGTGCCACACTCCTT GCCCTTTAAGGTG (SEQ ID NO: 263)	PDGFR-beta extracellular linker
YPPVIVEMNSSVEAIEGSHVSLLC GADSNPPPLLTWMRDGTVLREA VAESLLLELEEVTPAEDGVYACL AENAYGQDNRTVGLSVMYAPW KPTVNGTMVAVEGETVSILCSTQ SNPDPILTIFKEKQILSTVIYESELQ LELPAVSPEDDGEYWCVAENQY GQRATAFNLSVEFAPVLLLESHC AAARDTVQCLCVVKSNPEPSVAF ELPSRNVTVNESEREFVYSERSGL	TACCCTCCAGTGATCGTGGAAATG AACAGCAGCGTGGAAGCCATCGA GGGCTCTCATGTGTCTCTGCTGTG TGGCGCCGACAGCAATCCTCCTCC TCTGCTGACCTGGATGAGAGATGG CACCGTGCTGAGAGAAGCCGTGG CCGAATCTCTGCTGCTGGAACTGG AAGAAGTGACCCCTGCCGAGGAT GGCGTGTACGCTTGTCTGGCCGAG AATGCCTACGGCCAGGACAATAG	MAG hinge

Amino Acid Sequence	Nucleic Acid Sequence	Description
VLTSILTLRGQAQAPPRVICTARN LYGAKSLELPFQGAHRLMWAKIG P (SEQ ID NO: 264)	AACCGTGGGCCTGTCCGTGATGTA CGCCCCTTGGAAGCCTACCGTGAA CGGCACAATGGTGGCCGTGGAAG GCGAGACAGTGTCCATCCTGTGTA GCACCCAGAGCAACCCCGATCCT ATCCTGACCATCTTCAAAGAGAAG CAGATCCTGAGCACCGTGATCTAC GAGAGCGAACTGCAGCTCGAACT GCCCGCTGTGTCCCCAGAGGATGA TGGCGAATATTGGTGCGTGGCAG AGAACCAGTACGGCCAGAGAGCC ACCGCCTTCAACCTGAGCGTGGAA TTTGCTCCCGTGCTGCTGCTCGAG AGCCATTGTGCTGCCGCCAGAGAT ACCGTGCAGTGCCTGTGTGTGGTC AAGTCTAACCCCGAGCCTAGCGTG GCCTTTGAGCTGCCCAGCAGAAAC GTGACCGTGAATGAGAGCGAGCG CGAGTTCGTGTACAGCGAGAGAT CTGGACTGGTGCTGACCAGCATCC TGACACTGAGAGGACAGGCTCAG GCCCCTCCTAGAGTGATCTGCACC GCCAGAAATCTGTACGGCGCCAA GAGCCTGGAACTGCCATTTCAGGG CGCCCACAGACTCATGTGGGCCA AGATTGGACCT (SEQ ID NO: 265)	

A CAR can have a transmembrane domain, such as a CD8 transmembrane domain, a CD28 transmembrane domain a CD3zeta-chain transmembrane domain, a CD4 transmembrane domain, a 4-1BB transmembrane domain, an OX40 transmembrane domain, an ICOS transmembrane domain, a CTLA-4 transmembrane domain, a PD-1 transmembrane domain, a LAG-3 transmembrane domain, a 2B4 transmembrane domain, a BTLA transmembrane domain, an OX40 transmembrane domain, a DAP10 transmembrane domain, a DAP12 transmembrane domain, a CD16a transmembrane domain, a DNAM-1 transmembrane domain, a KIR2DS1 transmembrane domain, a KIR3DS1 transmembrane domain, an NKp44 transmembrane domain, an NKp46 transmembrane domain, an FceRlg transmembrane domain, an NKG2D transmembrane domain, fragments thereof, combinations thereof, or combinations of fragments thereof. A CAR can have a spacer region between the antigen-binding domain and the

transmembrane domain. Exemplary transmembrane domain sequences are provided in **Table 6C**.

Table 6C.

Amino Acid Sequence	Nucleotide sequence	Description
VAISTSTVLLCGLSAVSLLACYL (SEQ ID NO: 230)	GTGGCCATCAGCACAAGCACCG TGCTGCTGTGTGGACTGTCTGCC GTTTCTCTGCTGGCCTGCTACCT G (SEQ ID NO: 231)	IL-15Rα transmembrane domain
FWVLVVVGGVLACYSLLVTVAFIIF WV (SEQ ID NO: 232)	TTCTGGGTGCTCGTGGTTGTTGG CGGAGTGCTGGCCTGTTACTCTC TGCTGGTCACCGTGGCCTTCATC ATCTTTTGGGTC (SEQ ID NO: 233)	CD28 transmembrane domain
VAAILGLGLVLGLLGPLAIL (SEQ ID NO: 234)	GTGGCCGCCATTCTCGGACTGG GACTTGTTCTGGGACTGCTGGG ACCTCTGGCCATTCTGCT (SEQ ID NO: 235)	OX40 transmembrane domain*
VAAILGLGLVLGLLGPLAILL (SEQ ID NO: 244)	GTGGCCGCCATTCTCGGACTGG GACTTGTTCTGGGACTGCTGGG ACCTCTGGCCATTCTGCTG (SEQ ID NO: 245)	OX40 transmembrane domain
IYIWAPLAGTCGVLLLSLVIT (SEQ ID NO: 236)	ATCTACATCTGGGCCCCTCTGGC TGGAACATGCGGAGTGTTGCTG CTGAGCCTGGTCATCACC (SEQ ID NO: 237) OR ATCTACATCTGGGCCCCTCTGGC TGGAACATGTGGTGTCTTGCTGC TGAGCCTGGTCATCACC (SEQ ID NO: 338)	CD8 transmembrane domain
IYIWAPLAGTCGVLLLSLVITLYCN HR (SEQ ID NO: 242)	ATCTACATCTGGGCCCCTCTGGC TGGAACATGTGGTGTCCTGCTGC TGAGCCTGGTCATCACCCTGTAC TGCAACCACCGG (SEQ ID NO: 243)	CD8 FA transmembrane domain
MGLAFLVLVALVWFLVEDWLS (SEQ ID NO: 238)	ATGGGCCTCGCCTTTCTGGTGCT GGTGGCCCTTGTGTGGTTCCTGG TGGAAGATTGGCTGAGC (SEQ ID NO: 239)	NKp46 transmembrane domain
FLVIIVILSALFLGTLACFCV (SEQ ID NO: 240)	TTCCTGGTCATCATCGTGATCCT GAGCGCCCTGTTCCTGGGCACC CTGGCCTGTTTTTGCGTG (SEQ ID NO: 241)	2B4 transmembrane domain

In some embodiments, the CAR antigen-binding domain that binds to GPC3 includes a heavy chain variable (VH) region and a light chain variable (VL) region, wherein the VH includes: a heavy chain complementarity determining region 1 (CDR-H1) having the amino acid sequence of KNAMN (**SEQ ID NO: 199**), a heavy chain complementarity determining region 2 (CDR-H2) having the amino acid sequence of RIRNKTNNYATYYADSVKA (**SEQ ID NO: 200**), and a heavy chain complementarity determining region 3 (CDR-H3) having the amino

acid sequence of GNSFAY (**SEQ ID NO: 201**), and wherein the VL includes: a light chain complementarity determining region 1 (CDR-L1) having the amino acid sequence of KSSQSLLYSSNQKNYLA (**SEQ ID NO: 202**), a light chain complementarity determining region 2 (CDR-L2) having the amino acid sequence of WASSRES (**SEQ ID NO: 203**), and a light chain complementarity determining region 3 (CDR-L3) having the amino acid sequence of QQYYNYPLT (**SEQ ID NO: 204**). In some embodiments, the antigen-binding domain that binds to GPC3 includes a heavy chain complementarity determining region 1 (CDR-H1) having the amino acid sequence of KNAMN (**SEQ ID NO: 199**). In some embodiments, the antigen-binding domain that binds to GPC3 includes a heavy chain complementarity determining region 2 (CDR-H2) having the amino acid sequence of RIRNKTNNYATYYADSVKA (**SEQ ID NO: 200**). In some embodiments, the antigen-binding domain that binds to GPC3 includes a heavy chain complementarity determining region 3 (CDR-H3) having the amino acid sequence of GNSFAY (**SEQ ID NO: 201**). In some embodiments, the antigen-binding domain that binds to GPC3 includes a light chain complementarity determining region 1 (CDR-L1) having the amino acid sequence of KSSQSLLYSSNQKNYLA (**SEQ ID NO: 202**). In some embodiments, the antigen-binding domain that binds to GPC3 includes a light chain complementarity determining region 2 (CDR-L2) having the amino acid sequence of WASSRES (**SEQ ID NO: 203**). In some embodiments, the antigen-binding domain that binds to GPC3 includes a light chain complementarity determining region 3 (CDR-L3) having the amino acid sequence of QQYYNYPLT (**SEQ ID NO: 204**).

In some embodiments, the antigen-binding domain that binds to GPC3 includes a VH region having an amino acid sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to the amino acid sequence of

EVQLVETGGGMVQPEGSLKLSCAASGFTFNKNAMNWVRQAPGKGLEWVARIRNKTN NYATYYADSVKARFTISRDDSQSMLYLQMNNLKIEDTAMYYCVAGNSFA YWGQGTLVTVSA (SEQ ID NO: 205) or

EVQLVESGGGLVQPGGSLRLSCAASGFTFNKNAMNWVRQAPGKGLEWVGRIRNKTNN YATYYADSVKARFTISRDDSKNSLYLQMNSLKTEDTAVYYCVAGNSFAYWGQGTLVT VSA (SEQ ID NO: 206). An exemplary nucleic acid sequence encoding SEQ ID NO: 206 is

GAAGTGCAGCTGGTGGAATCTGGCGGAGGACTGGTTCAACCTGGCGGCTCTCTGAG ACTGTCTTGTGCCGCCAGCGGCTTCACCTTCAACAAGAACGCCATGAACTGGGTCCG ACAGGCCCCTGGCAAAGGCCTTGAATGGGTCGGACGGATCCGGAACAAGACCAAC AACTACGCCACCTACTACGCCGACAGCGTGAAGGCCAGGTTCACCATCTCCAGAGA TGACAGCAAGAACAGCCTGTACCTGCAGATGAACTCCCTGAAAACCGAGGACACCG

CCGTGTACTATTGCGTGGCCGGCAATAGCTTTGCCTACTGGGGACAGGGCACCCTG GTTACAGTTTCTGCT (SEQ ID NO: 222) or GAAGTGCAGCTGGTTGAATCAGGTGGCGGCCTGGTTCAACCTGGCGGATCTCTGAG ACTGAGCTGTGCCGCCAGCGGCTTCACCTTCAACAAGAACGCCATGAACTGGGTCC GACAGGCCCCTGGCAAAGGCCTTGAATGGGTCGGACGGATCCGGAACAAGACCAA CAACTACGCCACCTACTACGCCGACAGCGTGAAGGCCAGATTCACCATCAGCCGGG ACGACAGCAAGAACAGCCTGTACCTGCAGATGAACTCCCTGAAAACCGAGGACACC GCCGTGTATTATTGCGTGGCCGGCAACAGCTTTGCCTACTGGGGACAGGGAACCCT GGTCACCGTGTCTGCC (SEQ ID NO: 330). In certain embodiments, a nucleic acid encoding SEQ ID NO: 206 comprises a sequence that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 222 or SEQ ID NO: 330.

In some embodiments, the antigen-binding domain that binds to GPC3 includes a VL region having an amino acid sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to the amino acid sequence of DIVMSQSPSSLVVSIGEKVTMTCKSSQSLLYSSNQKNYLAWYQQKPGQSPKLLIYWASS RESGVPDRFTGSGSGTDFTLTISSVKAEDLAVYYCQQYYNYPLTFGAGTKLELK (SEQ ID NO: 207), or DIVMTQSPDSLAVSLGERATINCKSSQSLLYSSNQKNYLAWYQQKPGQPPKLLIYWASS RESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQQYYNYPLTFGQGTKLEIK (SEQ ID NO: 208). An exemplary nucleic acid sequence encoding SEQ ID NO: 208 is GACATCGTGATGACACAGAGCCCCGATAGCCTGGCCGTGTCTCTGGGAGAAAGAGC CACCATCAACTGCAAGAGCAGCCAGAGCCTGCTGTACTCCAGCAACCAGAAGAACT ACCTGGCCTGGTATCAGCAAAAGCCCGGCCAGCCTCCTAAGCTGCTGATCTATTGG GCCAGCTCCAGAGAAAGCGGCGTGCCCGATAGATTTTCTGGCTCTGGCAGCGGCAC CGACTTCACCCTGACAATTTCTAGCCTGCAAGCCGAGGACGTGGCCGTGTACTACTG CCAGCAGTACTACAACTACCCTCTGACCTTCGGCCAGGGCACCAAGCTGGAAATCA AA (SEQ ID NO: 221) or GACATCGTGATGACACAGAGCCCCGATAGCCTGGCCGTGTCTCTGGGAGAAAGAGC CACCATCAACTGCAAGAGCAGCCAGAGCCTGCTGTACTCCAGCAACCAGAAGAACT ACCTGGCCTGGTATCAGCAAAAGCCCGGCCAGCCTCCTAAGCTGCTGATCTATTGG GCCAGCTCCAGAGAAAGCGGCGTGCCCGATAGATTTTCTGGCTCTGGCAGCGGCAC CGACTTCACCCTGACAATTTCTAGCCTGCAAGCCGAGGACGTGGCCGTGTATTACTG CCAGCAGTACTACAACTACCCTCTGACCTTCGGCCAGGGCACCAAGCTGGAAATCA

AA (SEQ ID NO: 333) or

GACATCGTGATGACACAGAGCCCCGATAGCCTGGCCGTGTCTCTGGGAGAAAGAGC
CACCATCAACTGCAAGAGCAGCCAGAGCCTGCTGTACTCCAGCAACCAGAAGAACT
ACCTGGCCTGGTATCAGCAAAAGCCCGGCCAGCCTCCTAAGCTGCTGATCTATTGG
GCCAGCTCCAGAGAAAGCGGCGTGCCCGATAGATTTTCTGGCTCTGGCAGCGGCAC
CGACTTCACCCTGACAATTTCTAGCCTGCAAGCCGAGGACGTGGCCGTGTATTACTG
CCAGCAGTACTACAACTACCCTCTGACCTTCGGCCAGGGCACCAAGCTGGAAATCA
AG (SEQ ID NO: 336). In certain embodiments, a nucleic acid encoding SEQ ID NO: 208 comprises a sequence that is at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 221 or SEQ ID NO: 336.

In general, the ACP of the immunoresponsive cells described herein includes a synthetic transcription factor. A synthetic transcription factor is a non-naturally occurring protein that includes a DNA-binding domain and a transcriptional effector domain and is capable of modulating (i.e., activating or repressing) transcription through binding to a cognate promoter recognized by the DNA-binding domain. In some embodiments, the ACP is a transcriptional repressor. In some embodiments, the ACP is a transcriptional activator.

Engineered Cell Types

Also provided herein are engineered immunoresponsive cells. Immunoresponsive cells can be engineered to comprise any of the engineered nucleic acids described herein (*e.g.*, any of the engineered nucleic acids encoding the cytokines, membrane-cleavable chimeric proteins, and/or CARs described herein). Cells can be engineered to possess any of the features of any of the engineered cells described herein. In a particular aspect, provided herein are cells engineered to produce two cytokines and a CAR, where at least one of the cytokines is membrane-cleavable chimeric protein having the formula **S – C – MT** or **MT – C – S** described herein.

The engineered immunoresponsive cells include, but are not limited to, a T cell, a CD8+ T cell, a CD4+ T cell, a gamma-delta T cell, a cytotoxic T lymphocyte (CTL), a regulatory T cell, a viral-specific T cell, a Natural Killer T (NKT) cell, a Natural Killer (NK) cell, a B cell, a tumor-infiltrating lymphocyte (TIL), an innate lymphoid cell, a mast cell, an eosinophil, a basophil, a neutrophil, a myeloid cell, a macrophage, a monocyte, a dendritic cell, an erythrocyte, a platelet cell, a human embryonic stem cell (ESC), an ESC-derived cell, a pluripotent stem cell, a mesenchymal stromal cell (MSC), an induced pluripotent stem cell (iPSC), and an iPSC-derived cell.

A cell can be engineered to produce the proteins described herein using methods known to those skilled in the art. For example, cells can be transduced to engineer the tumor. In an embodiment, the cell is transduced using a virus.

In a particular embodiment, the cell is transduced using an oncolytic virus. Examples of oncolytic viruses include, but are not limited to, an oncolytic herpes simplex virus, an oncolytic adenovirus, an oncolytic measles virus, an oncolytic influenza virus, an oncolytic Indiana vesiculovirus, an oncolytic Newcastle disease virus, an oncolytic vaccinia virus, an oncolytic poliovirus, an oncolytic myxoma virus, an oncolytic reovirus, an oncolytic mumps virus, an oncolytic Maraba virus, an oncolytic rabies virus, an oncolytic rotavirus, an oncolytic hepatitis virus, an oncolytic rubella virus, an oncolytic dengue virus, an oncolytic chikungunya virus, an oncolytic respiratory syncytial virus, an oncolytic lymphocytic choriomeningitis virus, an oncolytic morbillivirus, an oncolytic lentivirus, an oncolytic replicating retrovirus, an oncolytic rhabdovirus, an oncolytic Seneca Valley virus, an oncolytic sindbis virus, and any variant or derivative thereof.

The virus, including any of the oncolytic viruses described herein, can be a recombinant virus that encodes one more transgenes encoding one or more proteins, such as any of the engineered nucleic acids described herein. The virus, including any of the oncolytic viruses described herein, can be a recombinant virus that encodes one more transgenes encoding one or more of the two or more proteins, such as any of the engineered nucleic acids described herein.

Also provided herein are engineered bacterial cells. Bacterial cells can be engineered to comprise any of the engineered nucleic acids described herein. Bacterial cells can be engineered to possess any of the features of any of the engineered cells described herein. In a particular aspect, provided herein are bacterial cells engineered to produce two or more of the proteins described herein. Bacterial cells can be engineered to produce one or more mammalian-derived proteins. Bacterial cells can be engineered to produce two or more mammalian-derived proteins. Examples of bacterial cells include, but are not limited to, *Clostridium beijerinckii*, *Clostridium sporogenes, Clostridium novyi, Escherichia coli*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Salmonella typhimurium*, and *Salmonella choleraesuis.*

An engineered cell can be a human cell. An engineered cell can be a human primary cell. An engineered primary cell can be a tumor infiltrating primary cell. An engineered primary cell can be a primary T cell. An engineered primary cell can be a hematopoietic stem cell (HSC). An engineered primary cell can be a natural killer (NK) cell. An engineered primary cell can be any somatic cell. An engineered primary cell can be a MSC. Human cells (*e.g.*, immune cells) can be engineered to comprise any of the engineered nucleic acids described herein. Human cells (*e.g.*, immune cells) can be engineered to possess any of the features of any of the engineered cells

described herein. In a particular aspect, provided herein are human cells (*e.g.*, immune cells) engineered to produce one or more of the proteins described herein. In a particular aspect, provided herein are human cells (*e.g.*, immune cells) engineered to produce two or more of the proteins described herein.

An engineered cell can be isolated from a subject (autologous), such as a subject known or suspected to have cancer. Cell isolation methods are known to those skilled in the art and include, but are not limited to, sorting techniques based on cell-surface marker expression, such as FACS sorting, positive isolation techniques, and negative isolation, magnetic isolation, and combinations thereof.

An engineered cell can be allogenic with reference to the subject being administered a treatment. Allogenic modified cells can be HLA-matched to the subject being administered a treatment. An engineered cell can be a cultured cell, such as an *ex vivo* cultured cell. An engineered cell can be an *ex vivo* cultured cell, such as a primary cell isolated from a subject. Cultured cell can be cultured with one or more cytokines.

Also provided herein are methods that include culturing the engineered cells of the present disclosure. Methods of culturing the engineered cells described herein are known. One skilled in the art will recognize that culturing conditions will depend on the particular engineered cell of interest. One skilled in the art will recognize that culturing conditions will depend on the specific downstream use of the engineered cell, for example, specific culturing conditions for subsequent administration of the engineered cell to a subject.

Methods of Engineering Cells

Also provided herein are compositions and methods for engineering immunoresponsive cells to produce one or more proteins of interest (*e.g.*, the cytokines, CARs, ACPs, and/or membrane-cleavable chimeric proteins having the formula **S – C – MT** or **MT – C – S** described herein).

In general, cells are engineered to produce proteins of interest through introduction (*i.e.*, delivery) of polynucleotides encoding the one or more proteins of interest or effector molecules, *e.g.*, the chimeric proteins described herein including the protein of interest or effector molecule, into the cell's cytosol and/or nucleus. For example, the polynucleotides encoding the one or more chimeric proteins can be any of the engineered nucleic acids encoding the cytokines, CARs, or membrane-cleavable chimeric proteins having the formula **S – C – MT** or **MT – C – S** described herein. Delivery methods include, but are not limited to, viral-mediated delivery, lipid-mediated transfection, nanoparticle delivery, electroporation, sonication, and cell

membrane deformation by physical means. One skilled in the art will appreciate the choice of delivery method can depend on the specific cell type to be engineered.

Viral-Mediated Delivery

Viral vector-based delivery platforms can be used to engineer cells. In general, a viral vector-based delivery platform engineers a cell through introducing (*i.e.,* delivering) into a host cell. For example, a viral vector-based delivery platform can engineer a cell through introducing any of the engineered nucleic acids described herein (*e.g.*, any of the exogenous polynucleotide sequences encoding the cytokines, CARs, ACPs, and/or the membrane-cleavable chimeric proteins having the formula **S – C – MT** or **MT – C – S** described herein, and/or any of the expression cassettes described herein containing a promoter and an exogenous polynucleotide sequence encoding the proteins, oriented from N-terminal to C-terminal). A viral vector-based delivery platform can be a nucleic acid, and as such, an engineered nucleic acid can also encompass an engineered virally-derived nucleic acid. Such engineered virally-derived nucleic acids can also be referred to as recombinant viruses or engineered viruses.

A viral vector-based delivery platform can encode more than one engineered nucleic acid, gene, or transgene within the same nucleic acid. For example, an engineered virally-derived nucleic acid, *e.g.*, a recombinant virus or an engineered virus, can encode one or more transgenes, including, but not limited to, any of the engineered nucleic acids described herein that encode one or more of the proteins described herein. The one or more transgenes encoding the one or more proteins can be configured to express the one or more proteins and/or other protein of interest. A viral vector-based delivery platform can encode one or more genes in addition to the one or more transgenes (*e.g.*, transgenes encoding the one or more proteins and/or other protein of interest), such as viral genes needed for viral infectivity and/or viral production (*e.g.*, capsid proteins, envelope proteins, viral polymerases, viral transcriptases, *etc.*), referred to as cis-acting elements or genes.

A viral vector-based delivery platform can comprise more than one viral vector, such as separate viral vectors encoding the engineered nucleic acids, genes, or transgenes described herein, and referred to as trans-acting elements or genes. For example, a helper-dependent viral vector-based delivery platform can provide additional genes needed for viral infectivity and/or viral production on one or more additional separate vectors in addition to the vector encoding the one or more proteins and/or other protein of interest. One viral vector can deliver more than one engineered nucleic acids, such as one vector that delivers engineered nucleic acids that are configured to produce two or more proteins and/or other protein of interest. More than one viral vector can deliver more than one engineered nucleic acids, such as more than one vector that

delivers one or more engineered nucleic acid configured to produce one or more proteins and/or other protein of interest. The number of viral vectors used can depend on the packaging capacity of the above mentioned viral vector-based vaccine platforms, and one skilled in the art can select the appropriate number of viral vectors.

In general, any of the viral vector-based systems can be used for the *in vitro* production of molecules, such as the proteins, effector molecules, and/or other protein of interest described herein, or used *in vivo* and *ex vivo* gene therapy procedures, *e.g.*, for *in vivo* delivery of the engineered nucleic acids encoding one or more proteins and/or other protein of interest. The selection of an appropriate viral vector-based system will depend on a variety of factors, such as cargo/payload size, immunogenicity of the viral system, target cell of interest, gene expression strength and timing, and other factors appreciated by one skilled in the art.

Viral vector-based delivery platforms can be RNA-based viruses or DNA-based viruses. Exemplary viral vector-based delivery platforms include, but are not limited to, a herpes simplex virus, a adenovirus, a measles virus, an influenza virus, a Indiana vesiculovirus, a Newcastle disease virus, a vaccinia virus, a poliovirus, a myxoma virus, a reovirus, a mumps virus, a Maraba virus, a rabies virus, a rotavirus, a hepatitis virus, a rubella virus, a dengue virus, a chikungunya virus, a respiratory syncytial virus, a lymphocytic choriomeningitis virus, a morbillivirus, a lentivirus, a replicating retrovirus, a rhabdovirus, a Seneca Valley virus, a sindbis virus, and any variant or derivative thereof. Other exemplary viral vector-based delivery platforms are described in the art, such as vaccinia, fowlpox, self-replicating alphavirus, marabavirus, adenovirus (*See, e.g.,* Tatsis *et al.*, Adenoviruses, *Molecular Therapy* (2004) 10, 616—629), or lentivirus, including but not limited to second, third or hybrid second/third generation lentivirus and recombinant lentivirus of any generation designed to target specific cell types or receptors (*See, e.g.,* Hu *et al.*, Immunization Delivered by Lentiviral Vectors for Cancer and Infectious Diseases, *Immunol Rev.* (2011) 239(1): 45-61, Sakuman e*t al.*, Lentiviral vectors: basic to translational, *Biochem J.* (2012) 443(3):603-18, Cooper *et al.*, Rescue of splicing-mediated intron loss maximizes expression in lentiviral vectors containing the human ubiquitin C promoter, *Nucl. Acids Res.* (2015) 43 (1): 682-690, Zufferey *et al.*, Self-Inactivating Lentivirus Vector for Safe and Efficient *In vivo* Gene Delivery, *J. Virol.* (1998) 72 (12): 9873-9880).

The sequences may be preceded with one or more sequences targeting a subcellular compartment. Upon introduction (*i.e.* delivery) into a host cell, infected cells (*i.e.*, an engineered cell) can express the proteins and/or other protein of interest. Vaccinia vectors and methods useful in immunization protocols are described in, *e.g.*, U.S. Pat. No. 4,722,848. Another vector is BCG (Bacille Calmette Guerin). BCG vectors are described in Stover *et al.* (Nature 351:456-

460 (1991)). A wide variety of other vectors useful for the introduction (*i.e.*, delivery) of engineered nucleic acids, *e.g.*, Salmonella typhi vectors, and the like will be apparent to those skilled in the art from the description herein.

The viral vector-based delivery platforms can be a virus that targets a cell, herein referred to as an oncolytic virus. Examples of oncolytic viruses include, but are not limited to, an oncolytic herpes simplex virus, an oncolytic adenovirus, an oncolytic measles virus, an oncolytic influenza virus, an oncolytic Indiana vesiculovirus, an oncolytic Newcastle disease virus, an oncolytic vaccinia virus, an oncolytic poliovirus, an oncolytic myxoma virus, an oncolytic reovirus, an oncolytic mumps virus, an oncolytic Maraba virus, an oncolytic rabies virus, an oncolytic rotavirus, an oncolytic hepatitis virus, an oncolytic rubella virus, an oncolytic dengue virus, an oncolytic chikungunya virus, an oncolytic respiratory syncytial virus, an oncolytic lymphocytic choriomeningitis virus, an oncolytic morbillivirus, an oncolytic lentivirus, an oncolytic replicating retrovirus, an oncolytic rhabdovirus, an oncolytic Seneca Valley virus, an oncolytic sindbis virus, and any variant or derivative thereof. Any of the oncolytic viruses described herein can be a recombinant oncolytic virus comprising one more transgenes (*e.g.*, an engineered nucleic acid) encoding one or more proteins and/or other protein of interest. The transgenes encoding the one or more proteins and/or other protein of interest can be configured to express the proteins and/or other protein of interest.

The viral vector-based delivery platform can be retrovirus-based. In general, retroviral vectors are comprised of cis-acting long terminal repeats with packaging capacity for up to 6-10 kb of foreign sequence. The minimum cis-acting LTRs are sufficient for replication and packaging of the vectors, which are then used to integrate the one or more engineered nucleic acids (*e.g.*, transgenes encoding the one or more proteins and/or other protein of interest) into the target cell to provide permanent transgene expression. Retroviral-based delivery systems include, but are not limited to, those based upon murine leukemia, virus (MuLV), gibbon ape leukemia virus (GaLV), Simian Immuno deficiency vims (SIV), human immuno deficiency vims (HIV), and combinations thereof (see, *e.g.*, Buchscher *et al.*, J. Virol. 66:2731-2739 (1992); Johann et ah, J. Virol. 66:1635-1640 (1992); Sommnerfelt *et al.*, Virol. 176:58-59 (1990); Wilson et ah, J. Virol. 63:2374-2378 (1989); Miller et al, J, Virol. 65:2220-2224 (1991); PCT/US94/05700). Other retroviral systems include the Phoenix retrovirus system.

The viral vector-based delivery platform can be lentivirus-based. In general, lentiviral vectors are retroviral vectors that are able to transduce or infect non-dividing cells and typically produce high viral titers. Lentiviral-based delivery platforms can be HIV-based, such as ViraPower systems (ThermoFisher) or pLenti systems (Cell Biolabs). Lentiviral-based delivery platforms can be SIV, or FIV-based. Other exemplary lentivirus-based delivery platforms are

described in more detail in U.S. Pat. Nos. 7,311,907; 7,262,049; 7,250,299; 7,226,780; 7,220,578; 7,211,247; 7,160,721; 7,078,031; 7,070,993; 7,056,699; 6,955,919, each herein incorporated by reference for all purposes.

The viral vector-based delivery platform can be adenovirus-based. In general, adenoviral based vectors are capable of very high transduction efficiency in many cell types, do not require cell division, achieve high titer and levels of expression, and can be produced in large quantities in a relatively simple system. In general, adenoviruses can be used for transient expression of a transgene within an infected cell since adenoviruses do not typically integrate into a host's genome. Adenovirus-based delivery platforms are described in more detail in Li *et al.*, Invest Opthalmol Vis Sci 35:2543 2549, 1994; Borras *et al.*, Gene Ther 6:515 524, 1999; Li and Davidson, PNAS 92:7700 7704, 1995; Sakamoto *et al.*, H Gene Ther 5:1088 1097, 1999; WO 94/12649, WO 93/03769; WO 93/19191; WO 94/28938; WO 95/11984 and WO 95/00655, each herein incorporated by reference for all purposes. Other exemplary adenovirus-based delivery platforms are described in more detail in U.S. Pat. Nos. 5585362; 6,083,716, 7,371,570; 7,348,178; 7,323,177; 7,319,033; 7,318,919; and 7,306,793 and International Patent Application WO96/13597, each herein incorporated by reference for all purposes.

The viral vector-based delivery platform can be adeno-associated virus (AAV)-based. Adeno-associated virus ("AAV") vectors may be used to transduce cells with engineered nucleic acids (*e.g.*, any of the engineered nucleic acids described herein). AAV systems can be used for the *in vitro* production of proteins of interest, such as the proteins described herein and/or effector molecules, or used *in vivo* and *ex vivo* gene therapy procedures, *e.g.*, for *in vivo* delivery of the engineered nucleic acids encoding one or more proteins and/or other protein of interest (see, *e.g.*, West *et al.*, Virology 160:38-47 (1987); U.S. Pat. Nos. 4,797,368; 5,436,146; 6,632,670; 6,642,051; 7,078,387; 7,314,912; 6,498,244; 7,906,111; US patent publications US 2003-0138772, US 2007/0036760, and US 2009/0197338; Gao, *et al.*, J. Virol, 78(12):6381-6388 (June 2004); Gao, et al, Proc Natl Acad Sci USA, 100(10):6081-6086 (May 13, 2003); and International Patent applications WO 2010/138263 and WO 93/24641; Kotin, Human Gene Therapy 5:793-801 (1994); Muzyczka, J. Clin. Invest. 94:1351 (1994), each herein incorporated by reference for all purposes). Exemplary methods for constructing recombinant AAV vectors are described in more detail in U.S. Pat. No, 5,173,414; Tratschin et ah, Mol. Cell. Biol. 5:3251-3260 (1985); Tratschin, et ah, Mol. Cell, Biol. 4:2072-2081 (1984); Hermonat & Muzyczka, PNAS 81:64666470 (1984); and Samuiski et ah, J. Virol. 63:03822-3828 (1989), each herein incorporated by reference for all purposes. In general, an AAV-based vector comprises a capsid protein having an amino acid sequence corresponding to any one of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV.Rh10, AAV11 and variants

thereof. In particular examples, an AAV-based vector has a capsid protein having an amino acid sequence corresponding to AAV2. In particular examples, an AAV-based vector has a capsid protein having an amino acid sequence corresponding to AAV8.

AAV vectors can be engineered to have any of the exogenous polynucleotide sequences encoding the proteins described herein, such as the cytokines, CARs, ACPs, and/or membrane-cleavable chimeric proteins described herein having the formula: **S – C – MT** or **MT – C – S.**

The viral vector-based delivery platform can be a virus-like particle (VLP) platform. In general, VLPs are constructed by producing viral structural proteins and purifying resulting viral particles. Then, following purification, a cargo/payload (*e.g.*, any of the engineered nucleic acids described herein) is encapsulated within the purified particle *ex vivo*. Accordingly, production of VLPs maintains separation of the nucleic acids encoding viral structural proteins and the nucleic acids encoding the cargo/payload. The viral structural proteins used in VLP production can be produced in a variety of expression systems, including mammalian, yeast, insect, bacterial, or *in vivo* translation expression systems. The purified viral particles can be denatured and reformed in the presence of the desired cargo to produce VLPs using methods known to those skilled in the art. Production of VLPs are described in more detail in Seow *et al.* (Mol Ther. 2009 May; 17(5): 767–777), herein incorporated by reference for all purposes.

The viral vector-based delivery platform can be engineered to target (*i.e.*, infect) a range of cells, target a narrow subset of cells, or target a specific cell. In general, the envelope protein chosen for the viral vector-based delivery platform will determine the viral tropism. The virus used in the viral vector-based delivery platform can be pseudotyped to target a specific cell of interest. The viral vector-based delivery platform can be pantropic and infect a range of cells. For example, pantropic viral vector-based delivery platforms can include the VSV-G envelope. The viral vector-based delivery platform can be amphotropic and infect mammalian cells. Accordingly, one skilled in the art can select the appropriate tropism, pseudotype, and/or envelope protein for targeting a desired cell type.

Lipid Structure Delivery Systems

Engineered nucleic acids (*e.g.*, any of the engineered nucleic acids described herein) can be introduced into a cell using a lipid-mediated delivery system. In general, a lipid-mediated delivery system uses a structure composed of an outer lipid membrane enveloping an internal compartment. Examples of lipid-based structures include, but are not limited to, a lipid-based nanoparticle, a liposome, a micelle, an exosome, a vesicle, an extracellular vesicle, a cell, or a tissue. Lipid structure delivery systems can deliver a cargo/payload (*e.g.*, any of the engineered nucleic acids described herein) *in vitro*, *in vivo*, or *ex vivo*.

A lipid-based nanoparticle can include, but is not limited to, a unilamellar liposome, a multilamellar liposome, and a lipid preparation. As used herein, a "liposome" is a generic term encompassing *in vitro* preparations of lipid vehicles formed by enclosing a desired cargo, *e.g.*, an engineered nucleic acid, such as any of the engineered nucleic acids described herein, within a lipid shell or a lipid aggregate. Liposomes may be characterized as having vesicular structures with a bilayer membrane, generally comprising a phospholipid, and an inner medium that generally comprises an aqueous composition. Liposomes include, but are not limited to, emulsions, foams, micelles, insoluble monolayers, liquid crystals, phospholipid dispersions, lamellar layers and the like. Liposomes can be unilamellar liposomes. Liposomes can be multilamellar liposomes. Liposomes can be multivesicular liposomes. Liposomes can be positively charged, negatively charged, or neutrally charged. In certain embodiments, the liposomes are neutral in charge. Liposomes can be formed from standard vesicle-forming lipids, which generally include neutral and negatively charged phospholipids and a sterol, such as cholesterol. The selection of lipids is generally guided by consideration of a desired purpose, *e.g.*, criteria for *in vivo* delivery, such as liposome size, acid lability and stability of the liposomes in the blood stream. A variety of methods are available for preparing liposomes, as described in, *e.g.*, Szokan e*t al.*, Ann. Rev. Biophys. Bioeng. 9; 467 (1980), U.S. Pat. Nos. 4,235,871, 4,501,728, 4,501,728, 4,837,028, and 5,019,369, each herein incorporated by reference for all purposes.

A multilamellar liposome is generated spontaneously when lipids comprising phospholipids are suspended in an excess of aqueous solution such that multiple lipid layers are separated by an aqueous medium. Water and dissolved solutes are entrapped in closed structures between the lipid bilayers following the lipid components undergoing self-rearrangement. A desired cargo (*e.g.*, a polypeptide, a nucleic acid, a small molecule drug, an engineered nucleic acid, such as any of the engineered nucleic acids described herein, a viral vector, a viral-based delivery system, *etc.*) can be encapsulated in the aqueous interior of a liposome, attached to a liposome via a linking molecule that is associated with both the liposome and the polypeptide/nucleic acid, interspersed within the lipid bilayer of a liposome, entrapped in a liposome, complexed with a liposome, or otherwise associated with the liposome such that it can be delivered to a target entity. Lipophilic molecules or molecules with lipophilic regions may also dissolve in or associate with the lipid bilayer.

A liposome used according to the present embodiments can be made by different methods, as would be known to one of ordinary skill in the art. Preparations of liposomes are described in further detail in WO 2016/201323, International Applications PCT/US85/01161

and PCT/US89/05040, and U.S. Patents 4,728,578, 4,728,575, 4,737,323, 4,533,254, 4,162,282, 4,310,505, and 4,921,706; each herein incorporated by reference for all purposes.

Liposomes can be cationic liposomes. Examples of cationic liposomes are described in more detail in U.S. Patent No. 5,962,016; 5,030,453; 6,680,068, U.S. Application 2004/0208921, and International Patent Applications WO03/015757A1, WO04029213A2, and WO02/100435A1, each hereby incorporated by reference in their entirety.

Lipid-mediated gene delivery methods are described, for instance, in WO 96/18372; WO 93/24640; Mannino & Gould-Fogerite, BioTechniques 6(7): 682-691 (1988); U.S. Pat. No. 5,279,833 Rose U.S. Pat. No. 5,279,833; WO91/06309; and Felgner *et al.*, Proc. Natl. Acad. Sci. USA 84: 7413-7414 (1987), each herein incorporated by reference for all purposes.

Exosomes are small membrane vesicles of endocytic origin that are released into the extracellular environment following fusion of multivesicular bodies with the plasma membrane. The size of exosomes ranges between 30 and 100 nm in diameter. Their surface consists of a lipid bilayer from the donor cell's cell membrane, and they contain cytosol from the cell that produced the exosome, and exhibit membrane proteins from the parental cell on the surface. Exosomes useful for the delivery of nucleic acids are known to those skilled in the art, *e.g.*, the exosomes described in more detail in U.S. Pat. No. 9,889,210, herein incorporated by reference for all purposes.

As used herein, the term "extracellular vesicle" or "EV" refers to a cell-derived vesicle comprising a membrane that encloses an internal space. In general, extracellular vesicles comprise all membrane-bound vesicles that have a smaller diameter than the cell from which they are derived. Generally extracellular vesicles range in diameter from 20 nm to 1000 nm, and can comprise various macromolecular cargo either within the internal space, displayed on the external surface of the extracellular vesicle, and/or spanning the membrane. The cargo can comprise nucleic acids (*e.g.*, any of the engineered nucleic acids described herein), proteins, carbohydrates, lipids, small molecules, and/or combinations thereof. By way of example and without limitation, extracellular vesicles include apoptotic bodies, fragments of cells, vesicles derived from cells by direct or indirect manipulation (*e.g.*, by serial extrusion or treatment with alkaline solutions), vesiculated organelles, and vesicles produced by living cells (*e.g.*, by direct plasma membrane budding or fusion of the late endosome with the plasma membrane). Extracellular vesicles can be derived from a living or dead organism, explanted tissues or organs, and/or cultured cells.

As used herein the term "exosome" refers to a cell-derived small (between 20-300 nm in diameter, more preferably 40-200 nm in diameter) vesicle comprising a membrane that encloses an internal space, and which is generated from the cell by direct plasma membrane budding or

by fusion of the late endosome with the plasma membrane. The exosome comprises lipid or fatty acid and polypeptide and optionally comprises a payload (*e.g.*, a therapeutic agent), a receiver (*e.g.*, a targeting moiety), a polynucleotide (*e.g.*, a nucleic acid, RNA, or DNA, such as any of the engineered nucleic acids described herein), a sugar (*e.g.*, a simple sugar, polysaccharide, or glycan) or other molecules. The exosome can be derived from a producer cell, and isolated from the producer cell based on its size, density, biochemical parameters, or a combination thereof. An exosome is a species of extracellular vesicle. Generally, exosome production/biogenesis does not result in the destruction of the producer cell. Exosomes and preparation of exosomes are described in further detail in WO 2016/201323, which is hereby incorporated by reference in its entirety.

As used herein, the term "nanovesicle" (also referred to as a "microvesicle") refers to a cell-derived small (between 20-250 nm in diameter, more preferably 30-150 nm in diameter) vesicle comprising a membrane that encloses an internal space, and which is generated from the cell by direct or indirect manipulation such that said nanovesicle would not be produced by said producer cell without said manipulation. In general, a nanovesicle is a sub-species of an extracellular vesicle. Appropriate manipulations of the producer cell include but are not limited to serial extrusion, treatment with alkaline solutions, sonication, or combinations thereof. The production of nanovesicles may, in some instances, result in the destruction of said producer cell. Preferably, populations of nanovesicles are substantially free of vesicles that are derived from producer cells by way of direct budding from the plasma membrane or fusion of the late endosome with the plasma membrane. The nanovesicle comprises lipid or fatty acid and polypeptide, and optionally comprises a payload (*e.g.*, a therapeutic agent), a receiver (*e.g.*, a targeting moiety), a polynucleotide (*e.g.*, a nucleic acid, RNA, or DNA, such as any of the engineered nucleic acids described herein), a sugar (*e.g.*, a simple sugar, polysaccharide, or glycan) or other molecules. The nanovesicle, once it is derived from a producer cell according to said manipulation, may be isolated from the producer cell based on its size, density, biochemical parameters, or a combination thereof.

Lipid nanoparticles (LNPs), in general, are synthetic lipid structures that rely on the amphiphilic nature of lipids to form membranes and vesicle like structures (Riley 2017). In general, these vesicles deliver cargo/payloads, such as any of the engineered nucleic acids or viral systems described herein, by absorbing into the membrane of target cells and releasing the cargo into the cytosol. Lipids used in LNP formation can be cationic, anionic, or neutral. The lipids can be synthetic or naturally derived, and in some instances biodegradable. Lipids can include fats, cholesterol, phospholipids, lipid conjugates including, but not limited to, polyethyleneglycol (PEG) conjugates (PEGylated lipids), waxes, oils, glycerides, and fat soluble

vitamins. Lipid compositions generally include defined mixtures of materials, such as the cationic, neutral, anionic, and amphipathic lipids. In some instances, specific lipids are included to prevent LNP aggregation, prevent lipid oxidation, or provide functional chemical groups that facilitate attachment of additional moieties. Lipid composition can influence overall LNP size and stability. In an example, the lipid composition comprises dilinoleylmethyl- 4-dimethylaminobutyrate (MC3) or MC3-like molecules. MC3 and MC3-like lipid compositions can be formulated to include one or more other lipids, such as a PEG or PEG-conjugated lipid, a sterol, or neutral lipids. In addition, LNPs can be further engineered or functionalized to facilitate targeting of specific cell types. Another consideration in LNP design is the balance between targeting efficiency and cytotoxicity.

Micelles, in general, are spherical synthetic lipid structures that are formed using single-chain lipids, where the single-chain lipid's hydrophilic head forms an outer layer or membrane and the single-chain lipid's hydrophobic tails form the micelle center. Micelles typically refer to lipid structures only containing a lipid mono-layer. Micelles are described in more detail in Quader *et al.* (Mol Ther. 2017 Jul 5; 25(7): 1501–1513), herein incorporated by reference for all purposes.

Nucleic-acid vectors, such as expression vectors, exposed directly to serum can have several undesirable consequences, including degradation of the nucleic acid by serum nucleases or off-target stimulation of the immune system by the free nucleic acids. Similarly, viral delivery systems exposed directly to serum can trigger an undesired immune response and/or neutralization of the viral delivery system. Therefore, encapsulation of an engineered nucleic acid and/or viral delivery system can be used to avoid degradation, while also avoiding potential off-target affects. In certain examples, an engineered nucleic acid and/or viral delivery system is fully encapsulated within the delivery vehicle, such as within the aqueous interior of an LNP. Encapsulation of an engineered nucleic acid and/or viral delivery system within an LNP can be carried out by techniques well-known to those skilled in the art, such as microfluidic mixing and droplet generation carried out on a microfluidic droplet generating device. Such devices include, but are not limited to, standard T-junction devices or flow-focusing devices. In an example, the desired lipid formulation, such as MC3 or MC3-like containing compositions, is provided to the droplet generating device in parallel with an engineered nucleic acid or viral delivery system and any other desired agents, such that the delivery vector and desired agents are fully encapsulated within the interior of the MC3 or MC3-like based LNP. In an example, the droplet generating device can control the size range and size distribution of the LNPs produced. For example, the LNP can have a size ranging from 1 to 1000 nanometers in diameter, *e.g.*, 1, 10, 50, 100, 500, or 1000 nanometers. Following droplet generation, the delivery vehicles encapsulating the

cargo/payload (*e.g.*, an engineered nucleic acid and/or viral delivery system) can be further treated or engineered to prepare them for administration.

Nanoparticle Delivery

Nanomaterials can be used to deliver engineered nucleic acids (*e.g.*, any of the engineered nucleic acids described herein). Nanomaterial vehicles, importantly, can be made of non-immunogenic materials and generally avoid eliciting immunity to the delivery vector itself. These materials can include, but are not limited to, lipids (as previously described), inorganic nanomaterials, and other polymeric materials. Nanomaterial particles are described in more detail in Riley *et al.* (Recent Advances in Nanomaterials for Gene Delivery—A Review. Nanomaterials 2017, 7(5), 94), herein incorporated by reference for all purposes.

Genomic Editing Systems

A genomic editing systems can be used to engineer a host genome to encode an engineered nucleic acid, such as an engineered nucleic acid encoding the cytokines, CARs, ACPs, and/or membrane-cleavable chimeric proteins having the formula **S – C – MT** or **MT – C – S** described herein. In general, a "genomic editing system" refers to any system for integrating an exogenous gene into a host cell's genome. Genomic editing systems include, but are not limited to, a transposon system, a nuclease genomic editing system, and a viral vector-based delivery platform.

A transposon system can be used to integrate an engineered nucleic acid, such as the cytokines, CARs, ACPs, and/or membrane-cleavable chimeric proteins having the formula **S – C – MT** or **MT – C – S** described herein, into a host genome. Transposons generally comprise terminal inverted repeats (TIR) that flank a cargo/payload nucleic acid and a transposase. The transposon system can provide the transposon in *cis* or in *trans* with the TIR-flanked cargo. A transposon system can be a retrotransposon system or a DNA transposon system. In general, transposon systems integrate a cargo/payload (*e.g.*, an engineered nucleic acid) randomly into a host genome. Examples of transposon systems include systems using a transposon of the Tc1/mariner transposon superfamily, such as a Sleeping Beauty transposon system, described in more detail in Hudecek *et al.* (Crit Rev Biochem Mol Biol. 2017 Aug;52(4):355-380), and U.S. Patent Nos. 6,489,458, 6,613,752 and 7,985,739, each of which is herein incorporated by reference for all purposes. Another example of a transposon system includes a PiggyBac transposon system, described in more detail in U.S. Patent Nos. 6,218,185 and 6,962,810, each of which is herein incorporated by reference for all purposes.

A nuclease genomic editing system can be used to engineer a host genome to encode an engineered nucleic acid, such as an engineered nucleic acid encoding the cytokines, CARs, ACPs, and/or the membrane-cleavable chimeric proteins having the formula **S – C – MT** or **MT – C – S** described herein. Without wishing to be bound by theory, in general, the nuclease-mediated gene editing systems used to introduce an exogenous gene take advantage of a cell's natural DNA repair mechanisms, particularly homologous recombination (HR) repair pathways. Briefly, following an insult to genomic DNA (typically a double-stranded break), a cell can resolve the insult by using another DNA source that has identical, or substantially identical, sequences at both its 5' and 3' ends as a template during DNA synthesis to repair the lesion. In a natural context, HDR can use the other chromosome present in a cell as a template. In gene editing systems, exogenous polynucleotides are introduced into the cell to be used as a homologous recombination template (HRT or HR template). In general, any additional exogenous sequence not originally found in the chromosome with the lesion that is included between the 5' and 3' complimentary ends within the HRT (*e.g.*, a gene or a portion of a gene) can be incorporated (*i.e.*, "integrated") into the given genomic locus during templated HDR. Thus, a typical HR template for a given genomic locus has a nucleotide sequence identical to a first region of an endogenous genomic target locus, a nucleotide sequence identical to a second region of the endogenous genomic target locus, and a nucleotide sequence encoding a cargo/payload nucleic acid (*e.g.*, any of the engineered nucleic acids described herein, such as any of the engineered nucleic acids encoding the cytokines, CARs, ACPs, and/or membrane-cleavable chimeric proteins having the formula **S – C – MT** or **MT – C – S** described herein).

In some examples, a HR template can be linear. Examples of linear HR templates include, but are not limited to, a linearized plasmid vector, a ssDNA, a synthesized DNA, and a PCR amplified DNA. In particular examples, a HR template can be circular, such as a plasmid. A circular template can include a supercoiled template.

The identical, or substantially identical, sequences found at the 5' and 3' ends of the HR template, with respect to the exogenous sequence to be introduced, are generally referred to as arms (HR arms). HR arms can be identical to regions of the endogenous genomic target locus (*i.e.*, 100% identical). HR arms in some examples can be substantially identical to regions of the endogenous genomic target locus. While substantially identical HR arms can be used, it can be advantageous for HR arms to be identical as the efficiency of the HDR pathway may be impacted by HR arms having less than 100% identity.

Each HR arm, *i.e.*, the 5' and 3' HR arms, can be the same size or different sizes. Each HR arm can each be greater than or equal to 50, 100, 200, 300, 400, or 500 bases in length. Although HR arms can, in general, be of any length, practical considerations, such as the impact

of HR arm length and overall template size on overall editing efficiency, can also be taken into account. An HR arms can be identical, or substantially identical to, regions of an endogenous genomic target locus immediately adjacent to a cleavage site. Each HR arms can be identical to, or substantially identical to, regions of an endogenous genomic target locus immediately adjacent to a cleavage site. Each HR arms can be identical, or substantially identical to, regions of an endogenous genomic target locus within a certain distance of a cleavage site, such as 1 base-pair, less than or equal to 10 base-pairs, less than or equal to 50 base-pairs, or less than or equal to 100 base-pairs of each other.

A nuclease genomic editing system can use a variety of nucleases to cut a target genomic locus, including, but not limited to, a Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) family nuclease or derivative thereof, a Transcription activator-like effector nuclease (TALEN) or derivative thereof, a zinc-finger nuclease (ZFN) or derivative thereof, and a homing endonuclease (HE) or derivative thereof.

A CRISPR-mediated gene editing system can be used to engineer a host genome to encode an engineered nucleic acid, such as an engineered nucleic acid encoding the cytokines, CARs, ACPs, and/or membrane-cleavable chimeric proteins having the formula **S – C – MT** or **MT – C – S** described herein. CRISPR systems are described in more detail in M. Adli ("The CRISPR tool kit for genome editing and beyond" Nature Communications; volume 9 (2018), Article number: 1911), herein incorporated by reference for all that it teaches. In general, a CRISPR-mediated gene editing system comprises a CRISPR-associated (Cas) nuclease and a RNA(s) that directs cleavage to a particular target sequence. An exemplary CRISPR-mediated gene editing system is the CRISPR/Cas9 systems comprised of a Cas9 nuclease and a RNA(s) that has a CRISPR RNA (crRNA) domain and a trans-activating CRISPR (tracrRNA) domain. The crRNA typically has two RNA domains: a guide RNA sequence (gRNA) that directs specificity through base-pair hybridization to a target sequence ("a defined nucleotide sequence"), *e.g.*, a genomic sequence; and an RNA domain that hybridizes to a tracrRNA. A tracrRNA can interact with and thereby promote recruitment of a nuclease (*e.g.*, Cas9) to a genomic locus. The crRNA and tracrRNA polynucleotides can be separate polynucleotides. The crRNA and tracrRNA polynucleotides can be a single polynucleotide, also referred to as a single guide RNA (sgRNA). While the Cas9 system is illustrated here, other CRISPR systems can be used, such as the Cpf1/Cas12 or Cas13 systems. Nucleases can include derivatives thereof, such as Cas9 functional mutants, *e.g.*, a Cas9 "nickase" mutant that in general mediates cleavage of only a single strand of a defined nucleotide sequence as opposed to a complete double-stranded break typically produced by Cas9 enzymes.

In general, the components of a CRISPR system interact with each other to form a Ribonucleoprotein (RNP) complex to mediate sequence specific cleavage. In some CRISPR systems, each component can be separately produced and used to form the RNP complex. In some CRISPR systems, each component can be separately produced *in vitro* and contacted (*i.e.*, "complexed") with each other *in vitro* to form the RNP complex. The *in vitro* produced RNP can then be introduced (*i.e.*, "delivered") into a cell's cytosol and/or nucleus, *e.g.*, a T cell's cytosol and/or nucleus. The *in vitro* produced RNP complexes can be delivered to a cell by a variety of means including, but not limited to, electroporation, lipid-mediated transfection, cell membrane deformation by physical means, lipid nanoparticles (LNP), virus like particles (VLP), and sonication. In a particular example, *in vitro* produced RNP complexes can be delivered to a cell using a Nucleofactor/Nucleofection® electroporation-based delivery system (Lonza®). Other electroporation systems include, but are not limited to, MaxCyte electroporation systems, Miltenyi CliniMACS electroporation systems, Neon electroporation systems, and BTX electroporation systems. CRISPR nucleases, *e.g.*, Cas9, can be produced *in vitro* (*i.e.*, synthesized and purified) using a variety of protein production techniques known to those skilled in the art. CRISPR system RNAs, *e.g.*, an sgRNA, can be produced *in vitro* (*i.e.*, synthesized and purified) using a variety of RNA production techniques known to those skilled in the art, such as *in vitro* transcription or chemical synthesis.

An *in vitro* produced RNP complex can be complexed at different ratios of nuclease to gRNA. An *in vitro* produced RNP complex can also be used at different amounts in a CRISPR-mediated editing system. For example, depending on the number of cells desired to be edited, the total RNP amount added can be adjusted, such as a reduction in the amount of RNP complex added when editing a large number of cells in a reaction.

In some CRISPR systems, each component (*e.g.*, Cas9 and an sgRNA) can be separately encoded by a polynucleotide with each polynucleotide introduced into a cell together or separately. In some CRISPR systems, each component can be encoded by a single polynucleotide (*i.e.*, a multi-promoter or multicistronic vector, *see* description of exemplary multicistronic systems below) and introduced into a cell. Following expression of each polynucleotide encoded CRISPR component within a cell (*e.g.*, translation of a nuclease and transcription of CRISPR RNAs), an RNP complex can form within the cell and can then direct site-specific cleavage.

Some RNPs can be engineered to have moieties that promote delivery of the RNP into the nucleus. For example, a Cas9 nuclease can have a nuclear localization signal (NLS) domain such that if a Cas9 RNP complex is delivered into a cell's cytosol or following translation of

Cas9 and subsequent RNP formation, the NLS can promote further trafficking of a Cas9 RNP into the nucleus.

The engineered cells described herein can be engineered using non-viral methods, *e.g.*, the nuclease and/or CRISPR mediated gene editing systems described herein can be delivered to a cell using non-viral methods. The engineered cells described herein can be engineered using viral methods, *e.g.*, the nuclease and/or CRISPR mediated gene editing systems described herein can be delivered to a cell using viral methods such as adenoviral, retroviral, lentiviral, or any of the other viral-based delivery methods described herein.

In some CRISPR systems, more than one CRISPR composition can be provided such that each separately target the same gene or general genomic locus at more than target nucleotide sequence. For example, two separate CRISPR compositions can be provided to direct cleavage at two different target nucleotide sequences within a certain distance of each other. In some CRISPR systems, more than one CRISPR composition can be provided such that each separately target opposite strands of the same gene or general genomic locus. For example, two separate CRISPR "nickase" compositions can be provided to direct cleavage at the same gene or general genomic locus at opposite strands.

In general, the features of a CRISPR-mediated editing system described herein can apply to other nuclease-based genomic editing systems. TALEN is an engineered site-specific nuclease, which is composed of the DNA- binding domain of TALE (transcription activator-like effectors) and the catalytic domain of restriction endonuclease FokI. By changing the amino acids present in the highly variable residue region of the monomers of the DNA binding domain, different artificial TALENs can be created to target various nucleotides sequences. The DNA binding domain subsequently directs the nuclease to the target sequences and creates a double-stranded break. TALEN-based systems are described in more detail in U.S. Ser. No. 12/965,590; U.S. Pat. No. 8,450,471; U.S. Pat. No. 8,440,431; U.S. Pat. No. 8,440,432; U.S. Pat. No. 10,172,880; and U.S. Ser. No. 13/738,381, all of which are incorporated by reference herein in their entirety. ZFN-based editing systems are described in more detail in U.S. Patent Nos. 6,453,242; 6,534,261; 6,599,692; 6,503,717; 6,689,558; 7,030,215; 6,794,136; 7,067,317; 7,262,054; 7,070,934; 7,361,635; 7,253,273; and U.S. Patent Publication Nos. 2005/0064474; 2007/0218528; 2005/0267061, all incorporated herein by reference in their entireties for all purposes.

Other Engineering Delivery Systems

Various additional means to introduce engineered nucleic acids (*e.g.*, any of the engineered nucleic acids described herein) into a cell or other target recipient entity, such as any of the lipid structures described herein.

Electroporation can used to deliver polynucleotides to recipient entities. Electroporation is a method of internalizing a cargo/payload into a target cell or entity's interior compartment through applying an electrical field to transiently permeabilize the outer membrane or shell of the target cell or entity. In general, the method involves placing cells or target entities between two electrodes in a solution containing a cargo of interest (*e.g.*, any of the engineered nucleic acids described herein). The lipid membrane of the cells is then disrupted, *i.e.*, permeabilized, by applying a transient set voltage that allows the cargo to enter the interior of the entity, such as the cytoplasm of the cell. In the example of cells, at least some, if not a majority, of the cells remain viable. Cells and other entities can be electroporated *in vitro*, *in vivo*, or *ex vivo*. Electroporation conditions (*e.g.*, number of cells, concentration of cargo, recovery conditions, voltage, time, capacitance, pulse type, pulse length, volume, cuvette length, electroporation solution composition, *etc.*) vary depending on several factors including, but not limited to, the type of cell or other recipient entity, the cargo to be delivered, the efficiency of internalization desired, and the viability desired. Optimization of such criteria are within the scope of those skilled in the art. A variety devices and protocols can be used for electroporation. Examples include, but are not limited to, Neon® Transfection System, MaxCyte® Flow Electroporation™, Lonza® Nucleofector™ systems, and Bio-Rad® electroporation systems.

Other means for introducing engineered nucleic acids (*e.g.*, any of the engineered nucleic acids described herein) into a cell or other target recipient entity include, but are not limited to, sonication, gene gun, hydrodynamic injection, and cell membrane deformation by physical means.

Compositions and methods for delivering engineered mRNAs *in vivo*, such as naked plasmids or mRNA, are described in detail in Kowalski *et al.* (Mol Ther. 2019 Apr 10; 27(4): 710–728) and Kaczmarek *et al.* (Genome Med. 2017; 9: 60.), each herein incorporated by reference for all purposes.

Delivery Vehicles

Also provided herein are compositions for delivering a cargo/payload (a "delivery vehicle").

The cargo can comprise nucleic acids (*e.g.*, any of the engineered nucleic acids described herein, such as any of the engineered nucleic acids described herein encoding the cytokines, CARs, ACPs, and/or membrane-cleavable chimeric proteins having the formula **S – C – MT** or **MT – C – S** described herein), as described above. The cargo can comprise proteins, carbohydrates, lipids, small molecules, and/or combinations thereof.

The delivery vehicle can comprise any composition suitable for delivering a cargo. The delivery vehicle can comprise any composition suitable for delivering a protein (*e.g.*, any of the proteins described herein). The delivery vehicle can be any of the lipid structure delivery systems described herein. For example, a delivery vehicle can be a lipid-based structure including, but not limited to, a lipid-based nanoparticle, a liposome, a micelle, an exosome, a vesicle, an extracellular vesicle, a cell, or a tissue. The delivery vehicle can be any of the nanoparticles described herein, such as nanoparticles comprising lipids (as previously described), inorganic nanomaterials, and other polymeric materials.

The delivery vehicle can be capable of delivering the cargo to a cell, such as delivering any of the proteins described herein to a cell. The delivery vehicle can be capable of delivering the cargo to a cell, such as delivering any of the proteins described herein to a cell. The delivery vehicle can be configured to target a specific cell, such as configured with a re-directing antibody to target a specific cell. The delivery vehicle can be capable of delivering the cargo to a cell *in vivo*.

The delivery vehicle can be capable of delivering the cargo to a tissue or tissue environment (*e.g.*, a tumor microenvironment), such as delivering any of the proteins described herein to a tissue or tissue environment *in vivo*. Delivering a cargo can include secreting the cargo, such as secreting any of the proteins described herein. Accordingly, the delivery vehicle can be capable of secreting the cargo, such as secreting any of the proteins described herein. The delivery vehicle can be capable of secreting the cargo to a tissue or tissue environment (*e.g.*, a tumor microenvironment), such as secreting any of the proteins described herein into a tissue or tissue environment. The delivery vehicle can be configured to target a specific tissue or tissue environment (*e.g.*, a tumor microenvironment), such as configured with a re-directing antibody to target a specific tissue or tissue environment.

Methods of Treatment

Further provided herein are methods that include delivering, or administering, to a subject (*e.g.*, a human subject) engineered cells as provided herein to produce *in vivo* at least one protein of interest produced by the engineered cells (*e.g.*, any of the cytokines, CARs, ACPs,

and/or membrane-cleavable chimeric proteins having the formula **S – C – MT** or **MT – C – S** described herein, or the secreted effector molecules provided for herein following protease cleavage of the chimeric protein). Further provided herein are methods that include delivering, or administering, to a subject (*e.g.*, a human subject) engineered cells as provided herein to produce *in vivo* at least two proteins of interest, *e.g.*, at least two of the cytokines, CARs, ACPs, and/or membrane-cleavable chimeric proteins having the formula **S – C – MT** or **MT – C – S** described herein, produced by the engineered cells.

Further provided herein are methods that include delivering, or administering, to a subject (*e.g.*, a human subject) any of the delivery vehicles described herein, such as any of the delivery vehicles described herein comprising any of the proteins of interest described herein, *e.g.*, any of the cytokines, CARs, ACPs, and/or membrane-cleavable chimeric proteins having the formula **S – C – MT** or **MT – C – S** described herein. Further provided herein are methods that include delivering, or administering, to a subject (*e.g.*, a human subject) any of the delivery vehicles described herein, such as any of the delivery vehicles described herein comprising two or more proteins of, *e.g.*, at least two of the cytokines, CARs, ACPs, and/or the membrane-cleavable chimeric proteins having the formula **S – C – MT** or **MT – C – S** described herein.

In some embodiments, the engineered cells or delivery vehicles are administered via intravenous, intraperitoneal, intratracheal, subcutaneous, intratumoral, oral, anal, intranasal (*e.g.*, packed in a delivery particle), or arterial (*e.g.*, internal carotid artery) routes. Thus, the engineered cells or delivery vehicles may be administered systemically or locally (*e.g.*, to a TME or via intratumoral administration). An engineered cell can be isolated from a subject, such as a subject known or suspected to have cancer. An engineered cell can be allogenic with reference to the subject being administered a treatment. Allogenic modified cells can be HLA-matched to the subject being administered a treatment. Delivery vehicles can be any of the lipid structure delivery systems described herein. Delivery vehicles can be any of the nanoparticles described herein.

Engineered cells or delivery vehicles can be administered alone or in combination with other treatments, either simultaneously or sequentially dependent upon the condition to be treated. For example, engineered cells or delivery vehicles can be administered in combination with one or more IMiDs described herein. FDA-approved IMiDs can be administered in their approved fashion. In another example, engineered cells or delivery vehicles can be administered in combination with a checkpoint inhibitor therapy. Exemplary checkpoint inhibitors include, but are not limited to, anti-PD-1 antibodies, anti-PD-L1 antibodies, anti-PD-L2 antibodies, anti-CTLA-4 antibodies, anti-LAG-3 antibodies, anti-TIM-3 antibodies, anti-TIGIT antibodies, anti-VISTA antibodies, anti-KIR antibodies, anti-B7-H3 antibodies, anti-B7-H4 antibodies, anti-

HVEM antibodies, anti-BTLA antibodies, anti-GAL9 antibodies, anti-A2AR antibodies, anti-phosphatidylserine antibodies, anti-CD27 antibodies, anti-TNFa antibodies, anti-TREMl antibodies, and anti-TREM2 antibodies. Illustrative immune checkpoint inhibitors include pembrolizumab (anti-PD-1; MK-3475/Keytruda® - Merck), nivolumamb (anti-PD-1; Opdivo® - BMS), pidilizumab (anti-PD-1 antibody; CT-011 – Teva/CureTech), AMP224 (anti-PD-1; NCI), avelumab (anti-PD-L1; Bavencio® - Pfizer), durvalumab (anti-PD-L1; MEDI4736/Imfinzi® - Medimmune/AstraZeneca), atezolizumab (anti-PD-L1; Tecentriq® - Roche/Genentech), BMS-936559 (anti-PD-L1 - BMS), tremelimumab (anti-CTLA-4; Medimmune/AstraZeneca), ipilimumab (anti-CTLA-4; Yervoy ® - BMS), lirilumab (anti-KIR; BMS), monalizumab (anti-NKG2A; Innate Pharma/AstraZeneca). In other examples, engineered cells or delivery vehicles can be administered in combination with TGFbeta inhibitors, VEGF inhibitors, or HPGE2. In another example, engineered cells or delivery vehicles can be administered in combination with an anti-CD40 antibody.

Some methods comprise selecting a subject (or patient population) having a tumor (or cancer) and treating that subject with engineered cells or delivery vehicles that modulate tumor-mediated immunosuppressive mechanisms.

The engineered cells or delivery vehicles of the present disclosure may be used, in some instances, to treat cancer, such as ovarian cancer. Other cancers are described herein. For example, the engineered cells may be used to treat bladder tumors, brain tumors, breast tumors, cervical tumors, colorectal tumors, esophageal tumors, gliomas, kidney tumors, liver tumors, lung tumors, melanomas, ovarian tumors, pancreatic tumors, prostate tumors, skin tumors, thyroid tumors, and/or uterine tumors. The engineered cells or delivery vehicles of the present disclosure can be used to treat cancers with tumors located in the peritoneal space of a subject.

The methods provided herein also include delivering a preparation of engineered cells or delivery vehicles. A preparation, in some embodiments, is a substantially pure preparation, containing, for example, less than 5% (*e.g.*, less than 4%, 3%, 2%, or 1%) of cells other than engineered cells. A preparation may comprise $1x10^5$ cells/kg to $1x10^7$ cells/kg cells. Preparation of engineered cells or delivery vehicles can include pharmaceutical compositions having one or more pharmaceutically acceptable carriers. For example, preparations of engineered cells or delivery vehicles can include any of the engineered viruses, such as an engineered AAV virus, or any of the engineered viral vectors, such as AAV vector, described herein.

In vivo Expression

The methods provided herein also include delivering a composition *in vivo* capable of producing the engineered cells described herein, *e.g.*, capable of delivering any of the

engineered nucleic acids described herein to a cell *in vivo*. Such compositions include any of the viral-mediated delivery platforms, any of the lipid structure delivery systems, any of the nanoparticle delivery systems, any of the genomic editing systems, or any of the other engineering delivery systems described herein capable of engineering a cell *in vivo*.

The methods provided herein also include delivering a composition *in vivo* capable of producing any of the proteins of interest described herein, *e.g.*, any of the cytokines, CARs, ACPs, and/or membrane-cleavable chimeric proteins having the formula **S – C – MT** or **MT – C – S** described herein. The methods provided herein also include delivering a composition *in vivo* capable of producing two or more of the proteins of interest described herein. Compositions capable of *in vivo* production of proteins of interest include, but are not limited to, any of the engineered nucleic acids described herein. Compositions capable of *in vivo* production proteins of interest can be a naked mRNA or a naked plasmid.

ADDITIONAL EMBODIMENTS

Provided below are enumerated embodiments describing specific embodiments of the invention:

Embodiment 1: An immunoresponsive cell comprising:

(a) a first engineered nucleic acid comprising

a first expression cassette comprising a first promoter operably linked to a first, exogenous polynucleotide sequence encoding a first cytokine, and

a second expression cassette comprising a second promoter operably linked to a second exogenous polynucleotide sequence encoding a chimeric antigen receptor (CAR) that binds to GPC3; and

(b) a second engineered nucleic acid comprising

a third expression cassette comprising a synthetic transcription factor-responsive promoter operably linked to a third exogenous polynucleotide sequence encoding a second cytokine, and

a fourth expression cassette comprising a fourth promoter operably linked to a fourth exogenous polynucleotide sequence encoding an activation-conditional control polypeptide (ACP), wherein the ACP comprises a synthetic transcription factor comprising a DNA-binding domain and a transcriptional effector domain,

wherein the ACP is capable of inducing expression of the third exogenous polynucleotide sequence by binding to the ACP-responsive promoter,

wherein at least one of the first exogenous polynucleotide sequence and the third

exogenous polynucleotide sequence encodes a membrane-cleavable chimeric protein, oriented from N-terminal to C-terminal, having the formula:

S – C – MT or **MT – C – S**

wherein

S comprises a secretable effector molecule comprising the first and/or second cytokine,

C comprises a protease cleavage site, and

MT comprises a cell membrane tethering domain, and

wherein **S – C – MT** or **MT – C – S** is configured to be expressed as a single polypeptide.

Embodiment 2: The immunoresponsive cell of embodiment 1, wherein the first expression cassette is configured to be transcribed in an opposite orientation relative to transcription of the second expression cassette.

Embodiment 3: The immunoresponsive cell of embodiment 2, wherein the first expression cassette and the second expression cassette are oriented within the first engineered nucleic acid in a head-to-head directionality.

Embodiment 4: The immunoresponsive cell of embodiment 1, wherein the first expression cassette is configured to be transcribed in a same orientation relative to the transcription of the second expression cassette.

Embodiment 5: The immunoresponsive cell of embodiment 4, wherein the first expression cassette and the second expression cassette are oriented within the first engineered nucleic acid in a head-to-tail directionality.

Embodiment 6: The immunoresponsive cell of any one of embodiments 1-5, wherein the first promoter comprises a constitutive promoter, an inducible promoter, or a synthetic promoter.

Embodiment 7: The immunoresponsive cell of embodiment 6, wherein the first promoter is a constitutive promoter selected from the group consisting of: CAG, HLP, CMV, EFS, SFFV, SV40, MND, PGK, UbC, hEF1aV1, hCAGG, hEF1aV2, hACTb, heIF4A1, hGAPDH, hGRP78, hGRP94, hHSP70, hKINb, and hUBIb.

Embodiment 8: The immunoresponsive cell of any one of embodiments 1-7, wherein the second promoter comprises a constitutive promoter, an inducible promoter, or a synthetic promoter.

Embodiment 9: The immunoresponsive cell of embodiment 8, wherein the second promoter is a constitutive promoter selected from the group consisting of: CAG, HLP, CMV, EFS, SFFV, SV40, MND, PGK, UbC, hEF1aV1, hCAGG, hEF1aV2, hACTb, heIF4A1, hGAPDH, hGRP78, hGRP94, hHSP70, hKINb, and hUBIb.

Embodiment 10: The immunoresponsive cell of any one of embodiments 1-9, wherein the third expression cassette is configured to be transcribed in an opposite orientation relative to transcription of the fourth expression cassette within the second engineered nucleic acid.

Embodiment 11: The immunoresponsive cell of any one of embodiments 1-10, wherein the third expression cassette and the fourth expression cassette are oriented within the second engineered nucleic acid in a head-to-head directionality.

Embodiment 12: The immunoresponsive cell of any one of embodiments 1-11, wherein the third expression cassette and the fourth expression cassette are oriented within the second engineered nucleic acid in a tail-to-tail directionality.

Embodiment 13: The immunoresponsive cell of any one of embodiments 1-11, wherein the fourth promoter comprises a constitutive promoter, an inducible promoter, or a synthetic promoter.

Embodiment 14: The immunoresponsive cell of embodiment 13, wherein the fourth promoter is a constitutive promoter selected from the group consisting of: CAG, HLP, CMV, EFS, SFFV, SV40, MND, PGK, UbC, hEF1aV1, hCAGG, hEF1aV2, hACTb, heIF4A1, hGAPDH, hGRP78, hGRP94, hHSP70, hKINb, and hUBIb.

Embodiment 15: An immunoresponsive cell comprising:

(a) a first engineered nucleic acid comprising
a first expression cassette comprising a first promoter operably linked to a first exogenous polynucleotide sequence encoding a first cytokine and a second exogenous polynucleotide sequence encoding a chimeric antigen receptor (CAR) that binds to GPC3, and
a second expression cassette comprising a synthetic transcription factor-responsive promoter operably linked to a third exogenous polynucleotide sequence encoding a second cytokine; and

(b) a second engineered nucleic acid comprising
a third expression cassette comprising a third promoter operably linked to fourth exogenous polynucleotide sequence encoding an activation-conditional control

polypeptide (ACP), wherein the ACP comprises a synthetic transcription factor comprising a DNA-binding domain and a transcriptional effector domain,

wherein the ACP is capable of inducing expression of the third exogenous polynucleotide sequence by binding to the ACP-responsive promoter,

wherein at least one of the first exogenous polynucleotide sequence and the third exogenous polynucleotide sequence encodes a membrane-cleavable chimeric protein, oriented from N-terminal to C-terminal, having the formula:

S – C – MT or **MT – C – S**

wherein

S comprises a secretable effector molecule comprising the first and/or second cytokine,

C comprises a protease cleavage site, and

MT comprises a cell membrane tethering domain, and

wherein **S – C – MT** or **MT – C – S** is configured to be expressed as a single polypeptide.

Embodiment 16: The immunoresponsive cell of embodiment 15, wherein transcription of the first expression cassette is oriented in the opposite direction relative to transcription of the second expression cassette within the first engineered nucleic acid.

Embodiment 17: The immunoresponsive cell of embodiment 16, wherein the first expression cassette and the second expression cassette are oriented within the first engineered nucleic acid in a head-to-head directionality.

Embodiment 18: The immunoresponsive cell of embodiment 15, wherein the first expression cassette is configured to be transcribed in a same orientation relative to transcription of the second expression cassette.

Embodiment 19: The immunoresponsive cell of embodiment 18, wherein the first expression cassette and the second expression cassette are oriented within the first engineered nucleic acid in a head-to-tail directionality.

Embodiment 20: An immunoresponsive cell comprising:

(a) a first engineered nucleic acid comprising

a first expression cassette comprising a first promoter operably linked to a first exogenous polynucleotide sequence encoding a chimeric antigen receptor (CAR) that

binds to GPC3 and a second exogenous polynucleotide sequence encoding a first cytokine; and

(b) a second engineered nucleic acid comprising

a second expression cassette comprising a synthetic transcription factor-responsive promoter operably linked to a third exogenous polynucleotide sequence encoding a second cytokine, and a third expression cassette comprising a third promoter operably linked to fourth exogenous polynucleotide sequence encoding an activation-conditional control polypeptide (ACP), wherein the ACP comprises a synthetic transcription factor comprising a DNA-binding domain and a transcriptional effector domain,

wherein the ACP is capable of inducing expression of the third exogenous polynucleotide sequence by binding to the ACP-responsive promoter,

wherein at least one of the second exogenous polynucleotide sequence and the third exogenous polynucleotide sequence encodes a membrane-cleavable chimeric protein, oriented from N-terminal to C-terminal, having the formula:

S – C – MT or **MT – C – S**

wherein

S comprises a secretable effector molecule comprising the first and/or second cytokine,

C comprises a protease cleavage site, and

MT comprises a cell membrane tethering domain, and

wherein **S – C – MT** or **MT – C – S** is configured to be expressed as a single polypeptide.

Embodiment 21: The immunoresponsive cell of embodiment 20, wherein transcription of the second expression cassette is oriented in the opposite direction relative to transcription of the third expression cassette within the first engineered nucleic acid.

Embodiment 22: The immunoresponsive cell of embodiment 20 or embodiment 21, wherein the second expression cassette and the third expression cassette are oriented within the second engineered nucleic acid in a head-to-head directionality.

Embodiment 23: The immunoresponsive cell of any one of embodiments 15-22, wherein the first promoter comprises a constitutive promoter, an inducible promoter, or a synthetic promoter.

Embodiment 24: The immunoresponsive cell of embodiment 23, wherein the first promoter is a constitutive promoter selected from the group consisting of: CAG, HLP, CMV, EFS, SFFV, SV40, MND, PGK, UbC, hEF1aV1, hCAGG, hEF1aV2, hACTb, heIF4A1, hGAPDH, hGRP78, hGRP94, hHSP70, hKINb, and hUBIb.

Embodiment 25: The immunoresponsive cell of any one of embodiments 15-24, wherein the first exogenous polynucleotide sequence and the second exogenous polynucleotide sequence are separated by a linker polynucleotide sequence.

Embodiment 26: The immunoresponsive cell of embodiment 25, wherein the linker polynucleotide sequence is operably associated with the translation of the first cytokine and the CAR as separate polypeptides.

Embodiment 27: The immunoresponsive cell of embodiment 26, wherein the linker polynucleotide sequence encodes one or more 2A ribosome skipping elements.

Embodiment 28: The immunoresponsive cell of embodiment 27, wherein the one or more 2A ribosome skipping elements are each selected from the group consisting of: P2A, T2A, E2A, F2A, and combinations thereof.

Embodiment 29: The immunoresponsive cell of embodiment 28, wherein the one or more 2A ribosome skipping elements comprises an E2A/T2A combination.

Embodiment 30: The immunoresponsive cell of embodiment 29, wherein the E2A/T2A combination comprises the amino acid sequence of SEQ ID NO: 281.

Embodiment 31: The immunoresponsive cell of embodiment 25 or embodiment 26, wherein the linker polynucleotide sequence encodes an Internal Ribosome Entry Site (IRES).

Embodiment 32: The immunoresponsive cell of any one of embodiments 25-31, wherein the linker polynucleotide sequence encodes a cleavable polypeptide.

Embodiment 33: The immunoresponsive cell of embodiment 32, wherein the cleavable polypeptide comprises a furin polypeptide sequence.

Embodiment 34: The immunoresponsive cell of any one of embodiments 15-33, wherein the third promoter comprises a constitutive promoter, an inducible promoter, or a synthetic promoter.

Embodiment 35: The immunoresponsive cell of embodiment 34, wherein the third promoter is a constitutive promoter selected from the group consisting of: CAG, HLP, CMV, EFS, SFFV, SV40, MND, PGK, UbC, hEF1aV1, hCAGG, hEF1aV2, hACTb, heIF4A1, hGAPDH, hGRP78, hGRP94, hHSP70, hKINb, and hUBIb.

Embodiment 36: The immunoresponsive cell of any one of embodiments 1-35, wherein the first cytokine is IL-15.

Embodiment 37: The immunoresponsive cell of embodiment 36, wherein the IL-15 comprises the amino acid sequence of SEQ ID NO: 285.

Embodiment 38: The immunoresponsive cell of any one of embodiments 1-36, wherein the second cytokine is selected from the group consisting of: IL12, an IL12p70 fusion protein, IL18, and IL21.

Embodiment 39: The immunoresponsive cell of embodiment 38, wherein the second cytokine is the IL12p70 fusion protein.

Embodiment 40: The immunoresponsive cell of embodiment 39, wherein the IL12p70 fusion protein comprises the amino acid sequence of SEQ ID NO: 293.

Embodiment 41: The immunoresponsive cell of any one of embodiments 1-35, wherein the first cytokine is IL12 or an IL12p70 fusion protein.

Embodiment 42: The immunoresponsive cell of any one of embodiments 1-36, wherein the second cytokine is selected from the group consisting of: IL15, IL18, and IL21.

Embodiment 43: The immunoresponsive cell of any one of embodiments 1-42, wherein the protease cleavage site is cleavable by a protease selected from the group consisting of: a Type 1 transmembrane protease, a Type II transmembrane protease, a GPI anchored protease, an ADAM8 protease, an ADAM9 protease, an ADAM10 protease, an ADAM12 protease, an ADAM15 protease, an ADAM17 protease, an ADAM19 protease, an ADAM20 protease, an ADAM21 protease, an ADAM28 protease, an ADAM30 protease, an ADAM33 protease, a BACE1 protease, a BACE2 protease, a SIP protease, an MT1-MMP protease, an MT3-MMP protease, an MT5-MMP protease, a furin protease, a PCSK7 protease, a matriptase protease, a matriptase-2 protease, an MMP9 protease, and an NS3 protease.

Embodiment 44: The immunoresponsive cell of embodiment 43, wherein the protease cleavage site is cleavable by an ADAM17 protease.

Embodiment 45: The immunoresponsive cell of any one of embodiments 1-44, wherein the protease cleavage site comprises a first region having the amino acid sequence of PRAE (SEQ ID NO: 176).

Embodiment 46: The immunoresponsive cell of any one of embodiments 1-45, wherein the protease cleavage site comprises a second region having the amino acid sequence of KGG (SEQ ID NO: 177).

Embodiment 47: The immunoresponsive cell of embodiment 46, wherein the first region is located N-terminal to the second region.

Embodiment 48: The immunoresponsive cell of any one of embodiments 1-47, wherein the protease cleavage site comprises the amino acid sequence of $PRAEX_1X_2KGG$ (SEQ ID NO: 178),
wherein X_1 is A, Y, P, S, or F, and
wherein X_2 is V, L, S, I, Y, T, or A.

Embodiment 49: The immunoresponsive cell of embodiment 48, wherein the protease cleavage site comprises the amino acid sequence of PRAEAVKGG (SEQ ID NO: 179).

Embodiment 50: The immunoresponsive cell of embodiment 48, wherein the protease cleavage site comprises the amino acid sequence of PRAEALKGG (SEQ ID NO: 180).

Embodiment 51: The immunoresponsive cell of embodiment 48, wherein the protease cleavage site comprises the amino acid sequence of PRAEYSKGG (SEQ ID NO: 181).

Embodiment 52: The immunoresponsive cell of embodiment 48, wherein the protease cleavage site comprises the amino acid sequence of PRAEPIKGG (SEQ ID NO: 182).

Embodiment 53: The immunoresponsive cell of embodiment 48, wherein the protease cleavage site comprises the amino acid sequence of PRAEAYKGG (SEQ ID NO: 183).

Embodiment 54: The immunoresponsive cell of embodiment 48, wherein the protease cleavage site comprises the amino acid sequence of PRAESSKGG (SEQ ID NO: 184).

Embodiment 55: The immunoresponsive cell of embodiment 48, wherein the protease cleavage site comprises the amino acid sequence of PRAEFTKGG (SEQ ID NO: 185).

Embodiment 56: The immunoresponsive cell of embodiment 48, wherein the protease cleavage site comprises the amino acid sequence of PRAEAAKGG (SEQ ID NO: 186).

Embodiment 57: The immunoresponsive cell of embodiment 48, wherein the protease cleavage site comprises the amino acid sequence of DEPHYSQRR (SEQ ID NO: 187).

Embodiment 58: The immunoresponsive cell of embodiment 48, wherein the protease cleavage site comprises the amino acid sequence of PPLGPIFNPG (SEQ ID NO: 188).

Embodiment 59: The immunoresponsive cell of embodiment 48, wherein the protease cleavage site comprises the amino acid sequence of PLAQAYRSS (SEQ ID NO: 189).

Embodiment 60: The immunoresponsive cell of embodiment 48, wherein the protease cleavage site comprises the amino acid sequence of TPIDSSFNPD (SEQ ID NO: 190).

Embodiment 61: The immunoresponsive cell of embodiment 48, wherein the protease cleavage site comprises the amino acid sequence of VTPEPIFSLI (SEQ ID NO: 191).

Embodiment 62: The immunoresponsive cell of any one of embodiments 1-44, wherein the protease cleavage site comprises the amino acid sequence of ITQGLAVSTISSFF (SEQ ID NO: 198).

Embodiment 63: The immunoresponsive cell of any one of embodiments 1-62, wherein the protease cleavage site is comprised within a peptide linker.

Embodiment 64: The immunoresponsive cell of any one of embodiments 1-62, wherein the protease cleavage site is N-terminal to a peptide linker.

Embodiment 65: The immunoresponsive cell of embodiment 63 or embodiment 64, wherein the peptide linker comprises a glycine-serine (GS) linker.

Embodiment 66: The immunoresponsive cell of any one of embodiments 1-62, wherein the cell membrane tethering domain comprises a transmembrane-intracellular domain or a transmembrane domain.

Embodiment 67: The immunoresponsive cell of embodiment 66, wherein the transmembrane-intracellular domain and/or transmembrane domain is derived from PDGFR-beta, CD8, CD28, CD3zeta-chain, CD4, 4-1BB, OX40, ICOS, CTLA-4, PD-1, LAG-3, 2B4, LNGFR, NKG2D, EpoR, TNFR2, B7-1, or BTLA.

Embodiment 68: The immunoresponsive cell of embodiment 67, wherein the transmembrane-intracellular domain and/or transmembrane domain is derived from B7-1.

Embodiment 69: The immunoresponsive cell of embodiment 68, wherein the transmembrane-intracellular domain and/or transmembrane domain comprises the amino acid sequence of SEQ ID NO: 219.

Embodiment 70: The immunoresponsive cell of any one of embodiments 1-67, wherein the cell membrane tethering domain comprises a post-translational modification tag, or motif

capable of post-translational modification to modify the chimeric protein to include a post-translational modification tag, wherein the post-translational modification tag is capable of association with a cell membrane.

Embodiment 71: The immunoresponsive cell of embodiment 70, wherein the post-translational modification tag comprises a lipid-anchor domain, optionally wherein the lipid-anchor domain is selected from the group consisting of: a GPI lipid-anchor, a myristoylation tag, and a palmitoylation tag.

Embodiment 72: The immunoresponsive cell of any one of embodiments 1-71, wherein the cell membrane tethering domain comprises a cell surface receptor, or a cell membrane-bound portion thereof.

Embodiment 73: The immunoresponsive cell of any one of embodiments 1-72, wherein the cytokine of the membrane-cleavable chimeric protein is tethered to a cell membrane of the cell.

Embodiment 74: The immunoresponsive cell of any one of embodiments 1-73, wherein the cell further comprises a protease capable of cleaving the protease cleavage site.

Embodiment 75: The immunoresponsive cell of embodiment 74, wherein the protease is endogenous to the cell.

Embodiment 76: The immunoresponsive cell of embodiment 74, wherein the protease is selected from the group consisting of: a Type 1 transmembrane protease, a Type II transmembrane protease, a GPI anchored protease, an ADAM8 protease, an ADAM9 protease, an ADAM10 protease, an ADAM12 protease, an ADAM15 protease, an ADAM17 protease, an ADAM19 protease, an ADAM20 protease, an ADAM21 protease, an ADAM28 protease, an ADAM30 protease, an ADAM33 protease, a BACE1 protease, a BACE2 protease, a SIP protease, an MT1-MMP protease, an MT3-MMP protease, an MT5-MMP protease, a furin protease, a PCSK7 protease, a matriptase protease, a matriptase-2 protease, and an MMP9 protease.

Embodiment 77: The immunoresponsive cell of embodiment 76, wherein the protease is an ADAM17 protease.

Embodiment 78: The immunoresponsive cell of any one of embodiments 74-77, wherein the protease is expressed on the cell membrane of the cell.

Embodiment 79: The immunoresponsive cell of embodiment 78, wherein the protease is capable of cleaving the protease cleavage site.

Embodiment 80: The immunoresponsive cell of embodiment 79, wherein cleavage of the protease cleavage site releases the cytokine of the membrane-cleavable chimeric protein from the cell membrane of the cell.

Embodiment 81: The immunoresponsive cell of any one of embodiments 1-19 and 23-80, wherein the first exogenous polynucleotide sequence encodes a membrane-cleavable chimeric protein.

Embodiment 82: The immunoresponsive cell of any one of embodiments 15-81, wherein the first exogenous polynucleotide sequence further comprises a polynucleotide sequence encoding a secretion signal peptide.

Embodiment 83: The immunoresponsive cell of any one of embodiments 20-80, wherein the second exogenous polynucleotide sequence encodes a membrane-cleavable chimeric protein.

Embodiment 84: The immunoresponsive cell of any one of embodiments 15-83, wherein the second exogenous polynucleotide sequence further comprises a polynucleotide sequence encoding a secretion signal peptide

Embodiment 85: The immunoresponsive cell embodiment 82 or embodiment 84, wherein the secretion signal peptide is derived from a protein selected from the group consisting of: IL-12, Trypsinogen-2, Gaussia Luciferase, CD5, IgKVII, VSV-G, prolactin, serum albumin preproprotein, azurocidin preproprotein, osteonectin (BM40), CD33, IL-6, IL-8, CCL2, TIMP2, VEGFB, osteoprotegerin, serpin-E1, GROalpha, CXCL12, IL-21, CD8, GMCSFRa, NKG2D, and IgE.

Embodiment 86: The immunoresponsive cell of embodiment 82, wherein the secretion signal peptide is derived from GMCSFRa.

Embodiment 87: The immunoresponsive cell of embodiment 86, wherein the secretion signal peptide comprises the amino acid sequence of SEQ ID NO: 216.

Embodiment 88: The immunoresponsive cell of embodiment 84, wherein the secretion signal peptide is derived from IgE.

Embodiment 89: The immunoresponsive cell of embodiment 88, wherein the secretion signal peptide comprises the amino acid sequence of SEQ ID NO: 218.

Embodiment 90: The immunoresponsive cell of any one of embodiments 15-89, wherein the third exogenous polynucleotide sequence further comprises a polynucleotide sequence encoding a secretion signal peptide.

Embodiment 91: The immunoresponsive cell of embodiment 90, wherein the secretion signal peptide is operably associated with the second cytokine.

Embodiment 92: The immunoresponsive cell of embodiment 82 or embodiment 91, wherein the secretion signal peptide is native to the second cytokine.

Embodiment 93: The immunoresponsive cell of embodiment 82 or embodiment 91, wherein the secretion signal peptide is non-native to the second cytokine.

Embodiment 94: The immunoresponsive cell of any one of embodiments 20-93, wherein the third exogenous polynucleotide sequence encodes a membrane-cleavable chimeric protein.

Embodiment 95: The immunoresponsive cell of embodiment 94, wherein the second expression cassette further comprises a polynucleotide sequence encoding a secretion signal peptide.

Embodiment 96: The immunoresponsive cell of any one of embodiments 15-95, wherein the secretion signal peptide is operably associated with the first cytokine.

Embodiment 97: The immunoresponsive cell of embodiment 96, wherein the secretion signal peptide is native to the first cytokine.

Embodiment 98: The immunoresponsive cell of embodiment 96, wherein the secretion signal peptide is non-native to the first cytokine.

Embodiment 99: The immunoresponsive cell of any one of embodiments 15-98, wherein the first exogenous polynucleotide sequence encodes a first membrane-cleavable chimeric protein and the third exogenous polynucleotide sequence encodes a second membrane-cleavable chimeric protein.

Embodiment 100: The immunoresponsive cell of any one of embodiments 20-98, wherein the second exogenous polynucleotide sequence encodes a first membrane-cleavable chimeric protein and the third exogenous polynucleotide sequence encodes a second membrane-cleavable chimeric protein.

Embodiment 101: The immunoresponsive cell of any one of embodiments 1-100, wherein the CAR comprises an antigen-binding domain comprising a heavy chain variable (VH) region and a light chain variable (VL) region,

wherein the VH comprises:

a heavy chain complementarity determining region 1 (CDR-H1) having the amino acid sequence of KNAMN (SEQ ID NO: 199),

a heavy chain complementarity determining region 2 (CDR-H2) having the amino acid sequence of RIRNKTNNYATYYADSVKA (SEQ ID NO: 200), and

a heavy chain complementarity determining region 3 (CDR-H3) having the amino acid sequence of GNSFAY (SEQ ID NO: 201), and

wherein the VL comprises:

a light chain complementarity determining region 1 (CDR-L1) having the amino acid sequence of KSSQSLLYSSNQKNYLA (SEQ ID NO: 202),

a light chain complementarity determining region 2 (CDR-L2) having the amino acid sequence of WASSRES (SEQ ID NO: 203), and

a light chain complementarity determining region 3 (CDR-L3) having the amino acid sequence of QQYYNYPLT (SEQ ID NO: 204).

Embodiment 102: The immunoresponsive cell of embodiment 101, wherein the VH region comprises an amino acid sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to the amino acid sequence of EVQLVETGGGMVQPEGSLKLSCAASGFTFNKNAMNWVRQAPGKGLEWVARIR NKTNNYATYYADSVKARFTISRDDSQSMLYLQMNNLKIEDTAMYYCVAGNSFA YWGQGTLVTVSA (SEQ ID NO: 205) or EVQLVESGGGLVQPGGSLRLSCAASGFTFNKNAMNWVRQAPGKGLEWVGRIR NKTNNYATYYADSVKARFTISRDDSKNSLYLQMNSLKTEDTAVYYCVAGNSFA YWGQGTLVTVSA (SEQ ID NO: 206).

Embodiment 103: The immunoresponsive cell of embodiment 101, wherein the VH region comprises an amino acid sequence with at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to the amino acid sequence of SEQ ID NO: 206.

Embodiment 104: The immunoresponsive cell of any one of embodiments 101-103, wherein the VL region comprises an amino acid sequence with at least 90 %, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to the amino acid sequence of DIVMSQSPSSLVVSIGEKVTMTCKSSQSLLYSSNQKNYLAWYQQKPGQSPKLLIY WASSRESGVPDRFTGSGSGTDFTLTISSVKAEDLAVYYCQQYYNYPLTFGAGTK LELK (SEQ ID NO: 207), or DIVMTQSPDSLAVSLGERATINCKSSQSLLYSSNQKNYLAWYQQKPGQPPKLLIY

WASSRESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQQYYNYPLTFGQGTK LEIK (SEQ ID NO: 208).

Embodiment 105: The immunoresponsive cell of embodiment 104, wherein the VL region comprises an amino acid sequence with at least 90 %, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identity to the amino acid sequence of SEQ ID NO: 208.

Embodiment 106: The immunoresponsive cell of any one of embodiments 101-98, wherein the antigen-binding domain comprises a single chain variable fragment (scFv).

Embodiment 107: The immunoresponsive cell of any one of embodiments 101-106, wherein the VH and VL are separated by a peptide linker.

Embodiment 108: The immunoresponsive cell of embodiment 107, wherein the peptide linker comprises a glycine-serine (GS) linker.

Embodiment 109: The immunoresponsive cell of embodiment 108, wherein the GS linker comprises the amino acid sequence of (GGGGS)3 (SEQ ID NO: 223).

Embodiment 110: The immunoresponsive cell of embodiment 107, wherein the scFv comprises the structure VH-L-VL or VL-L-VH, wherein VH is the heavy chain variable domain, L is the peptide linker, and VL is the light chain variable domain.

Embodiment 111: The immunoresponsive cell of any one of embodiments 1-110, wherein the CAR comprises one or more intracellular signaling domains, and each of the one or more intracellular signaling domains is selected from the group consisting of: a CD3zeta-chain intracellular signaling domain, a CD97 intracellular signaling domain, a CD11a-CD18 intracellular signaling domain, a CD2 intracellular signaling domain, an ICOS intracellular signaling domain, a CD27 intracellular signaling domain, a CD154 intracellular signaling domain, a CD8 intracellular signaling domain, an OX40 intracellular signaling domain, a 4-1BB intracellular signaling domain, a CD28 intracellular signaling domain, a ZAP40 intracellular signaling domain, a CD30 intracellular signaling domain, a GITR intracellular signaling domain, an HVEM intracellular signaling domain, a DAP10 intracellular signaling domain, a DAP12 intracellular signaling domain, a MyD88 intracellular signaling domain, a 2B4 intracellular signaling domain, a CD16a intracellular signaling domain, a DNAM-1 intracellular signaling domain, a KIR2DS1 intracellular signaling domain, a KIR3DS1 intracellular signaling domain, a NKp44 intracellular signaling domain, a NKp46

intracellular signaling domain, a FceRlg intracellular signaling domain, a NKG2D intracellular signaling domain, and an EAT-2 intracellular signaling domain

Embodiment 112: The immunoresponsive cell of embodiment 111, wherein the one or more intracellular signaling domains comprises an OX40 intracellular signaling domain.

Embodiment 113: The immunoresponsive cell of embodiment 112, wherein the OX40 intracellular signaling domain comprises the amino acid sequence of SEQ ID NO: 269.

Embodiment 114: The immunoresponsive cell of embodiment 111, wherein the one or more intracellular signaling domains comprises a CD28 intracellular signaling domain.

Embodiment 115: The immunoresponsive cell of embodiment 114, wherein the CD28 intracellular signaling domain comprises the amino acid sequence of SEQ ID NO: 267.

Embodiment 116: The immunoresponsive cell of embodiment 111, wherein the one or more intracellular signaling domains comprises a CD3z intracellular signaling domain.

Embodiment 117: The immunoresponsive cell of embodiment 116, wherein the CD3z intracellular signaling domain comprises an amino acid sequence of SEQ ID NO: 277 or SEQ ID NO: 279.

Embodiment 118: The immunoresponsive cell of any one of embodiments 1-117, wherein the CAR comprises a transmembrane domain, and the transmembrane domain is selected from the group consisting of: a CD8 transmembrane domain, a CD28 transmembrane domain a CD3zeta-chain transmembrane domain, a CD4 transmembrane domain, a 4-1BB transmembrane domain, an OX40 transmembrane domain, an ICOS transmembrane domain, a CTLA-4 transmembrane domain, a PD-1 transmembrane domain, a LAG-3 transmembrane domain, a 2B4 transmembrane domain, a BTLA transmembrane domain, an OX40 transmembrane domain, a DAP10 transmembrane domain, a DAP12 transmembrane domain, a CD16a transmembrane domain, a DNAM-1 transmembrane domain, a KIR2DS1 transmembrane domain, a KIR3DS1 transmembrane domain, an NKp44 transmembrane domain, an NKp46 transmembrane domain, an FceRlg transmembrane domain, and an NKG2D transmembrane domain.

Embodiment 119: The immunoresponsive cell of embodiment 118, wherein the transmembrane domain is an OX40 transmembrane domain.

Embodiment 120: The immunoresponsive cell of embodiment 119, wherein the OX40 transmembrane domain comprises the amino acid sequence of SEQ ID NO: 244.

Embodiment 121: The immunoresponsive cell of embodiment 118, wherein the transmembrane domain is a CD8 transmembrane domain.

Embodiment 122: The immunoresponsive cell of embodiment 121, wherein the CD8 transmembrane domain comprises an amino acid sequence of SEQ ID NO: 236 or SEQ ID NO: 242.

Embodiment 123: The immunoresponsive cell of any one of embodiments 118-122, wherein the CAR comprises a spacer region between the antigen-binding domain and the transmembrane domain.

Embodiment 124: The immunoresponsive cell of embodiment 123, wherein the spacer region is derived from a protein selected from the group consisting of: CD8, CD28, IgG4, IgG1, LNGFR, PDGFR-beta, and MAG.

Embodiment 125: The immunoresponsive cell of embodiment 124, wherein the spacer region is a CD8 hinge.

Embodiment 126: The immunoresponsive cell of embodiment 125, wherein the CD8 hinge comprises the amino acid sequence of SEQ ID NO: 226 or SEQ ID NO: 228.

Embodiment 127: The immunoresponsive cell of any one of embodiments 1-123, wherein the ACP comprises a DNA binding domain and a transcriptional effector domain.

Embodiment 128: The immunoresponsive cell of embodiment 127, wherein the transcriptional effector domain comprises a transcriptional activator domain.

Embodiment 129: The immunoresponsive cell of embodiment 128, wherein the transcriptional activator domain is selected from the group consisting of: a Herpes Simplex Virus Protein 16 (VP16) activation domain; an activation domain comprising four tandem copies of VP16, a VP64 activation domain; a p65 activation domain of NFκB; an Epstein-Barr virus R transactivator (Rta) activation domain; a tripartite activator comprising the VP64, the p65, and the Rta activation domains (VPR activation domain); a histone acetyltransferase (HAT) core domain of the human E1A-associated protein p300 (p300 HAT core activation domain).

Embodiment 130: The immunoresponsive cell of embodiment 129, wherein the transcriptional activator domain comprises a VPR activation domain.

Embodiment 131: The immunoresponsive cell of embodiment 131, wherein the VPR activation domain comprises the amino acid sequence of SEQ ID NO: 325.

Embodiment 132: The immunoresponsive cell of embodiment 128, wherein the transcriptional effector domain comprises a transcriptional repressor domain.

Embodiment 133: The immunoresponsive cell of embodiment 132, wherein the transcriptional repressor domain is selected from the group consisting of: a Krüppel associated box (KRAB) repression domain; a truncated Krüppel associated box (KRAB) repression domain; a Repressor Element Silencing Transcription Factor (REST) repression domain; a WRPW motif of the hairy-related basic helix-loop-helix repressor proteins, the motif is known as a WRPW repression domain; a DNA (cytosine-5)-methyltransferase 3B (DNMT3B) repression domain; and an HP1 alpha chromoshadow repression domain.

Embodiment 134: The immunoresponsive cell of any one of embodiments 127-133, wherein the DNA binding domain comprises a zinc finger (ZF) protein domain.

Embodiment 135: The immunoresponsive cell of embodiment 134, wherein the ZF protein domain is modular in design and comprises an array of zinc finger motifs.

Embodiment 136: The immunoresponsive cell of embodiment 134, wherein the ZF protein domain comprises an array of one to ten zinc finger motifs.

Embodiment 137: The immunoresponsive cell of embodiment 136, wherein the ZF protein domain comprises the amino acid sequence of SEQ ID NO: 320.

Embodiment 138: The immunoresponsive cell of any one of embodiments 1-136, wherein the ACP further comprises a repressible protease and one or more cognate cleavage sites of the repressible protease.

Embodiment 139: The immunoresponsive cell of embodiment 138, wherein the repressible protease is hepatitis C virus (HCV) nonstructural protein 3 (NS3).

Embodiment 140: The immunoresponsive cell of embodiment 139, wherein the NS3 protease comprises the amino acid sequence of SEQ ID NO: 321.

Embodiment 141: The immunoresponsive cell of embodiment 138 or embodiment 139, wherein the cognate cleavage site of the repressible protease comprises an NS3 protease cleavage site.

Embodiment 142: The immunoresponsive cell of embodiment 141, wherein the NS3 protease cleavage site comprises a NS3/NS4A, a NS4A/NS4B, a NS4B/NS5A, or a NS5A/NS5B junction cleavage site.

Embodiment 143: The immunoresponsive cell of any one of embodiments 139-142, wherein the NS3 protease is repressible by a protease inhibitor.

Embodiment 144: The immunoresponsive cell of embodiment 143, wherein the protease inhibitor is selected from the group consisting of: simeprevir, danoprevir, asunaprevir, ciluprevir, boceprevir, sovaprevir, paritaprevir, telaprevir, grazoprevir, glecaprevir, and voxiloprevir.

Embodiment 145: The immunoresponsive cell of embodiment 144, wherein the protease inhibitor is grazoprevir (GRZ).

Embodiment 146: The immunoresponsive cell of any one of embodiments 1-145, wherein the ACP further comprises a nuclear localization signal (NLS).

Embodiment 147: The immunoresponsive cell of embodiment 146, wherein the NLS comprises the amino acid sequence of SEQ ID NO: 296.

Embodiment 148: The immunoresponsive cell of any one of embodiments 138-144, wherein the one or more cognate cleavage sites of the repressible protease are localized between the DNA binding domain and the transcriptional effector domain.

Embodiment 149: The immunoresponsive cell of any one of embodiments 1-148, wherein the ACP further comprises a hormone binding domain of estrogen receptor variant ERT2.

Embodiment 150: The immunoresponsive cell of any one of embodiments 1-149, wherein the ACP-responsive promoter is a synthetic promoter.

Embodiment 151: The immunoresponsive cell of any one of embodiments 1-150, wherein the ACP-responsive promoter comprises an ACP binding domain sequence and a minimal promoter sequence.

Embodiment 152: The immunoresponsive cell of embodiment 151, wherein the ACP binding domain sequence comprises one or more zinc finger binding sites.

Embodiment 153: The immunoresponsive cell of any one of embodiments 1,15, or 20, wherein the first engineered nucleic acid comprises a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 309.

Embodiment 154: The immunoresponsive cell of any one of embodiments 1,15, or 20, wherein the first engineered nucleic acid comprises a nucleotide sequence at least 90%,

at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 326.

Embodiment 155: The immunoresponsive cell of any one of embodiments 1,15, or 20, wherein the first engineered nucleic acid comprises a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 310.

Embodiment 156: The immunoresponsive cell of any one of embodiments 1,15, or 20, wherein the first engineered nucleic acid comprises a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 327.

Embodiment 157: The immunoresponsive cell of any one of embodiments 1,15, or 20, wherein the first engineered nucleic acid comprises a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 314.

Embodiment 158: The immunoresponsive cell of any one of embodiments 1,15, or 20, wherein the first engineered nucleic acid comprises a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 315.

Embodiment 159: The immunoresponsive cell of any one of embodiments 1-11 or 20-152, wherein the second engineered nucleic acid comprises a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 317.

Embodiment 160: The immunoresponsive cell of any one of embodiments 1-11 or 20-152, wherein the second engineered nucleic acid comprises a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 318.

Embodiment 161: An immunoresponsive cell comprising:

a) a first engineered nucleic acid comprising the nucleotide sequence of SEQ ID NO: 310; and

b) a second engineered nucleic acid comprising the nucleotide sequence of SEQ ID NO: 317.

Embodiment 162: An immunoresponsive cell comprising:

a) a first engineered nucleic acid comprising the nucleotide sequence of SEQ ID NO: 327; and

c) a second engineered nucleic acid comprising the nucleotide sequence of SEQ ID NO: 317.

Embodiment 163: The immunoresponsive cell of any one of embodiments 1-162, wherein the cell is selected from the group consisting of: a T cell, a CD8+ T cell, a CD4+ T cell, a gamma-delta T cell, a cytotoxic T lymphocyte (CTL), a regulatory T cell, a viral-specific T cell, a Natural Killer T (NKT) cell, a Natural Killer (NK) cell, a B cell, a tumor-infiltrating lymphocyte (TIL), an innate lymphoid cell, a mast cell, an eosinophil, a basophil, a neutrophil, a myeloid cell, a macrophage, a monocyte, a dendritic cell, an erythrocyte, a platelet cell, a human embryonic stem cell (ESC), an ESC-derived cell, a pluripotent stem cell, a mesenchymal stromal cell (MSC), an induced pluripotent stem cell (iPSC), and an iPSC-derived cell.

Embodiment 164: The immunoresponsive cell of any one of embodiments 1-162, wherein the cell is a Natural Killer (NK) cell.

Embodiment 165: The immunoresponsive cell of embodiment 163 or embodiment 164, wherein the cell is autologous.

Embodiment 166: The immunoresponsive cell of embodiment 163 of embodiment 164, wherein the cell is allogeneic.

Embodiment 167: An engineered nucleic acid comprising:

a first expression cassette comprising a first promoter operably linked to a first, exogenous polynucleotide sequence encoding IL15, and

a second expression cassette comprising a second promoter operably linked to a second exogenous polynucleotide sequence encoding a chimeric antigen receptor (CAR) that binds to GPC3,

wherein the first exogenous polynucleotide sequence encodes a membrane-cleavable chimeric protein, oriented from N-terminal to C-terminal, having the formula:

S – C – MT or **MT – C – S**

wherein

S comprises a secretable effector molecule comprising the IL15,

C comprises a protease cleavage site, and

MT comprises a cell membrane tethering domain, and

wherein **S – C – MT** or **MT – C – S** is configured to be expressed as a single polypeptide.

Embodiment 168: The engineered nucleic acid of embodiment 167, wherein

a) the first expression cassette and the second expression cassette are oriented within the first engineered nucleic acid in a head-to-tail directionality,

b) the first exogenous polynucleotide sequence and the second exogenous polynucleotide sequence are separated by a linker polynucleotide sequence comprising an E2A/T2A ribosome skipping element, and

c) the CAR that binds to GPC3 comprises a CD28 intracellular signaling domain or an OX40 intracellular signaling domain.

Embodiment 169: An engineered nucleic acid comprising:

a first expression cassette comprising a first promoter operably linked to a first, exogenous polynucleotide sequence encoding a chimeric antigen receptor (CAR) that binds to GPC3 and a second exogenous polynucleotide sequence encoding IL15,

wherein the first exogenous polynucleotide sequence encodes a membrane-cleavable chimeric protein, oriented from N-terminal to C-terminal, having the formula:

S – C – MT or **MT – C – S**

wherein

S comprises a secretable effector molecule comprising the IL15,

C comprises a protease cleavage site, and

MT comprises a cell membrane tethering domain, and

wherein **S – C – MT** or **MT – C – S** is configured to be expressed as a single polypeptide.

Embodiment 170: The engineered nucleic acid of embodiment 169, wherein

a) the first exogenous polynucleotide sequence and the second exogenous polynucleotide sequence are separated by a linker polynucleotide sequence comprising an E2A/T2A ribosome skipping element, and

b) the CAR that binds to GPC3 comprises a CD28 intracellular signaling domain or an OX40 intracellular signaling domain.

Embodiment 171: The engineered nucleic acid of any one of embodiments 167-170, wherein the engineered nucleic acid comprises a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 309.

Embodiment 172: The engineered nucleic acid of any one of embodiments 167-170, wherein the engineered nucleic acid comprises a nucleotide sequence at least 90%, at least 91%,

at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 326.

Embodiment 173: The engineered nucleic acid of any one of embodiments 167-170,, wherein the engineered nucleic acid comprises a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 310.

Embodiment 174: The engineered nucleic acid of any one of embodiments 167-170,, wherein the engineered nucleic acid comprises a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 327.

Embodiment 175: The engineered nucleic acid of any one of embodiments 167-170,, wherein the engineered nucleic acid comprises a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 314.

Embodiment 176: The engineered nucleic acid of any one of embodiments 167-170,, wherein the engineered nucleic acid comprises a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 315.

Embodiment 177: An engineered nucleic acid comprising the nucleotide sequence of SEQ ID NO: 310.

Embodiment 178: An engineered nucleic acid comprising the nucleotide sequence of SEQ ID NO: 327.

Embodiment 179: An engineered nucleic acid comprising:

a first expression cassette comprising a synthetic transcription factor-responsive promoter operably linked to a first exogenous polynucleotide sequence encoding an IL12p70 fusion protein, and

a second expression cassette comprising a second promoter operably linked to a second exogenous polynucleotide sequence encoding an activation-conditional control polypeptide (ACP), wherein the ACP comprises a synthetic transcription factor comprising a DNA-binding domain and a transcriptional effector domain,

wherein the ACP is capable of inducing expression of the first exogenous polynucleotide sequence by binding to the ACP-responsive promoter,

wherein the first exogenous polynucleotide sequence encodes a membrane-cleavable chimeric protein, oriented from N-terminal to C-terminal, having the formula:

S – C – MT or **MT – C – S**

wherein

S comprises a secretable effector molecule comprising the IL12p70 fusion protein,

C comprises a protease cleavage site, and

MT comprises a cell membrane tethering domain, and

wherein **S – C – MT** or **MT – C – S** is configured to be expressed as a single polypeptide.

Embodiment 180: The engineered nucleic acid of embodiment 179, wherein

a) the first expression cassette and the second expression cassette are oriented within the first engineered nucleic acid in a head-to-head directionality, and

b) the ACP comprises a DNA binding domain and a transcriptional effector domain, wherein the transcriptional activator domain comprises a VPR activation domain.

Embodiment 181: The engineered nucleic acid of embodiment 179 or 180, wherein the engineered nucleic acid comprises a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 317.

Embodiment 182: The engineered nucleic acid of embodiment 179 or 180, wherein the engineered nucleic acid comprises a nucleotide sequence at least 90%, at least 91%, at least 92%, at least 93%, at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, or at least 99% identical to SEQ ID NO: 318.

Embodiment 183: An engineered nucleic acid comprising the nucleotide sequence of SEQ ID NO: 317.

Embodiment 184: An expression vector comprising the engineered nucleic acid of any one of embodiments 167-183.

Embodiment 185: An immunoresponsive cell comprising the engineered nucleic acid of any one of embodiments 167-183 or the expression vector of embodiment 184.

Embodiment 186: A pharmaceutical composition comprising the immunoresponsive cell of any one of embodiments 1-166 or 185, the engineered nucleic acid of any one of embodiments 167-183, or the expression vector of embodiment 184, and a pharmaceutically acceptable carrier, pharmaceutically acceptable excipient, or a combination thereof.

Embodiment 187: A method of treating a subject in need thereof, the method comprising administering a therapeutically effective dose of any of the immunoresponsive cells of any one of embodiments 1-166 or 185 ,the engineered nucleic acid of any one of embodiments 167-183, the expression vector of embodiment 184, or the pharmaceutical composition of embodiment 186.

Embodiment 188: A method of stimulating a cell-mediated immune response to a tumor cell in a subject, the method comprising administering to a subject having a tumor a therapeutically effective dose of any of the immunoresponsive cells of any one of embodiments 1-166 or 185 ,the engineered nucleic acid of any one of embodiments 167-183, the expression vector of embodiment 184, or the pharmaceutical composition of embodiment 186.

Embodiment 189: A method of reducing tumor volume in a subject, the method comprising administering to a subject having a tumor a composition comprising any of the immunoresponsive cells of any one of embodiments 1-166 or 185 ,the engineered nucleic acid of any one of embodiments 167-183, the expression vector of embodiment 184, or the pharmaceutical composition of embodiment 186.

Embodiment 190: A method of providing an anti-tumor immunity in a subject, the method comprising administering to a subject in need thereof a therapeutically effective dose of any of the immunoresponsive cells of any one of embodiments 1-166 or 185 ,the engineered nucleic acid of any one of embodiments 167-183, the expression vector of embodiment 184, or the pharmaceutical composition of embodiment 186.

Embodiment 191: The method of any one of embodiments 188-190, wherein the tumor comprises a GPC3-expressing tumor.

Embodiment 192: The method of any one of embodiments 188-191, wherein the tumor is selected from the group consisting of: hepatocellular carcinoma (HCC), ovarian clear cell carcinoma, melanoma, squamous cell carcinoma of the lung, hepatoblastoma, nephroblastoma (Wilms tumor), and yolk sac tumor.

Embodiment 193: A method of treating a subject having cancer, the method comprising administering a therapeutically effective dose of any of the immunoresponsive cells of any one of embodiments 1-166 or 185 ,the engineered nucleic acid of any one of embodiments 167-183, the expression vector of embodiment 184, or the pharmaceutical composition of embodiment 186.

Embodiment 194: The method of embodiment 193, wherein the cancer comprises a GPC3-expressing cancer.

Embodiment 195: The method of embodiment 193 or embodiment 194, wherein the cancer is selected from the group consisting of: hepatocellular carcinoma (HCC), ovarian clear cell carcinoma, melanoma, squamous cell carcinoma of the lung, hepatoblastoma, nephroblastoma (Wilms tumor), and yolk sac tumor.

Embodiment 196: The method of any one of embodiments 187-195, wherein the administering comprises systemic administration.

Embodiment 197: The method of any one of embodiments 187-195, wherein the administering comprises intratumoral administration.

Embodiment 198: The method of any one of embodiments 187-197, wherein the immunoresponsive cell is derived from the subject.

Embodiment 199: The method of any one of embodiments 187-198, wherein the immunoresponsive cell is allogeneic with reference to the subject.

EXAMPLES

Below are examples of specific embodiments for carrying out the present invention. The examples are offered for illustrative purposes only, and are not intended to limit the scope of the present invention in any way. For example, the experiments described and performed below demonstrate the general utility of engineering cells to secrete payloads (*e.g.*, effector molecules) and delivering those cells to induce an immunogenic response against tumors.

Efforts have been made to ensure accuracy with respect to numbers used (*e.g.*, amounts, temperatures, etc.), but some experimental error and deviation should, of course, be allowed for.

Example 1: Expression and Function of an anti-GPC3 CAR + IL15 Bidirectional Construct

Protein expression, cellular activation, and killing activity of cells transduced with anti-GPC3 CAR + IL15 bidirectional constructs were assessed. A cartoon diagram of the bidirectional orientation of the constructs is shown in **FIG. 1**.

Materials and Methods

Primary, donor-derived NK cells were transduced (50,000 to 100,000 cells/transduction) in a non-TC treated retronectin coated plate with lentivirus (at a multiplicity of infection, MOI, of 40) or retrovirus (SinVec, approximately 400µl each) encoding constructs having a first expression cassette encoding an anti-GPC3 CAR and a second expression cassette encoding

IL15, with the two expression cassettes in a head-to-head bidirectional orientation. Constructs varied in the intracellular domains of the CAR, having 4-1BB and CD3-zeta signaling domains (41BBz), CD28 and CD3-zeta signaling domains (CD28z), OX40 and CD3-zeta signaling domains (OX40z) or a KIR3DS1 signaling domain (KIR3DS1), and transductions using either a lentivirus or a retrovirus system were compared for each construct. As a control, transductions were also performed with retroviruses and lentiviruses encoding each of the same CARs, but without the IL15 expression cassette ("CAR-only). After transduction, NK cells were rested in the same plate for 3 days before transfer to a 24-well non-adherent cell-optimized plate. NK cells were expanded to a total of 5 ml with a first cytokine spike-in on day 7 following transduction and a second cytokine spike-in on day 15 (each spike-in included 500 IU/ml IL12 for the CAR+IL15 transductions and the CAR-only transductions, and 10ng/ml IL15 for the CAR only constructs).

On days five and seven following transduction, CAR expression was assessed by flow cytometry for each construct. Day seven CAR expression from cells transduced with lentivirus encoding a bidirectional CAR + IL15 bidirectional construct and cells transduced with a lentivirus encoding the CAR-only is shown in **FIG. 2**. Day seven CAR expression from cells transduced with retrovirus encoding a bidirectional CAR + IL15 bidirectional construct and cells transduced with a retrovirus encoding the CAR-only is shown in **FIG. 3**. Day fifteen CAR expression from cells transduced with lentivirus encoding a bidirectional CAR + IL15 bidirectional construct and cells transduced with a lentivirus encoding the CAR-only is shown in **FIG. 4**. Day fifteen CAR expression from cells transduced with retrovirus encoding a bidirectional CAR + IL15 bidirectional construct and cells transduced with a retrovirus encoding the CAR-only is shown in **FIG. 5**.

On day seven following transduction, a payload assay was conducted to assess IL15 levels for each construct. 200,000 cells per well were plated in 200µl media (NK MACs complete media with IL2) in a 96-well plate. NK cells were incubated for 48 hours, and then IL15 levels were assessed by immunoassay. IL15 expression is shown in **FIG. 6**.

Co-culture killing assays were then performed. 25,000 target cells (a Huh7 mKate cell line or a HepG2 mKate cell line) per well were plated in a 96-well plate. Effector cells (the NK cells expressing each construct) were added to the plate at effector to target (E to T) cell ratios of 1:1 or 0.5:1, and the cells were cultured with NK MACs complete media without cytokines in a total volume of 200µl. Two to three days following co-culture, real-time, fluorescence-based assays to measure mKate levels were performed to assess target cell killing. Killing by lentivirus-transduced NK cells expressing each construct is shown in **FIG. 7**, and killing by retrovirus-transduced NK cells expressing each construct is shown in **FIG. 8**.

Results

CAR expression from NK cells transduced with each construct was assessed. As shown in **FIG. 2**, at day seven transduced NK cells had measurable CAR expression for each construct, with at least 10% of cells in each transduced population positive for CAR expression. As shown in **FIG. 3**, at day fifteen lentivirus-transduced NK cells had measurable CAR expression for each construct (top panel), with at least 20% of cells in each transduced population positive for CAR expression. Additionally, as shown in **FIG. 3**, retrovirus-transduced NK cells expressing the 28z CAR + IL15 bidirectional construct had measurable CAR expression, with at least 42% of cells in the transduced population positive for CAR expression.

IL15 expression by NK cells transduced with each construct was also assessed. Assay of IL15 expression by non-transduced cells and Ox40z CAR-only cells was performed as a negative control. As shown in **FIG. 6**, retrovirus-transduced NK cells expressing bidirectional CAR + IL15 had statistically significant increase in IL15 production over reciprocal lentivirus-transduced NK cells.

Killing by NK cells transduced with each construct was then assessed. As shown in **FIG. 7**, lentivirus-transduced NK cells expressing the CAR + IL15 bidirectional construct had statistically significant increased killing over lentivirus-transduced NK cells expressing the CAR alone (without the IL15 expression cassette). As shown in **FIG. 8**, retrovirus-transduced NK cells expressing the CAR + IL15 bidirectional construct had statistically significant increased killing over retrovirus-transduced NK cells expressing the CAR alone (without the IL15 expression cassette).

Example 2: Expression of IL12 from Bidirectional Constructs Encoding a Regulatable IL12 and a Synthetic Transcription Factor

IL12 expression was assessed from NK cells transduced to express bidirectional constructs including a first expression cassette encoding a regulatable IL12 and a second expression cassette encoding a synthetic transcription factor. The regulatable IL12 is operably linked to a synthetic transcription factor-responsive promoter, which includes a ZF-10-1 binding site and a minimal promoter sequence (YBTATA). The synthetic transcription factor includes a DNA binding domain (an array of six zinc finger motifs known as ZF-10-1) and a transcriptional activation domain (Vpr). Between the DNA biding domain and the transcriptional activation domain is a protease domain (NS3) and cognate cleavage site for the protease. In the absence of an inhibitor of the protease, the protease induces cleavage at the cleavage site, resulting in a non-functional synthetic transcription factor. In the presence of the protease inhibitor, the synthetic transcription factor is not cleaved and is thus capable of modulating expression of the IL12. Constructs tested included IL12 expression cassettes having mRNA destabilization elements in

the 3' untranslated region. A cartoon diagram of the bidirectional orientation of the constructs is shown in **FIG. 9**.

Materials and Methods

Bidirectional constructs including two expression cassettes, a first expression cassette encoding a regulatable IL12 and a second expression cassette encoding a small molecule-regulatable synthetic transcription factor, were produced. A first construct lacks an mRNA destabilization element ("WT"), and four constructs each include a different mRNA destabilization element added to the 5' non-coding region. The four destabilization elements used were: 1) an AU-rich motif ("AU" or "1XAU"); 2) a stem-loop destabilization element ("SLDE" or "1XSLDE"); 3) a tandem AU motif and SLDE motif ("AuSLDE" or "1X AuSLDE"); and 4) two repeated AuSLDE motifs (2X AuSLDE). The destabilization elements were added to attempt to reduce leakiness of IL12 expression in the absence of the small molecule regulator of the synthetic promoter (e.g., grazoprevir).

Primary, donor-derived NK cells were expanded for ten days and grown in IL21 and IL15, with K562 feeder cells, and then were transduced with a multiplicity of infection (MOI) of 40 (as determined by infection units titer) in a retronectin-coated 24 well plate, following Bx795 pre-treatment. Transduction was performed with spinoculation, at 800g for 2 hours at 32°C.

On day three following transduction, NK cells were counted and seeded at 1e6 cells/mL with no drug or 0.1uM grazoprevir (GRZ) for 24 hours.

On day four following transduction (with 24 hours of drug treatment), supernatant was harvested and analyzed for IL12 levels by immunoassay. IL12 concentrations for each cell type and condition are shown in **FIG. 10**.

Results

As shown in **FIG. 10**, NK cells transduced with each construct demonstrated increased IL12 expression following treatment with grazoprevir, as compared to the absence of drug. NK cells transduced with the IL12 lacking a destabilization element ("WT") had greater than 19-fold induction of IL12 expression following treatment with grazoprevir. However, NK cells transduced with constructs that included destabilization tags demonstrated about a 457-fold, 58-fold, 50-fold, and 89-fold induction of IL12 upon treatment with grazoprevir for 2X AuSLDE, 1X AuSLDE, 1X AU, and 1X SLDE, respectively. Additionally, each of the destabilization tags decreased the baseline IL12 expression in the absence of grazoprevir. Furthermore, the construct encoding an IL12 with a 2X AuSLDE destabilization element resulted in a non-detectable level of IL12 expression in the absence of grazoprevir.

Example 3: Expression and Function of anti-GPC3 CAR + IL15 Bidirectional Constructs

Protein expression, cellular activation, and killing activity of cells transduced with anti-GPC3 CAR + cleavable release IL15 bidirectional constructs were assessed. The expression cassette encoding the cleavable release IL15 includes a chimeric polypeptide including the IL15 and a transmembrane domain. Between the IL15 and the transmembrane domain is a protease cleavage domain that is cleavable by a protease endogenous to NK cells. A cartoon diagram of the bidirectional construct encoding a cleavable release IL15 is shown in **FIG. 11**.

Briefly, primary, donor-derived NK cells were transduced with viral vectors encoding constructs having a first expression cassette encoding an anti-GPC3 CAR and a second expression cassette encoding a cleavable release IL15 expression cassette, with the two expression cassettes in a head-to-head bidirectional orientation.

Culture Supernatant: Spinoculation of NK cells was performed (day 0). A partial culture media exchange was performed on days 1, 2, and 6. Cell culture supernatant was harvested on day 8.

Flow cytometry: On day 10 following transduction, CAR and mbIL15 expression was assessed by flow cytometry for each construct. NK cells were stained with an IL-15 primary antibody and PE-secondary, and rhGPC3-FITC and Sytox blue (viability stain). Cells were run on Cytoflex and analyzed using Flowjo for CAR/mbIL15 expression.

Payload assay: On day 7 or 8 following transduction, a payload assay was conducted to assess IL15 levels for each construct. 200,000 cells per well were plated in 200 µl media (NK MACs complete media with IL2 only) in a 96-well plate, run in duplicates. Cells were incubated for 48 hours, and then cleaved IL15 levels were assessed by Luminex immunoassay.

Serial killing assay: Co-culture killing assays were performed. About 25,000 target cells (a Huh7 mKate cell line or a HepG2 mKate cell line) per well were plated in a 96-well plate. Effector cells (the NK cells expressing each construct) were added to the plate at effector to target (E to T) cell ratios of 1:1 in triplicates, and the cells were cultured with NK MAC complete media (no cytokines) in a total volume of 200 µl. Real-time, fluorescence-based assays were used to measure mKate to assess target cell killing in a serial-killing assay performed at 37° C; initial killing was at day 9 post-transduction, serial one was at day 11 post-transduction, and serial 2 was at day 14 post transduction.

Over 150 IL15 cleavable release (crIL15) constructs were designed, and 33 constructs were selected for experimental testing. (see **Table 7A**). Each construct was tested in two viral backbones (*e.g.*, SB06250 and SB06256, as shown in **Table 7A**). A summary of expression and killing activity of cells expressing a subset of bicistronic constructs is shown in **Table 7B**. Full-

length sequences of a subset of constructs are shown in **Table 7C**. A summary of bicistronic constructs tested and their functional activities is provided in **FIG. 12**.

Table 7A.

Construct	SB# (CD3 Senti)	SB# (CD3mut)
GPC3-CAR (41BB) 2A crIL15(Tace10)	SB06250	SB06290
	SB06256	SB06296
GPC3-CAR (OX40) 2A crIL15(Tace10)	SB06251	SB06291
	SB06257	SB06297
GPC3-CAR (CD28) 2A crIL15(Tace10)	SB06252	SB06292
	SB06258	SB06298
crIL15(Tace10) 2A GPC3-CAR (41BB)	SB06253	SB06293
	SB06259	SB06299
crIL15(Tace10) 2A GPC3-CAR (OX40)	SB06254	SB06294
	SB06260	SB06300
crIL15(Tace10) 2A GPC3-CAR (CD28)	SB06255	SB06295
	SB06261	SB06301
crIL15(TaceOPT) 2A GPC3-CAR (41BB)	SB06685	SB06688
	SB06691	SB06694
crIL15(TaceOPT) 2A GPC3-CAR (OX40)	SB06686	SB06689
	SB06692	SB06695
crIL15(TaceOPT) 2A GPC3-CAR (CD28)	SB06687	SB06690
	SB06693	SB06696

Table 7B.

Virus	SB#	CAR%	IL15%	IL15(pg/ml)	%Target cell growth (round3)[a]	Hinge	TM	Co-stim	CD3z	IL15	Description
Retrovec	SB06252	76.7 60.6	64.8 51.2	151 117	n/a 70	CD8FA	CD8FA	CD28	wt	Tace10	CAR 2A crIL15
SinVec	SB06258	66.8 52.5	38.5 30.6	84 74	n/a 62						
Retrovec	SB06255	59.8 37.5	67.6 41.0	54 68	n/a 81.4						crIL15 2A CAR
Retrovec	SB06251	64.2 44.2	30.9 18.5	17 65	11.2* 22	CD8 S2L	OX40	OX40	wt	Tace1 0	CAR 2A crIL15
SinVec	SB06257	78.3 55.8	30.1 15.8	53 40	59* 39						
Retrovec	SB06254	67.5 48.9	52.2 30.1	137 43	89* 74						crIL15 2A CAR
Retrovec	SB06294	60 71	39 58	50 27	8	CD8 S2L	OX40	OX40	CD3z- Alt	Tace1 0	crIL15 2A CAR

a Normalized to Target cells alone

* crIL-15 control did not function as expected

** crIL-15 control did not killed as*

Table 7C.

Construct	Full nucleotide sequence
SB06251	aagctttgctcttaggagtttcctaatacatcccaaactcaaatatataaagcatttgacttgttctatgccctaggggcgggggaagcta agccagctttttttaacatttaaaatgttaattccattttaaatgcacagatgtttttatttcataagggtttcaatgtgcatgaatgctgcaatattc ctgttaccaaagctagtataaataaaaatagataaacgtggaaattacttagagtttctgtcattaacgtttccttcctcagttgacaacataaa tgcgctgctgagaagccagtttgcatctgtcaggatcaatttcccattatgccagtcatattaattactagtcaattagttgatttttatttttgac atatacatgtgaaagaccccacctgtaggtttggcaagctagcttaagtaacgccattttgcaaggcatggaaaaatacataactgagaat agaaaagttcagatcaaggtcaggaacagatggaacagctgaatatgggccaaacaggatatctgtggtaagcagttcctgccccggc tcagggccaagaacagatggaacagctgaatatgggccaaacaggatatctgtggtaagcagttcctgccccggctcagggccaaga acagatggtccccagatgcggtccagccctcagcagtttctagagaaccatcagatgtttccagggtgccccaaggacctgaaatgacc ctgtgccttatttgaactaaccaatcagttcgcttctcgcttctgttcgcgcgcttctgctcccgagctcaataaaagagcccacaacccct cactcggcgcgccagtcctccgattgactgagtcgcccgggtacccgtgtatccaataaaccctcttgcagttgcatccgacttgtggtct cgctgttccttgggagggtctcctctgagtgattgactacccgtcagcgggggtctttcatttgggggctcgtccgagatcgggagaccc ctgcccagggaccaccgacccaccaccgggaggtaagctggccagcaacttatctgtgtctgtccgattgtctagtgtctatgactgattt tatgcgcctgcgtcggtactagttagctaactagctctgtatctggcggacccgtggtggaactgacgagttcggaacacccggccgca accctgggagacgtcccagggacttcgggggccgtttttgtggcccgacctgagtcctaaaatcccgatcgtttaggactctttggtgca ccccccttagaggagggatatgtggttctggtaggagacgagaacctaaaacagttcccgcctccgtctgaatttttgctttcggtttggga ccgaagccgcgccgcgcgtcttgtctgctgcagcatcgttctgtgttgtctctgtctgactgtgtttctgtatttgtctgaaaatatggatcttat atggggcacccccgcccccttgtaaacttccctgaccctgacatgacaagagttactaacagcccctctctccaagctcacttacaggctct ctacttagtccagcacgaagtctggagacctctggcggcagcctaccaagaacaactggaccgaccggtggtacctcacccttaccga gtcggcgacacagtgtgggtccgccgacaccagactaagaacctagaacctcgctggaaaggaccttacacagtcctgctgaccacc cccaccgccctcaaagtagacggcatcgcagcttggatacacgccgcccacgtgaaggctgccgaccccgggggtggaccatcctct agactgccggatccGCCGCCACCATGCTGCTGCTGGTCACATCTCTGCTGCTGTGCGAGCT GCCCCATCCTGCCTTTCTGCTGATCCCTCACATGGACATCGTGATGACACAGAGCC CCGATAGCCTGGCCGTGTCTCTGGGAGAAAGAGCCACCATCAACTGCAAGAGCAG CCAGAGCCTGCTGTACTCCAGCAACCAGAAGAACTACCTGGCCTGGTATCAGCAA AAGCCCGGCCAGCCTCCTAAGCTGCTGATCTATTGGGCCAGCTCCAGAGAAAGCG GCGTGCCCGATAGATTTTCTGGCTCTGGCAGCGGCACCGACTTCACCCTGACAATT TCTAGCCTGCAAGCCGAGGACGTGGCCGTGTACTACTGCCAGCAGTACTACAACT ACCCTCTGACCTTCGGCCAGGGCACCAAGCTGGAAATCAAAGGCGGCGGAGGATC TGGCGGAGGTGGAAGTGGCGGAGGCGGATCTGAAGTGCAGCTGGTTGAATCAGG TGGCGGCCTGGTTCAACCTGGCGGATCTCTGAGACTGAGCTGTGCCGCCAGCGGC TTCACCTTCAACAAGAACGCCATGAACTGGGTCCGACAGGCCCCTGGCAAAGGCC TTGAATGGGTCGGACGGATCCGGAACAAGACCAACAACTACGCCACCTACTACGC CGACAGCGTGAAGGCCAGATTCACCATCAGCCGGGACGACAGCAAGAACAGCCT GTACCTGCAGATGAACTCCCTGAAAACCGAGGACACCGCCGTGTATTATTGCGTG GCCGGCAACAGCTTTGCCTACTGGGGACAGGGAACCCTGGTCACCGTGTCTGCCA CAACAACCCCTGCTCCTAGACCTCCTACACCAGCTCCTACAATCGCCCTGCAGCCT CTGTCTCTGAGGCCAGAAGCTTGTAGACCAGCTGCTGGCGGAGCCGTGCATACAA GAGGACTGGACTTCGCCTGTGATGTGGCCGCCATTCTCGGACTGGGACTTGTTCTG GGACTGCTGGGACCTCTGGCCATTCTGCTGGCTCTGTATCTGCTGCGGAGGGACCA

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NK cells comprising CARs comprising OX40 transmembrane (TM) and co-stimulatory (co-stim) domains, SB06251, SB06257, and SB06254, were assessed for expression of constructs as described above. Results as determined by flow cytometry are shown in **FIG. 13A and FIG. 13B**. Secreted IL-15 was measured as described above; results are summarized in **FIG. 14A** and **FIG. 14B**. To assess killing of the target cell population, cell growth was determined as described above (**FIG. 15A** and **FIG. 15B**).

Serial killing by the NK cells comprising SB06257 was also assessed. Target cells were added at Days 0, 2, and 5, and Huh7 target cell count was calculated using an Incucyte. Results are shown in **FIG. 16**.

NK cells comprising CARs comprising CD28 co-stimulatory (co-stim) domains, SB06252, SB06258, and SB06255, were assessed for expression of constructs as described above. Results as determined by flow cytometry FACS are shown in **FIG. 17A** and **FIG. 17B**. Secreted IL-15 was measured as described above; results are summarized in **FIG. 18A** and **FIG. 18B**. To assess killing of the target cell population, cell growth was determined as described above (**FIG. 19A** and **FIG. 19B**).

Serial killing by the NK cells comprising SB06252 and SB06258 was also assessed. Target cells were added at Days 0, 2, and 5, and Huh7 target cell count was calculated using an Incucyte. Results are shown in **FIG. 20**.

Screening for bicistronic constructs

0.5e6 NK donor 7B cells were expanded in the presence of fresh irradiated mbIL21/IL15 K562 feeder cells on retronectin coated non-TC 24-well plates. Spinoculation was performed at

800g at 32°C for 2 hr. For viral transduction, 300 µl of virus added, for a total transduction volume of 500 µl.

Cells were cultured in the same plate for the entire expansion period, in 2 ml final volume. Three partial media exchanges were performed as described above before assessing expression and using the cells in functional assays. Results of expression and cytotoxicity against target cells are shown in **Table 8**. As shown, SB06261, SB6294, and SB6298 showed good CAR and IL-15 expression levels as determined by flow and good cytotoxicity in serial killing assay (n=2). Flow cytometry expression data is shown in **FIG. 21A** and **FIG. 21B**, IL-15 levels are shown in **FIG. 22A** and **FIG. 22B**, and cell growth of the target cell population (as a measure of cell killing by the NK cells) is shown in **FIG. 23A** and **FIG. 23B**.

Due to its high CAR and IL-15 expression and performance in functional assays, SB06294, a retroviral vector with crIL15 2A OX40 CAR design, was selected for further study.

Table 8.

SB#	Virus	Insert 1	2A	Insert 2	Expt #	CAR%	mbIL15%	Double + %	sIL15 (pg/mL)	Round 1	Round 2	Round 3
6261	Sinvec	crIL15 TACE-10	E2A T2A	CD28	CARn-173	37	32	24.5	62	9.8	8.5	16.6
					CARn-174	63.3	49	46	36	9.5	39	100
6294	**Retrovec**	**crIL15 TACE-10**	**E2A T2A**	**OX40-CD3 alt**	**CARn-173**	**59.8**	**38.7**	**32.8**	**50**	**8.8**	7.7	**8**
					CARn-174	**74**	**53**	**52**	**27**	**9.2**	**32**	**98**
6298	Sinvec	CD28-CD3 alt	E2A T2A	crIL15 TACE-10	CARn-173	48.7	27.9	23.1	75	5.3	3.6	6.2
					CARn-174	65	39	39	82	13.8	41	98

Analysis of TACE-OPT constructs

Bicistronic TACE-OPT constructs comprising a TACE10 cleavage site, were analyzed for CAR and IL-15 expression, CNA assay, and payload assay for secreted cytokines, as described above. A TACE10 cleavage site was modified to increase cleavage kinetics, resulting in "TACE-OPT," which results in higher cytokine secretion levels as compared to the parent TACE10. Tricistronic constructs were analyzed for CAR and IL-15 expression, and IL-12 induction.

Briefly, 0.5e6 NK donor 7B cells were expanded in the presence of fresh irradiated mbIL21/IL15 K562 feeder cells on retronectin coated non-TC 24-well plates. Spinoculation was performed at 800g at 32°C for 2 hr. For viral transduction, 300 µl of virus was added, for a total transduction volume of 500 µl.

Bicistronic constructs SB6691 (comprising 41BB co-stimulatory domain), SB6692 (comprising OX40 co-stimulatory domain), and SB6693 (comprising CD28 co-stimulatory domain) were assessed by flow cytometry for expression of CAR and IL-15 (**FIG. 24A**). Copy number of each construct per cell is shown in **Table 9**. IL-15 secretion was quantified as described above at 48 hours and 24 weeks post-tranduction (**FIG. 24B**). While the TACE-OPT constructs tested have similar expression levels and cytokine secretion, SB06692 (comprising an OX40 co-stimulatory domain) has the highest CAR expression.

Table 9.

COPY #	YP7 [CAR] (copies/cell)	IL-15 (copies/cell)	WPRE (copies/cell)
SB06691	116.6	120.2	147.2
SB06692	308.3	318.3	313.0
SB06693	48.8	49.4	57.6

SB06258, SB06257, SB06294 and SB06692 demonstrated high CAR expression, high crIL-15 expression (both membrane-bound and secreted), and high serial killing function *in vitro*.

Example 4: Expression of IL12 from Bidirectional Constructs Encoding a Regulatable, Cleavable-Release IL12 and a Synthetic Transcription Factor

IL12 expression was assessed for NK cells transduced with bidirectional constructs encoding regulatable, cleavable release IL12 and a synthetic transcription factor, with transductions performed as described in Example 3 above. The regulatable, cleavable IL12 is operably linked to a synthetic transcription factor-responsive promoter, which includes a ZF-10-1 binding site and a minimal promoter sequence. The synthetic transcription factor includes a DNA binding domain and a transcriptional activation domain. Between the DNA binding domain and the transcriptional activation domain is a protease domain that is regulatable by a protease inhibitor and cognate cleavage site for the protease. In the absence of an inhibitor of the protease, the protease induces cleavage at the cleavage site, resulting in a non-functional synthetic transcription factor. In the presence of the protease inhibitor, the synthetic transcription factor is not cleaved and is thus capable of modulating expression of the cleavable IL12. The expression cassette encoding the cleavable release IL12 includes a chimeric polypeptide including the IL12 and a transmembrane domain. Between the IL12 and the transmembrane domain is a protease cleavage domain that is cleavable by a protease endogenous to NK cells. A cartoon diagram of the bidirectional constructs encoding cleavable release 12 is shown in **FIG.25**. Parameters of the constructs tested herein are summarized in **Table 10**. Designs tested include: cleavable-release IL12 (crIL12) regulated constructs (32 constructs tested), soluble IL12 (sIL12) regulated and/or WPRE and polyA + different destabilizing domains (32 constructs tested), destabilizing domain and/or WPRE and polyA (26 constructs tested). Initial studies demonstrated toxicity generally due to leaky expression of IL-12, resulting in poor NK cell viability and expansion following transduction (data not shown). A screen was designed to discovere constructs that could overcome or reduce IL-12 associated toxicity by modifying the parameters in Table 10. A summary of screening criteria for is shown in **Table 11A**. Suitable candidates SB05058 and SB05042 (both gammaretroviral vectors) and SB04599 (lentiviral vector) were identified. A summary of these candidates is provided in **Table 11B**.

Table 10.

Parameters tested				
Promoter	**ZF copies**	**IL12**	**Effector domain orientation**	**Modifications**
SFFV	10-1	crIL12 CD16	N-terminal	UTR
SV40	5-7	crIL12 TACE 10	C-terminal	Destabilizing domains
		sIL12		Remove WPRE/PolyA

Table 11A.

Screen	
Metrics	**Recommendations**
IL12 induction at 0.1uM GRZ in 24hours in vitro	>50-fold >1000 pg/ml
NK cell viability Day 10 post-transduction	>75%
Fold-expansion in 10 days (mid-scale, 6 well G-rex)	> 10-fold (research)

Table 11B.

Candidates					
Viral vector	**SB#**	**ZF**	**IL12**	**NS3 promoter**	**Effector domain orientation**
Gamma retro	SB05058	5-7	CD16 crIL12	SV40	C-terminal
Gamma retro	SB05042	5-7	CD16 crIL12	SV40	N-terminal
Lenti	SB04599	10-1	1X SLDE sIL12	SFFV	C-terminal

Assessment of gammaretroviral vectors and lentiviral vectors was performed. A grazoprevir (GRZ) dose response assay measuring IL12 secretion demonstrated that both gammaretroviral constructs showed higher sensitivity to GRZ as compared to the lentiviral construct (**FIG. 26** and **Table 12A**).

Table 12A.

[GRZ] µM	SB04599	SB05042	SB05058
2	1762.68	10629.99	7167.37
0.6	1387.37	8722.87	10922.93
0.16	514.02	2031.82	1470.22
0.05	112.14	173.44	151.69
0.013	4.80	31.57	29.72
0.004	u.d	28.48	35.83
0.001	u.d	28.48	14.83
0	u.d	11.27	17.56

u.d = <5 pg/ml, undetectable

Construct expression and cellular viability were determined 10-days following transduction of NK cells. Results are shown in **Table 12B** and demonstrate an above 10-fold cellular expansion in mid-scale plates, above 85% viability, and greater than 2 copies/cell. Gammaretroviral vectors displayed higher transduction efficiency of NK cells than lentiviral vectors, particularly for the bidirectional vectors tested.

Table 12B.

Viral vector	SB#	MOI	Viability (%)	Fold expansion	CNA (avg copies/cell)
	NV	n/a	88	29.9	n/a
Lenti	4599	29.7	89	19.6	1.0
Gamma retro	5042	83.5	89	15.1	1.6
Gamma retro	5058	0.8	86	11.6	1.8

Additionally, IL12 induction was assessed *in vivo*. Briefly, mice were injected intravenously with transduced NK cells at a dose of 15e6 cells in a 200µL volume. Blood was collected 24 hours after injection and assayed for IL12 expression levels. SB05042 and SB05058 showed the highest IL12 fold-induction. No induction was observed in 10 mg/kg dose groups (data not shown). The percentage of %hNKs in mouse blood was determined to be less than 2% for all constructs. Results are summarized in **Table 12C**. IL12 levels are shown in **FIG. 27A** and fold change is shown in **FIG. 27B**.

Table 12C.

Viral vector	SB#	- GRZ (pg/ml)	+ GRZ (pg/ml)	Fold-change
	NV	0.6	1.35	1.35
Lenti	4599	1.35	14.16	9.30
Gamma retro	5042	0.71	49.0	48.99
Gamma retro	5058	1.0	117.62	118.12

The gammaretroviral vectors (SB05042 and SB05058) demonstrated superior IL12 induction *in vitro* compared to the lentiviral vector (SB04599), while maintaining good viability and cell growth post-transduction. Importantly, both gammaretroviral vectors tested showed IL12 induction in NK cells *in vivo*.

Full-length sequences of constructs described in this Example are shown in **Table 13**.

Table 13.

Construct	Full nucleotide sequence
SB05042 (B7-1 (TM) - CD16 TACE (cleavage site) - IL12 - YB_TATA ZFBD (syn prmoter) - A2 (insulator) - SV40 (promoter) - Syn TF (NLS + miniVPR activation domain + NS3 protease + ZFBD DNA binding domain)	aagcttggaattcgagcttgcatgcctgcaggtcgttacataacttacggtaaatggcccgcctggctgaccgcccaacgacccccgcccattgacgtcaataatgacgtatgttcccatagtaacgccaatagggactttccattgacgtcaatgggtggagtatttacggtaaactgcccacttggcagtacatcaagtgtatcatatgccaagtacgccccctattgacgtcaatgacggtaaatggcccgcctggcattatgcccagtacatgaccttatgggactttcctacttggcagtacatctacgtattagtcatcgctattaccatggtgatgcggttttggcagtacatcaatgggcgtggatagcggtttgactcacggggatttccaagtctccaccccattgacgtcaatgggagtttgttttggcaccaaaatcaacgggactttccaaaatgtcgtaacaactccgccccattgacgcaaatgggcggtaggcgtgtacggtgggaggtctatataagcagagctcaataaaagagcccacaaccccctcactcggcgcgccagtcctccgattgactgagtcgcccgggtacccgtgtatccaataaaccctcttgcagttgcatccgacttgtggtctcgctgttccttgggagggtctcctctgagtgattgactacccgtcagcgggggtctttcatttgggggctcgtccgagatcgggagacccctgcccagggaccaccgacccaccaccgggaggtaagctggccagcaacttatctgtgtctgtccgattgtctagtgtctatgactgattttatgcgcctgcgtcggtactagttagctaactagctctgtatctggcggacccgtggtggaactgacgagttcggaacacccggccgcaaccctgggagacgtcccagggacttcgggggccgtttttgtggcccgacctgagtcctaaaatcccgatcgtttaggactctttggtgcacccccctttagaggagggatatgtggttctggtaggagacgagaacctaaaacagttcccgcctccgtctgaatttttgctttcggtttgggaccgaagccgcgccgcgcgtcttgtctgctgcagcatcgttctgtgttgtctctgtctgactgtgtttctgtatttgtctgaaaatatgggccccccctcgagtccccagcatgcctgctattctcttcccaatcctcccccttgctgtcctgccccaccccaccccccagaatagaatgacacctactcagacaatgcgatgcaatttcctcattttattaggaaaggacagtgggagtggcaccttccagggtcaaggaaggcacgggggaggggcaaacaacagatggctggcaactagaaggcacagttacttaCACAGGCCGCACAGATTCTCTCCGCAGCCGTTCGTTTCTTCTCCGCTCTCTGCACCGAGGGGCGAAGCAGTAGGTCAGGCAACAGATCACGAAGATGCCGTTCACGGAGATCAGTGTGATGGCCCAGCTTGGCAGCAGTTGAAGAGATCCACCGCCACTGCCACCGCCGCCACTACCGCCACCAAAGAAGCTGGAGATTGTAGACACGGCGAGTCCCTGTGTAATTCCAGATCCTCCGCCTCCGCTACCACCTCCGCCGCTAGAGGCGTTCAGGTAGCTCATCACTCTGTCGATGGTCACGGCTCTG

	ATCCGGAAGGCGTGCAGCAGGATGCACAGCTTGATCTTGGTCTTGTAGAAGTC
	GGGTTCTTCCAGGCTAGACTTCTGGGGCACTGTCTCGCTGTTGAAGTTCAGGGC
	CTGCATCAGCTCGTCGATCACGGCCAGCATATTCTGGTCCAGGAAGATCTGCC
	GCTTGGGGTCCATCAGCAGCTTGGCGTTCATGGTCTTGAATTCCACCTGGTACA
	TCTTCAGGTCCTCGTAGATGCTGCTCAGGCACAGGGCCATCATGAAGGAGGTC
	TTTCTGCTGGCCAGGCAAGAGCCGTTGGTGATGAAGCTGGTTTCCCGGCTGTTC
	AGGCAGCTCTCGTTCTTGGTCAGTTCCAGAGGCAGGCAGGCTTCCACGGTGCT
	GGTCTTATCCTTGGTGATGTCCTCGTGGTCGATTTCCTCGCTGGTGCAGGGGTA
	GAATTCCAGGGTCTGTCTGGCCTTCTGCAGCATGTTGGACACGGCTCTCAGCAG
	GTTCTGGCTGTGGTGCAGACAAGGGAACATGCCAGGATCAGGAGTGGCCACAG
	GCAGGTTTCTAGATCCGCCGCCAGATCCACCACCTGATCCGCCACCGCTTCCTC
	CGCCAGAACATGGCACGCTGGCCCATTCGCTCCAAGAGCTGCTGTAGTACCGG
	TCCTGGGCTCTGACGCTGATGCTGGCGTTCTTTCTGCAGATCACGGTGGCGCTG
	GTCTTGTCGGTGAACACCCGGTCCTTTTTCTCGCGCTTGGACTTGCCCTGCACTT
	GCACGCAAAAGGTCAGGCTGAAGTAGCTGTGGGGTGTAGACCAGGTGTCGGG
	GTACTCCCAGGACACTTCCACCTGTCTGCTGTTCTTCAGAGGCTTCAGCTGCAG
	GTTCTTTGGAGGATCGGGCTTGATGATGTCCCGGATGAAAAAGCTGGAGGTGT
	AGTTCTCGTACTTCAGCTTGTGCACGGCGTCCACCATCACTTCGATAGGCAGAG
	ACTCTTCGGCGGCTGGACAGGCGCTGTCCTCTTGGCATTCCACGCTGTACTCGT
	ATTCTTTGTTGTCGCCCCGCACTCTTTCGGCAGACAGTGTAGCGGCGCCACATG
	TAACGCCCTGAGGATCACTGCTGCCTCTGCTGGACTTCACGCTGAAGGTCAGGT
	CGGTGCTGATGGTGGTCAGCCACCAACATGTGAACCGGCCGCTGTAGTTCTTG
	GCCTCGCATCTCAGGAAGGTCTTGTTCTTGGGCTCTTTCTGGTCCTTCAGGATG
	TCGGTGCTCCAAATGCCATCCTCTTTCTTGTGGAGCAGCAGCAGGCTGTGGCTC
	AGCACTTCTCCGCCTTTGTGACAGGTGTACTGGCCGGCGTCGCCAAACTCTTTC
	ACTTGGATGGTCAGGGTCTTGCCGCTGCCGAGCACCTCGCTAGACTGATCCAGT
	GTCCAGGTGATGCCGTCCTCTTCAGGGGTATCGCAGGTCAGCACCACCATCTCG
	CCAGGAGCATCGGGATACCAGTCCAGTTCCACCACGTACACGTCTTTCTTCAGC
	TCCCAGATGGCCACCAGAGGAGAGGCCAGGAACACCAGGCTGAACCAGCTGA
	TGACCAGCTGCTGGTGACACATCATGGTGGCGACACCGGTACGCGTTGGCCCC
	CATTATATACCCTCTAGAACTAGTtatccactccgtgtaagggagagtgagcctcttacgaatcTTCGG
	CGTCGACTGCTTCattccgaggcgactgataccTTCGGCGTCGACTGCTTCatacgaaggcagtccga
	ttcTTCGGCGTCGACTGCTTCaacctttactgagacgggacTTCGGCGTCGACTGCTTCaaaggc
	gttgcgaatcctcatgcgattgttacgaaacccgTTAATTAAAGAGCGAGATTCCGTCTCAAAGAAA
	AAAAAAGTAATGAAATGAATAAAATGAGTCCTAGAGCCAGTAAATGTCGTAA
	ATGTCTCAGCTAGTCAGGTAGTAAAAGGTCTCAACTAGGCAGTGGCAGAGCAG
	GATTCAAATTCAGGGCTGTTGTGATGCCTCCGCAGACTCTGAGCGCCACCTGGT
	GGTAATTTGTCTGTGCCTCTTCTGACGTGGAAGAACAGCAACTAACACACTAA
	CACGGCATTTACTATGGGCCAGCCATTGTCCATCTAGATGGccgataaaataaaagattttat
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	GTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGAAGTATGCAAAGCATGCATC
	TCAATTAGTCAGCAACCAGGTGTGGAAAGTCCCCAGGCTCCCCAGCAGGCAGA

	AGTATGCAAAGCATGCATCTCAATTAGTCAGCAACCATAGTCCCGCCCCTAAC
	TCCGCCCATCCCGCCCCTAACTCCGCCCAGTTCCGCCCATTCTCCGCCCCATGG
	CTGACTAATTTTTTTTATTTATGCAGAGGCCGAGGCCGCCTCTGCCTCTGAGCT
	ATTCCAGAAGTAGTGAGGAGGCTTTTTTGGAGGCCTAGGCTTTTGCAAAGGAT
	CCGCCACCATGCCCAAGAAAAAGCGGAAGGTGGACGCCCTGGACGACTTCGAT
	CTGGATATGCTGGGCAGCGACGCTCTGGATGATTTTGACCTGGACATGCTCGG
	CTCTGATGCACTCGACGATTTCGACCTCGATATGTTGGGATCTGATGCCCTTGA
	TGACTTTGATCTCGACATGTTGATCAATAGCCGGTCCAGCGGCAGCCCCAAGA
	AGAAGAGAAAAGTCGGCTCTGGCGGCGGATCTGGCGGTTCTGGATCTGTTTTG
	CCCCAAGCTCCTGCTCCTGCACCAGCTCCAGCTATGGTTTCTGCTCTGGCTCAG
	GCTCCAGCTCCTGTGCCTGTTCTTGCTCCTGGACCTCCTCAGGCTGTTGCTCCAC
	CAGCACCTAAACCTACACAGGCCGGCGAGGGAACACTGTCTGAAGCTCTGCTG
	CAGCTCCAGTTCGACGACGAAGATCTGGGAGCCCTGCTGGGCAATAGCACAGA
	TCCTGCCGTGTTCACCGATCTGGCCAGCGTGGACAATAGCGAGTTCCAGCAGC
	TCCTGAACCAGGGCATTCCTGTGGCTCCTCACACCACCGAGCCTATGCTGATGG
	AATACCCCGAGGCCATCACCAGACTGGTCACCGGTGCTCAAAGACCACCTGAT
	CCGGCTCCAGCACCTCTTGGAGCACCTGGACTGCCTAATGGACTGCTGTCTGGC
	GACGAGGACTTCAGCTCTATCGCCGACATGGATTTCAGCGCCCTGCTCAGTGG
	CGGTGGAAGCGGAGGAAGTGGCAGCGATCTTTCTCACCCTCCACCTAGAGGCC
	ACCTGGACGAGCTGACAACCACACTGGAATCCATGACCGAGGACCTGAACCTG
	GACAGCCCTCTGACACCCGAGCTGAACGAGATCCTGGACACCTTCCTGAACGA
	CGAGTGTCTGCTGCACGCCATGCACATCTCTACCGGCCTGAGCATCTTCGACAC
	CAGCCTGTTTGAGGATGTCGTGTGCTGCCACAGCATCTACGGCAAGAAGAAGG
	GCGACATCGACACCTACCGGTACATCGGCAGCTCTGGCACAGGCTGTGTGGTC
	ATCGTGGGCAGAATCGTGCTGTCTGGCAGCGGAACAAGCGCCCCTATCACAGC
	CTATGCTCAGCAGACAAGAGGCCTGCTGGGCTGCATCATCACAAGCCTGACCG
	GCAGAGACAAGAACCAGGTGGAAGGCGAGGTGCAGATCGTGTCTACAGCTAC
	CCAGACCTTCCTGGCCACCTGTATCAATGGCGTGTGCTGGGCCGTGTATCACGG
	CGCTGGAACCAGAACAATCGCCTCTCCTAAGGGCCCCGTGATCCAGATGTACA
	CCAACGTGGACCAGGACCTCGTTGGCTGGCCTGCTCCTCAAGGCAGCAGAAGC
	CTGACACCTTGCACCTGTGGCTCCAGCGATCTGTACCTGGTCACCAGACACGCC
	GACGTGATCCCTGTCAGAAGAAGAGGGGATTCCAGAGGCAGCCTGCTGAGCCC
	TAGACCTATCAGCTACCTGAAGGGCTCTAGCGGCGGACCTCTGCTTTGTCCTGC
	TGGACATGCCGTGGGCCTGTTTAGAGCCGCCGTGTGTACAAGAGGCGTGGCCA
	AAGCCGTGGACTTCATCCCCGTGGAAAACCTGGAAACCACCATGCGGAGCCCC
	GTGTTCACCGACAATTCTAGCCCTCCAGCCGTGACACTGACACACCCCATCACC
	AAGATCGACAGAGAGGTGCTGTACCAAGAGTTCGACGAGATGGAAGAGTGCA
	GCCAGCACATGTCTAGACCTGGCGAGAGGCCCTTCCAGTGCCGGATCTGCATG
	CGGAACTTCAGCAACATGAGCAACCTGACCAGACACACCCGGACACACACAG
	GCGAGAAGCCTTTTCAGTGCAGAATCTGTATGCGCAATTTCTCCGACAGAAGC
	GTGCTGCGGAGACACCTGAGAACCCACACCGGCAGCCAGAAACCATTCCAGTG
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Example 5: Screening of GPC3 CAR / IL15 Expression Constructs

Assessment of the expression and function of the GPC3 CAR/IL15 expression constructs in NK cells was performed. 2e6 NK cells were plated into a 6-well non-TC treated, retronectin coated plate. A single viral transduction via spinoculation (MOI = 15) was performed on plated NK cells. The NK cells were transduced using lentivirus or retrovirus containing the expression construct. Expression of the CAR and membrane IL15 were assessed as seen in **FIG. 28A.** NK cells transduced with constructs **SB06257, SB06258, SB06294,** and **SB06692** exhibited expression of greater than 65% of cells in the gated population. In addition, **FIG. 28A** shows the measured copy numbers of YP7 and IL15 of each transduced NK cell population.

In addition to CAR expression being assessed, secreted IL-15 was also measured using the same expression constructs. To measure the levels of secreted IL-15, 200,000 transduced NK

cells were suspended in 200 μL of MACS media in the presence of IL2. Secreted IL-15 was measured 48 hours after transduction. The concentrations of secreted IL-15 were measured for each construct and the results are shown in **FIG. 28B.**

Serial killing by NK cells transduced with the constructs was also assessed. Target cells were added at Days 0, 2, and 5, and target cell killing was measured over the course of the study. Results for serial NK cell killing of HepG2 target cells are shown in **FIG. 28C** and **FIG. 29A**. **FIG. 29B** shows results of serial NK cell killing of HuH-7 target cells.

Table 14 shows the exemplary constructs and their components used in this study.

Table 14

Construct	Base Vector	Co-Stim	Orientation
SB06257	SinVec	OX40	CAR 2A crIL15 (T10)
SB06258	SinVec	CD28	CAR 2A crIL15 (T10)
SB06294	RetroVec	OX40	crIL15 2A CAR (T10)
SB06692	SinVec	OX40	crIL15 2A CAR (T-OPT)

Example 6: Measuring GPC3 CAR / IL15 Expression and Function in Expanded NK cells

In this study, the expression and function of GPC3 CAR/IL15 were measured for NK cells that were expanded using the G-Rex (Gas rapid expansion) system.

7-day-old donor-derived 7B NK cells (mbIL21/IL15 K562 feeders) were transduced and expanded in two different G-Rex experimental methods. Experiment 1 transduced 7-day donor 7B NK cells (mbIL21/IL15 K562 feeders) in G-Rex 6M culture containers for 11 days and harvested 11 days after transduction. Experiment 2 transduced 7-day donor 7B NK cells (mbIL21/IL15 K562 feeders) in G-Rex 1L culture containers for 7 days and harvested 10 days after transduction. **FIG. 30A** demonstrated the effects of the different expansion conditions have on the expression of different proteins of interest in the engineered NK cells. **FIG. 30B** shows the serial killing assay measurements from the NK Cells derived from the different experiments.

Table 15 shows a summary of the study performed in **Example 6.** The top number corresponds to results obtained from NK cells expanded using the method of Experiment 1. The bottom number corresponds to results obtained from NK cells expended using the method of Experiment 2.

Table 15

SB#	Back bone	Co-stim	IL15	Orientation	% GPC3 CAR	% mIL15	pg/ml sIL15	1st round - % HepG2 killing	2nd round- % HepG2 killing	3rd round- % HepG2 killing	CAN (copies per cell)	MOI
NV	-	-	-	-	1.02 0.2	1.37 1.9	4.9 4.9	0 77.2	0 11.0	0 3.9	-	-
6257	SinVec	OX40	Tace10	CAR/ crIL15	37.5 57.4	1.69 10.3	5.1 17.0	71.6 81.2	37.2 78.8	17.8 83.2	23.3 23.9	30.6 30.6
6258	SinVec	CD28	Tace10	CAR/ crIL15	36.8 70.7	10.7 35.9	5.5 56.7	18.3 87.6	1.4 79.0	0 73.0	39.2 54.1	15.5 15.5
6294	RetroVec	OX40	Tace10	crIL15/ CAR	78.4 91.9	58.9 63.9	26.2 60.1	58.5 85.2	33.2 83.8	12.5 84.2	41.7 35.0	10.5 8.8
6692	SinVec	OX40	TaceOPT	crIL15/ CAR	- 78.8	- 16.9	- 104.5	- 83.4	- 83.0	- 83.2	- 47.5	- 15.0

Example 7: Assessment of GPC3 CAR / IL15 Bicistronic Constructs in a Xenograft Tumor Model

The in vivo function of selected engineered NK cells was assessed using a HepG2 xenotransplantation tumor model. Two studies were conducted: a double NK dose and a triple NK dose.

Double NK Dose In vivo Xenograft Tumor Model

The tumor was implanted in NSG mice at day 0. Mice were randomized at day 9. NK cells were injected twice over the course of the study on days 10 and 17. **Table 16** summarizes the study set-up.

Table 16: Summary of double NK dosing in vivo xenograft tumor model

Tumor model	Group Name	# NKs per dose	Dose day(s)
IP HepG2, 6e6	PBS	-	10, 17
	No virus (NV)	30e6	
	SB06257		
	SB06258		
	SB06294*		

** Due to cell # limitation, second dose was ~15e6*

For this survival study, Jackson Labs NSG mice were also injected with 50,000 IU rhIL2 per mouse twice per week. Bioluminescence imaging (BLI), body weight, and overall health measurements were conducted twice a week. Upon euthanizing mice, tumor were collected, weighed, and formalin fixed paraffin embedded (FFPE) for histology. IP fluid and cells were collected from the IP space and the % NK cells were assessed by flow cytometry. FIG. 31 summarizes the results the fold change in normalized mean BLI measurement in the HepG2 xenotransplantation tumor model. SB06258 showed the lowest normalized mean BLI compared to other treatment groups and was found to be statistically significant compared to the no virus (NV) group. **FIG. 32A** shows a survival curve of animals and **FIG. 32B** shows a summary of the median survival of each of the treatment groups. Each of the different CAR constructs tested were found to be statistically significant compared to un-engineered NK cells.

FIG. 33 shows a time course of the mice treated with different CAR-NK cells as measured and observed through bioluminescence imaging (BLI). The animals shown here were imaged 3 days, 10 days, 34 days, 48 days, and 69 days after treatment. In **FIG. 34**, BLI measurements were normalized to day 10 (first dose).

Triple Dosing – In Vivo HepG2 Xenograft Tumor Model

The in vivo function of selected engineered NK cells was assessed using a HepG2 xenotransplantation tumor model. The tumor was implanted in NSG mice at day 0 in another in vivo experiments. Mice were randomized at day 9 and day 20. 30e6 NK cells were injected (IP) three times over the course of the study on days 10, 15, and 22. **Table 17** summarizes the study set-up. On day 21, half of the mice were euthanized. The other half were euthanized on day 50 of the study. Upon euthanizing mice, tumor were collected, weighed, and formalin fixed paraffin embedded (FFPE) for histology.

Table 17: Study Design of HepG2 xenograft model

Tumor model	Group Name	# NKs per dose	NK dose days
IP 6e6 HepG2	PBS	-	10, 15, 22
	No virus (NV)	IP 30e6	
	SB06257		
	SB06258		
	SB06294		
	SB06692		

For this survival study, Jackson Labs NSG mice were also injected with 50,000 IU rhIL2 per mouse twice per week. Bioluminescence imaging (BLI), body weight, and overall health measurements were conducted twice a week. IP fluid and cells were collected from the IP space and the % NK cells were assessed by flow cytometry. **FIG. 35A** shows a representative BLI image at day 23 of the study. **FIG. 35B** summarizes the results the fold change in normalized mean BLI measurement in the HepG2 xenograft tumor model.

The fold change of BLI measurements were assessed at different stages of the experiments to assess the effect of a single or double dose of the engineered NK cells had an effect. **FIG. 36A** shows the fold change of BLI measurements on day 13, in which the mice had undergone one dose of the engineered NK cells. **FIG. 36B** shows the fold change of BLI measurements on day 20, in which the mice had undergone two doses of the engineered NK cells.

Comparison of the results from the two *in vivo* experiments are presented in FIG. 37A and FIG. 37B. In FIG. 37A, the different CAR constructs were tested in a xenograft model, plotting fold change of BLI over the course of the study. As seen in FIG. 37A and FIG. 37B, the two *in vivo* experiments exhibit differences in antitumor function of SB06257 and SB06258. GPC3 CAR- crIL-15 NK cell therapy shows statically significant *in vivo* anti-tumor efficacy compared to unengineered NK cells in an IP HCC (HepG2+luciferase) xenotransplantation model. All 3 groups treated with GPC3 CAR-crIL-15 engineered NK cells show significant increased survival over untreated (PBS) and unengineered NK cell-treated groups.

In vivo Xenograft model – Intratumoral Injection of NK cells

Another experimental approach was used to demonstrate NK-mediated anti-tumor killing for an HepG2 (HCC) subcutaneous xenograft tumor model. In this survival study, mice were injected three times with 3e6 NK cells on days 20, 25, and 32. **FIG. 38A** demonstrates tumor growth in mice in the absence or presence of injected engineered NK cells. GPC3 CAR- crIL-15 NK cell therapy shows significant in vivo anti-tumor efficacy compared to unengineered NK cells injected intratumorally (IT) within a subcutaneous HCC (HepG2+luciferase) xenotransplantation model. NK cells transduced with SB05605 show significantly increased survival over untreated (PBS) and unengineered NK cell-treated groups. **Table 18** provides the constructs used for intratumoral injection of NK cells. SB05009 and SB06205 contain IL15 and the GPC3 CAR that are separate, and their expression is driven by separate promoters (SV40 promoter = GPC3 CAR, hPGK promoter = IL15). In addition, these constructs are oriented such that the reading frames are oriented in opposing directions.

Table 18

SEQ ID NO:	Construct	Sequence
328	SB05009	aagcttgaattcgagcttgcatgcctgcaggtcgttacataacttacggtaaatggcccgcctgg ctgaccgcccaacgacccccgcccattgacgtcaataatgacgtatgttcccatagtaacgcca atagggactttccattgacgtcaatgggtggagtatttacggtaaactgcccacttggcagtacat caagtgtatcatatgccaagtacgccccctattgacgtcaatgacggtaaatggcccgcctggc attatgcccagtacatgaccttatgggactttcctacttggcagtacatctacgtattagtcatcgct attaccatggtgatgcggttttggcagtacatcaatgggcgtggatagcggtttgactcacgggg atttccaagtctccaccccattgacgtcaatgggagtttgttttggcaccaaaatcaacgggacttt ccaaaatgtcgtaacaactccgccccattgacgcaaatgggcggtaggcgtgtacggtggga ggtctatataagcagagctcaataaaagagcccacaacccctcactcggcgcgccagtcctcc

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		ggggcgccagtcctccgattgactgagtcgcccggccgcttcgagcagacatgataagatacattgatgagtttggacaaaccacaactagaatgcagtgaaaaaaatgctttatttgtgaaatttgtgatgctattgctttatttgtaaccattataagctgcaataaacaagttaacaacaacaattgcattcatttatgtttcaggttcaggggggagatgtgggaggttttttaaagcaagtaaaacctctacaaatgtggtaaaatcgataaggatcgggtacccgtgtatccaataaaccctcttgcagttgcatccgacttgtggtctcgctgttccttgggagggtctcctctgagtgattgactacccgtcagcgggggtctttcacacatgcagcatgtatcaaaattaatttggtttttttttcttaagctgtgccttctagttgccagccatctgttgtttgcccctcccccgtgccttccttgaccctggaaggtgccactcccactgtcctttcctaataaaatgaggaaattgcatcgcattgtctgagtaggtgtcattctattctgggggggtggggtggggcaggacagcaaggggaggattgggaagacaatagcaggcatgctggggatgcggtgggctctatggagatcccgcggtacctcgcgaatgcatctagatccaatggccttttttggcccagacatgataagatacattgatgagtttggacaaaccacaactagaatgcagtgaaaaaaatgctttatttgtgaaatttgtgatgctattgctttatttgtaaccattataagctgcaataaacaagttgcggccgcttagccctcccacacataaccagagggcagcaattcacgaatcccaactgccgtcggctgtccatcactgtccttcactatggctttgatcccaggatgcagatcgagaagcacctgtcggcaccgtccgcagggctcaagatgcccctgttctcatttccgatcgcgacgatacaagtcaggttgccagctgccgcagcagcagcagtgcccagcaccacgagttctgcacaaggtcccccagtaaaatgatatacatttgacaccagtgaagatgcggccgtcgctagagagagctgcgctggcgacgctgtagtcttcagagatggggatgctgttgattgtagccgttgctctttcaatgagggtggattcttcttgagacaaaggcttggccatgcggccgccgctcggtgttcgaggccacacgcgtcaccttaatatgcgaagtggacctcggaccgcgccgccccgactgcatctgcgtgttcgaattcgccaatgacaagacgctgggcggggtttgtgtcatcatagaactaaagacatgcaaatatatttcttccgggggggtaccggcctttttggccATTGGatcggatctggccaaaaaggcccttaagtatttacattaaatggccatagtacttaaagttacattggcttccttgaaataaacatggagtattcagaatgtgtcataaatatttctaattttaagatagtatctccattggctttctacttttttcttttatttttttttgtcctctgtcttccatttgttgttgttgttgtttgtttgttgttggttggttggttaatttttttttaaagatcctacactatagttcaagctagactattagctactctgtaacccagggtgaccttgaagtcatgggtagcctgctgttttagccttccccacatctaagattacaggtatgagctatcatttttggtatattgattgattgattgatgtgtgtgtgtgtgattgtgtttgtgtgtgtgaTtgtgTaTatgtgtgtatggTtgtgtgtgaTtgtgtgtatgtatgTTtgtgtgtgaTtgTgtgtgtgaTtgtgcatgtgtgtgtgtgtgaTtgtgtTtatgtgtatgaTtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgttgtgTaTaTatatttatggtagtgagagGcaacgctccggctcaggtgtcaggttggttttgagacagagtctttcacttagcttggaattcactggccgtcgttttacaacgtcgtgactgggaaaaccctggcgttacccaacttaatcgccttgcagcacatccccctttcgccagctggcgtaatagcgaagaggcccgcaccgatcgcccttcccaacagttgcgcagcctgaatggcgaatggcgcctgatgcggtattttctccttac

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		gctggcacgacaggtttcccgactggaaagcgggcagtgagcgcaacgcaattaatgtgagtt agctcactcattaggcaccccaggctttacactttatgcttccggctcgtatgttgtgtggaattgt gagcggataacaatttcacacaggaaacagctatgaccatgattacgcc

Example 8: Assessment of Grazoprevir induction of IL12 in natural killer cells

For this study, the induction of IL12 was measured in the presence and absence of grazoprevir, an inhibitor of the HCV NS3 protease. The construct used in this study has been previously described in Example 2. Two regulatable IL-12 constructs demonstrated controlled crIL-12 expression by GRZ in a dose-response manner and show low donor-to-donor variability The tested construct candidates resulted in low IL-12 basal levels in the absence of GRZ (less than 100 pg/ml) and greater than 100-fold induction of IL-12 by 0.1 μM of GRZ ($p=<0.0001$). FIG. 39A-39B show two different time points (24 hours and 72 hours, respectively) after addition of GRZ to NK cells expressing the SB05042 and SB05058 constructs.

To assess whether the grazoprevir can be used to transition the circuit in an on to off or off to on state in a mouse model, the following study was designed. On day 0, NK cells were injected (IV) in the presence of grazoprevir or vehicle. On days 1, 9, and 10, another dose of grazoprevir or vehicle was injected. Mice were bled on days 2, 9, and 11 to assess expression of IL-12. FIG. 40 shows the results of the study. On day 2, IL12 expression increased in the presence of 20, 50, and 100 mg/kg GRZ as compared to the control. On day 9, where GRZ administration has not occurred for 8 days, expression of IL12 is decreased as compared to sampling on day 2. On day 11, expression has increased once again in relation to the control.

Example 9: Assessment of Co-transduction of GPC3 CAR / IL15 and Regulated IL12 constructs

Function and expression of GPC3 CAR, IL15 and IL12 were assessed in NK cells that were co-transduced with GPC/IL15 constructs and the regulated IL12 construct.

Expression of GPC3 CAR / IL15

Three construct combinations were tested: 1) SB05042 + SB0257, 2) SB05042 + SB06258, and 3) SB05042 and SB06294. NK cells co-transduced with SB05042 + SB06257 or SB05042 + SB06258 expressed GPC3 CAR and IL15 populations and similar copies per cell. NK cells co-transduced with SB06294 exhibited a higher double positive (GPC+/IL15+)

population with a slight decrease in CAR only population and with similar copies per cell (**FIG. 41**)

Expression of secreted IL12 and IL15

Expression of secreted IL12 and IL15 were measured in NK cells in the presence or absence of grazoprevir was tested. 200,000 transduced NK cells were suspended in 200 μL of NK MACS media supplemented with IL-2. Grazoprevir was added to "+" conditions at a molar concentration of 0.1 μM. NK cells were incubated for 48 hours at 37C prior to measurement of the supernatant for IL15 (**FIG. 42A**) and IL12 (**FIG. 42B**) concentration. IL15 expression increased slightly in the presence of grazoprevir, with the co-transduced NK cells showing statistically significant IL15 expression in the presence of GRZ. NK cells co-transduced with SB05042 +SB06257 expressed 2201 pg/mL IL12 in the presence of grazoprevir, as compared to 12 pg/mL in the absence of grazoprevir (1100-fold induction). SB05042 +SB06258 cotransduction exhibited 1003-fold induction in the presence of grazoprevir. SB05042 +SB06294 co transduction exhibited 736-fold induction. The three co-transduction combinations were statistically significant compared to NK cells transduced with SB05042 alone. Assessing IL12 expression, NK cells transduced with SB05042 alone showed induction of IL12 in the presence of grazoprevir, showing an 390-fold increase in expression.

Cytokine Secretion during Serial Killing (Huh7)

Serial killing of target cells were carried out as previously described using NK cells singly transduced or co-transduced with GPC3 CAR/IL15 (SB06257, SB06258, SB06294) and /or IL12 constructs (SB05042).

Co-transduced samples maintained low amounts of IL12 induction into the 3rd round in the presence of GRZ. Overall cytokine secretion decreases overtime in both IL12 and IL15 (FIG. 43). In the presence of grazoprevir, SB05042 and SB05042 + SB06257 transductions showed significant induction of IL12 expression in the first round of killing. In the second round, the three co-transductions with the different GPC3 CAR expressing constructs (SB06257, SB06258, SB06294) and SB05042 showed statistically significant induction of IL12. In the third round, only SB05042 + SB06257 and SB05042 + SB06294 showed significant IL12 induction.

Serial Killing Assays with Co-transduced NK cells

The cell killing effect of NK cells that were co-transduced with GPC3 CAR/IL15 (SB06257, SB06258, SB06294) and /or IL12 constructs (SB05042) were assessed using a serial killing assay. NK cells co-transduced with SB05042 + SB06258 (**FIG. 44A**), SB05042 + SB06257 (**FIG. 44B**) and SB05042 + SB06294 (**FIG. 44C**) were used in a serial killing assay in

which GRZ was added at the first and third rounds of cell killing. When co-cultured with HepG2 we see a greater difference between +/- GRZ (induced IL12 or not) as compared to huh7. **FIG. 44D** shows a combination of the data shown in **FIGs. 44A-44C.**

Example 10: Selection of GPC3 CAR / IL15 clones

Selection of clones were performed by transducing NK cells that have stably integrated the expression construct. A lower MOI was used (MOI=3) was used for clonal selection of SB06258. A control transient transduction (MOI = 15) was also performed used in SB06258 and SB07273 (identical to SB06258 but contains a kanamycin resistance marker instead of an ampicillin resistance marker). 8 days after transduction, the cells were assessed. The copies per cell was lower in the PCB clones as compared to the transient transduction using SB06258 (FIG. 45A). CAR expression was relatively constant across the different PCB clones (FIG. 45B), as well as the IL15+ population (FIG. 45C). Secreted IL15 of PCB clones was measured to be greater than 30 pg/mL (FIG. 45D).

Flow cytometry was also used to assess the expression of the GPC3 CAR and IL15 in the PCM clones. As a control, SB07473 was used to transduced NK cells at an MOI=15. PCB clones were transduced at an MOI of 3.0. For all PCR clones, GPC3 CAR expression was greater than 20% (FIG. 46A).

For select clones, SB05042 was also co-transduced to assess the expression of the GPC3 CAR, membrane bound IL15 and membrane bound IL12 9 days after transduction. Clone 3 (MOI=3.0) and clone 4 (MOI=3.0) was co-transduced with SB05042 (MOI = 0.05). During co-transduction, there was similar expression of the GPC3 CAR and membrane bound IL12 (FIG. 46B). **Table 19** shows a summary of the expression levels of the PCB clones transduced with SB06258.

Table 19

			PCB Clones							
	6258	**7473**	**2**	**3**	**4**	**5**	**6**	**7**	**9**	**10**
Copy #	19.8	3.9		8.88	6.2	10.7	14.4	7.9	9	12.1
	19.2	2.4				10.4	10.9	6.2		
	24.2		8.3	11.6	10.2					
CAR %	59.5	22.5		35.2	29	40.9	43.1	36.7	38.8	46.3
	59.5	16.5				46.8	41.5	31.6		

	75.1	37.1	46.8	54.6	44.8					
memb-IL-15	32.7	9.23		15.2	13.4	18.8	20.1	15.4	16.1	21.6
	20.4	9.9				14.6	19.2	15.2		
	55.1	20.6	25.5	36.3	31.5					
Sec-IL15	73.0	11.9	30.6	49.9	39.9	51.0	51.5	33.8		
	63.8	13.9	29.8	44.8	30.4	45.8	46.5	29.0		
	67.6	13.5	28.3	52.4	35.4	47.1	51.3	29.8		

Table 20

SEQ ID NO	Construct	Sequence
326	SB07472	aagcttggaattcgagcttgcatgcctgcaggtcgttacataacttacggtaaatggcccgcct ggctgaccgcccaacgacccccgcccattgacgtcaataatgacgtatgttcccatagtaacg ccaatagggactttccattgacgtcaatgggtggagtatttacggtaaactgcccacttggcagt acatcaagtgtatcatatgccaagtacgccccctattgacgtcaatgacggtaaatggcccgcc tggcattatgcccagtacatgaccttatgggactttcctacttggcagtacatctacgtattagtca tcgctattaccatggtgatgcggttttggcagtacatcaatgggcgtggatagcggtttgactca cggggatttccaagtctccaccccattgacgtcaatgggagtttgttttggcaccaaaatcaac gggactttccaaaatgtcgtaacaactccgccccattgacgcaaatgggcggtaggcgtgtac ggtgggaggtctatataagcagagctcaataaaagagcccacaacccctcactcggcgcgc cagtcctccgattgactgagtcgcccgggtacccgtgtatccaataaaccctcttgcagttgcat ccgacttgtggtctcgctgttccttgggagggtctcctctgagtgattgactacccgtcagcgg gggtctttcatttgggggctcgtccgagatcgggagacccctgcccagggaccaccgaccca ccaccgggaggtaagctggccagcaacttatctgtgtctgtccgattgtctagtgtctatgactg attttatgcgcctgcgtcggtactagttagctaactagctctgtatctggcggacccgtggtgga actgacgagttcggaacacccggccgcaaccctgggagacgtcccagggacttcgggggc cgtttttgtggcccgacctgagtcctaaaatcccgatcgtttaggactctttggtgcacccccctt agaggagggatatgtggttctggtaggagacgagaacctaaaacagttcccgcctccgtctg aatttttgctttcggtttgggaccgaagccgcgccgcgcgtcttgtctgctgcagcatcgttctgt gttgtctctgtctgactgtgtttctgtatttgtctgaaaatatgggccccccctcgaggtaacgcca ttttgcaaggcatggaaaaataccaaaccaagaatagagaagttcagatcaagggcgggtac atgaaaatagctaacgttgggccaaacaggatatctgcggtgagcagtttcggccccggccc ggggccaagaacagatggtcaccgcagtttcggccccggcccgaggccaagaacagatgg tccccagatatggcccaaccctcagcagtttcttaagacccatcagatgtttccaggctccccc aaggacctgaaatgaccctgcgccttatttgaattaaccaatcagcctgcttctcgcttctgttcg cgcgcttctgcttcccgagctctataaaagagctcacaacccctcactcggcgcgccagtcct ccgacagactgagtcgcccgggGCCGCCACCATGCTGCTGCTGGTCA CATCTCTGCTGCTGTGCGAGCTGCCCCATCCTGCCTTTCT GCTGATCCCTCACATGGACATCGTGATGACACAGAGCCC CGATAGCCTGGCCGTGTCTCTGGGAGAAAGAGCCACCAT CAACTGCAAGAGCAGCCAGAGCCTGCTGTACTCCAGCA ACCAGAAGAACTACCTGGCCTGGTATCAGCAAAAGCCC GGCCAGCCTCCTAAGCTGCTGATCTATTGGGCCAGCTCC AGAGAAAGCGGCGTGCCCGATAGATTTTCTGGCTCTGGC AGCGGCACCGACTTCACCCTGACAATTTCTAGCCTGCAA

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		attgattgattgattgatgtgtgtgtgtgattgtgtttgtgtgtgtgaTtgtgTaTatg tgtgtatggTtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgtgttgtgTaTaTatatttatggt agtgagagGcaacgctccggctcaggtgtcaggttggtttttgagacagagtctttcactta gcttggaattcactggccgtcgttttacaacgtcgtgactgggaaaaccctggcgttaccca acttaatcgccttgcagcacatccccctttcgccagctggcgtaatagcgaagaggcccgca ccgatcgcccttcccaacagttgcgcagcctgaatggcgaatggcgcctgatgcggtattttc tccttacgcatctgtgcggtatttcacaccgcatatggtgcactctcagtacaatctgctctga tgccgcatagttaagccagccccgacacccgccaacacccgctgacgcgccctgacgggct tgtctgctcccggcatccgcttacagacaagctgtgaccgtctccgggagctgcatgtgtcag aggttttcaccgtcatcaccgaaacgcgcgagacgaaagggcctcgtgatacgcctatttttt ataggttaatgtcatgataataatggtttcttagacgtcaggtggcacttttcggggaaatgt gcgcggaacccctatttgtttatttttctaaatacattcaaatatgtatccgctcatgagacaa taaccctgataaatgcttcaataatattgaaaaaggaagagtatgagccatattcaacggg aaacgtcgaggccgcgattaaattccaacatggatgctgatttatatgggtataaatgggct cgcgataatgtcgggcaatcaggtgcgacaatctatcgcttgtatgggaagcccgatgcgc cagagttgtttctgaaacatggcaaaggtagcgttgccaatgatgttacagatgagatggtc agactaaactggctgacggaatttatgcctcttccgaccatcaagcattttatccgtactcct gatgatgcatggttactcaccactgcgatccccggaaaaacagcattccaggtattagaag aatatcctgattcaggtgaaaatattgttgatgcgctggcagtgttcctgcgccggttgcattc gattcctgtttgtaattgtccttttaacagcgatcgcgtatttcgtctcgctcaggcgcaatcac gaatgaataacggtttggttgatgcgagtgattttgatgacgagcgtaatggctggcctgttg aacaagtctggaaagaaatgcataaacttttgccattctcaccggattcagtcgtcactcat ggtgatttctcacttgataaccttatttttgacgaggggaaattaataggttgtattgatgttg gacgagtcggaatcgcagaccgataccaggatcttgccatcctatggaactgcctcggtga gttttctccttcattacagaaacggctttttcaaaaatatggtattgataatcctgatatgaat aaattgcagtttcatttgatgctcgatgagtttttctaactgtcagaccaagtttactcatatat actttagattgatttaaaacttcatttttaatttaaaaggatctaggtgaagatccttttttgata atctcatgaccaaaatcccttaacgtgagttttcgttccactgagcgtcagaccccgtagaa aagatcaaaggatcttcttgagatcctttttttctgcgcgtaatctgctgcttgcaaacaaaa aaaccaccgctaccagcggtggtttgtttgccggatcaagagctaccaactctttttccgaag gtaactggcttcagcagagcgcagataccaaatactgtTcttctagtgtagccgtagttagg ccaccacttcaagaactctgtagcaccgcctacatacctcgctctgctaatcctgttaccagt ggctgctgccagtggcgataagtcgtgtcttaccgggttggactcaagacgatagttaccgg ataaggcgcagcggtcgggctgaacgggggttcgtgcacacagcccagcttggagcgaa cgacctacaccgaactgagatacctacagcgtgagctatgagaaagcgccacgcttcccga agggagaaaggcggacaggtatccggtaagcggcagggtcggaacaggagagcgcacg agggagcttccagggggaaacgcctggtatctttatagtcctgtcgggtttcgccacctctga cttgagcgtcgatttttgtgatgctcgtcaggggggcggagcctatggaaaaacgccagca acgcggccttttacggttcctggccttttgctggccttttgctcacatgttctttcctgcgttat cccctgattctgtggataaccgtattaccgcctttgagtgagctgataccgctcgccgcagcc gaacgaccgagcgcagcgagtcagtgagcgaggaagcggaagagcgcccaatacgcaa accgcctctccccgcgcgttggccgattcattaatgcagctggcacgacaggtttcccgactg gaaagcgggcagtgagcgcaacgcaattaatgtgagttagctcactcattaggcaccccag gctttacactttatgcttccggctcgtatgttgtgtggaattgtgagcggataacaatttcaca caggaaacagctatgac catgattacgcc

Table 21:

SB ID	Description	Backbone	Seq Type	SS	scFV	Linker	scFV	Hinge	TM	Co-stim ICD	CD3z ICD	E2A	SS	IL15	Cleavage Site	TM domain
SB06251				GM-CSF-Ra	hPY7 VL	(GGGGS)3	hPY7 VH	CD8 S2L	OX40	OX40	CD3z	E2A/T2A	IgE	IL15	LR1 split N term linker + Tace10	B7-1
	GM-CSF-Ra (SS) - aGPC3 hPY7 vL - (GGGGS)3 - aGPC3 hPY7 - CD8 S2L (Hinge) - OX40 (TM) - OX40 (ICD) - CD3z (ICD) - E2A T2A - IgE (SS) - IL-15 - Tace10 (cleavage site) - B7-1 (TM)		DNA	ATGCTGCTGCTGGTCACATCTCTGCTGTGCGAGCTGCCCCATCCTGCCTTTCTGCTGATCCCT	GACATCGTGATGACACAGAGCCCCGATAGCCTGGCCGTGTCTCTGGGAGAAAGAGCCACCATCAACTGCAAGAGCAGCCAGAGCCTGCTGTACTCCAGCAACCAGAAGAACTACCTGGCCTGGTATCAGCAAAAGCCCGGCCAGCCTCCTAAGCTGCTGATCTATTGGGCCAGCTCCAGAGAAAGCGGCGTGCCCGATAGATTTTCTGGCTCTGGCAGCGGCACCGACTTCACCCTGACAATTTCTAGCCTGCAAGCCGAGGACGTGGCCGTGTACTACTGCCAGCAGTACTACAACTACCCTCTGACCTTCGGCCAGGGCACCAAGCTGGAAATCAAA	GGCGGCGGAGGATCTGGCGGAGGTGGAAGTGGCGGAGGCGGATCT	GAAGTGCAGCTGGTTGAATCAGGTGGCGGCCTGGTTCAACCTGGCGGATCTCTGAGACTGAGCTGTGCCGCCAGCGGCTTCACCTTCAACAAGAACGCCATGAACTGGGTCCGACAGGCCCCTGGCAAAGGCCTTGAATGGGTCGGACGGATCCGGAACAAGACCAACAACTACGCCACCTACTACGCCGACAGCGTGAAGGCCAGATTCACCATCAGCCGGGACGACAGCAAGAACAGCCTGTACCTGCAGATGAACTCCCTGAAAACCGAGGACACCGCCGTGTATTATTGCGTGGCCGGCAACAGCTTTGCCTACTGGGGACAGGGAACCCTGGTCACCGTGTCTGCC	ACAACAACCCCTGCTCCTAGACCTCCTACACCAGCTCCTACAATCGCCCTGCAGCCTCTGTCTCTGAGGCCAGAAGCTTGTAGACCAGCTGCTGGCGGACGCCGTGCATAGCAAGAGGACTTCGCCTGTGAT	GTGGCCGCCATTCTCGGACTTGGGACTGCTGGGACCTCTGGCCATTCTGCT	GCTCTGTATCTGCTGAGCTCACAACCAGACTGCCTCCTGATGCTCACAAGCCTCCAGGCGGAGGCAGCTTCAGAACCCCTATCCAAGAGGACAGGCCGACGCTCACAGCACCCTGGCCAAGAATT	AGAGTGAAGTTCAGCAGGAAGCGCCGACGCACCCGCCTATAAGCAGGGACAGAACCAGCTGTACAACGAGCTGAACCTGGGGAGAAGAGAAGAGTACGACGTGCTGGACAAGCGGAGAGGCAGAGATCCTGAGATGGGCGGCAAGCCCAGACGGAAGAATCCTCAAGAGGGCCTGTATAATGAGCTGCAGAAAGACAAGATGGCCGAGGCCTACAGCGAGATCGGAATGAAGGGCGAGCGCAGAAGAGGCAAGGGACACGATGGACTGTACCAGGGCCTGAGCACCGCCACCAAGGATACCTATGATGCCCTGCACATGCAGGCCCTGCCTCCAAGA	GGTAGCGGCAACTACGCCCTGCTGAAACTGGCCGGCGACGTGGAAGTCTAATCCTGGACCTGGATCTGGCGAGGGACGTGGAGGAAAACCCTGGACCT	ATGGACTGGAATCCTGGGTTCGCCAAGAGTGCA	AATTGGGTCAACGTGATCAGCGACCTGAAGAAGATCGAGGACCTGATCCAGAGCATGCACATCGACGCCACACTGTACACCGAGAGCGACGTGCACCCTAGCTGTAAAGTGACCGCCATGAAGTGCTTTCTGCTGGAACTGCAAGTGATCAGCCTGGAAAGCGGCGACGCCAGCATCCACGACACCGTGGAAAACCTGATCATCCTGGCCAACAACAGCCTGAGCAGCAACGGCAATGTGACCGACGTCCGGCTGCAAAGAGTGCGAGGAACTGGAAGAGAAGAATATCAAAGAGTTCCTGCAGAGCTTCGTGCACATCGTGCAGATGTTCATCAACACAAGC	TCTGGCGGCGGAGGATCTGGCGGAGGTGGAAGCGGAGTTACACCCGAGCCTATCTTCAGCCTGATCGGAGGCGGTAGCGGAGGCGGAGTGGTGGCGGATCTCTGCAA	CTGCTGCCTATCGCTGGGCCATCACACTGATCTCCGTGAACGGCATCTTCGTGATCTGCTGCCTGACCTACTGCTTCGCCCCTAGATGCAGAGAGCGGCGGAGAAACGAACGGCTGAGAAGAGAATCTGTGCGGCCCGTT
	SEQ ID NO			**217**	**221**	**224**	**330**	**227**	**235**	**270**	**278**	**282**	**214**	**286**	**288**	**331**
			AA	MLLLVTSLLLCELPHPAFLLIP	DIVMTQSPDSLAVSLGERATINCKSSQSLLYSSNQKNYLAWYQQKPGQPPKLLIYWASSRESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQQYYNYPLTFGQGTKLEIK	GGGGSGGGGSGGGGS	EVQLVESGGGLVQPGGSLRLSCAASGFTFNKNAMNWVRQAPGKGLEWVGRIRNKTNNYATYYADSVKARFTISRDDSKNSLYLQMNSLKTEDTAVYYCVAGNSFAYWGQGTLVTVSA	TTTPAPRPPTPAPTIALQPLSLRPEACRPAAGGAVHTRGLDFACD	VAAILGLGLVLGLLGPLAIL	ALYLLRRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKI	RVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR	GSGQCTNYALLKLAGDVESNPGPGSGEGRGSLLTCGDVEENPGP	MDWTWILFLVAAATRVHS	NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMKCFLLELQVISLESGDASIHDTVENLILANNSLSSNGNVTESGCKECEELEEKNIKEFLQSFVHIVQMFINTS	SGGGGSGGGGSG**VTPEPIFSLIG**GGSGGGSGGGSLQ	LLPSWAITLISVNGIFVICCLTYCFAPRCRRRRNERLRRESVRPV
	SEQ ID NO			**216**	**208**	**223**	**206**	**226**	**234**	**269**	**277**	**281**	**218**	**285**	**287**	**219**

SB06252																				
	GM-CSF-Ra (SS) - aGPC3 hPY7 vH - (GGGGS)3 - aGPC3 hPY7 vL - CD8FA (Hinge) - CD8FA (TM) - CD28 (ICD) - CD3z (ICD) - E2A T2A - IgE (SS) - IL-15 - Tace10 (cleavage site) - B7-1 (TM)																			
	SEQ ID NO		217	222	332	333	229	243	268	280	282	214	286	288	331					
		AA	MLLL VTSL LLCE LPHP AFLL IP	EVQLVESGGGLV QPGGSLRLSCAAS GFTFNKNAMNW VRQAPGKGLEWV GRIRNKTNNYAT YYADSVKARFTIS RDDSKNSLYLQM NSLKTEDTAVYY CVAGNSFAYWGQ GTLVTVSA	GGGGSG GGGSGG GGS	DIVMTQSPDSLAV SLGERATINCKSS QSLLYSSNQKNYL AWYQQKPGQPPK LLIYWASSRESGV PDRFSGSGSGTDF RDDSKNSLYLQM VYYCQQYYNYPL TFGQGTKLEIK	GALSNS IMYFSH FVPVFL PAKPTT TPAPRP PTPAPTI ASQPLS LRPEAC RPAAG GAVHT RGLDFA CD	IYIWA PLAGT CGVLL LSLVI TLYCN HR	RSKRSR LLHSDY MNMTP RRPGPT RKHYQ PYAPPR DFAAY RS	RVKFSRSADAPA YQQGQNQLYNEL NLGRREEYDVLD KRRGRDPEMGGK PRRKNPQEGLYN ELQKDKMAEAYS EIGMKGERRRGK GHDGLYQGLSTA TKDTYDALHMQA LPPR	GSG**QCTNYA** **LLKLAGDVE** **SNPGP**GSGE **GRGSLLTCG** **DVEENPGP**	MDWT WILFL VAAAT RVHS	NWVNVISDLKKIEDLIQ SMHIDATLYTESDVHPS CKVTAMKCFLLELQVI SLESGDASIHDTVENLII LANNSLSSNGNVTESG CKECEELEEKNIKEFLQ SFVHIVQMFINTS	SGGGGSGGG GSG**VTPEPIF** **SLIG**GGSGGGG SGGGGSLQ	LLPSWAITLIS VNGIFVICCL TYCFAPRCRE RRRNERLRRE SVRPV					
	SEQ ID NO		216	206	223	208	228	242	267	279	281	218	285	287	219					

SB06257				GM-CSF-Ra	hPY7 VL	(GGGGS)3	hPY7 VH	CD8 S2L	OX40	OX40	CD3z	E2A/T2A	IgE	IL15	LR1 split N term linker + Tace10	B7-1
	GM-CSF-Ra (SS) - aGPC3 hPY7 vL - (GGGGS)3 - aGPC3 hPY7 vH - CD8 S2L (Hinge) - OX40 (TM) - OX40 (ICD) - CD3z (ICD) - E2A T2A - IgE (SS) - IL-15 - Tace10 (cleavage site) - B7-1 (TM)	SinVec	DNA	ATGC TGCT GCTG GTCA CATC TCTG CTGC TGTG CGA GCTG CCCC ATCC TGCC TTTC TGCT GATC CCT	GACATCGTGATG ACACAGAGCCCC GATAGCCTGGCC GTGTCTCTGGGA GAAAGAGCCACC ATCAACTGCAAG AGCAGCCAGAGC CTGCTGTACTCC AGCAACCAGAAG AACTACCTGGCC TGGTATCAGCAA AAGCCCGGCCAG CCTCCTAAGCTG CTGATCTATTGG GCCAGCTCCAGA GAAAGCGGCGTG CCCGATAGATTT TCTGGCTCTGGC AGCGGCACCGAC TTCACCCTGACA ATTTCTAGCCTG CAAGCCGAGGAC GTGGCCGTGTAC TACTGCCAGCAG TACTACAACTAC CCTCTGACCTTC GGCCAGGGCACC AAGCTGGAAATC AAA	GGCGGC GGAGGA TCTGGCG GAGGTG GAAGTG GCGGAG GCGGAT	GAAGTGCAGCTG GTTGAATCAGGT GGCGGCCTGGTT CAACCTGGCGGA TCTCTGAGACTG AGCTGTGCCGCC AGCGGCTTCACC TTCAACAAGAAC GCCATGAACTGG GTCCGACAGGCC CCTGGCAAAGGC CTTGAATGGGTC GGACGGATCCGG AACAAGACCAAC AACTACGCCACC TACTACGCCGAC AGCCGGGACGAC AGCAAGAACAGC CTGTACCTGCAG ATGAACTCCCTG AAAACCGAGGAC ACCGCCGTGTAT TATTGCGTGGCC GCCTACTGGGGA CAGGGAACCCTG GTCACCGTGTCT GCC	ACAAC CCGCC ATTCT CGGA GACTT GTTCT GGGA CTGCT TGATGC GCCTCC TACAAT GCCCCT GCAGC CCTCGT CTCTGA GGCCA GAAGC TTGTAG ACCAG CAAGA TGCTAG GGCGAC GCGGA GGCTT ATTCT GCTG CTGCTC CTAGA CCTCCT ACACC AGCTCC TACAAT CGCCCT GCAGC CCTCGT CTCTGA GGCCA GAAGC TTGTAG ACCAG CTGCTG GGACT GGACT GGACTTI CGCCTG TGAT	GTGG CCGCC ATTCT CGGA GACTT GTTCT GGGA CTGCT GCTTA GGGA CCTCT GGCC ATTCT GCTG CTGCT GGGA CCTCT GGCC ATTCT GCTG CTGCT GGGA CCTCT GGCC ATTCT GCTG CTGCT

SB06258				GM-CSF-Ra	hPY7 VH	(GGGGS)3	hPY7 VL	CD8FA	CD8FA	CD28	CD3z	E2A/T2A	IgE	IL15	LR1 split N term linker + Tace10	B7-1
	GM-CSF-Ra (SS) - aGPC3 hPY7 vH - (GGGGS)3 - aGPC3 hPY7 vL - CD8FA (Hinge) - CD8 (TM) - CD28 (ICD) - CD3z (ICD) - E2A T2A - IgE (SS) - IL-15 - Tace10 (cleavage site) - B7-1 (TM)	SinVec	DNA	ATGC TGCT GCTG GTCA CATC TCTG CTGC TGTG CGA GCTG CCCC ATCC TGCC TTTC TGCT GATC CCT	GAAGTGCAGCTG GTTGAATCAGGT GGCGGCCTGGTT CAACCTGGCGGA TCTCTGAGACTG AGCTGTGCCGCC AGCGGCTTCACC TTCAACAAGAAC GCCATGAACTGG GTCCGACAGGCC CCTGGCAAAGGC CTTGAATGGGTC GGACGGATCCGG AACAAGACCAAC AACTACGCCACC TACTACGCCGAC AGCGTGAAGGCC AGATTCACCATC AGCCGGGACGAC AGCAAGAACAGC CTGTACCTGCAG ATGAACTCCCTG AAAACCGAGGAC ACCGCCGTGTAT TATTGCGTGGCC GGCAACAGCTTT GCCTACTGGGGA CAGGGAACCCTG GTCACCGTGTCT GCC	GGCGGC GGAGGA TCTGGCG GAGGTG GAAGTG GCGGAG GCGGAT CT	GACATCGTGATT GACACAGAGCC CCGATAGCCTG GCCGTGTCTCT GGGAGAAAGA GCCACCATCAA CTGCAAGAGCA GCCAGAGCCTG CTGTACTCCAG CAACCAGAAGA ACTACCTGGCC TGGTATCAGCA AAAGCCCGGCC AGCCTCCTAAG CTGCTGATCTA TTGGGCCAGCT GGCGTGCCCGA TAGATTTCTGG CTCTGGCAGCG GCACCGACTTC ACCCTGACAAT TACCCTCTGAC CTTCGGCCAGG GCACCAAGCTG GAAATCAAA	GGCGCC CTGAGC AACAGC ATCATGT ACTTCAG CCACTTC GTGCCC GTGTTTC CCTGCCGC CAAGCC TACAAC AACCCCT GCCAGC GACCTCC TGCCAGC CTGCTAC AGCCTC TGTCTCT GAGGCC AGAAGC TGCATAC TTGTAGA CCTGCTG CAGGCG GAGCCG TTTCGCC TGCGAC	ATCTA CATCT GGGC AGCT TGGCT GGAA CATGT GGTGT CCTGC TGCTG AGCCT GGTC G ACTGC ACTAC AACC ACCG	CGGAG GAAGC AGACT GCTGC ACAGC GACTA CATGA ACCCCT AGACG GCCCG GACCT ACCAG AAAGC ACTACC AGCTT ACGCTC CTCCTA GAGAC TTCGCC GAGCTC TTCGCC GCCTAC GAAGC CTGCTC GAGGCC AGAAGC CCTGCTG CAGGCG GAGCCG TGCATAC AAGAGG ACTGGA TTTCGCC TGCGAC	AGAGTGAAGTTC AGCAGATCCGCC GATGCTCCCGCC TATCAGCAGGGA CAGAACCAGCTG TACAACGAGCTG AACCTGGGCAGA AGAGAAGAGTAC CGACGTGCATGA AAGAAGAGTTC AAGCGGAGAGGC ATGGGCGACAAG CCCAGACGGAAG AATCCTCAAGAG GGCCTGTATAAT GAGCTGCAGAAA GACAAGATGGCC GAGATCGGAATG AAGGGCGAGCGC AGAAGAGGCAA GGGACACGATGG ACTGTACCAGGG CCTGACACCGC CACCAAGGATAC CTATGATGCCCT GCACATGCAGGC CCTGCCTCCAAG A	GGTAGCGGC CAGTGTACC AACTACGCC CTGCTGAAA CTGGCCGGC GACCTGGAT GACCTGGAG GAGCAAGAGGG GACCT GACCT	ATGGA CTGGA CCTGG ATCCT GTTTC TGGTG GCCGC TGCCA CAAGA GTGCA CAGC	AATTGGGTCAACGTG ATCAGCGACCTGAAG AAGATCGAGGACCTG ATCCAGAGCATGCAC ATCGACGCCCACACTGT ACACCGAGAGCGACG TGCACCCTAGCTGTAA AGTGACCGCCATGAA GTGCTTTCTGCTGGAA CTGCAAGTGATCAGCC TGGAAAGCGGCGACG CCAGCATCCACGACA CCGTGGAAAACCTGA TCATCCTGGCCAACAA CAGCCTGAGCAGCAA CGGCAATGTGACCGA GTCCGGCTGCAAAGA GTGCGAGGAACTGGA AGAGAAGAATATCAA AGAGTTCCTGCAGAG CTTCGTGCACATCGTG CAGATGTTCATCAACA CAAGC	TCTGGCGCGGC GGAGGATCT GGCGGAGGTI GCCGAAGCGGA GGATCCTATC TTCAGCCTG AGTGGCCTGA CCTACTGCTT CGCCCCTAG ATGCAGAGA GCGGCGGAG AAACGAACG GCTGAGAAG AGAATCTGT GCGGCCCGT T	CTGCTGCCT AGCTGGGCC ATCACACTG ATCTCCGTG AACGGCATC TTCGTGATCI GCTGCCTGA CCTACTGCTT
	SEQ ID NO			217	330	224	221	229	243	268	280	282	214	286	288	331
			AA	MLLL VTSL LLCE LPHP AFLL IP	EVQLVESGGGLV QPGGSLRLSCAAS GFTFNKNAMNW VRQAPGKGLEWV GRIRNKTNNYAT YYADSVKARFTIS RDDSKNSLYLQM NSLKTEDTAVYY CVAGNSFAYWGQ GTLVTVSA	GGGGSG GGGSGG GGS	DIVMTQSPDSLA VSLGERATINCK SSQSLLYSSNQK NYLAWYQQKPG QPPKLLIYWASS RESGVPDRFSGS GSGTDFTLTISSL QAEDVAVYYCQ QYYNYPLTFGQ GTKLEIK	GALSNSI MYFSHFV PVFLPAK PTTTPAP RPPTPAP TIASQPLS LRPEACR PAAGGA VHTRGL DFACD	IYTWA PLAGT CGVLL LSLVI TLYCN HR	RSKRSR LLHSDY MNMTP RRPGPT RKHYQ PYAPPR DFAAY RS	RVKFSRSADAPA YQQQQNQLYNEL NLGRREEYDVLD KRRGRDPEMGGK PRRKNPQEGLYN ELQKDKMAEAYS EIGMKGERRRGK GHDGLYQGLSTA TKDTYDALHMQA LPPR	**GSG**QCTNYA LLKLAGDVES NPGP**GSG**EGR GSLLTCGDVE ENPGP	MDWT WILFL VAAAT RVHS	NWVNVISDLKKIEDLIQ SMHIDATLYTESDVHPS CKVTAMKCFLLELQVI SLESGDASIHDTVENLII LANNSLSSNGNVTESG CKECEELEEKNIKEFLQ SFVHIVQMFINTS	SGGGGSGGG **GSGVTPEPIF SLIG**GGSGGG GSGGGSLQ	LLPSWAITLIS VNGIFVICCL TYCFAPRCRE RRRNERLRRE SVRPV
	SEQ ID NO			216	206	223	208	228	242	267	279	281	218	285	287	219

SB06298																	
			GM-CSF-Ra	hPY7 VH	(GGGGS)3	hPY7 VL	CD8FA	CD8FA	CD28	CD3z mut	E2A/T2A	IgE	IL15	LR1 split N term linker + Tace10	B7-1
	GM-CSF-Ra (SS) - aGPC3 hPY7 vH - (GGGGS)3 - aGPC3 hPY7 vL - CD8FA (Hinge) - CD8 (TM) - CD28 (tCD) - CD3z mut (ICD) - E2A T2A - IgE (SS) - IL-15 - Tace10 (cleavage site) - B7-1 (TM)	SinVec	DNA	ATGC TGCT GCTG GTCA CATC TCTG CTGC TGTG CGA GCTG CCCC ATCC TGCC TTTC TGCT GATC CCT	GAAGTGCAGCTG GTGGAATCTGGC GGAGGACTGGTT CAACCTGGCGGC TCTCTGAGACTG TCTTGTGCCGCC AGCGGCTTCACC TTCAACAAGAAC GCCATGAACTGG GTCCGACAGGCC CCTGGCAAAGGC CTTGAATGGGTC GGACGGATCCGG AACAAGACCAAC AACTACGCCACC TACTACGCCGAC AGCGTGAAGGCC ATGAACTCCCTG AAAACCGAGGAC ACCGCCGTGTAC TATTGCGTGGCC GGCAATAGCTTT GCCTACTGGGGA CAGGGCACCCTG GTTACAGTTTCT GCT	332	GGCGGC GGAGGA AGCGGA GGATCC GGTGGT GGTGGA TCT	333	GACATCGTGATG GACACAGAGCC CCGATAGCCTG GCCGTGTCTCT GGGAGAAAGA GCCACCATCAA CTGCAAGAGCA GCCAGAGCCTG CTGTACTCCAG CAACCAGAAGA ACTACCTGGCC TGGTATCAGCA AAAGCCCGGCC AGCCTCCTAAG CTGCTGATCTA TTGGGCCAGCT AGCGTGCCGAC TAGATTTCTGG CTCTGGCAGCG GCACCGACTTC ACCCTGACAAT TTCTAGCCTGC AAGCCGAGGAC GTGGCCGTGTA AGTACTACAAC TACCCTCTGAC CTTCGGCCAGG GCACCAAGCTG GAAATCAAA	243	229	268	334	284	214	286	288	331
		AA	MLLL VTSL LLCE LPHP AFLL IP	EVQLVESGGGLV QPGGSLRLSCAAS GFTFNKNAMNW VRQAPGKGLEWV GRIRNKTNNYAT YYADSVKARFTIS RDDSKNSLYLQM NSLKTEDTAVYY CVAGNSFAYWGQ GTLVTVSA	GGGGSG GGGSGG GGS	DIVMTQSPDSLA VSLGERATINCK SSQSLLYSSNQK NYLAWYQQKPG QPPKLLIYWASS RESGVPDRFSGS GSGTDFTLTISSL QAEDVAVYYCQ QYYNYPLTFGQ GTKLEIK	208	GALSNSI MYFSHFV PVFLPAK PTTTPAP RPPTPAP TIASQPLS LRPEACR PAAGGA VHTRGL DFACD	228	IYTWA PLAGT CGVLL LSLVI TLYCN HR	242	RSKRSR LLHSDY MNMTP RRPGPT RKHYQ PYAPPR DFAAY RS	267	RVKFSRSADAPA YKQQQNQLYNEL NLGRREEYDVLD KRRGRDPEMGGK PRRKNPQEGLYN ELQKDKMAEAYS EIGMKGERRRGK GHDGLYQGLSTA TKDTYDALHMQA LPPR	277	QCTNYALLK LAGDVESNPG PGSGEGRGSL LTCGDVEENP GP	283	MDWT WLFL VAAAT RVHS	218	NWVNVISDLKKIEDLIQ SMHIDATLYTESDVHPS CKVTAMKCFLLELQVI SLESGDASIHDTVENLII LANNSLSSNGNVTESG CKECEELEEKNIKEFLQ SFVHIVQMFINTS	285	287	219
	SEQ ID NO		216	206	223	208	228	242	267	277	283	218	285	287	219

SB ID	Description	Backbone	Seq Type	SS	IL15	Cleavage Site	TM domain	E2A	SS	scFV VL	Linker	scFV VH	Hinge	TM	Co-stim ICD	CD3z ICD
SB06254				IgE	IL15	LR1 split N term linker + Tace10	B7-1	E2A/T2A	GM-CSF-Ra	hPY7 VL	(GGGGS)3	hPY7 VH	CD8 S2L	OX40	OX40	CD3z
	IgE (SS) - IL-15 Tace10 (cleavage site) - B7-1 (TM) - E2A T2A - GM-CSF-Ra (SS) - aGPC3 hPY7 vL - (GGGGS)3 - aGPC3 hPY7 vH - CD8 S2L (Hinge) - OX40 (TM) - OX40 (ICD) - CD3z (ICD)		DNA	ATGG ACTG GACC TGGA TCCT GTTT CTGG TGGC CGCT GCCA CAA GAGT GCAC AGC	AATTGGGTCAAC GTGATCAGCGAC CTGAAGAAGATC GAGGACCTGATC CAGAGCATGCAC ATCGACGCCACA CTGTACACCGAG AGCGACGTGCAC CCTAGCTGTAAA GTGACCGCCATG AAGTGCTTTCTG CTGGAACTGCAA GTGATCAGCCTG GAAAGCGGCGAC GCCAGCATCCAC GACACCGTGGAA AACCTGATCATC CTGGCCAACAAC AGCCTGAGCAGC AACGGCAATGTG ACCGAGTCCGGC TGCAAAGAGTGC GAGGAACTGGAA GAGAAGAATATC AAAGAGTTCCTG CAGAGCTTCGTG CACATCGTGCAG ATGTTCATCAAC ACAAGC	TCTGGCG GCGGAG GATCTG GCGGAG GTGGAA GCGGAG TTACACC CGAGCC TATCTTC ATCGGA GGCGGT GCGGGA GGCGGA GGAAGT GGTGGC GGATCTC TGCAA	CTGCTGCC TAGCTGG GCCATCA CACTGAT CTCCGTG AACGGCA TCTTCGTG ATCTGCTG CCTGACCT ACTGCTTC GCCCCTA GATGCAG AGAGCGG CGGAGAA ACGAACG GCTGAGA AGAGAAT CTGTGCG GCCCGTT	CAGTGT ACCAA CTACGC CCTGCT GAAAC TGGCC GGCGA CGTGG AATCTA ATCCTG GACCT GGATCT GGCGA GGGAC GCGGG GTGGA GGAAA ACCCTG GACCT	ATGCT GCTGC TGGTC ACATC TCTGC TGCTG TGCG AGCT GCCCC AGCAACCAGAAG AACTACCTGGCC GCCTT TCTGC TGATC CCT	GACATCGTGATG ACACAGAGCCCC AACTACCTGGCC GATAGCCTGGCC GTGTCTCTGGGA GAAAGAGCCACC ATCAACTGCAAG AGCAGCCAGAGC CTGCTGTACTCC AGCAACCAGAAG AACTACCTGGCC TGGTATCAGCAA AAGCCCGGCCAG CCTCCTAAGCTG CTGATCTATTGG GCCAGCTCCAGA GAAAGCGGCGTG CCCGATAGATTT TCTGGCTCTGGC AGCGGCACCGAC TTCACCCTGACA ATTTCTAGCCTG CAAGCCGAGGAC GTGGCCGTGTAC TACTGCCAGCAG TACTACAACTAC CCTCTGACCTTC GGCCAGGGCACC AAGCTGGAAAATC AAA	GGCGGCG GAGGATC TGGCGGA GGTGGAA GTGGCGG AGGCGGA TCT	GAAGTGCAGCTG GTTGAATCAGGT GGCGGCCTGGTT CAACCTGGCGGA TCTCTGAGACTG AGCTGTGCCGCC ATCAACTGCCAC AGCGGCTTCACC TTCAACAAGAAC GCCATGAACTGG GTCCGACAGGCC CCTGGCAAAGGC CTTGAATGGGTC GGACGGATCCGG AACAAGACCAAC AACTACGCCACC TACTACGCCGAC AGCGTGAAGGCC AGATTCACCATC AGCCGGGACGAC AGCAAGAACAGC CTGTACCTGCAG ATGAACTCCCTG AAAACCGAGGAC ACCGCCGTGTAT TATTGCGTGGCC GGCAACAGCTTT GCCTACTGGGGA CAGGGAACCCTG GTCACCGTGTCT GCC	ACAACAAC CCCTGCTCC TAGACCTC CTACACCA GCTCCTAC AATCGCCC TGCAGCCT CTGTCTCTG AGGCCAGA AGCTTGTA GACCAGCT CTTCGCCTG TGAT	GTGG CCGCC ATTCT CGGA CTGG GACTT GTTCT GGGA CTGCT GGGA TT	GCTCTGTATCTGCT GCGGAGGGACCAA AGACTGCCTCCTGA TGCTCACAAGCCTC CAGGCGGAGGCAG CTTCAGAACCCCTA TCCAAGAGGAACAG GCCGACGCTCACAG CACCCTGGCCAAGA	AGAGTGAAGTTC AGCAGAAGCGCC GACGCACCCGCC TATAAGCAGGGA CAGAACCAGCTG TACAACGAGCTG AACCTGGGGAGA AGAGAAGAGTAC GACGTGCTGGAC AAGCGGAGAGGC AGAGATCCTGAG ATGGGCGGCAAG CCCAGACGGAAG AATCCTCAAGAG GGCCTGTATAAT GAGCTGCAGAAA GACAAGATGGCC GAGGCCTACAGC GAGATCGGAATG AAGGGCGAGCGC AGAAGAGGCAA GGGACACGATGG ACTGTACCAGGG CCTGAGCACCGC CACCAAGGATAC CTATGATGCCCT GCACATGCAGGC CCTGCCTCCAAG A
	SEQ ID NO			214	286	288	331	284	217	221	224	330	227	245	270	278
			AA	MDW TWIL FLVA AATR VHS	NWVNVISDLKKIE DLIQSMHIDATLY TESDVHPSCKVTA MKCFLLELQVISL ESGDASIHDTVEN LILANNSLSSNGN VTESGCKECEELE EKNIKEFLQSFVHI VQMFINTS	SGGGSGG GGGSGV TPEPIS LIGGGSG GGGSGG GSLQ	LLPSWAIT LISVNGIFV ICCLTYCF APRCRERR RNERLRRE SVRPV	QCTNY ALLKLA GDVESN PGPGSG EGRGSL LTCGDV EENPGP	MLLL VTSLL LCELP HPAFL LIP	DIVMTQSPDSLAV SLGERATINCKSS QSLLYSSNQKNYL AWYQQKPGQPPK LLIYWASSRESGV PDRFSGSGSGTDF TLTISSLQAEDVA VYYCQQYYNYPL TFGQGTKLEIK	GGGGSGG GGSGGGG S	EVQLVESGGGLV QPGGSLRLSCAAS GFTFNKNAMNW VRQAPGKGLEWV GRIRNKTNNYAT YYADSVKARFTIS RDDSKNSLYLQM NSLKTEDTAVYY CVAGNSFAYWGQ GTLVTVSA	TTTPAPRPP TPAPTIALQP LSLRPEACR PAAGGAVH TRGLDFACD	VAAIL GLGLV LGLLG PLAIL L	ALYLLRRDQRLPPD AHKPPGGGSFRTPIQ EEQADAHSTLAKI	RVKFSRSADAPA YKQGQNQLYNEL NLGRREEYDVLD KRRGRDPEMGGK PRKNPQEGLYN ELQKDKMAEAYS EIGMKGERRRGK GHDGLYQGLSTA TKDTYDALHMQA LPPR
	SEQ ID NO			218	285	287	219	283	216	208	223	206	226	244	269	277

SB06255			IgE	IL15	LR1 split N term linker + Tace10	B7-1	E2A/T2A	GM-CSF-Ra	hPY7 VH	(GGGGS)3	hPY7 VL	CD8FA	CD8FA	CD28	CD3z
	IgE (SS) - IL-15 Tace10 (cleavage site) - B7-1 (TM) - E2A T2A - GM-CSF-Ra (SS) - aGPC3 hPY7 vL - (GGGGS)3 - aGPC3 hPY7 vH - CD8FA (Hinge) - CD8FA (TM) -CD28 (ICD) - CD3z (ICD)	DNA	ATGG ACTG GACC TGGA TCCT GTTT CTGG TGGC CGCT GCCA CAA GAGT GCAC AGC	AATTGGGTCAAC GTGATCAGCGAC CTGAAGAAGATC GAGGACCTGATC CAGAGCATGCAC ATCGACGCCACA CTGTACACCGAG AGCGACGTGCAC CCTAGCTGTAAA GTGACCGCCATG AAGTGCTTTCTG CTGGAACTGCAA GTGATCAGCCTG GAAAGCGGCGAC GCCAGCATCCAC GACACCGTGGAA AACCTGATCATC CTGGCCAACAAC AGCCTGAGCAGC AACGGCAATGTG ACCGAGTCCGGC TGCAAAGAGTGC GAGGAACTGGAA GAGAAGAATATC AAAGAGTTCCTG CAGAGCTTCGTG CACATCGTGCAG ATGTTCATCAAC ACAAGC	TCTGGCGG GCGGAG GATCTG GCGGAG GTGGAA GCGGAG TTACACC CGAGCC TATCTTC AGCCTG ATCGGA GGCGGA GGAAGT GGTGGC GGATCTC TGCAA	CTGCTGCC TAGCTGG GCCATCA CACTGAT CTCCGTG AACGGCA ATCTCGTG ATCTGCTG CCTGACCT ACTGCTTC GCCCCTA GATGCAG AGAGCGG CGGAGAA ACGAACG GCTGAGA AGAGAAT CTGTGCG GCCCGTT	LLPSWAIT LISVNGIFV **TPEPIFS** APRCRERR RNERLRRE SVRPV	QCTNY ALLKLA GDVESN PGPGSG EGRGSL LTCGDV EENPGP	ATGCT GCTGC TGGTC ACATC TCTGC TGCTG TGCG AGCT GCCCC ATCCTT GACCT GCCTT TCTGC TGATC CCT	MLLL VTSLL LCELP HPAFL LIP	GAAGTGCAGCTG GTGGAATCTGGC GGAGGACTGGTT TCTCTGAGACTG TCTTGTGCCGCC AGCGGCTTCACC TTCAACAAGAAC GCCATGAACTGG GTCCGACAGGCC CCTGGCAAAGGC CTTGAATGGGTC GGACGGATCCGG AACAAGACCAAC AACTACGCCACC TACTACGCCGAC AGCGTGAAGGCC AGTTCACCATC TCCAGAGATGAC AGCAAGAACAGC CTGTACCTGCAG ATGAACTCCCTG AAAACCGAGGAC ACCGCCGTGTAC TATTGCGTGGCC GGCAATAGCTTT GCCTACTGGGGA CAGGGCACCCTG GTTACAGTTTCT GCT	GGCGGCG GAGGAAG CGGAGGC GGAGGAT CCGGTGG TGGTGGA TCT	GACATCGTGATG ACACAGAGCCCC GATAGCCTGGCC GTGTCTCTGGGA GAAAGAGCCACC ATCAACTGCAAG AGCAGCCAGAGC CTGCTGTACTCC AGCAACCAGAAG AACTACCTGGCC TGGTATCAGCAA AAGCCCGGCCAG CCTCCTAAGCTG CTGATCTATTGG GCCAGCTCCAGA GAAAGCGGCGTG CCCGATAGATTT TCTGGCTCTGGC AGCGGCACCGAC TTCACCCTGACA ATTTCTAGCCTG CAAGCCGAGGAC GTGGCCGTGTAT TACTGCCAGCAG TACTACAACTAC CCTCTGACCTTC GGCCAGGGCACC AAGCTGGAAATC AAA	GGCGCCCT GAGCAACA GCATCATG CACTTCAGC CACTTCGTG CTGCCCGC CAAGCCTA CAACAACC CCTGCTCCT AGACCTCC TACACCAG CTCCTACA ATCGCCAG CCAGCCTCT GTCTCTGA GGCCAGAA GCTTGTAG ACCTGCTG CAGGCGGA GCCGTGCA GACTGGAT TTCGCCTGC GAC	ATCTA CATCT GGGC CCCTC TGGCT GGAA CATGT GGTGT CCTGC TGCTG AGCCT GGTC CATGC ACTGC AACC ACCG G	CGGAGCAAGAGAA GCAGACTGCTGCAC AGCGACTACATGAA GGCCCGGACCTACC AGAAAGCACTACCA GCCTTACGCTCCTC CTAGAGACTTCGCC GCCTACCGGTCC	AGAGTGAAGTTC AGCAGATCCGCC GATGCTCCCGCC TATCAGCAGGGA CAGAACCAGCTG TACAACGAGCTG AACCTGGGGAGA AGAGAAGAGTAC GACGTGCTGGAC AAGCGGAGAGGC AGAGATCCTGAG ATGGGCGGCAAG CCCAGACGGAAG AATCCTCAAGAG GGCCTGTATAAT GAGCTGCAGAAA GACAAGATGGCC GAGGCCTACAGC GAGATCGGAATG AAGGGCGAGCGC AGAAGAGGCAA GGGACACGATGG ACTGTACCAGGG CCTGAGCACCGC CACCAAGGATAC CTATGATGCCCT GCACATGCAGGC CCTGCCTCCAAG A
	SEQ ID NO		**214**	**286**	**288**	**331**	**284**	**217**	**222**	**332**	**333**	**229**	**243**	**268**	**280**
		AA	MDW TWIL FLVA AATR VHS	NWVNVISDLKKIE DLIQSMHIDATLY TESDVHPSCKVTA MKCFLLELQVISL ESGDASHIDTVEN LIILANNSLSSNGN VTESGCKECEELE EKNIKEFLQSFVHI VQMFINTS	SGGGGSG GGGSGV **TPEPIFS** **LI**GGGSG GGGSGG GSLQ	LLPSWAIT LISVNGIFV ICCLTYCF APRCRERR RNERLRRE SVRPV	QCTNY ALLKLA GDVESN PGPGSG EGRGSL LTCGDV EENPGP	MLLL VTSLL LCELP HPAFL LIP	EVQLVESGGGLV QPGGSLRLSCAAS GFTFNKNAMNW VRQAPGKGLEWV GRIRNKTNNYAT YYADSVKARFTIS RDDSKNSLYLQM NSLKTEDTAVYY CVAGNSFAYWGQ GTLVTVSA	GGGGSGG GGSGGGG S	DIVMTQSPDSLAV SLGERATINCKSS QSLLYSSNQKNYL AWYQQKPGQPPK LLIYWASSRESGV PDRFSGSGSGTDF TLTISSLQAEDVA VYYCQQYYNYPL TFGQGTKLEIK	GALSNSIMY FSHFVPVFL PAKPTTTPA PRPPTPAPTI ASQPLSLRP EACRPAAG GAVHTRGL DFACD	IYIWA PLAGT CGVLL LSLVI TLYCN HR	RSKRSRLLHSDYMN MTPRRPGPTRKHYQ PYAPPRDFAAYRS	RVKFSRSADAPA YQQGQNQLYNEL NLGRREEYDVLD KRRGRDPEMGGK PRRKNPQEGLYN ELQKDKMAEAYS EIGMKGERRRGK GHDGLYQGLSTA TKDTYDALHMQA LPPR
	SEQ ID NO		**218**	**285**	**287**	**219**	**283**	**216**	**206**	**223**	**208**	**228**	**242**	**267**	**279**

SB06294																		
	IgE (SS) - IL-15 Tace10 (cleavage site) - B7-1 (TM) - E2A T2A - GM-CSF-Ra (SS) - aGPC3 hPY7 vL - (GGGGS)3 - aGPC3 hPY7 vH - CD8 S2L (Hinge) - OX40 (TM) - OX40 (ICD) - CD3z mut (ICD)	RetroVec	DNA	ATGG ACTG GACC TGGA TCCT GTTT CTGG TGGC CGCT GCCA CAA GAGT GCAC AGC	AATTGGGTCAAC GTGATCAGCGAC CTGAAGAAGATC GAGGACCTGATC CAGAGCATGCAC ATCGACGCCACA CTGTACACCGAG AGCGACGTGCAC CCTAGCTGTAAA GTGACCGCCATG AAGTGCTTTCTG CTGGAACTGCAA GTGATCAGCCTG GAAAGCGGCGAC GCCAGCATCCAC GACACCGTGGAA AACCTGATCATC CTGGCCAACAAC AGCCTGAGCAGC AACGGCAATGTG ACCGAGTCCGGC TGCAAAGAGTGC GAGGAACTGGAA GAGAAGAATATC AAAGAGTTCCTG CAGAGCTTCGTG CACATCGTGCAG ATGTTCATCAAC ACAAGC	LR1 split N term linker + Tace10	B7-1	E2A/T2A	GM-CSF-Ra	hPY7 VL	(GGGGS)3	hPY7 VH	CD8 S2L	OX40	OX40	CD3z mut
	SEQ ID NO			214	286	288	331	284	217	221	224	330	227	245	270	334
			AA	MDW TWIL FLVA AATR VHS	NWVNVISDLKKIE DLIQSMHIDATLY TESDVHPSCKVTA MKCFLLELQVISL ESGDASHIDTVEN LIILANNSLSSNGN VTESGCKECEELE EKNIKEFLQSFVHI VQMFINTS	SGGGGSG GGGSGV **TPEPIFS** **LIG**GGSG GGGSGG GSLQ	LLPSWAIT LISVNGIFV ICCLTYCF APRCRERR RNERLRRE SVRPV	QCTNY ALLKLA GDVESN PGPGSG EGRGSL LTCGDV EENPGP	MLLL VTSLL LCELP HPAFL LIP	DIVMTQSPDSLAV SLGERATINCKSS QSLLYSSNQKNYL AWYQQKPGQPPK LLIYWASSRESGV PDRFSGSGSGTDF TLTISSLQAEDVA VYYCQQYYNYPL TFGQGTKLEIK	GGGGSGG GGSGGGG S	EVQLVESGGGLV QPGGSLRLSCAAS GFTFNKNAMNW VRQAPGKGLEWV GRIRNKTNNYAT YYADSVKARFTIS RDDSKNSLYLQM NSLKTEDTAVYY CVAGNSFAYWGQ GTLVTVSA	TTTPAPRPP TPAPTIAIQP LSLRPEACR PAAGGAVH TRGLDFACD	VAAIL GLGLV LGLLG PLAIL L	ALYLLRRDQRLPPD AHKPPGGGSFRTPIQ EEQADAHSTLAKI	RVKFSRSADAPA **YKQGQNQLYNEL** NLGRREEYDVLD KRRGRDPEMGGK PRRKNPQEGLYN ELQKDKMAEAYS EIGMKGERRRGK GHDGLYQGLSTA TKDTYDALHMQA LPPR
	SEQ ID NO			218	285	287	219	283	216	208	223	206	226	244	269	277

SB06692			IgE	IL15	TaceOPT + LR1 linker	B7-1	E2A/T2A	GM-CSF-Ra	hPY7 VL	(GGGGS)3	hPY7 VH	CD8 S2L	OX40	OX40	CD3z
	IgE (SS) - IL-15 - TaceOPT (cleavage site) - B7-1 (TM) - E2A T2A - GM-CSF-Ra (SS) - aGPC3 hPY7 vL - (GGGGS)3 - aGPC3 hPY7 vH - CD8 (Hinge) - OX40 (TM) - OX40 (ICD) - CD3z (ICD)	SinVec	DNA	ATGG ACTG GACC TGGA TCCT GTTT CTGG TGGC CGCT GCCA CAA GAGT GCAC AGC	AATTGGGTCAAC GTGATCAGCGAC CTGAAGAAGATC GAGGACCTGATC CAGAGCATGCAC ATCGACGCCACA CTGTACACCGAG AGCGACGTGCAC CCTAGCTGTAAA GTGACCGCCATG AAGTGCTTTCTG CTGGAACTGCAA GTGATCAGCCTG GAAAGCGGCGAC GCCAGCATCCAC GACACCGTGGAA AACCTGATCATC CTGGCCAACAAC AGCCTGAGCAGC AACGGCAATGTG ACCGAGTCCGGC TGCAAAGAGTGC GAGGAACTGGAA GAGAAGAATATC AAAGAGTTCCTG CAGAGCTTCGTG CACATCGTGCAG ATGTTCATCAAC ACAAGC	CCCAGA GCCGAG GCTCTGA AAGGCG GATCAG GCGGCG GTGGTA GTGGAG GCGGAG GCTCAG GCGGCG GAGGTT CCGGAG GTGGCG GTTCCGG CGGAGG ATCTCTT CAAT	CTGCTGCC TAGCTGG GCCATCA CACTGAT CTCCGTG AACGGCA TCTTCGTG ATCTGCTG CCTGACCT ACTGCTTC GCCCCTA GATGCAG AGACGCG CGGAGAA ACGAACG GCTGAGA AGAGAAT CTGTGCG GCCCGTT	CAGTGT ACCAA CTACGC CCTGCT GAAAC TGGCC GGCGA CGTGG AATCTA ATCCTG GACCT GGATCT GGCGA GGGAC GCGGG AGTCTA CTGAC GTGTG GAGAC GTGGA GAAAA ACCCTG GACCT	ATGCT GCTGC TGGTC ACATC TCTGC TGCTG TGCG AGCT GCCCC ATCCT GCCTT TCTGC TGATC	GACATCGTGATG ACACAGAGCCCC GATAGCCTGGCC GTGTCTCTGGGA GAAAGAGCCACC ATCAACTGCAAG AGCAGCCAGAGC CTGCTGTACTCC AGCAACCAGAAG AACTACCTGGCC TGGTATCAGCAA AAGCCCGGCCAG CCTCCTAAGCTG CTGATCTATTGG GCCAGCTCCAGA GAAAGCGGCGTG CCCGATAGATTT TCTGGCTCTGGC AGCGGCACCGAC TTCACCCTGACA ATTTCTAGCCTG CAAGCCGAGGAC GTGGCCGTGTAC TACTGCCAGCAG TACTACAACTAC CCTCTGACCTTC GGCCAGGGCACC AAGCTGGAAATC AAA	GGCGGCG GAGGATC TGGCGGA GGTGGAA GTGGCGG AGGCGGA TCT	GAAGTGCAGCTG GTTGAATCAGGT GGCGGCCTGGTT CAACCTGGCGGA TCTCTGAGACTG AGCTGTGCCGCC AGCGGCTTCACC TTCAACAAGAAC GCCATGAACTGG GTCCGACAGGCC CCTGGCAAAGGC CTTGAATGGGTC GGACGGATCCGG AACAAGACCAAC AACTACGCCACC TACTACGCCGAC AGCGTGAAGGCC AGATTCACCATC AGCCGGGACGAC AGCAAGAACAGC CTGTACCTGCAG ATGAACTCCCTG ACCGCCGTGTAT TATTGCGTGGCC GGCAACAGCTTT GCCTACTGGGGA CAGGGAACCCTG GTCACCGTGTCT GCC	ACAACAAC CCCTGCTCC TAGACCTC CTACACCA GCTCCTAC AATCGCCC TGCAGCCT CTGTCTCTG AGGCCAGA AGCTTGTA GACCAGCT GCTGGCGG AGCCGTGC ATACAAGA GGACTGGA CTTCGCCTG TGAT TTTPAPRPP TPAPTIALQP LSLRPEACR PAAGGAVH TRGLDFACD	GTGG CCGCC ATTCT CGGA CTGG GACTT GTTCT GGGA CTGCT GGGA CCTCT GGCC ATTCT GCTG VAAIL GLGLV LGLLG PLAIL L	GCTCTGTATCTGCT GCGGAGGGACCAA AGACTGCCTCCTGA TGCTCACAAGCCTC CAGGCGGAGGCAG CTTCAGAACCCCTA TCCAAGAGGAACAG GCCGACGCTCACAG CACCCTGGCCAAGA TT ALYLLRRDQRLPPD AHKPPGGGSFRTPIQ EEQADAHSTLAKI	AGAGTGAAGTTC AGCAGAAGCGCC GACGCACCCGCC TATAAGCAGGGA CAGAACCAGCTG TACAACGAGCTG AACCTGGGGAGA AGAGAAGAGTAC GACGTGCTGGAC AAGCGGAGAGGC AGAGATCCTGAG ATGGGCGGCAAG CCCAGACGGAAG AATCCTCAAGAG GGCCTGTATAAT GAGCTGCAGAAA GACAAGATGGCC GAGGCCTACAGC GAGATCGGAATG AAGGGCGAGCGC AGAAGAGGCAA GGGACACGATGG ACTGTACCAGGG CCTGAGCACCGC CACCAAGGATAC CTATGATGCCCT GCACATGCAGGC CCTGCCTCCAAG A
	SEQ ID NO			214	286	292	331	284	217	221	224	330	227	245	270	278
			AA	MDW TWIL FLVA AATR VHS	NWVNVISDLKKIE DLIQSMHIDATLY TESDVHPSCKVTA MKCFLLELQVISL ESGDASIHDTVEN LIILANNSLSSNGN VTESGCKECEELE EKNIKEFLQSFVHI VQMFINTS	**PRAEAL** **KGG**SGG GGSGGG GSGGGGS GGGGSG GGSLQ	LLPSWAIT LISVNGIFV ICCLTYCF APRCRERR RNERLRRE SVRPV	QCTNY ALLKLA GDVESN PGPGSG EGRGSL LTCGDV EENPGP	MLLL VTSLL LCFLP HPAFL LIP	DIVMTQSPDSLAV SLGERATINCKSS QSLLYSSNQKNYL AWYQQKPGQPPK LLIYWASSRESGV PDRFSGSGSGTDF TLTISSLQAEDVA VYYCQQYYNYPL TFGQGTKLEIK	GGGGSGG GGSGGGG S	EVQLVESGGGLV QPGGSLRLSCAAS GFTFNKNAMNW VRQAPGKGLEWV GRIRNKTNNYAT YYADSVKARFTIS RDDSKNSLYLQM NSLKTEDTAVYY CVAGNSFAYWGQ GTLVTVSA	206	226	244	269	277

SB06261			IgE	IL15	TaceOPT + LR1 linker	B7-1	E2A/T2A	GM-CSF-Ra	hPY7 VH	(GGGGS)3	hPY7 VL	CD8FA	CD8FA	CD28	CD3z
	IgE (SS) - IL-15 Tace10 (cleavage site) - B7-1 (TM) - E2A T2A - GM-CSF-Ra (SS) - aGPC3 hPY7 vL - (GGGGS)3 - aGPC3 hPY7 vH - CD8FA (Hinge) - CD8FA (TM) -CD28 (ICD) - CD3z (ICD)	SinVec	DNA	ATGG ACTG GACC TGGA TCCT GTTT CTGG TGGC CGCT GCCA CAA GAGT GCAC AGC	AATTGGGTCAAC GTGATCAGCGAC CTGAAGAAGATC GAGGACCTGATC CAGAGCATGCAC ATCGACGCCACA CTGTACACCGAG AGCGACGTGCAC CCTAGCTGTAAA GTGACCGCCATG AAGTGCTTTCTG CTGGAACTGCAA GTGATCAGCCTG GAAAGCGGCGAC GCCAGCATCCAC GACACCGTGGAA AACCTGATCATC CTGGCCAACAAC AGCCTGAGCAGC AACGGCAATGTG ACCGAGTCCGGC TGCAAAGAGTGC GAGGAACTGGAA GAGAAGAATATC AAAGAGTTCCTG CAGAGCTTCGTG CACATCGTGCAG ATGTTCATCAAC ACAAGC	TCTGGCG GCGGAG GATCTG GCGGAG GTGGAA GCGGAG TTACACC CGAGCC TATCTTC AGCCTG ATCGGA GGCGGA GGAAGT GGTGGC GGATCTC TGCAA	CTGCTGCC TAGCTGG GCCATCA CACTGAT CTCCGTG AACGCCA TCTTCGTG ATCTGCTG CCTGACCT ACTGCTTC GCCCCTA GATGCAG AGACGCG CGGACAA ACGAACG GCTGAGA AGAGAAT CTGTGCG GCCCCGTT	CAGTGT ACCAA CTACGC CCTGCT GAAAC TGGCC GGCGA CGTGG AATCTA ATCCTG GACCT GGATCT GGCGA GGGAC GCGGG AGTCTA CTGAC GTGTG GAGAC GTGGA GGAAA ACCCTG GACCT	ATGCT GCTGC TGGTC ACATC TCTGC TGCTG TGCG AGCT GCCCC ATCCT GCCTT TCTGC TGATC CCT	GAAGTGCAGCTG GTGGAATCTGGC GGAGGACTGGTT CAACCTGGCGGC TCTCTGAGACTG TCTTGTGCCGCC AGCGGCTTCACC TTCAACAAGAAC GCCATGAACTGG GTCCGACAGGCC CCTGGCAAAGGC CTTGAATGGGTC GGACGGATCCGG AACAAGACCAAC AACTACGCCACC TACTACGCCGAC AGCGTGAAGGCC AGGTTCACCATC TCCAGAGATGAC AGCAAGAACAGC CTGTACCTGCAG ATGAACTCCCTG AAAACCGAGGAC ACCGCCGTGTAC TATTGCGTGGCC GGCAATAGCTTT GCCTACTGGGGA CAGGGCACCCTG GTTACAGTTTCT GCT	GGCGGCG GAGGAAG CGGAGGC GGAGGAT CCGGTGG TGGTGGA TCT	GACATCGTGATG ACACAGAGCCCC GATAGCCTGGCC GTGTCTCTGGGA GAAAGAGCCACC ATCAACTGCAAG AGCAGCCAGAGC CTGCTGTACTCC AGCAACCAGAAG AACTACCTGGCC TGGTATCAGCAA AAGCCCGGCCAG CCTCCTAAGCTG CTGATCTATTGG GCCAGCTCCAGA GAAAGCGGCGTG CCCGATAGATTT TCTGGCTCTGGC AGCGGCACCGAC TTCACCCTGACA ATTTCTAGCCTG CAAGCCGAGGAC GTGGCCGTGTAT TACTGCCAGCAG TACTACAACTAC CCTCTGACCTTC GGCCAGGGCACC AAGCTGGAAATC AAA	GGCGCCCT GAGCAACA GATCATG TACTTCAGC CACTTCGTG CCGCTGTTT CTGCCCGC CAAGCCTA CAACAACC CCTGCTCCT AGACCTCC TACACCAG CTCCTACA ATCGCCAG CCAGCCTCT GTCTCTGA GGCCAGAA GACTGGAT TTCGCCTGC GAC	ATCTA CATCT GGGC CCCTC TGGCT GGAA CATGT GGTGT CCTGC TGCTG AGCCT GGTC ATCAC CCTGT ACTGC AACC ACCG G	CGGAGCAAGAGAA GCAGACTGCTGCAC AGCGACTACATGAA CATGACCCCTAGAC GGCCCGGACCTACC AGAAAGCACTACCA GCCTTACGCTCCTC CTAGAGACTTCGCC GCCTACCGGTCC	AGAGTGAAGTTC AGCAGATCCGCC GATGCTCCCGCC TATCAGCAGGGA CAGAACCAGCTG TACAACGAGCTG AACCTGGGGAGA AGAGAAGAGTAC GACGTGCTGGAC AAGCGGAGAGGC AGAGATCCTGAG ATGGGCGGCAAG CCCAGACGGAAG AATCCTCAAGAG GGCCTGTATAAT GAGCTGCAGAAA GACAAGATGGCC GAGGCCTACAGC GAGATCGGAATG AAGGGCGAGCGC AGAAGAGGCAA GGGACACGATGG ACTGTACCAGGG CCTGAGCACCGC CACCAAGGATAC CTATGATGCCCT GCACATGCAGGC CCTGCCTCCAAG A
	SEQ ID NO			214	286	288	331	284	217	222	332	333	229	243	268	280
			AA	MDW TWIL FLVA AATR VHS	NWVNVISDLKKIE DLIQSMHIDATLY TESDVHPSCKVTA MKCFLLELQVISL ESGDASIHDTVEN LIILANNSLSSNGN VTESGCKECEELE EKNIKEFLQSFVHI VQMFINTS	SGGGGSG GGGSGV **TPEPIFS** **LIG**GGSG GGGSGG GSLQ	LLPSWAIT LISVNGIFV ICCLTYCF APRCRERR RNERLRRE SVRPV	QCTNY ALLKLA GDVESN PGPGSG EGRGSL LTCGDV EENPGP	MLLL VTSLL LCFLP HPAFL LIP	EVQLVESGGGLV QPGGSLRLSCAAS GFTFNKNAMNW VRQAPGKGLEWV GRIRNKTNNYAT YYADSVKARFTIS RDDSKNSLYLQM NSLKTEDTAVYY CVAGNSFAYWGQ GTLVTVSA	GGGGSGG GGSGGGG S	DIVMTQSPDSLAV SLGERATINCKSS QSLLYSSNQKNYL AWYQQKPGQPPK LLIYWASSRESGV PDRFSGSGSGTDF TLTISSLQAEDVA VYYCQQYYNYPL TFGQGTKLEIK	GALSNSIMY FSHFVPVFL PAKPTTTPA PRPPTPAPTI ASQPLSLRP EACRPAAG GAVHTRGL DFACD	IYIWA PLAGI CGVLL LSLVI TLYCN HR	RSKRSRLLHSDYMN MTPRRPGPTRKHYQ PYAPPRDFAAYRS	RVKFSRSADAPA YQQGQNQLYNEL NLGRREEYDVLD KRRGRDPEMGGK PRRKNPQEGLYN ELQKDKMAEAYS EIGMKGERRRGK GHDGLYQGLSTA TKDTYDALHMQA LPPR
	SEQ ID NO			218	285	287	219	283	216	206	223	208	228	242	267	279

SB05009			IgE	IL15	TaceOPT + LR1 linker	B7-1	E2A/T2A	GM-CSF-Ra	hPY7 VH	(GGGGS)3	hPY7 VL	CD8FA	CD8FA	CD28	CD3z
	(IgE (SS) - IL-15 Tace10 (cleavage site) - B7-1 (TM)) (Reverse Orientation) - GM-CSF-Ra (SS) - aGPC3 hPY7 vL - (GGGGS)3 - aGPC3 hPY7 vH - CD8FA (Hinge) - CD8FA (TM) -CD28 (ICD) - CD3z (ICD)	SinVec	DNA	ATGG ACTG GACC TGGA TCCT GTTT CTGG TGGC CGCT GCCA CAA GAGT GCAC AGC	AATTGGGTCAAC GTGATCAGCGAC CTGAAGAAGATC GAGGACCTGATC CAGAGCATGCAC ATCGACGCCACA CTGTACACCGAG AGCGACGTGCAC CCTAGCTGTAAA GTGACCGCCATG AAGTGCTTTCTG CTGGAACTGCAA GTGATCAGCCTG GAAAGCGGCGAC GCCAGCATCCAC GACACCGTGGAA AACCTGATCATC CTGGCCAACAAC AGCCTGAGCAGC AACGGCAATGTG ACCGAGTCCGGC TGCAAAGAGTGC GAGGAACTGGAA GAGAAGAATATC AAAGAGTTCCTG CAGAGCTTCGTG CACATCGTGCAG ATGTTCATCAAC ACAAGC	TCTGGCG GCGGAG GATCTG GCGGAG GTGGAA GCGGAG TTACACC CGAGCC TATCTTC AGCCTG ATCGGA GGCGGT AGCGGA GGCGGA GGAAGT GGTGGC GGATCTC TGCAA SGGGGSG GGGSGV **TPEPIFS** **LIG**GGSGG GGGSGG GSLQ	CTGCTGCC TAGCTGG GCCATCA CACTGAT CTCCGTG AACGGCA TCTTCGTG ATCTGCTG CCTGACCT ACTGCTTC GCCCCTA GATGCAG AGACGCG CGGAGAA ACGAACG GCTGAGA AGAGAAT CTGTGCG GCCCGTT LLPSWATT LISVNGIFV ICCLTYCF APRCRERR RNERLRRE SVRPV	None	ATGCT GCTGC TGGTC ACATC TCTGC TGCTG TGCG AGCT GCCCC ATCCT GCCTT TCTGC TGATC CCT MLLL VTSLL LCFLP HPAFL LIP	GAAGTGCAGCTG GTGGAATCTGGC GGAGGACTGGTT CAACCTGGCGGC TCTCTGAGACTG TCTTGTGCCGCC AGCGGCTTCACC TTCAACAAGAAC GCCATGAACTGG GTCCGACAGGCC CCTGGCAAAGGC CTTGAATGGGTC GGACGGATCCGG AACAAGACCAAC AACTACGCCACC TACTACGCCGAC AGCGTGAAGGCC AGGTTCACCATC TCCAGAGATGAC AGCAAGAACAGC CTGTACCTGCAG ATGAACTCCCTG AAAACCGAGGAC ACCGCCGTGTAC TATTGCGTGGCC GGCAATAGCTTT GCCTACTGGGGA CAGGGCACCCTG GTTACAGTTTCT GCT EVQLVESGGGLV QPGGSLRLSCAAS GFTFNKNAMNW VRQAPGKGLEWV GRIRNKTNNYAT YYADSVKARFTIS RDDSKNSLYLQM NSLKTEDTAVYY CVAGNSFAYWGQ GTLVTVSA	GGCGGCG GAGGAAG CGGAGGC GGAGGAT CCGGTGG TGGTGGA TCT GGGGSGG GGSGGGG S	GACATCGTGATG ACACAGAGCCCC GATAGCCTGGCC GTGTCTCTGGGA GAAAGAGCCACC ATCAACTGCAAG AGCAGCCAGAGC CTGCTGTACTCC AGCAACCAGAAG AACTACCTGGCC TGGTATCAGCAA AAGCCCGGCCAG CCTCCTAAGCTG CTGATCTATTGG GCCAGCTCCAGA GAAAGCGGCGTG CCCGATAGATTT TCTGGCTCTGGC AGCGGCACCGAC TTCACCCTGACA ATTTCTAGCCTG CAAGCCGAGGAC GTGGCCGTGTAT TACTGCCAGCAG TACTACAACTAC CCTCTGACCTTC GGCCAGGGCACC AAGCTGGAAATC AAA DIVMTQSPDSLAV SLGERATINCKSS QSLLYSSNQKNYL AWYQQKPGQPPK LLIYWASSRESGV PDRFSGSGSGTDF TLTISSLQAEDVA VYYCQQYYNYPL FGQGTKLEIK	GCCCTGAG CAACAGCA TCATGTACT TCAGCCAC TTCGTGCCC GTGTTTCTG CCCGCCAA GCCTACAA CAACCCCT GCTCCTAG ACCTCCTAC ACCAGCTC CTACAATC GCCAGCCA GCCTCTGTC TCTGAGGC CAGAAGCT TGTAGACC TGCTGCAG GCGGAGCC GTGCATAC AAGAGGAC TGGATTTCG CCTGCGAC ALSNSIMYF SIFVPVFLP AKPTTTPAP RPPTPAPTIA SQPLSLRPE ACRPAAGG AVHTRGLD FACD	ATCTA CATCT GGGC CCCTC TGGCT GGAA CATGT GGTGT CCTGC TGCTG AGCCT GGTC ATCAC CCTGT ACTGC AACC ACCG G IYIWA PLAGI CGVLL LSLVI TLYCN HR	CGGAGCAAGAGAA GCAGACTGCTGCAC AGCGACTACATGAA CATGACCCCTAGAC GGCCCGGACCTACC AGAAAGCACTACCA GCCTTACGCTCCTC CTAGAGACTTCGCC GCCTACCGGTCC RSKRSRLLHSDYMN MTPRRPGPTRKHYQ PYAPPRDFAAYRS	AGAGTGAAGTTC AGCAGATCCGCC GATGCTCCCGCC TATCAGCAGGGA CAGAACCAGCTG TACAACGAGCTG AACCTGGGGAGA AGAGAAGAGTAC GACGTGCTGGAC AAGCGGAGAGGC AGAGATCCTGAG ATGGGCGGCAAG CCCAGACGGAAG AATCCTCAAGAG GGCCTGTATAAT GAGCTGCAGAAA GACAAGATGGCC GAGGCCTACAGC GAGATCGGAATG AAGGGCGAGCGC AGAAGAGGCAA GGGACACGATGG ACTGTACCAGGG CCTGAGCACCGC CACCAAGGATAC CTATGATGCCCT GCACATGCAGGC CCTGCCTCCAAG A RVKFSRSADAPA YQQGQNQLYNEL NLGRREEYDVLD KRRGRDPEMGGK PRRKNPQEGLYN ELQKDKMAEAYS EIGMKGERRRGK GHDGLYQGLSTA TKDTYDALHMQA LPPR
	SEQ ID NO			**214**	**286**	**288**	**331**		**217**	**222**	**332**	**333**	**335**	**243**	**268**	**280**
			AA	MDW TWIL FLVA AATR VHS	NWVNVISDLKKIE DLIQSMHIDATLY TESDVHPSCKVTA MKCFLLELQVISL ESGDASIHDTVEN LIILANNSLSSNGN VTESGCKECEELE EKNIKEFLQSFVHI VQMFINTS											
	SEQ ID NO			**218**	**285**	**287**	**219**		**216**	**206**	**223**	**208**	**336**	**242**	**267**	**279**

SB05605			IgE	IL15	TaceOPT + LR1 linker	B7-1	E2A/T2A	GM-CSF-Ra	hPY7 VH	(GGGGS)3	hPY7 VL	Hinge CD8a	CD8TA	41BB	CD3z
	(IgE (SS) - IL-15 Tace10 (cleavage site) - B7-1 (TM) (Reverse Orientation) - GM-CSF-Ra (SS) - aGPC3 hPY7 vL - (GGGGS)3 - aGPC3 hPY7 vH - CD8a (Hinge) - CD8 (TM) - 41BB (ICD) - CD3z (ICD)		ATGG ACTG GACC TGGA TCCT GTTT CTGG TGGC CGCT GCCA CAA GAGT GCAC AGC	AATTGGGTCAAC GTGATCAGCGAC CTGAAGAAGATC GAGGACCTGATC CAGAGCATGCAC ATCGACGCCACA CTGTACACCGAG AGCGACGTGCAC CCTAGCTGTAAA GTGACCGCCATG AAGTGCTTTCTG CTGGAACTGCAA GTGATCAGCCTG GAAAGCGGCGAC GCCAGCATCCAC GACACCGTGGAA AACCTGATCATC CTGGCCAACAAC AGCCTGAGCAGC AACGGCAATGTG ACCGAGTCCGGC TGCAAAGAGTGC GAGGAACTGGAA GAGAAGAATATC AAAGAGTTCCTG CAGAGCTTCGTG CACATCGTGCAG ATGTTCATCAAC ACAAGC	TCTGGCG GCGGAG GATCTG GCGGAG GTGGAA GCGGAG TTACACC CGAGCC TATCTTC AGCCTG ATCGGA GGCGGA GGAAGT GGTGGC GGATCTC TGCAA	CTGCTGCC TAGCTGG GCCATCA CACTGAT CTCCGTG AACGCCA TCTTCGTG ATCTGCTG CCTGACCT ACTGCTTC GCCCCTA GATGCAG AGACGCG CGGAGAA ACGAACG GCTGAGA AGAGAAT CTGTGCG GCCCGTT	None	ATGCT GCTGC TGGTC ACATC TCTGC TGCTG TGCG AGCT GCCCC ATCCT GCCTT TCTGC TGATC CCT	GAAGTGCAGCTG GTGGAATCTGGC GGAGGACTGGTT CAACCTGGCGGC TCTCTGAGACTG TCTTGTGCCGCC AGCGGCTTCACC TTCAACAAGAAC GCCATGAACTGG GTCCGACAGGCC CCTGGCAAAGGC CTTGAATGGGTC GGACGGATCCGG AACAAGACCAAC AACTACGCCACC TACTACGCCGAC AGCGTGAAGGCC AGGTTCACCATC TCCAGAGATGAC AGCAAGAACAGC CTGTACCTGCAG ATGAACTCCCTG AAAACCGAGGAC ACCGCCGTGTAC TATTGCGTGGCC GGCAATAGCTTT GCCTACTGGGGA CAGGGCACCCTG GTTACAGTTTCT GCT	GGCGGCG GAGGAAG CGGAGGC GGAGGAT CCGGTGG TGGTGGA TCT	GACATCGTGATG ACACAGAGCCCC GATAGCCTGGCC GTGTCTCTGGGA GAAAGAGCCACC ATCAACTGCAAG AGCAGCCAGAGC CTGCTGTACTCC AGCAACCAGAAG AACTACCTGGCC TGGTATCAGCAA AAGCCCGGCCAG CCTCCTAAGCTG CTGATCTATTGG GCCAGCTCCAGA GAAAGCGGCGTG CCCGATAGATTT TCTGGCTCTGGC AGCGGCACCGAC TTCACCCTGACA ATTTCTAGCCTG CAAGCCGAGGAC GTGGCCGTGTAT TACTGCCAGCAG TACTACAACTAC CCTCTGACCTTC GGCCAGGGCACC AAGCTGGAAATC AAG	ACCACCAC ACCAGCTC CTCGGCCA CCAACTCC AGCTCCAA CAATTGCC AGCCAGCC TCTGTCTCT GAGGCCCG AAGCTTGT AGACCTGC TGCAGGCG GAGCCGTG CATACAAG AGGACTGG ATTTCGCCT GCGAC	ATCTA CATCT GGGC CCCTC TGGCT GGAA CATGT GGTGT CTTGC TGCTG AGCCT GGTC ATCAC C	AAGCGGGGCAGAA AGAAGCTGCTGTAC ATCTTCAAGCAGCC CTTCATGCGGCCCG TGCAGACCACACAA GAGGAAGATGGCTG CAGCTGTCGGTTCC CCGAGGAAGAAGA AGGCGGCTGCGAGC TG	AGAGTGAAGTTC AGCAGGAGCGCA GACGCCCCCGCG TACAAGCAGGGC CAGAACCAGCTC TATAACGAGCTC AATCTAGGACGA AGAGAGGAGTAC GATGTTTTGGAC AAGAGACGTGGC CGGGACCCTGAG ATGGGGGGAAAG CCGAGAAGGAAG AACCCTCAGGAA GGCCTGTACAAT GAACTGCAGAAA GATAAGATGGCG GAGGCCTACAGT GAGATTGGGATG AAAGGCGAGCGC CGGAGGGGCAAG GGGCACGATGGC CTTTACCAGGGT CTCAGTACAGCC ACCAAGGACACC TACGACGCCCTT CACATGCAGGCC CTGCCCCCTCGC
	SEQ ID NO		214	286	288	331		217	222	332	336	337	338	339	334
			MDW TWIL FLVA AATR VHS	NWVNVISDLKKIE DLIQSMHIDATLY TESDVHPSCKVTA MKCFLLELQVISL ESGDASIHDTVEN LIILANNSLSSNGN VTESGCKECEELE EKNIKEFLQSFVHI VQMFINTS	SGGGGSG GGGSGV TPEPITSLI GGGSGG GGSGGGS LQ	LLPSWATT LISVNGIFV ICCLTYCF APRCRERR RNERLRRE SVRPV	None	MLLL VTSLL LCFLP HPAFL LIP	EVQLVESGGGLV QPGGSLRLSCAAS GFTFNKNAMNW VRQAPGKGLEWV GRIRNKTNNYAT YYADSVKARFTIS RDDSKNSLYLQM NSLKTEDTAVYY CVAGNSFAYWGQ GTLVTVSA	GGGGSGG GGSGGGG S	DIVMTQSPDSLAV SLGERATINCKSS QSLLYSSNQKNYL AWYQQKPGQPPK LLIYWASSRESGV PDRFSGSGSGTDF TLTISSLQAEDVA VYYCQQYYNYPL TFGQGTKLEIK	TTTPAPRPP TPAPTIASQP LSLRPEACR PAAGGAVH TRGLDFACD	IYIWA PLAGI CGVLL LSLVI T	KRGRKKLLYIFKQPF MRPVQTTQEEDGCS CRFPEEEEGGCEL	RVKFSRSADAPA YKQGQNQLYNEL NLGRREEYDVLD KRRGRDPEMGGK PRRKNPQEGLYN ELQKDKMAEAYS EIGMKGERRRGK GHDGLYQGLSTA TKDTYDALHMQA LPPR
	SEQ ID NO		218	285	287	219		216	206	223	208	196	236	271	277

Table 22:

SB ID	Description	Backbone	Seq Type	TM domain	Cleavage Site	IL-12		Syn promoter	Insulator	Promoter		SynTF		
						IL12p70		YB_TATA 4X ZF5 BD	A2	SV40	SV40 NLS	miniVPR	NS3	ZF5-7 DBD
SB05042	B7-1 (TM) - CD16 TACE (cleavage site) - IL12 - YB_TATA ZFBD (syn prmoter) - A2 (insulator) - SV40 (promoter) - Syn TF (NLS + miniVPR activation domain + NS3 protease + ZFBD DNA binding domain)	SinVec	DNA	CTGCT GCCA AGCT GGGC CATCA CACTG ATCTC CGTG AACG GCATC TTCGT GATCT GTTGC CTGAC CTACT GCTTC GCCCC TCGGT GCAG AGAG CGGA GAAG AAAC GAAC GGCT GCGG AGAG AATCT GTGC GGCCT GTG	LR1 split N term linker + CD16 TACE	AGCGGCG GAGGTGG TAGCGGA GGCGGAG GATCTGG AATTACA CAGGGAC TCGCCGT GTCTACA ATCTCCA GCTTCTTT GGTGGCG GTAGTGG CGGCGGT GGCAGTG GCGGTGG ATCTCTTC AA	ATGTGTCACCAGCAGCTGGTCATCAGCTG GTTCAGCCTGGTGTTCCTGGCCTCTCCTCT GGTGGCCATCTGGGAGCTGAAGAAAGAC GTGTACGTGGTGGAACTGGACTGGTATCC CGATGCTCCCTGGCGAGATGGTGGTGCTGA CCTGCGATACCCCTGAAGAGGACGGCATC ACCTGGACACTGGATCAGTCTAGCGAGGT GCTCGGCAGCGGCAAGACCCTGACCATCC AAGTGAAAGAGTTTGGCGACGCCGGCCA GTACACCTGTCACAAAGGCGGAGAAGTGC TGAGCCACAGCCTGCTGCTCCACAAG AAAGAGGATGGCATTTGGAGCACCGACAT CCTGAAGGACCAGAAAGAGCCCAAGAAC ACACTGTCTGCCGAAAGAGTGCGGGGCGA CAACAAAGAATACGAGTACAGCGTGGAA TGCCAAGAGGACAGCGCCTGTCCAGCCGC CGAAGAGTCTCTGCCTATCGAAGTGATGG TGGACGCCGTGCACAAGCTGAAGTACGAG AACTACACCTCCAGCTTTTCATCCGGGA CATCATCAAGCCCGATCCTCCAAAGAACC TGCAGCTGAAGCCTCTGAAGAACAGCAGA CAGGTGGAAGTGTCCTGGGAGTACCCCGA CACCTGGTCTACACCCCACAGCTACTTCA GCCTGACCTTTTGCGTGCAAGTGCAGGGC AAGTCCAAGCGCGAGAAAAAGGGACCGGG TGTTCACCGACAAGACCAGCGCCACCGTG ATCTGCAGAAAGAACGCCAGCATCAGCGT CAGAGCCCAGGACCGGTACTACAGCAGCT CTTGGAGCGAATGGGCCAGCGTGCCATGT TCTGGCGGAGGAAGCGGTGGCGGATCAG GTGGTGGATCTGGCGGCGGATCTAGAAAC CTGCCTGTGGCCACTCCTGATCCTGGCAT GTTCCTTGTCTGCACCACAGCCAGAACC TGCTGAGAGCCGTGTCCAACATGCTGCAG AAGGCCAGACAGACCCTGGAATTCTACCC CTGCACCAGCGAGGAAATCGACCACGAG GACATCACCAAGGATAAGACCAGCACCGT GGAAGCCTGCCTGCCTCTGGAACTGACCA AGAACGAGAGCTGCCTGAACAGCCGGGA AACCACGCTTCATCACCAACGGCTCTTGCC TGGCCACGAAAGAACCTCCTTCATGATG GCCCTGTGCCTGAGCAGCATCTACGAGGA CCTGAAGATGTACCAGGTGGAATTCAAGA	AATTAAcg ggtttcgtaacaa tcgcatggaggatt cgcaatcgcctt GAAGCAG TCGACGC CGAAgtccc gtctcagtaaag gttGAAGCA GTCGACG ttcgcatggaggatt CCGAAgaat cggaatgcctc gtatGAAGC AGTCGAC gattcgtaagag GCGGA GGCAT AATGGGG GCCAACG CGTACCG GTGTC	ACAAT GGCTG GTCCAT AAGTAA ATGCC GTGTTA GTGTGT TAGTTG CTGTTC CATTCAC GTCAG AAGAG GCACA GCTCA GACAA GAACA CTTTAC ATTACC CCAGG TGGGC CATGCT CAGCC CTGCCA AGTTG AGACC TTTTAC TACCTG ACTAG CTGAG ACATTT CTCATT TTATTC ATTTCA TTACTT TTTTTT GAATCT CGCTCT	GTGTGTC AGTTAGG GTGTGGA AAGTCCC CAGGCTC CCCAGCA GGCAGAA GTATGCA AAGCATG CATCTCA ATTAGTC AGCAACC AAGGTGTG GAAAGTC CCCAGGC TCCCCAG CAGGCAG AAGTATG CAAAGCA TGCATCTC CAGCAAC CATAGTC CCGCCCT ACTTCCG GAACTCG TTGAAT TTGAAT AACTCCG CCCATCC GCCCCTA CCAGTTCC GCCCATTC TCCGCCC ATGGCTG TCTGCTC TTTTATT ACGAC AGGCCGA GGCCGCC TGAGCTA TTCCAGA AGTAGTG AGGAGGC TTTTTGG AGGCCTA GGCTTTG CAAA	SV40 NLS CCAA GAA AAA GCG GAA GGTG ATGC	GACGCCCTGGACGACTT CGATCTGGATATGCTGG GCAGCGACGCTCTGGAT GATTTTGACCTGGACAT GCTCGGCTCTGATGCAC GATATGTTGGGATCTGA TGCCCTGATGACTTTG AATCTCGAACATGTTGATC CAGCCCCAAGAAGAAG AGAAAAGTCGGCTCTGG CGGCGGATCTGGCGGTT CTGGATCTGTTTTGCCC CAAGCTCCTGCTCCTGC ACCAGCTCCAGCTATGG TTTCTGCTCTGGCTCAG GCTCCAGCTCCTGTGCC TGTTCTTGCTCCTGGAC CTCCTCAGGCTGTTGCT CCACCAGCACCTAAACC TACACAGGCCGGCGAG GGAACACTGTCTGAAGC TCTGCTGCAGCTCCAGT TCGACGACGAAGATCTG GCAGCAGAAGCCTGAC ACCTTGCACCTGTGGCT TAGCACAGATCCTGCCG TGTTCACCGATCTGGCC AGCCGTGGACAATAGCG GTCGATCCCTGTCGAGCC AGTTCCAGCAGCTCCTG AACCAGGGCATTCCTGT GGCTCCTCACACCACCG AGCCTATGCTGATGGAA TACCCCGAGGCCATCAC CAGACTGGTCACCGGTG CTCAAAGACCACCTGAT GCTCTATCGCCGACATG GATTTCAGCGCCCTGCT CAGTGGCGGTGGAAGC GGAGGAAGTGGCAGCG TGGAATCCATGACCGAG	GAGGATGTCGTGTGCTG CCACAGCATCTACGGCA GAAGAAGGGCGACAT CGACACCTACCGGTACA TCGGCAGCTCTGGCACA GGCTGTGTGGTCATCGT CTGGCAGCGGAACAAG CGCCCCTATCACAGCCT ATGCTCAGCAGACAAG CGTGCTGCGGAGACA CCTGAGAACCCACAC ATTCCAGTGTCGCAT GCAGATCGTGTCTACAG CTACCCAGACCCTTCCA TCTGGCCCGGCACAC CGGCACCCTGCACAG ACACACAAGAACCCA TTTCCAATGTAGAAT CTGCATGCGAAATTT TTCCCAGCGGCCTAA CTGACCAGGCATCT GAGGACCCACCTGAG AGGATCT	ATGTCTAGACCTGGC GAGAGGCCCTTCCAG TGCCGGATCTGCATG CGGAACTTCAGCAAC ATGAGCAACCTGACC AGACACACCCGGACA CACACAGGCGAGAA GCCTTTTCAGTGCAA AATCTGTATGCGCAA TTTCTCGACAGAAG AGGCCTGCTGGGAGACA CCTGAGAACCCACAC ATTCCAGTGTCGCAT GTGTATGAGAAACTT TAGCGACCCCTCCAA CGTGCTGCGGAGACA CAGAACACATACCGG GGAAAAACCCTTTCA ATCACTACTGGCTCCCA GTGTAGGATATGCAT GAGGAATTTTTCCGA GCGGCACCCTGAGGC ACATACTGGCTCCCA AAAGCCGTTCCAATG TCGGATATGTATGCG CAACTTTAGCCAGAG CGGCACCCTGCACAG ACACACAAGAACCCA TACTGGCGAGAAACC AGGCATGTAGAATT CTGCATGCGAAATTT TTCCCAGCGGCCTAA CTGACCAGGCATCT GAGGACCCACCTGAG AGGATCT

| | | |

| | | AA | LLPSW AITLIS VNGIF VICCL TYCFA PRCRE RRRNF RLRRE SVRPV | SGGGGSG GGGSG**GITQ GLAVSTIS SFF**GGGSG GGGSGGG SLQ | MCHQQLVISWFSLVFLASPLVAIWELKKDV YVVELDWYPDAPGEMVVLTCDTPEEDGIT WTLDQSSEVLGSGKTLTIQVKEFGDAGQYT CHKGGEVLSHSLLLLHKKEDGIWSTDILKD QKEPKNKTFLRCEAKNYSGRFTCWWLTTIS TDLTFSVKSSRGSSDPQGVTCGAATLSAERV RGDNKEYEYSVECQEDSACPAAEESLPIEV MVDAVHKLKYENYTSSFFIRDIIKPDPPKNL QLKPLKNSRQVEVSWEYPDTWSTPHSYFSL TFCVQVQGKSKREKKDRVFTDKTSATVICR KNASISVRAQDRYYSSSWSEWASVPCSGGG SGGGSGGGSGGGSRNLPVATPDPGMFPCLH HSQNLLRAVSNMLQKARQTLEFYPCTSEEI DHEDITKDKTSTVEACLPLELTKNESCLNSR ETSFITNGSCLASRKTSFMMALCLSSIYEDL KMYQVEFKTMNAKLLMDPKRQIFLDQNML AVIDELMQALNFNSETVPQKSSLEEPDFYKT KIKLCILLHAFRIRAVTIDRVMSYLNAS | | |

	SEQ ID NO				57	299	300	17	297	341	342	343
					GTCGGAAGAACGCGTCAATCAGCGTCCGG GCGCAGGATAGATACTACTCGTCTCCTG GAGCGAATGGGCCAGCGTGCCTTGTTCCG GTGGCGGATCAGGCGGAGGTTCAGGAGG AGGCTCCGGAGGAGGTTCCCGGAACCTCC CTGTGGCAACCCCCGACCCTGGAATGTTC CCGTGCCTACACCACTCCCAAAACCTCCT GAGGGCTGTGTCGAACATGTTGCAGAAGG CCCGCCAGACCCTTGAGTTCTACCCTGC ACCTCGGAAGAAATTGATCACGAGGACAT CACCAAGGACAAGACCTCGACCGTGGAA GCCTGCCTGCCGCTGGAAACTGACCAAGAA CGAATCGTGTCTGAACTCCCGCGAGACAA GCTTTATCACTAACGGCAGCTGCCTGGCG TCGAGAAAGACCTCATTCATGATGGCGCT CTGTCTTTCCTCGATCTACGAAGATCTGAA GATGTATCAGGTCGAGTTCAAGACCATGA ACGCCAAGCTGCTCATGGACCCGAAGCGG CAGATCTTCCTGGACCAGAATATGCTCGC CGTGATTGATGAACTGATGCAGGCCCTGA ATTTCAACTCCGAGACTGTGCCTCAAAAG TCCAGCCTGGAAGAACCGGACTTCTACAA GACCAAGATCAAGCTGTGCATCCTGTTGC ACGCTTTCCGCATTCGAGCCGTGACCATT GACCGCGTGATGTCCTACCTGAACGCCAG T	TACCTG ACTAG CTGAG ACATTT ACGAC ATTTAC TGGCTC TAGGA CTCATT TTATTC ATTTCA TTACTT TTTTTT TCTTTG AGACG GAATCT CGCTCT	cgccagtcctcc gacagactgagt cgcccggg			GCGCACACACCTGAGA GGCAGC	CTAGACCTATCAGCTAC CTGAAGGGCAGCTCTGG CGGACCTCTGCTTTGTC CTGCTGGACATGCCGTG GGCCTGTTTAGAGCCGC CGTGTGTACAAGAGGCG TGGCCAAAGCCGTGGAC TTCATCCCCGTGGAAAA CCTGGAAACCACCATGC GGAGCCCCGTGTTCACC GACAATTCTAGCCCTCC AGCCGTGACACTGACAC ACCCCATCACCAAGATC GACAGAGAGGTGCTGT ACCAAGAGTTCGACGA GATGGAAGAGTGCAGC CAGCAC	ACGGACCTCGCATCA GTTGACAATAGTGAA TTTCAACAACTTCTT AACCAGGGAATACCG GTTGCGCCCCATACG ACGGAACCTATGCTG ATGGAGTACCCTGAA GCTATAACCAGACTC GTAACTGGCGCCCAA CGCCCGCCCGACCCG GCTCCTGCGCCGCTG GGTGCGCCCGGGTCTT CCGAATGGTCTTCTC TCAGGGGACGAAGAT TTCAGTTCCATTGCG GATATGGACTTTTCC GCGCTCCTGAGTGGG GGTGGCTCTGGAGGC TCTGGTTCCGACCTC AGCCATCCTCCACCG AGAGGACACCTCGAC GAGCTGACAACCACC CTCGAAAGTATGACG GAAGATCTGAACTTG GATTCCCCCCTTACC CCAGAACTGAATGAA ATCCTCGATACGTTC TTGAACGATGAGTGC CTTTTGCACGCCATG CATATATCAACAGGT TTGTCTATCTTCGAC ACGTCCCTCTTTTGA

		AA									
	SEQ ID NO				293			296	342	321	325
					MCHQQLVISWFSLVFLASPLVAIWELKKDV			MPK	SRPGERPFQCRICMRNFS	EDVVCCHSIYGKKKGDI	DALDDFDLDMLGSDA
					YVVELDWYPDAPGEMVVLTCDTPEEDGIT			KKR	RRHGLDRHTRTHTGEKP	DTYRYIGSSGTGCVVIVG	LDDFDLDMLGSDALD
					WTLDQSSEVLGSGKTLTIQVKEFGDAGQYT			KV	FQCRICMRNFSDHSSLKR	RIVLSGSGTSAPITAYAQ	DFDLDMLGSDALDDF
					CHKGGFVLSHSLLLLHKKEDGIWSTDILKD				HLRTHTGSQKPFQCRICM	QTRGLLGCIITSLTGRDK	DLDMLINSRSSGSPKK
					QKEPKNKTFLRCEAKNYSGRFTCWWLTTIS				RNFSVRHNLTRHLRTHT	NQVEGEVQIVSTATQTFL	KRKVGSGGGSGGSGS
					TDLTFSVKSSRGSSDPQGVTCGAATLSAERV				GEKPFQCRICMRNFSDHS	ATCINGVCWAVVHGAGT	VLPQAPAPAPAPAMV
					RGDNKEYEYSVECQEDSACPAAEESLPIEV				NLSRHLKTHTGSQKPFQC	RTIASPKGPVIQMYTNVD	SALAQAPAPVPVLAPG
					MVDAVHKLKYENYTSSFFIRDIIKPDPPKNL				RICMRNFSQRSSLVRHLR	QDLVGWPAPQGSRSLTP	PPQAVAPPAPKPTQAG
					QLKPLKNSRQVEVSWEYPDTWSTPHSYFSL				THTGEKPFQCRICMRNFS	CTCGSSDLYLVTRHADVI	EGTLSEALLQLQFDDE
					TFCVQVQGKSKREKKDRVFTDKTSATVICR				ESGHLKRHLRTHLRGS	PVRRRGDSRGSLLSPRPIS	DLGALLGNSTDPAVFT
					KNASISVRAQDRYYSSSWSEWASVPCSGGG					YLKGSSGGPLLCPAGHA	DLASVDNSEFQQLLN
					SGGGSGGGSGGGSRNLPVATPDPGMFPCLH					VGLFRAAVCTRGVAKAV	QGIPVAPHTTEPMLME
					HSQNLLRAVSNMLQKARQTLEFYPCTSEEI					DFIPVENLETTMRSPVFT	YPEAITRLVTGAQRPP
					DHEDITKDKTSTVEACLPLELTKNESCLNSR					DNSSPPAVTLTHPITKIDR	DPAPAPLGAPGLPNGL
					ETSFITNGSCLASRKTSFMMALCLSSIYEDL					EVLYQEFDEMEECSQH	LSGDEDFSSIADMDFS
					KMYQVEFKTMNAKLLMDPKRQIFLDQNML						ALLSGGGSGGSGSDLS
					AVIDELMQALNFNSETVPQKSSLEEPDFYKT						HPPPRGHLDELTTTLE
					KIKLCILLHAFRIRAVTIDRVMSYLNAS						SMTEDLNLDSPLTPEL
											NEILDTFLNDECLLHA
											MHISTGLSIFDTSLF

Interpretations

All references, patents and patent applications disclosed herein are incorporated by reference with respect to the subject matter for which each is cited, which in some cases may encompass the entirety of the document.

The indefinite articles "a" and "an," as used herein in the specification and in the claims, unless clearly indicated to the contrary, should be understood to mean "at least one."

It should also be understood that, unless clearly indicated to the contrary, in any methods claimed herein that include more than one step or act, the order of the steps or acts of the method is not necessarily limited to the order in which the steps or acts of the method are recited.

In the claims, as well as in the specification above, all transitional phrases such as "comprising," "including," "carrying," "having," "containing," "involving," "holding," "composed of," and the like are to be understood to be open-ended, *i.e.*, to mean including but not limited to. Only the transitional phrases "consisting of" and "consisting essentially of" shall be closed or semi-closed transitional phrases, respectively, as set forth in the United States Patent Office Manual of Patent Examining Procedures, Section 2111.03.

CLAIMS

What is claimed is:

1. An immunoresponsive cell comprising:

a) a first engineered nucleic acid comprising a first expression cassette comprising a first promoter operably linked to a first exogenous polynucleotide sequence encoding a chimeric antigen receptor (CAR) that binds to GPC3 and a second exogenous polynucleotide sequence encoding a first cytokine; and

b) a second engineered nucleic acid comprising a second expression cassette comprising a synthetic transcription factor-responsive promoter operably linked to a third exogenous polynucleotide sequence encoding a second cytokine, and a third expression cassette comprising a third promoter operably linked to fourth exogenous polynucleotide sequence encoding an activation-conditional control polypeptide (ACP), wherein the ACP comprises a synthetic transcription factor comprising a DNA-binding domain and a transcriptional effector domain,

wherein the ACP is capable of inducing expression of the third exogenous polynucleotide sequence by binding to the ACP-responsive promoter,
wherein at least one of the second exogenous polynucleotide sequence and the third exogenous polynucleotide sequence encodes a membrane-cleavable chimeric protein, oriented from N-terminal to C-terminal, having the formula:
S – C – MT or **MT – C – S**
wherein
S comprises a secretable effector molecule comprising the first and/or second cytokine,
C comprises a protease cleavage site, and
MT comprises a cell membrane tethering domain,
wherein **S – C – MT** or **MT – C – S** is configured to be expressed as a single polypeptide,
optionally wherein transcription of the second expression cassette is oriented in the opposite direction relative to transcription of the third expression cassette within the first engineered nucleic acid, and optionally wherein the second expression cassette and the third expression cassette are oriented within the second engineered nucleic acid in a head-to-head directionality.

2. The immunoresponsive cell of claim 1, wherein:

a) the first promoter comprises a constitutive promoter, an inducible promoter, or a synthetic promoter, optionally wherein the first promoter is a constitutive promoter selected from the group consisting of: CAG, HLP, CMV, EFS, SFFV, SV40, MND, PGK, UbC, hEF1aV1, hCAGG, hEF1aV2, hACTb, heIF4A1, hGAPDH, hGRP78, hGRP94, hHSP70, hKINb, and hUBIb; and/or

b) the first exogenous polynucleotide sequence and the second exogenous polynucleotide sequence are separated by a linker polynucleotide sequence, optionally wherein the linker polynucleotide sequence is operably associated with the translation of the first cytokine and the CAR as separate polypeptides, optionally wherein the linker polynucleotide sequence encodes one or more 2A ribosome skipping elements, optionally wherein the one or more 2A ribosome skipping elements are each selected from the group consisting of: P2A, T2A, E2A, F2A, and combinations thereof, optionally wherein the one or more 2A ribosome skipping elements comprises an E2A/T2A combination, optionally wherein the E2A/T2A combination comprises the amino acid sequence of SEQ ID NO: 281; and/or

c) the third promoter comprises a constitutive promoter, an inducible promoter, or a synthetic promoter, optionally wherein the third promoter is a constitutive promoter selected from the group consisting of: CAG, HLP, CMV, EFS, SFFV, SV40, MND, PGK, UbC, hEF1aV1, hCAGG, hEF1aV2, hACTb, heIF4A1, hGAPDH, hGRP78, hGRP94, hHSP70, hKINb, and hUBIb; and/or

d) the first cytokine is IL-15, optionally wherein the IL-15 comprises the amino acid sequence of SEQ ID NO: 285, and the second cytokine is selected from the group consisting of: IL12, an IL12p70 fusion protein, IL18, and IL21, optionally wherein the second cytokine is the IL12p70 fusion protein, optionally wherein the IL12p70 fusion protein comprises the amino acid sequence of SEQ ID NO: 293.

3. The immunoresponsive cell of claim 1 or claim 2, wherein:

a) the protease cleavage site is cleavable by a protease selected from the group consisting of: a Type 1 transmembrane protease, a Type II transmembrane protease, a GPI anchored protease, an ADAM8 protease, an ADAM9 protease, an ADAM10 protease, an ADAM12 protease, an ADAM15 protease, an ADAM17 protease, an ADAM19 protease, an ADAM20 protease, an ADAM21 protease, an ADAM28 protease, an ADAM30 protease, an ADAM33 protease, a BACE1 protease, a BACE2

protease, a SIP protease, an MT1-MMP protease, an MT3-MMP protease, an MT5-MMP protease, a furin protease, a PCSK7 protease, a matriptase protease, a matriptase-2 protease, an MMP9 protease, and an NS3 protease; or

b) the protease cleavage site is cleavable by an ADAM17 protease; or

c) the protease cleavage site comprises a first region having the amino acid sequence of PRAE (SEQ ID NO: 176) and/or the protease cleavage site comprises a second region having the amino acid sequence of KGG (SEQ ID NO: 177),
optionally wherein the first region is located N-terminal to the second region; or

d) the protease cleavage site comprises the amino acid sequence of PRAEX1X2KGG (SEQ ID NO: 178),
wherein X1 is A, Y, P, S, or F, and
wherein X2 is V, L, S, I, Y, T, or A; or

e) the protease cleavage site comprises the amino acid sequence of PRAEAVKGG (SEQ ID NO: 179); or

f) the protease cleavage site comprises the amino acid sequence of PRAEALKGG (SEQ ID NO: 180); or

g) the protease cleavage site comprises the amino acid sequence of PRAEYSKGG (SEQ ID NO: 181); or

h) the protease cleavage site comprises the amino acid sequence of PRAEPIKGG (SEQ ID NO: 182); or

i) the protease cleavage site comprises the amino acid sequence of PRAEAYKGG (SEQ ID NO: 183); or

j) the protease cleavage site comprises the amino acid sequence of PRAESSKGG (SEQ ID NO: 184); or

k) the protease cleavage site comprises the amino acid sequence of PRAEFTKGG (SEQ ID NO: 185); or

l) the protease cleavage site comprises the amino acid sequence of PRAEAAKGG (SEQ ID NO: 186); or

m) the protease cleavage site comprises the amino acid sequence of DEPHYSQRR (SEQ ID NO: 187); or

n) the protease cleavage site comprises the amino acid sequence of PPLGPIFNPG (SEQ ID NO: 188); or

o) the protease cleavage site comprises the amino acid sequence of PLAQAYRSS (SEQ ID NO: 189); or

p) the protease cleavage site comprises the amino acid sequence of TPIDSSFNPD (SEQ ID NO: 190); or

q) the protease cleavage site comprises the amino acid sequence of VTPEPIFSLI (SEQ ID NO: 191); or

r) the protease cleavage site comprises the amino acid sequence of ITQGLAVSTISSFF (SEQ ID NO: 198),

optionally wherein the protease cleavage site is comprised within a peptide linker, optionally wherein the protease cleavage site is N-terminal to a peptide linker, and/or optionally wherein the peptide linker comprises a glycine-serine (GS) linker.

4. The immunoresponsive cell of any one of claims 1-3, wherein:

a) the cell membrane tethering domain comprises a transmembrane-intracellular domain or a transmembrane domain, optionally wherein the transmembrane-intracellular domain and/or transmembrane domain is derived from PDGFR-beta, CD8, CD28, CD3zeta-chain, CD4, 4-1BB, OX40, ICOS, CTLA-4, PD-1, LAG-3, 2B4, LNGFR, NKG2D, EpoR, TNFR2, B7-1, or BTLA, optionally wherein the transmembrane-intracellular domain and/or transmembrane domain is derived from B7-1, optionally wherein the transmembrane-intracellular domain and/or transmembrane domain comprises the amino acid sequence of SEQ ID NO: 219; and/or

b) the cell membrane tethering domain comprises a post-translational modification tag, or motif capable of post-translational modification to modify the chimeric protein to include a post-translational modification tag, wherein the post-translational modification tag is capable of association with a cell membrane, optionallywherein the post-translational modification tag comprises a lipid-anchor domain, optionally wherein the lipid-anchor domain is selected from the group consisting of: a GPI lipid-anchor, a myristoylation tag, and a palmitoylation tag; and/or

c) the cell membrane tethering domain comprises a cell surface receptor, or a cell membrane-bound portion thereof; and/or

d) the cytokine of the membrane-cleavable chimeric protein is tethered to a cell membrane of the cell; and/or

e) wherein the cell further comprises a protease capable of cleaving the protease cleavage site, optionally wherein the protease is endogenous to the cell, optionally wherein the protease is selected from the group consisting of: a Type 1 transmembrane protease, a Type II transmembrane protease, a GPI anchored protease, an ADAM8 protease, an ADAM9 protease, an ADAM10 protease, an ADAM12 protease, an ADAM15 protease, an ADAM17 protease, an ADAM19 protease, an ADAM20 protease, an ADAM21 protease, an ADAM28 protease, an ADAM30 protease, an ADAM33 protease, a BACE1 protease, a BACE2 protease, a SIP protease, an MT1-MMP protease, an MT3-MMP protease, an MT5-MMP protease, a furin protease, a PCSK7 protease, a matriptase protease, a matriptase-2 protease, and an MMP9 protease, optionally wherein the protease is an ADAM17 protease, optionally wherein the protease is expressed on the cell membrane of the cell, optionally wherein the protease is capable of cleaving the protease cleavage site, optionally wherein cleavage of the protease cleavage site releases the cytokine of the membrane-cleavable chimeric protein from the cell membrane of the cell; and/or

f) the first exogenous polynucleotide sequence encodes a membrane-cleavable chimeric protein; and/or

g) the first exogenous polynucleotide sequence further comprises a polynucleotide sequence encoding a secretion signal peptide, optionally wherein the secretion signal peptide is derived from a protein selected from the group consisting of: IL-12, Trypsinogen-2, Gaussia Luciferase, CD5, IgKVII, VSV-G, prolactin, serum albumin preproprotein, azurocidin preproprotein, osteonectin (BM40), CD33, IL-6, IL-8, CCL2, TIMP2, VEGFB, osteoprotegerin, serpin-E1, GROalpha, CXCL12, IL-21, CD8, GMCSFRa, NKG2D, and IgE, optionally wherein the secretion signal peptide is derived from GMCSFRa, optionally wherein the secretion signal peptide comprises the amino acid sequence of SEQ ID NO: 216, optionally wherein the secretion signal peptide is operably associated with the CAR; and/or

h) the second exogenous polynucleotide sequence further comprises a polynucleotide sequence encoding a secretion signal peptide, optionally wherein the secretion signal peptide is derived from a protein selected from the group consisting of: IL-12, Trypsinogen-2, Gaussia Luciferase, CD5, IgKVII, VSV-G, prolactin, serum albumin preproprotein, azurocidin preproprotein, osteonectin (BM40), CD33, IL-6, IL-8,

CCL2, TIMP2, VEGFB, osteoprotegerin, serpin-E1, GROalpha, CXCL12, IL-21, CD8, GMCSFRa, NKG2D, and IgE, optionally wherein the secretion signal peptide is derived from IgE, optionally wherein the secretion signal peptide comprises the amino acid sequence of SEQ ID NO: 218, optionally wherein the secretion signal peptide is operably associated with the first cytokine; and/or

i) the third exogenous polynucleotide sequence encodes a membrane-cleavable chimeric protein; and/or

j) the second exogenous polynucleotide sequence encodes a first membrane-cleavable chimeric protein and the third exogenous polynucleotide sequence encodes a second membrane-cleavable chimeric protein.

5. The immunoresponsive cell of any one of claims 1-4, wherein:

a) the CAR comprises an antigen-binding domain comprising a heavy chain variable (VH) region and a light chain variable (VL) region,

wherein the VH comprises:

a heavy chain complementarity determining region 1 (CDR-H1) having the amino acid sequence of KNAMN (SEQ ID NO: 199),
a heavy chain complementarity determining region 2 (CDR-H2) having the amino acid sequence of RIRNKTNNYATYYADSVKA (SEQ ID NO: 200), and
a heavy chain complementarity determining region 3 (CDR-H3) having the amino acid sequence of GNSFAY (SEQ ID NO: 201), and

wherein the VL comprises:

a light chain complementarity determining region 1 (CDR-L1) having the amino acid sequence of KSSQSLLYSSNQKNYLA (SEQ ID NO: 202),

a light chain complementarity determining region 2 (CDR-L2) having the amino acid sequence of WASSRES (SEQ ID NO: 203), and

a light chain complementarity determining region 3 (CDR-L3) having the amino acid sequence of QQYYNYPLT (SEQ ID NO: 204); and/or

b) the VH region comprises the amino acid sequence of
EVQLVETGGGMVQPEGSLKLSCAASGFTFNKNAMNWVRQAPGKGLEWVA
RIRNKTNNYATYYADSVKARFTISRDDSQSMLYLQMNNLKIEDTAMYYCVA
GNSFA YWGQGTLVTVSA (SEQ ID NO: 205) or
EVQLVESGGGLVQPGGSLRLSCAASGFTFNKNAMNWVRQAPGKGLEWVGR

IRNKTNNYATYYADSVKARFTISRDDSKNSLYLQMNSLKTEDTAVYYCVAG NSFAYWGQGTLVTVSA (SEQ ID NO: 206); and/or

c) the VH region comprises the amino acid sequence of SEQ ID NO: 206; and/or

d) the VL region comprises the amino acid sequence of DIVMSQSPSSLVVSIGEKVTMTCKSSQSLLYSSNQKNYLAWYQQKPGQSPKL LIYWASSRESGVPDRFTGSGSGTDFTLTISSVKAEDLAVYYCQQYYNYPLTF GAGTKLELK (SEQ ID NO: 207), or DIVMTQSPDSLAVSLGERATINCKSSQSLLYSSNQKNYLAWYQQKPGQPPKL LIYWASSRESGVPDRFSGSGSGTDFTLTISSLQAEDVAVYYCQQYYNYPLTFG QGTKLEIK (SEQ ID NO: 208); and/or

e) the VL region comprises the amino acid sequence of SEQ ID NO: 208,

optionally wherein the antigen-binding domain comprises a single chain variable fragment (scFv),
optionally wherein the VH and VL are separated by a peptide linker,
optionally wherein the peptide linker comprises a glycine-serine (GS) linker,
optionally wherein the GS linker comprises the amino acid sequence of (GGGGS)3 (SEQ ID NO: 223),
optionally wherein the scFv comprises the structure VH-L-VL or VL-L-VH, wherein VH is the heavy chain variable domain, L is the peptide linker, and VL is the light chain variable domain,
optionally wherein the CAR comprises one or more intracellular signaling domains, and each of the one or more intracellular signaling domains is selected from the group consisting of: a CD3zeta-chain intracellular signaling domain, a CD97 intracellular signaling domain, a CD11a-CD18 intracellular signaling domain, a CD2 intracellular signaling domain, an ICOS intracellular signaling domain, a CD27 intracellular signaling domain, a CD154 intracellular signaling domain, a CD8 intracellular signaling domain, an OX40 intracellular signaling domain, a 4-1BB intracellular signaling domain, a CD28 intracellular signaling domain, a ZAP40 intracellular signaling domain, a CD30 intracellular signaling domain, a GITR intracellular signaling domain, an HVEM intracellular signaling domain, a DAP10 intracellular signaling domain, a DAP12 intracellular signaling domain, a MyD88 intracellular signaling domain, a 2B4 intracellular signaling domain, a CD16a intracellular signaling domain, a DNAM-1 intracellular signaling domain, a KIR2DS1 intracellular signaling domain, a KIR3DS1 intracellular signaling domain, a NKp44 intracellular signaling domain, a NKp46

intracellular signaling domain, a FceRlg intracellular signaling domain, a NKG2D intracellular signaling domain, and an EAT-2 intracellular signaling domain,
optionally wherein the one or more intracellular signaling domains comprises a CD28 intracellular signaling domain, wherein the CD28 intracellular signaling domain comprises the amino acid sequence of SEQ ID NO: 267,
optionally wherein the one or more intracellular signaling domains comprises a CD3z intracellular signaling domain, wherein the CD3z intracellular signaling domain comprises the amino acid sequence of SEQ ID NO: 277 or SEQ ID NO: 279,
optionally wherein the CAR comprises a transmembrane domain, and the transmembrane domain is selected from the group consisting of: a CD8 transmembrane domain, a CD28 transmembrane domain a CD3zeta-chain transmembrane domain, a CD4 transmembrane domain, a 4-1BB transmembrane domain, an OX40 transmembrane domain, an ICOS transmembrane domain, a CTLA-4 transmembrane domain, a PD-1 transmembrane domain, a LAG-3 transmembrane domain, a 2B4 transmembrane domain, a BTLA transmembrane domain, an OX40 transmembrane domain, a DAP10 transmembrane domain, a DAP12 transmembrane domain, a CD16a transmembrane domain, a DNAM-1 transmembrane domain, a KIR2DS1 transmembrane domain, a KIR3DS1 transmembrane domain, an NKp44 transmembrane domain, an NKp46 transmembrane domain, an FceRlg transmembrane domain, and an NKG2D transmembrane domain,
optionally wherein the transmembrane domain is a CD8 transmembrane domain, wherein the CD8 transmembrane domain comprises the amino acid sequence of SEQ ID NO: 236 or SEQ ID NO: 242,
optionally wherein the CAR comprises a spacer region between the antigen-binding domain and the transmembrane domain, wherein the spacer region is derived from a protein selected from the group consisting of: CD8, CD28, IgG4, IgG1, LNGFR, PDGFR-beta, and MAG, optionally wherein the spacer region is a CD8 hinge comprising the amino acid sequence of SEQ ID NO: 226 or SEQ ID NO: 228

6. The immunoresponsive cell of any one of claims 1-5, wherein:

a) the ACP comprises a DNA binding domain and a transcriptional effector domain, wherein the transcriptional effector domain comprises a transcriptional activator domain, optionally wherein the transcriptional activator domain is selected from the group consisting of: a Herpes Simplex Virus Protein 16 (VP16) activation domain; an activation domain comprising four tandem copies of VP16, a VP64 activation domain; a p65 activation domain of NFκB; an Epstein-Barr virus R transactivator

(Rta) activation domain; a tripartite activator comprising the VP64, the p65, and the Rta activation domains (VPR activation domain); a histone acetyltransferase (HAT) core domain of the human E1A-associated protein p300 (p300 HAT core activation domain), optionally wherein the transcriptional activator domain comprises a VPR activation domain comprising the amino acid sequence of SEQ ID NO: 325; and/or

b) the DNA binding domain comprises a zinc finger (ZF) protein domain, wherein the ZF protein domain is modular in design and comprises an array of zinc finger motifs, optionally wherein the ZF protein domain comprises an array of one to ten zinc finger motifs, optionally wherein the ZF protein domain comprises the amino acid sequence of SEQ ID NO: 320; and/or

c) the ACP further comprises a repressible protease and one or more cognate cleavage sites of the repressible protease, optionally wherein the repressible protease is hepatitis C virus (HCV) nonstructural protein 3 (NS3) comprising the amino acid sequence of SEQ ID NO: 321, optionally wherein the cognate cleavage site of the repressible protease comprises an NS3 protease cleavage site comprising a NS3/NS4A, a NS4A/NS4B, a NS4B/NS5A, or a NS5A/NS5B junction cleavage site, optionally wherein the NS3 protease is repressible by a protease inhibitor selected from the group consisting of: simeprevir, danoprevir, asunaprevir, ciluprevir, boceprevir, sovaprevir, paritaprevir, telaprevir, grazoprevir, glecaprevir, and voxiloprevir, optionally wherein the one or more cognate cleavage sites of the repressible protease are localized between the DNA binding domain and the transcriptional effector domain; and/or

d) the ACP further comprises a nuclear localization signal (NLS) comprising the amino acid sequence of SEQ ID NO: 296; and/or

e) the ACP further comprises a hormone binding domain of estrogen receptor variant ERT2; and/or

f) the ACP-responsive promoter is a synthetic promoter comprising an ACP binding domain sequence and a minimal promoter sequence, optionally wherein the ACP binding domain sequence comprises one or more zinc finger binding sites.

7. The immunoresponsive cell of any one of claims 1-6, wherein:

a) the first engineered nucleic acid comprises the nucleotide sequence of SEQ ID NO: 309, the nucleotide sequence of SEQ ID NO: 326, the nucleotide sequence of SEQ

ID NO: 310, the nucleotide sequence of SEQ ID NO: 327, the nucleotide sequence of SEQ ID NO: 314, or the nucleotide sequence of SEQ ID NO: 315; and

b) the second engineered nucleic acid comprises the nucleotide sequence of SEQ ID NO: 317 or the nucleotide sequence of SEQ ID NO: 318.

8. The immunoresponsive cell of any one of claims 1-7, wherein the cell is selected from the group consisting of: a T cell, a CD8+ T cell, a CD4+ T cell, a gamma-delta T cell, a cytotoxic T lymphocyte (CTL), a regulatory T cell, a viral-specific T cell, a Natural Killer T (NKT) cell, a Natural Killer (NK) cell, a B cell, a tumor-infiltrating lymphocyte (TIL), an innate lymphoid cell, a mast cell, an eosinophil, a basophil, a neutrophil, a myeloid cell, a macrophage, a monocyte, a dendritic cell, an erythrocyte, a platelet cell, a human embryonic stem cell (ESC), an ESC-derived cell, a pluripotent stem cell, a mesenchymal stromal cell (MSC), an induced pluripotent stem cell (iPSC), and an iPSC-derived cell,

optionally wherein the cell is autologous or the cell is allogeneic.

9. An engineered nucleic acid comprising:

a first expression cassette comprising a first promoter operably linked to a first, exogenous polynucleotide sequence encoding a chimeric antigen receptor (CAR) that binds to GPC3 and a second exogenous polynucleotide sequence encoding IL15,

wherein the first exogenous polynucleotide sequence encodes a membrane-cleavable chimeric protein, oriented from N-terminal to C-terminal, having the formula:

S – C – MT or **MT – C – S**

wherein

S comprises a secretable effector molecule comprising the IL15,

C comprises a protease cleavage site, and

MT comprises a cell membrane tethering domain, and

wherein **S – C – MT** or **MT – C – S** is configured to be expressed as a single polypeptide,

optionally wherein the first exogenous polynucleotide sequence and the second exogenous polynucleotide sequence are separated by a linker polynucleotide sequence comprising an E2A/T2A ribosome skipping element,

optionally wherein the CAR that binds to GPC3 comprises a CD28 intracellular signaling domain,

optionally wherein the engineered nucleic acid comprises a nucleotide sequence selected from the group consisting of SEQ ID NOs: 309, 326, 310, 327, 314 and 315.

10. An engineered nucleic acid comprising:

a first expression cassette comprising a synthetic transcription factor-responsive promoter operably linked to a first exogenous polynucleotide sequence encoding an IL12p70 fusion protein, and

a second expression cassette comprising a second promoter operably linked to a second exogenous polynucleotide sequence encoding an activation-conditional control polypeptide (ACP), wherein the ACP comprises a synthetic transcription factor comprising a DNA-binding domain and a transcriptional effector domain,

wherein the ACP is capable of inducing expression of the first exogenous polynucleotide sequence by binding to the ACP-responsive promoter,

wherein the first exogenous polynucleotide sequence encodes a membrane-cleavable chimeric protein, oriented from N-terminal to C-terminal, having the formula:

S – C – MT or **MT – C – S**

wherein

S comprises a secretable effector molecule comprising the IL12p70 fusion protein,

C comprises a protease cleavage site, and

MT comprises a cell membrane tethering domain, and

wherein **S – C – MT** or **MT – C – S** is configured to be expressed as a single polypeptide,

optionally wherein the first expression cassette and the second expression cassette are oriented within the first engineered nucleic acid in a head-to-head directionality,

optionally wherein the ACP comprises a DNA binding domain and a transcriptional effector domain, wherein the transcriptional activator domain comprises a VPR activation domain,

optionally wherein the engineered nucleic acid comprises a nucleotide sequence selected from the group consisting of SEQ ID Nos: 317 and 318.

11. An expression vector comprising the engineered nucleic acid of claim 9 or claim 10.

12. An immunoresponsive cell comprising the engineered nucleic acid of claim 9 or claim 10, or the expression vector of claim 11.

13. A pharmaceutical composition comprising the immunoresponsive cell of any one of claims 1-8 or 12, the engineered nucleic acid of claim 9 or claim 10, or the expression vector of claim 11, and a pharmaceutically acceptable carrier, pharmaceutically acceptable excipient, or a combination thereof.

14. A method of stimulating a cell-mediated immune response to a tumor cell, reducing tumor volume, or providing an anti-tumor immunity in a subject, the method comprising administering to a subject in need thereof a therapeutically effective dose of any of the immunoresponsive cells of any one of claims 1-8 or 12, the engineered nucleic acid of claim 9 or claim 10, the expression vector of claim 11, or the pharmaceutical composition of claim 13,

optionally wherein the tumor comprises a GPC3-expressing tumor, optionally wherein the tumor is selected from the group consisting of: hepatocellular carcinoma (HCC), ovarian clear cell carcinoma, melanoma, squamous cell carcinoma of the lung, hepatoblastoma, nephroblastoma (Wilms tumor), and yolk sac tumor,

optionally wherein the administering comprises systemic administration or intratumoral administration,

optionally wherein the immunoresponsive cell is derived from the subject or is allogeneic with reference to the subject.

15. A method of treating a subject having cancer, the method comprising administering a therapeutically effective dose of any of the immunoresponsive cells of any one of claims 1-8 or 12, the engineered nucleic acid of claim 9 or claim 10, the expression vector of claim 11, or the pharmaceutical composition of claim 13,

optionally wherein the cancer comprises a GPC3-expressing cancer,

optionally wherein the cancer is selected from the group consisting of: hepatocellular carcinoma (HCC), ovarian clear cell carcinoma, melanoma, squamous cell carcinoma of the lung, hepatoblastoma, nephroblastoma (Wilms tumor), and yolk sac tumor,

optionally wherein the administering comprises systemic administration or intratumoral administration,

optionally wherein the immunoresponsive cell is derived from the subject or is allogeneic with reference to the subject.

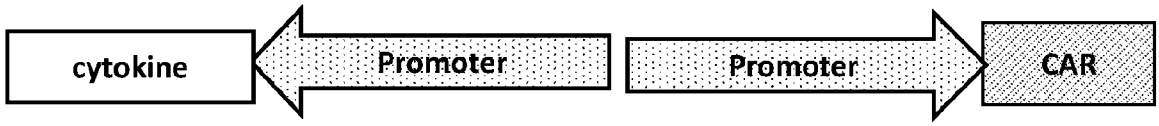


FIG. 1A

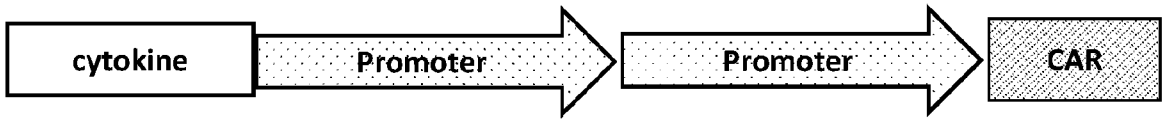


FIG. 1B

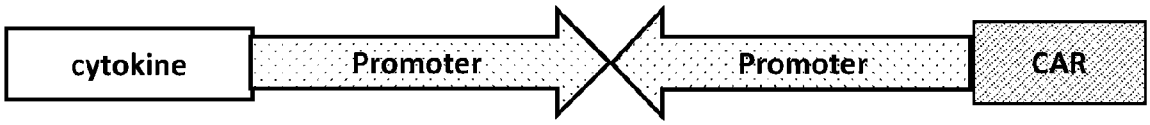


FIG. 1C

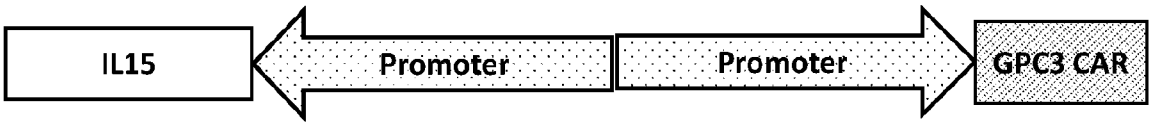
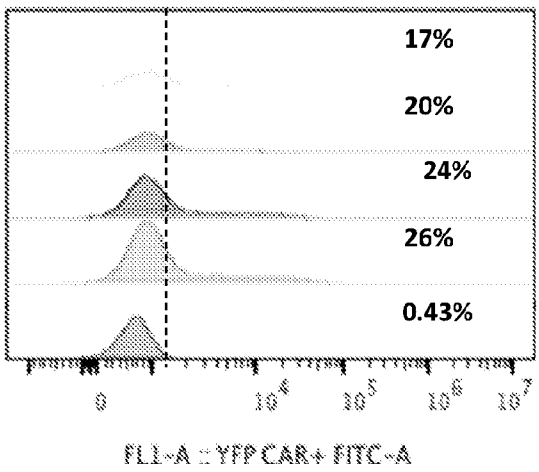


FIG. 1D

Lentiviral constructs (CAR + IL15)	KIR3DS1z + IL15
	CD28Z + IL15
	OX40Z + IL15
	41BBz + IL15
	NV



Lentiviral constructs (CAR only)	KIR3DS1z
	CD28Z
	OX40Z
	41BBz
	NV

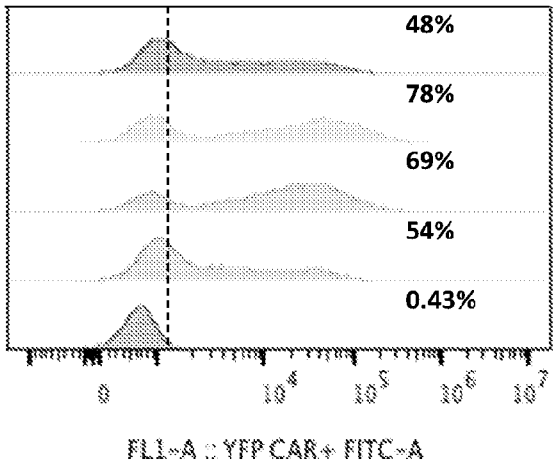
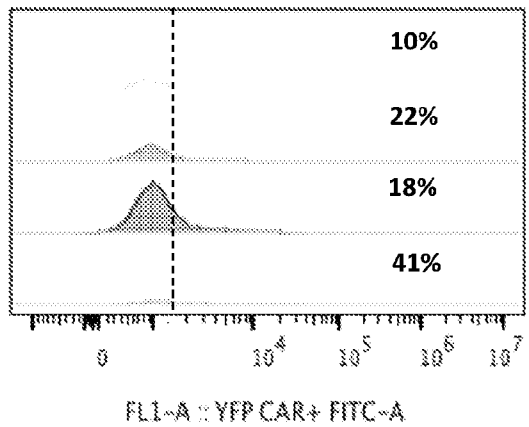


FIG. 2

Retroviral constructs (CAR + IL15)	KIR3DS1z + IL15
	CD28Z + IL15
	OX40Z + IL15
	41BBz + IL15



Retroviral constructs (CAR only)	KIR3DS1z
	CD28Z
	OX40Z
	41BBz
	NV

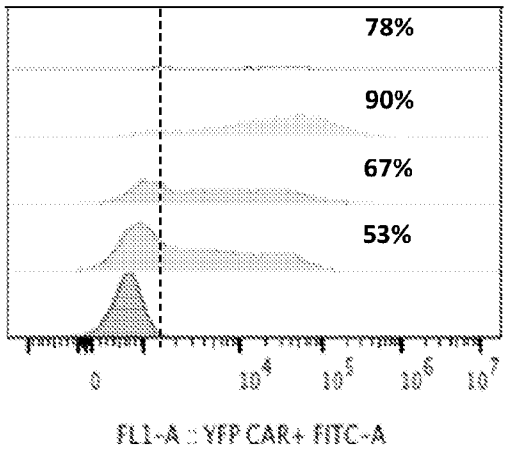
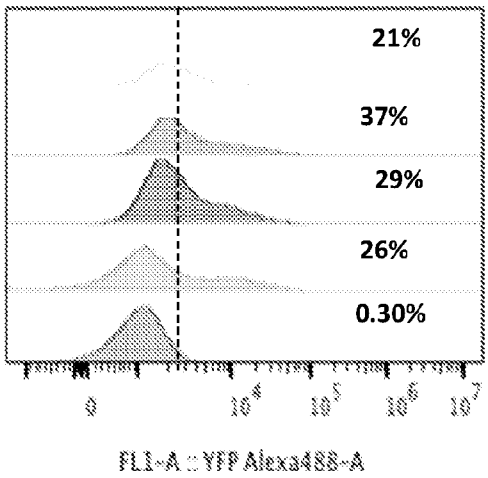


FIG. 3

Lentiviral constructs (CAR + IL15)	KIR3DS1z + IL15
	CD28Z + IL15
	OX40Z + IL15
	41BBz + IL15
	NV



Lentiviral constructs (CAR only)	KIR3DS1z
	CD28Z
	OX40Z
	41BBz
	NV

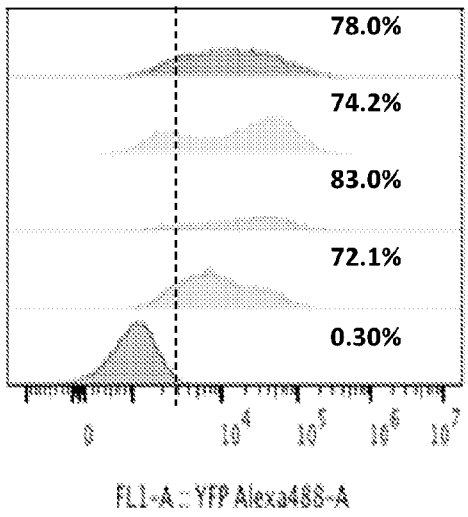
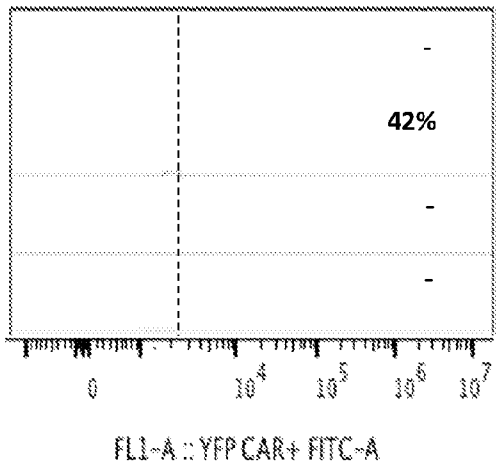


FIG. 4

Retroviral constructs (CAR + IL15)	KIR3DS1z + IL15
	CD28Z + IL15
	OX40Z + IL15
	41BBz + IL15



Retroviral constructs (CAR only)	KIR3DS1z
	CD28Z
	OX40Z
	41BBz
	NV

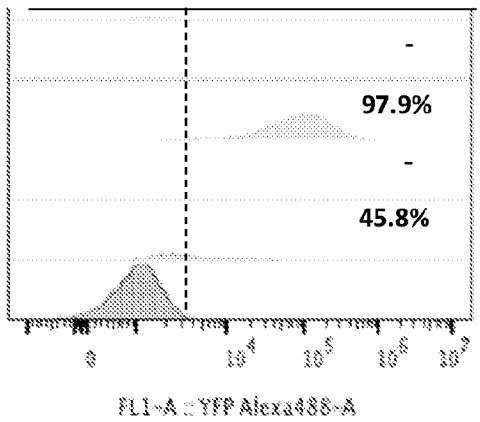


FIG. 5

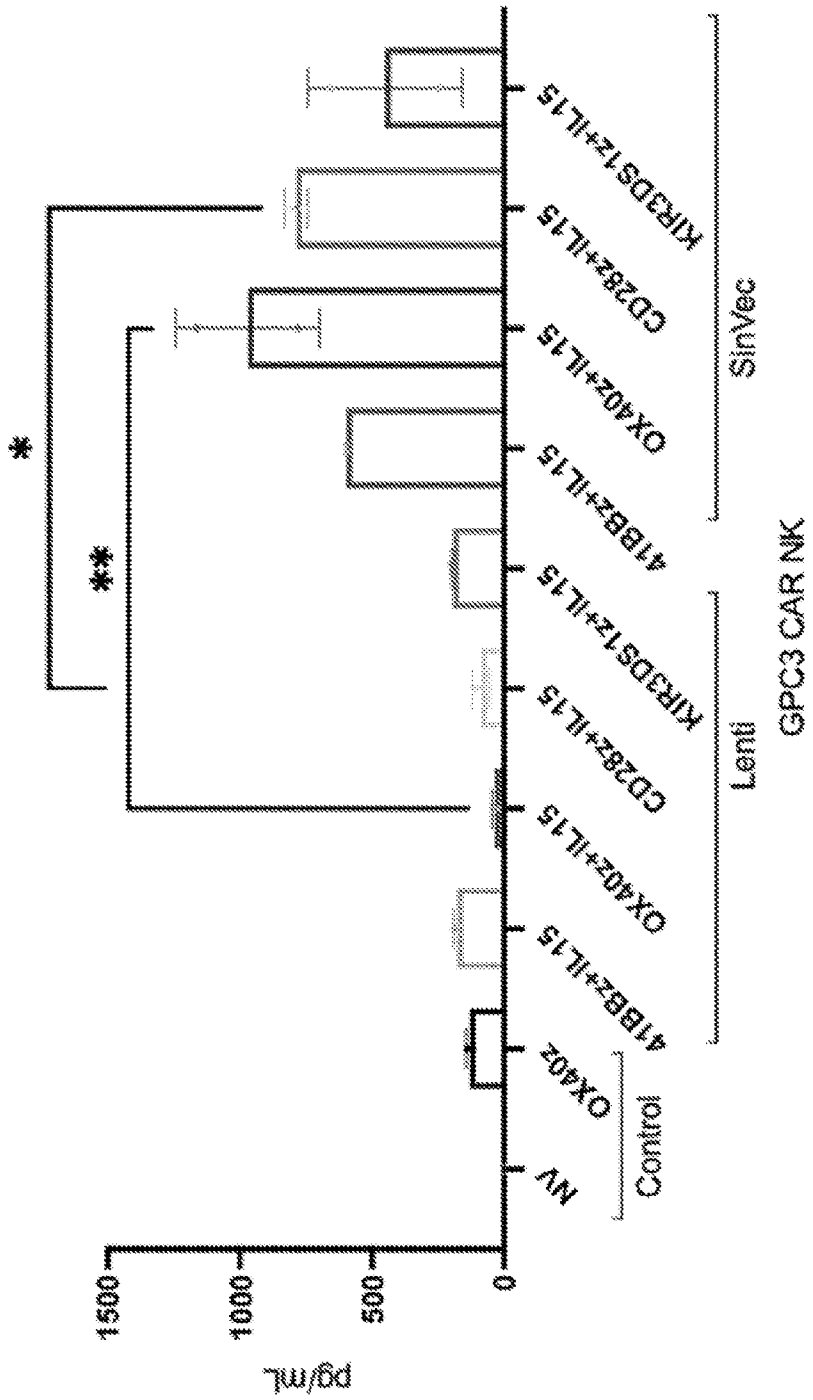

pg/mL
1500
1000
500
0
NV
OX40Z
4IBBz+IL15
OX40z+IL15
CD28z+IL15
KIR3DS1z+IL15
4IBBz+IL15
OX40z+IL15
CD28z+IL15
KIR3DS1z+IL15
4IBBz+IL15
OX40z+IL15
CD28z+IL15
KIR3DS1z+IL15
Control
Lenti
SinVec
GPC3 CAR NK
*
**

FIG. 6

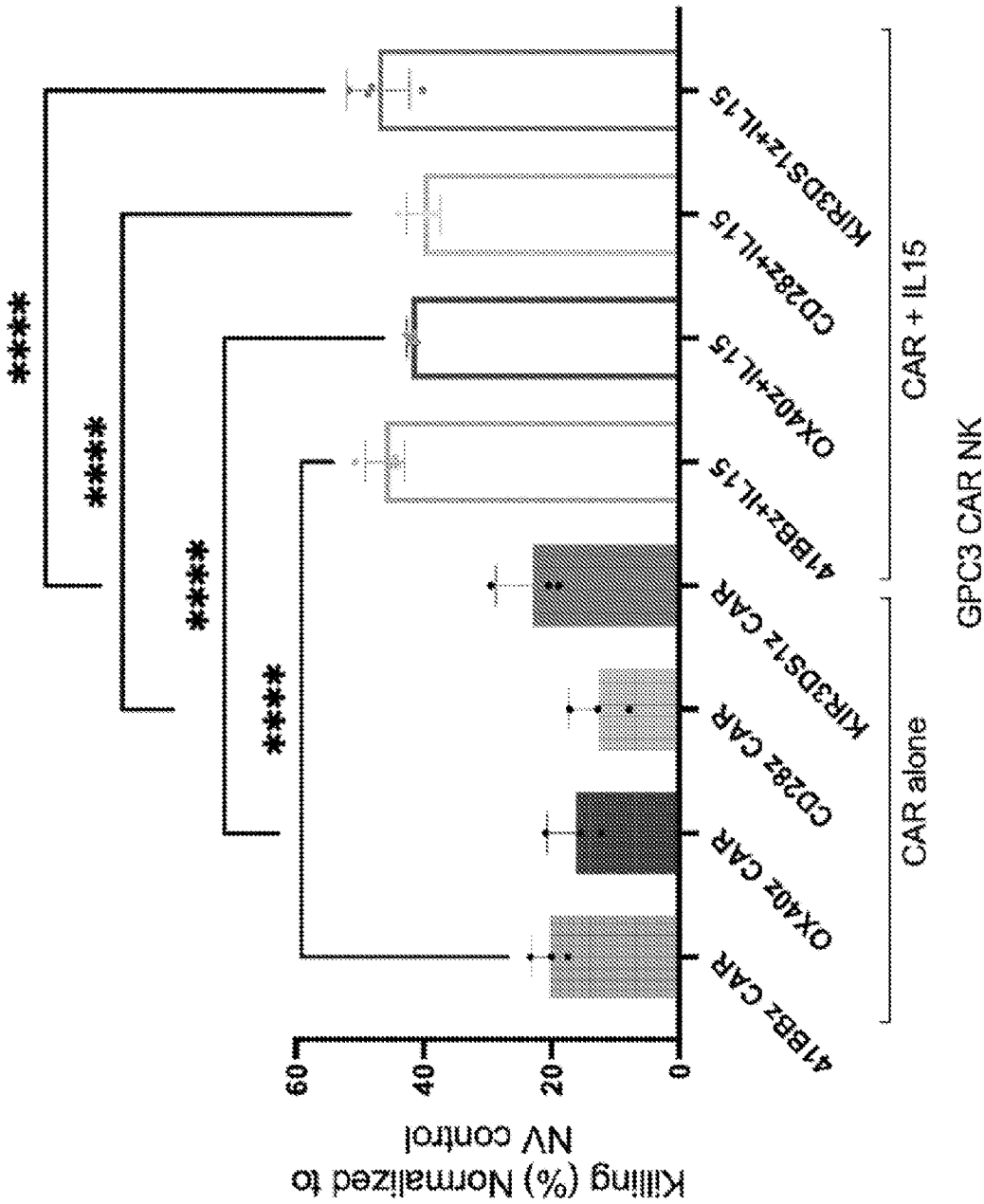

Killing (%) Normalized to NV control
0
20
40
60
4-1BBz CAR
OX40z CAR
CD28z CAR
KIR3DS1z CAR
4-1BBz+IL15
OX40z+IL15
CD28z+IL15
KIR3DS1z+IL15
CAR alone
CAR + IL15
GPC3 CAR NK
****
****
****
****

FIG. 7

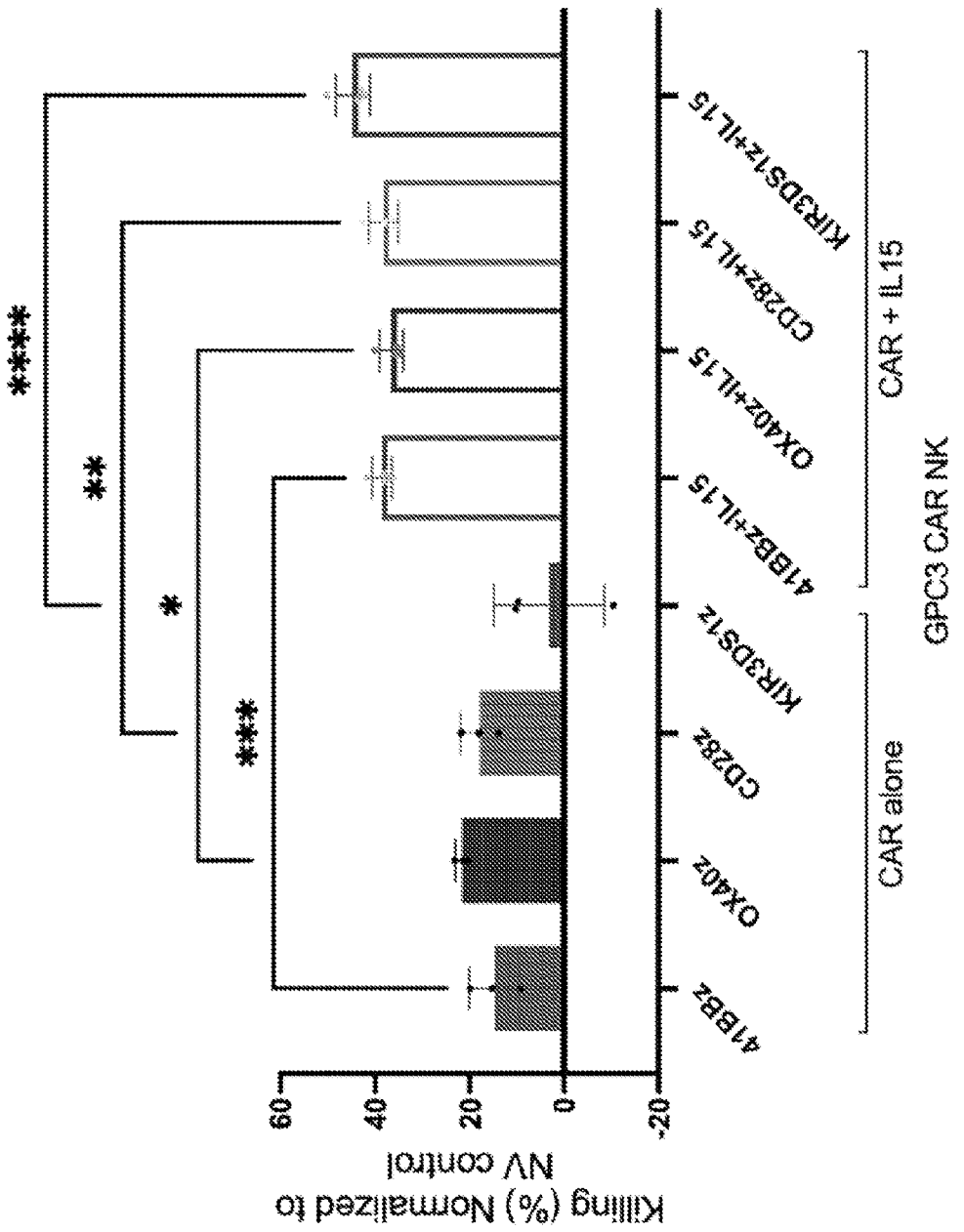

Killing (%) Normalized to NV control
60
40
20
0
-20
4
*
**
***
****
4-1BBz
OX40z
CD28z
KIR3DS1z
4-1BBz+IL-15
OX40z+IL-15
CD28z+IL-15
KIR3DS1z+IL-15
CAR alone
CAR + IL15
GPC3 CAR NK

FIG. 8

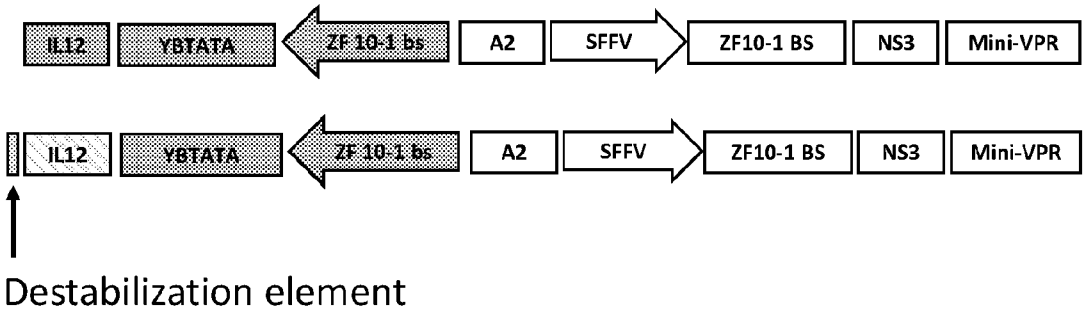


FIG. 9

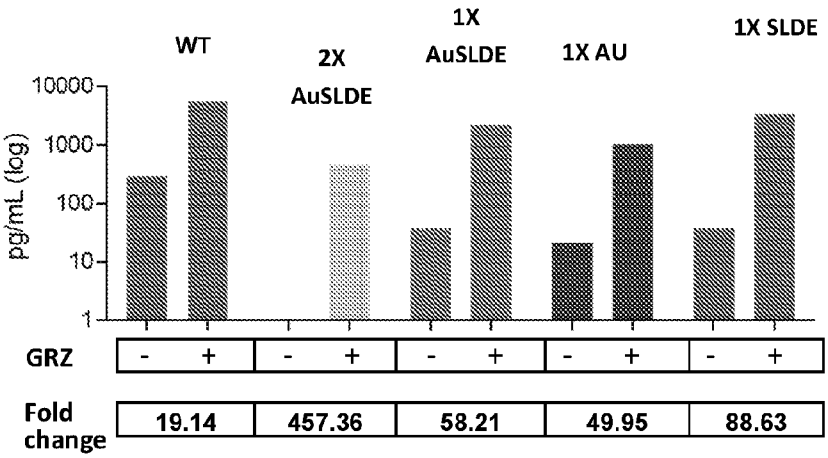


FIG. 10

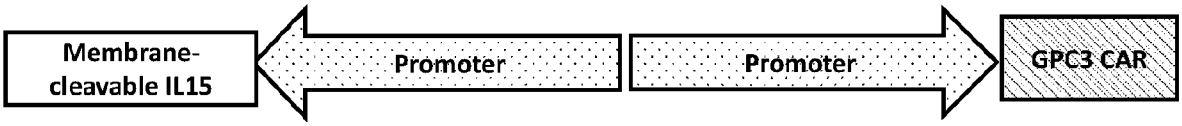
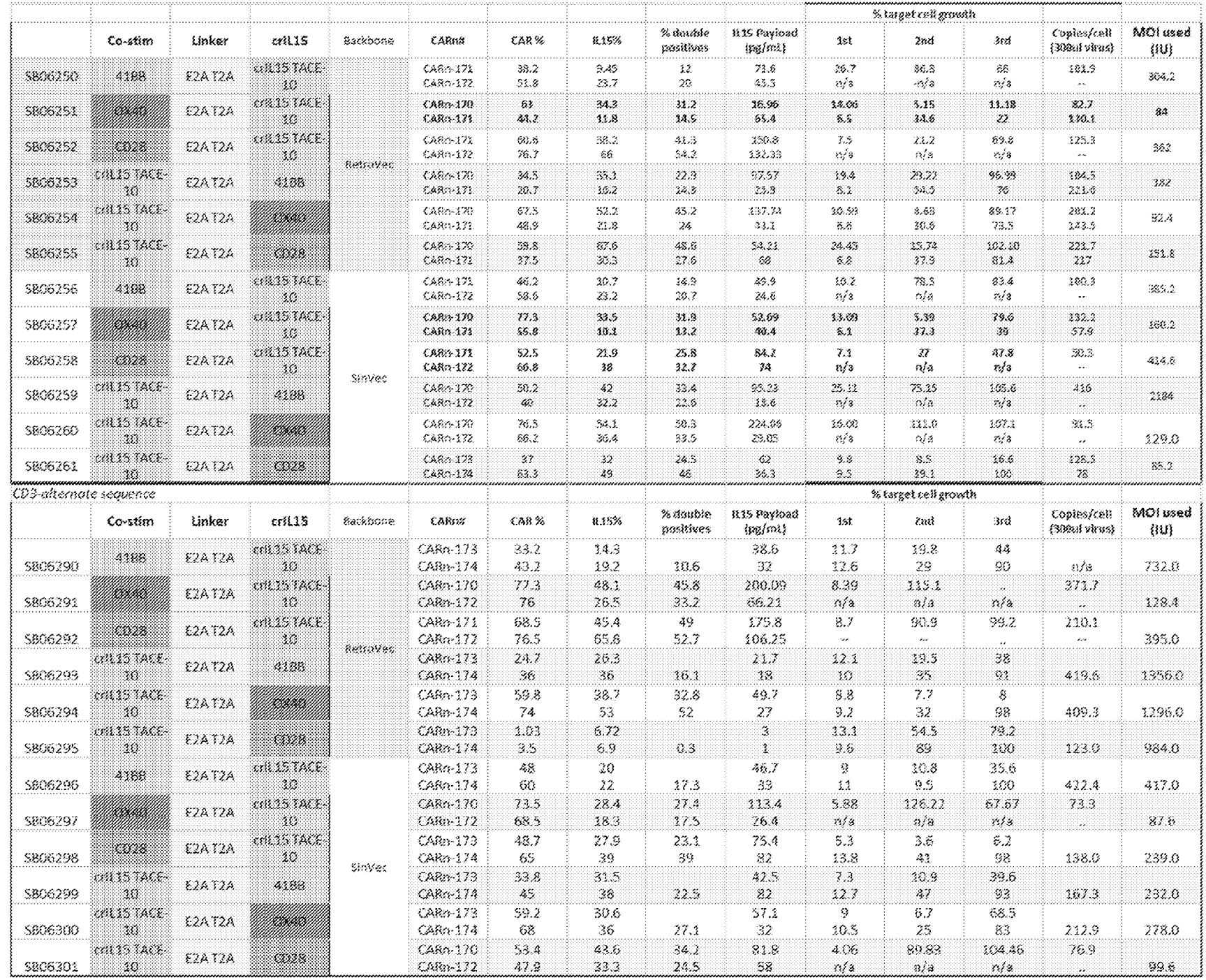


FIG. 11



	Co-stim	Linker	crIL15	Backbone	CARn#	CAR %	IL15%	% double positives	IL15 Payload (pg/ml)	% target cell growth 1st	2nd	3rd	Copies/cell (300ul virus)	MOI used (IU)
SB06250	41BB	E2A T2A	crIL15 TACE-10	RetroVec	CARn-171	38.2	9.45	12	71.6	36.7	86.5	86	181.9	304.2
					CARn-172	51.8	23.7	20	43.5	n/a	n/a	n/a	--	
SB06251	CD40	E2A T2A	crIL15 TACE-10		CARn-170	61	34.3	31.2	16.96	14.06	5.15	11.18	82.7	84
					CARn-171	44.2	11.8	14.5	65.4	6.5	14.6	22	130.1	
SB06252	CD28	E2A T2A	crIL15 TACE-10		CARn-171	60.6	38.2	41.5	150.8	7.5	21.2	69.8	125.3	382
					CARn-172	76.7	66	54.2	132.33	n/a	n/a	n/a	--	
SB06253	crIL15 TACE-10	E2A T2A	41BB		CARn-170	34.5	35.1	22.8	97.57	19.4	28.22	96.99	184.5	382
					CARn-171	20.7	16.2	14.3	23.9	8.1	34.5	76	221.6	
SB06254	crIL15 TACE-10	E2A T2A	CD40		CARn-170	67.5	52.2	45.2	137.74	10.99	8.68	89.17	284.2	82.4
					CARn-171	48.9	21.8	24	43.1	6.6	30.6	76.5	143.5	
SB06255	crIL15 TACE-10	E2A T2A	CD28		CARn-170	38.8	67.6	48.8	54.21	24.43	15.78	102.18	221.7	151.8
					CARn-171	37.5	30.3	27.6	60	6.8	37.3	81.4	227	
SB06256	41BB	E2A T2A	crIL15 TACE-10	SinVec	CARn-171	46.2	10.7	14.9	49.9	10.2	78.5	83.4	180.3	365.2
					CARn-172	58.6	23.2	20.7	24.6	n/a	n/a	n/a	--	
SB06257	CD40	E2A T2A	crIL15 TACE-10		CARn-170	77.3	33.5	31.8	52.69	13.09	5.38	79.6	132.2	168.2
					CARn-171	55.8	10.1	13.2	48.4	6.1	37.3	38	57.9	
SB06258	CD28	E2A T2A	crIL15 TACE-10		CARn-171	52.5	21.9	25.8	84.2	7.1	37	47.8	80.3	414.6
					CARn-172	66.8	38	32.7	34	n/a	n/a	n/a	--	
SB06259	crIL15 TACE-10	E2A T2A	41BB		CARn-170	58.2	42	33.4	95.23	25.11	75.15	105.6	416	2184
					CARn-172	40	32.2	22.6	18.6	n/a	n/a	n/a	--	
SB06260	crIL15 TACE-10	E2A T2A	CD40		CARn-170	76.5	54.1	50.3	224.09	16.08	111.6	107.1	91.5	129.0
					CARn-172	66.2	36.4	33.5	29.05	n/a	n/a	n/a	--	
SB06261	crIL15 TACE-10	E2A T2A	CD28		CARn-173	37	32	24.5	62	9.8	8.5	16.6	128.5	86.2
					CARn-174	63.3	49	48	36.3	8.5	39.1	100	78	

CD3-alternate sequence

	Co-stim	Linker	crIL15	Backbone	CARn#	CAR %	IL15%	% double positives	IL15 Payload (pg/ml)	% target cell growth 1st	2nd	3rd	Copies/cell (300ul virus)	MOI used (IU)
SB06290	41BB	E2A T2A	crIL15 TACE-10	RetroVec	CARn-173	33.2	14.3		38.6	11.7	19.8	44		
					CARn-174	43.2	19.2	10.6	32	12.6	29	90	n/a	732.0
SB06291	CD40	E2A T2A	crIL15 TACE-10		CARn-170	77.3	48.1	45.8	200.09	8.39	115.1	..	371.7	
					CARn-172	76	26.5	33.2	66.21	n/a	n/a	n/a	..	128.4
SB06292	CD28	E2A T2A	crIL15 TACE-10		CARn-171	68.5	45.4	49	175.8	8.7	90.9	99.2	210.1	
					CARn-172	76.5	65.8	52.7	106.25	--	--	..	--	395.0
SB06293	crIL15 TACE-10	E2A T2A	41BB		CARn-173	24.7	26.3		21.7	12.1	19.5	38		
					CARn-174	36	36	16.1	18	10	35	91	419.6	1356.0
SB06294	crIL15 TACE-10	E2A T2A	CD40		CARn-173	59.8	38.7	32.8	49.7	8.8	7.7	8		
					CARn-174	74	53	52	27	9.2	32	98	409.3	1296.0
SB06295	crIL15 TACE-10	E2A T2A	CD28		CARn-173	1.03	6.72		3	13.1	54.5	79.2		
					CARn-174	3.5	6.9	0.3	1	9.6	89	100	123.0	984.0
SB06296	41BB	E2A T2A	crIL15 TACE-10	SinVec	CARn-173	48	20		46.7	9	10.8	35.6		
					CARn-174	60	22	17.3	33	11	9.5	100	422.4	417.0
SB06297	CD40	E2A T2A	crIL15 TACE-10		CARn-170	73.5	28.4	27.4	113.4	5.88	126.22	67.67	73.3	
					CARn-172	68.5	18.3	17.5	26.4	n/a	n/a	n/a	..	87.6
SB06298	CD28	E2A T2A	crIL15 TACE-10		CARn-173	48.7	27.9	23.1	75.4	5.3	3.6	6.2		
					CARn-174	65	39	39	82	13.8	41	98	138.0	239.0
SB06299	crIL15 TACE-10	E2A T2A	41BB		CARn-173	33.8	31.5		42.5	7.3	10.9	39.6		
					CARn-174	45	38	22.5	82	12.7	47	93	167.3	232.0
SB06300	crIL15 TACE-10	E2A T2A	CD40		CARn-173	59.2	30.6		57.1	9	6.7	68.5		
					CARn-174	68	36	27.1	32	10.5	25	83	212.9	278.0
SB06301	crIL15 TACE-10	E2A T2A	CD28		CARn-170	53.4	43.6	34.2	81.8	4.06	89.82	104.46	76.9	
					CARn-172	47.9	33.3	24.5	58	n/a	n/a	n/a	..	99.6

FIG. 12

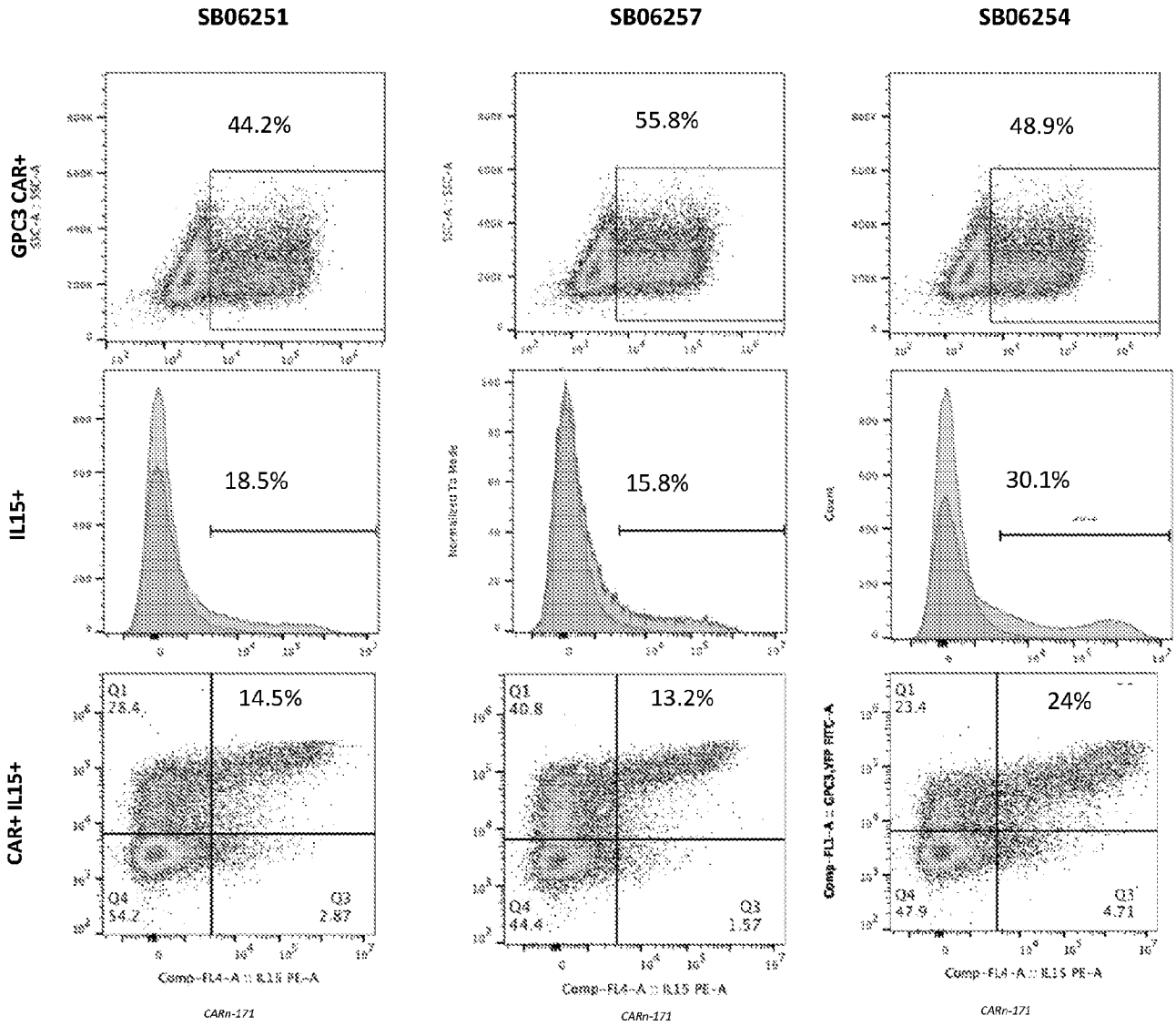

SB06251
SB06257
SB06254
GPC3 CAR+
IL15+
CAR+ IL15+
44.2%
55.8%
48.9%
18.5%
15.8%
30.1%
14.5%
13.2%
24%
Q1
28.4
Q4
54.2
Q3
2.87
Q1
40.8
Q4
44.4
Q3
1.57
Q1
23.4
Q4
47.9
Q3
4.71
Comp-FL4-A :: IL15 PE-A
CARn-171

FIG. 13A

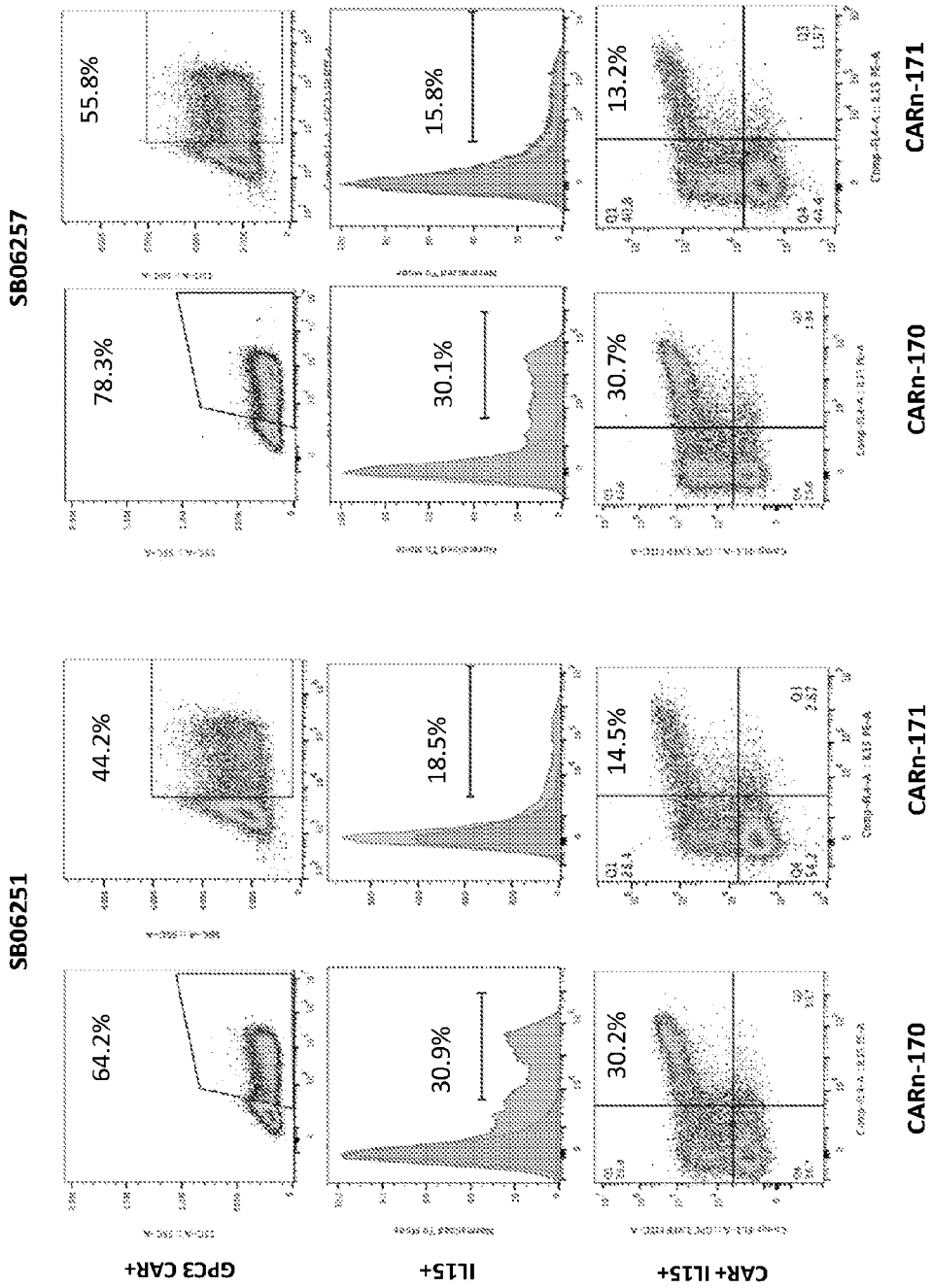

SB06251
SB06257
CARn-170
CARn-171
CARn-170
CARn-171
GPC3 CAR+
IL15+
CAR+ IL15+
64.2%
44.2%
78.3%
55.8%
30.9%
18.5%
30.1%
15.8%
30.2%
14.5%
30.7%
13.2%

FIG. 13B

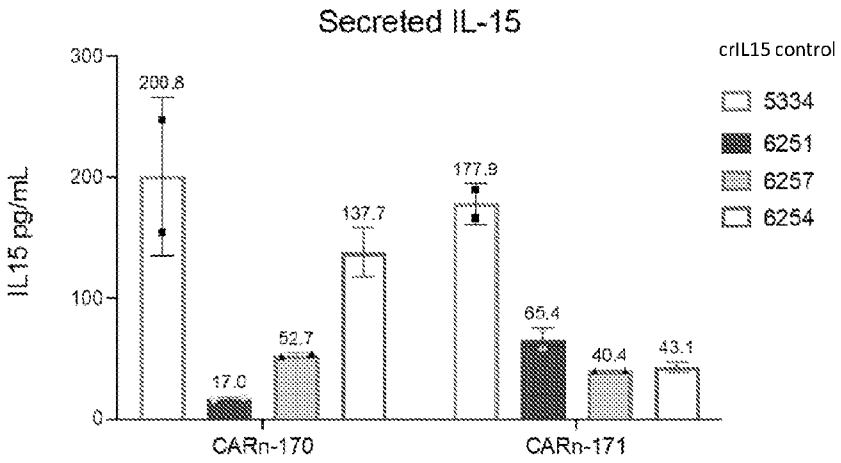


FIG. 14A

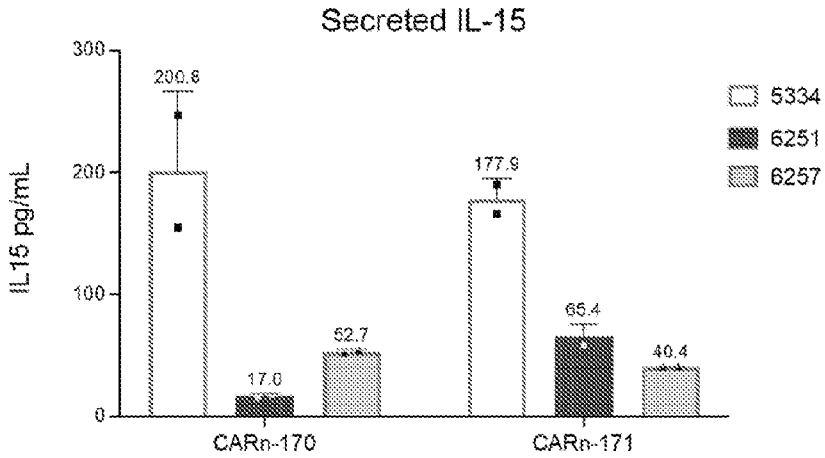


FIG. 14B

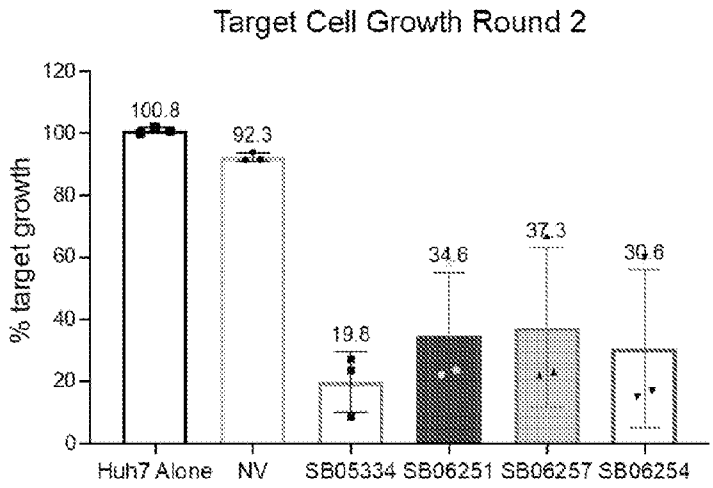


FIG. 15A

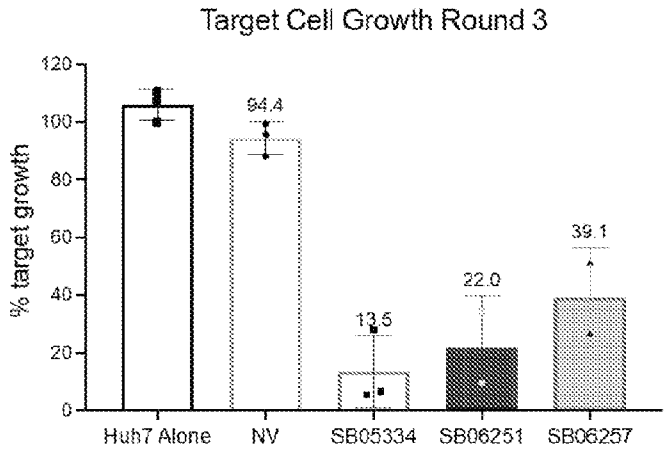


FIG. 15B

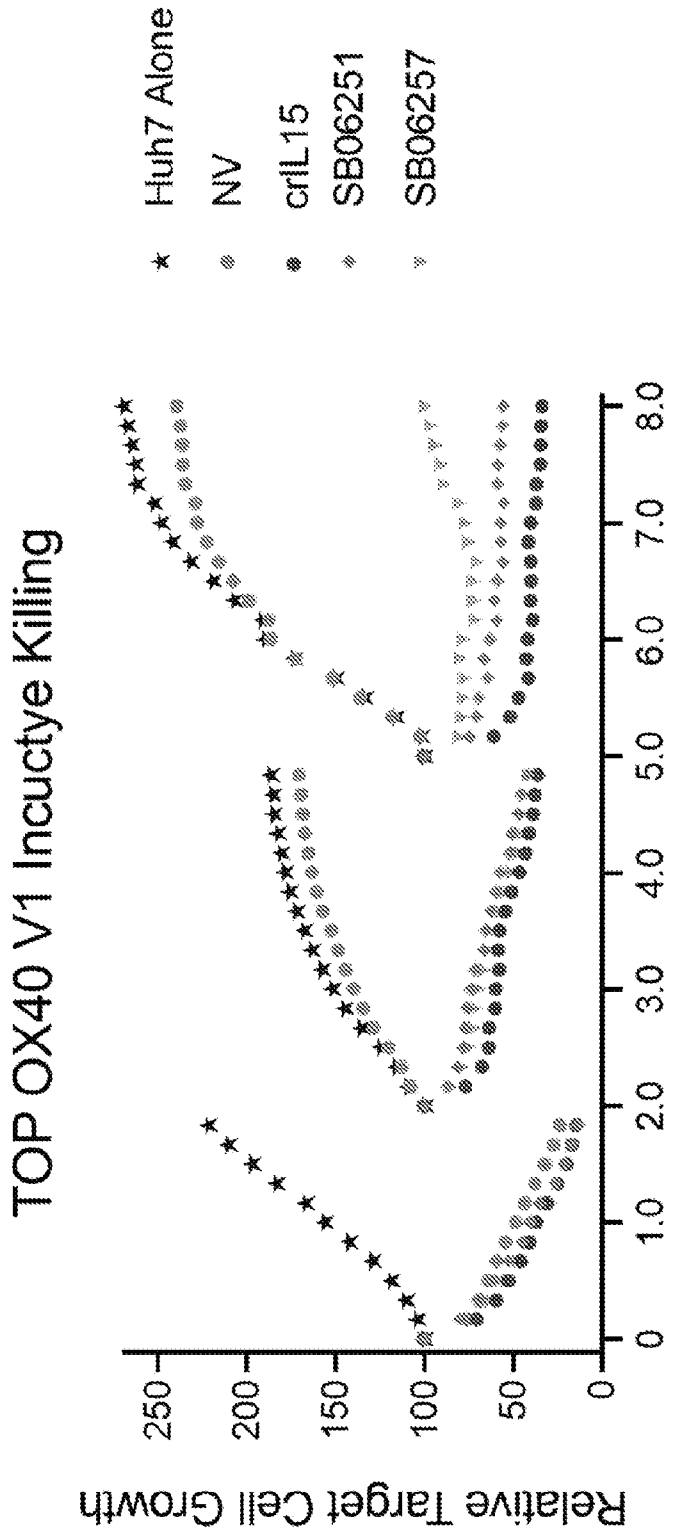

TOP OX40 V1 Incuctye Killing
Huh7 Alone
NV
crIL15
SB06251
SB06257
Relative Target Cell Growth
250
200
150
100
50
0
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
Days

FIG. 16

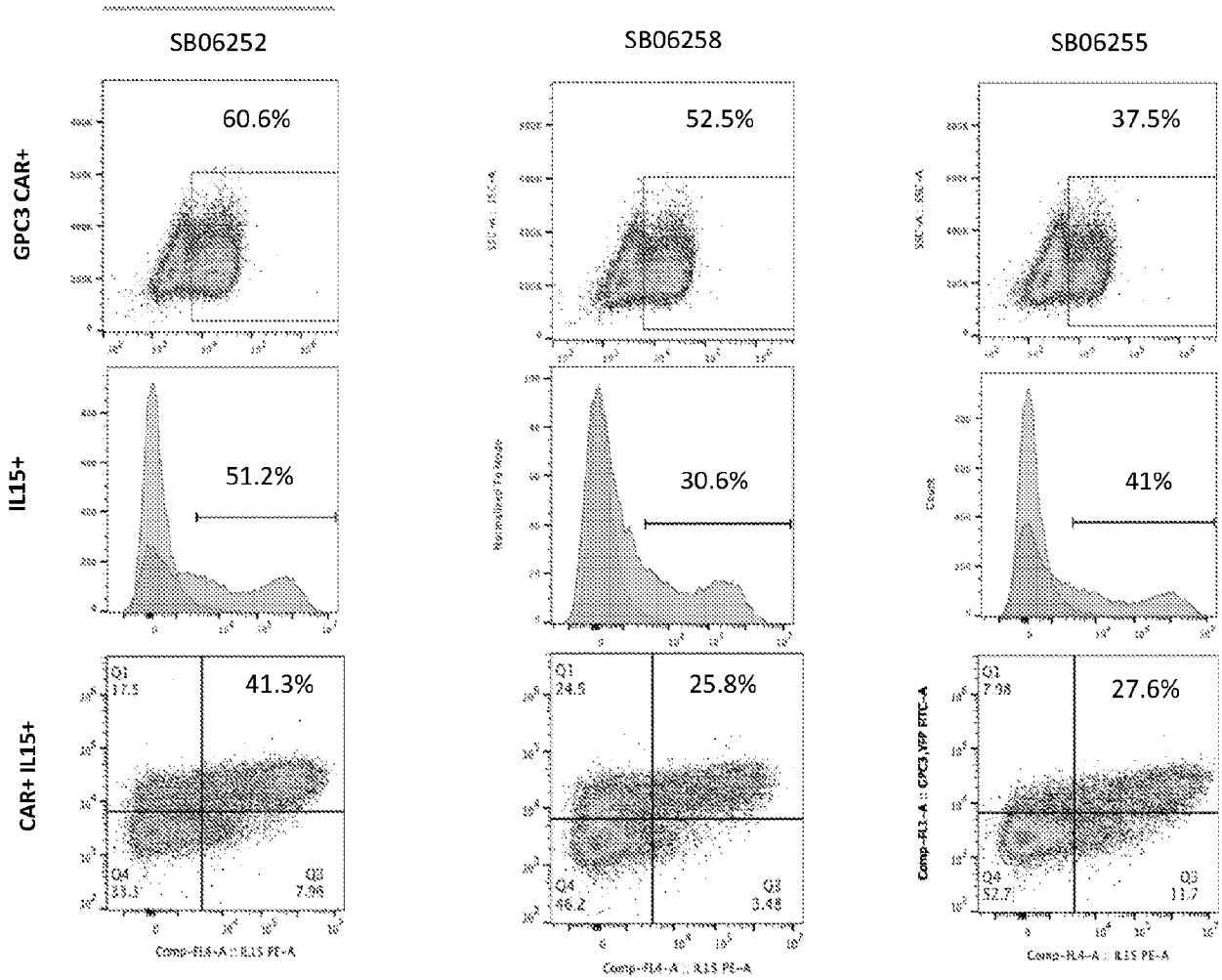

SB06252
SB06258
SB06255
GPC3 CAR+
IL15+
CAR+ IL15+
60.6%
52.5%
37.5%
51.2%
30.6%
41%
41.3%
25.8%
27.6%

FIG. 17A

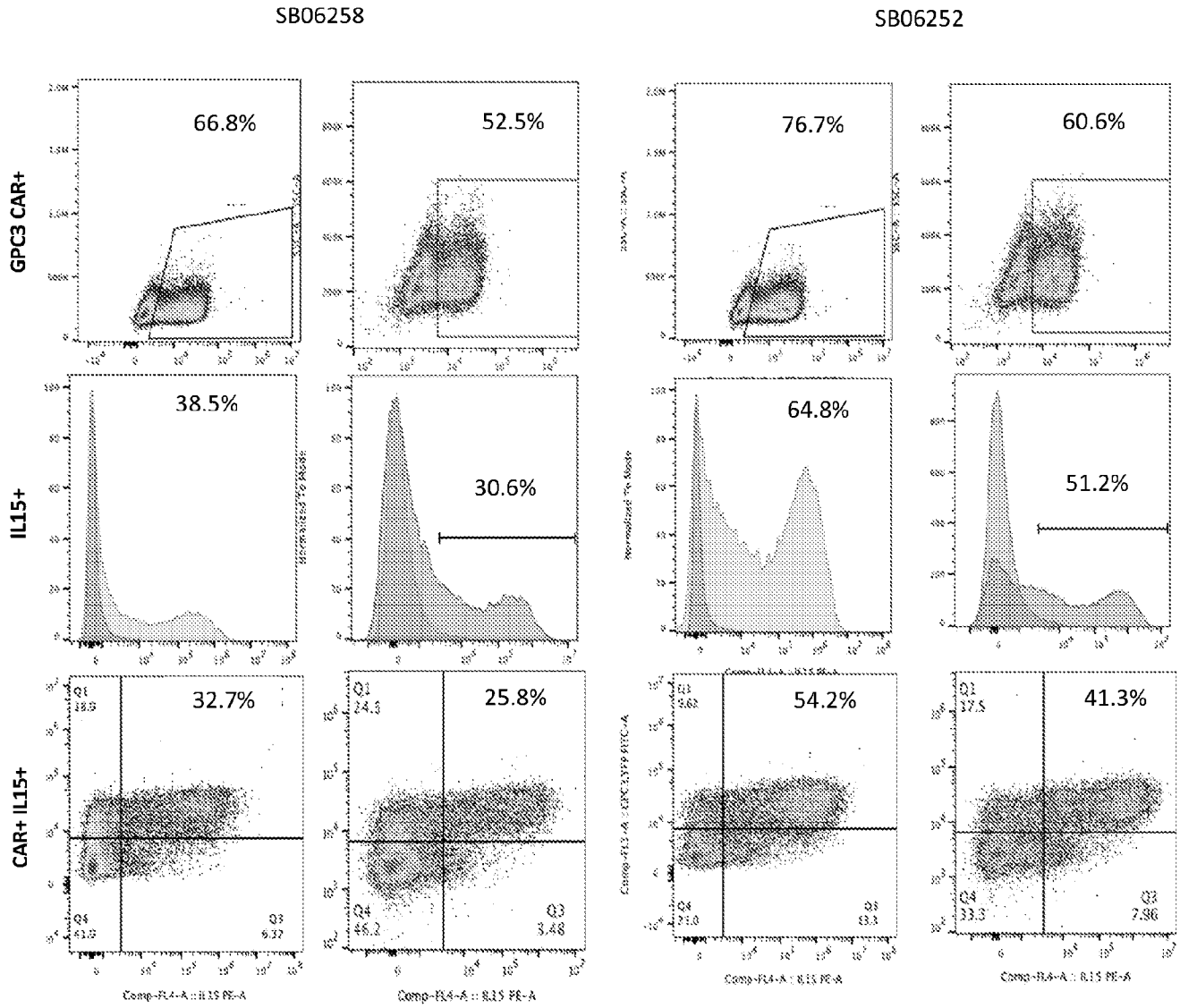

SB06258
SB06252
GPC3 CAR+
IL15+
CAR+ IL15+
66.8%
52.5%
76.7%
60.6%
38.5%
30.6%
64.8%
51.2%
32.7%
25.8%
54.2%
41.3%

FIG. 17B

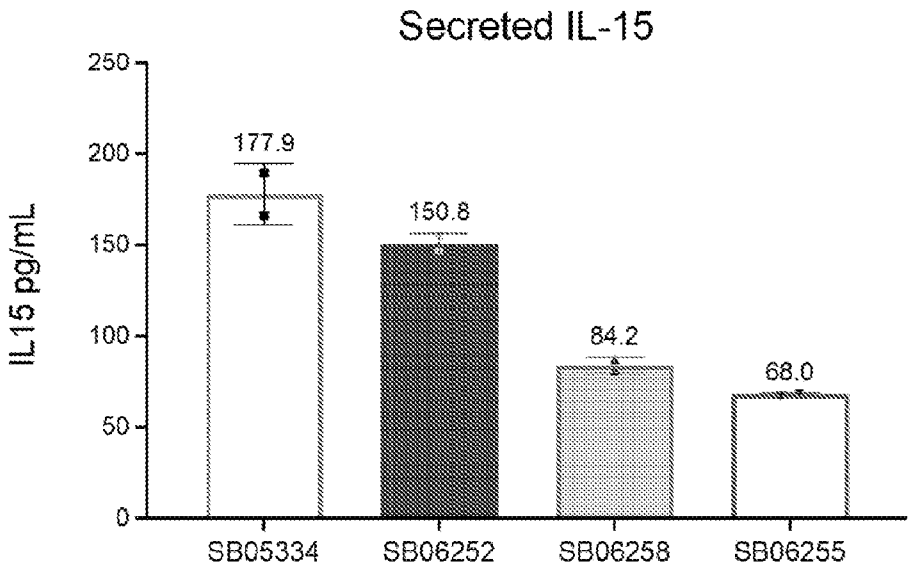


FIG. 18A

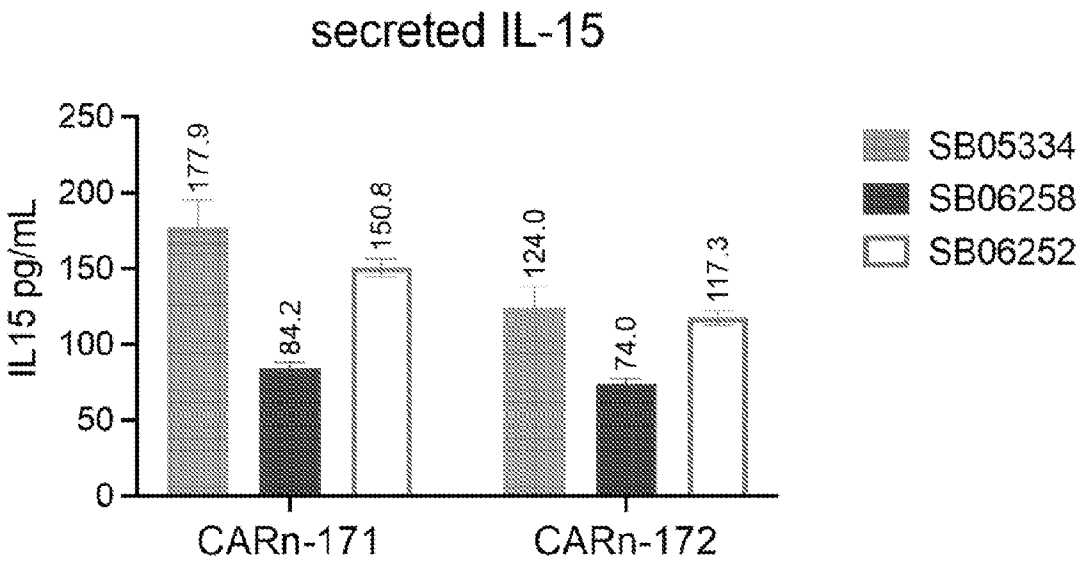


FIG. 18B

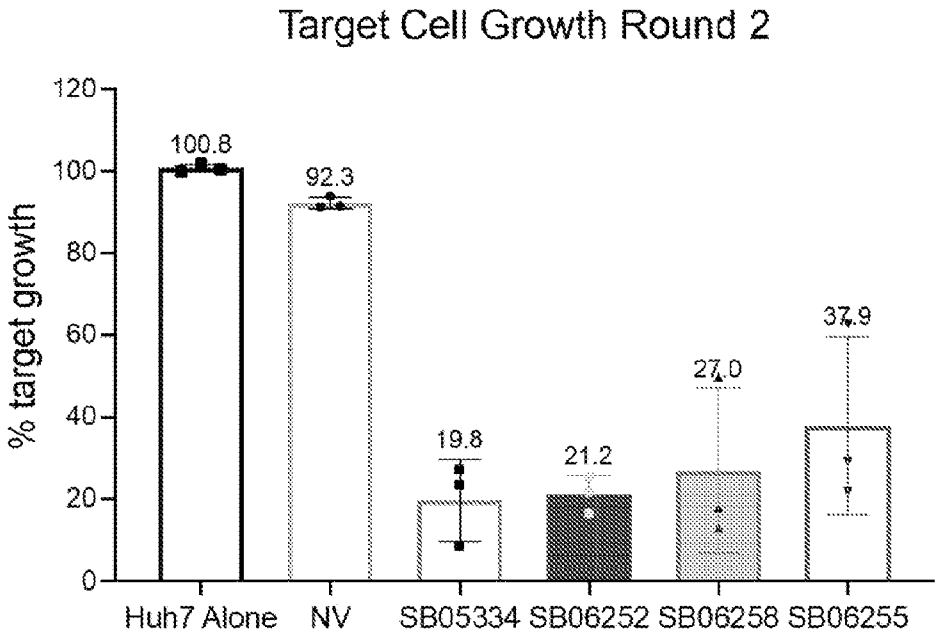


FIG. 19A

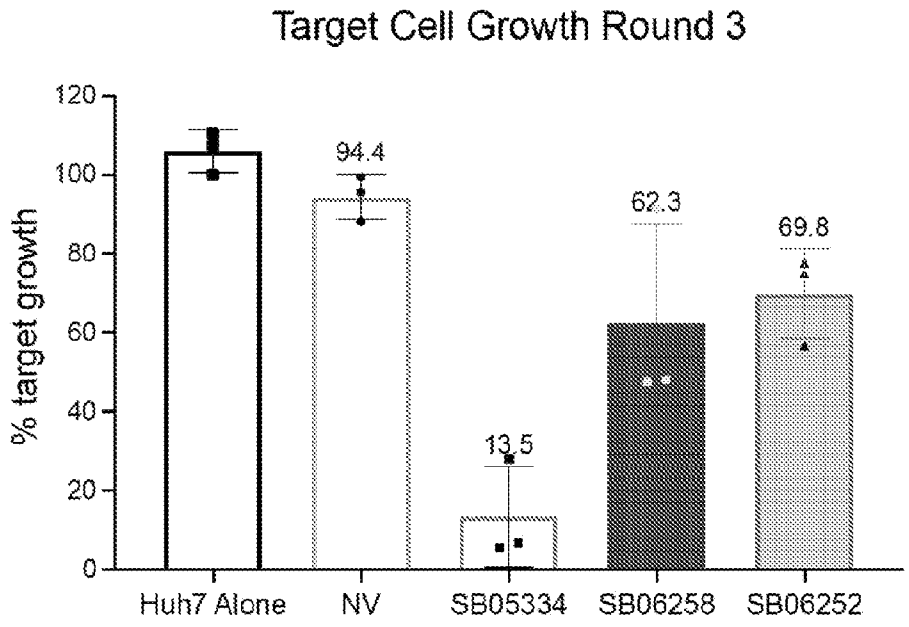


FIG. 19B

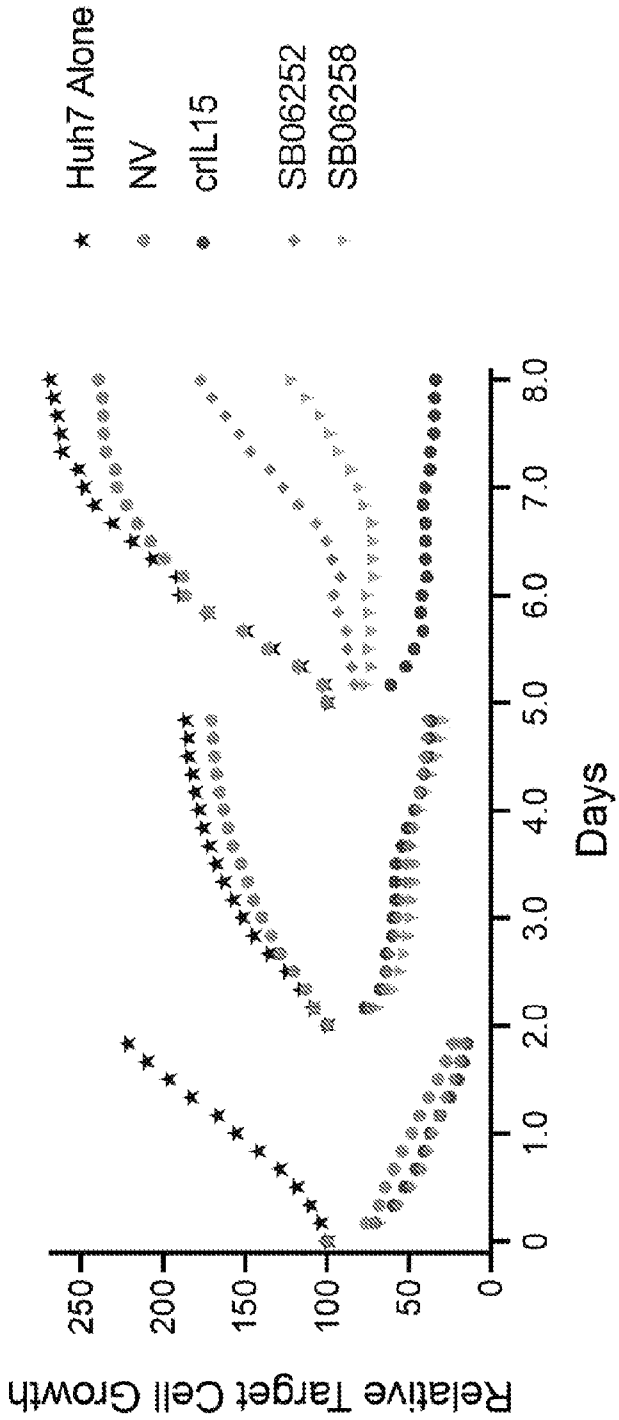

TOP CD28 V1 Incuctye Killing
Huh7 Alone
NV
crlL15
SB06252
SB06258
Relative Target Cell Growth
250
200
150
100
50
0
0
1.0
2.0
3.0
4.0
5.0
6.0
7.0
8.0
Days

FIG. 20

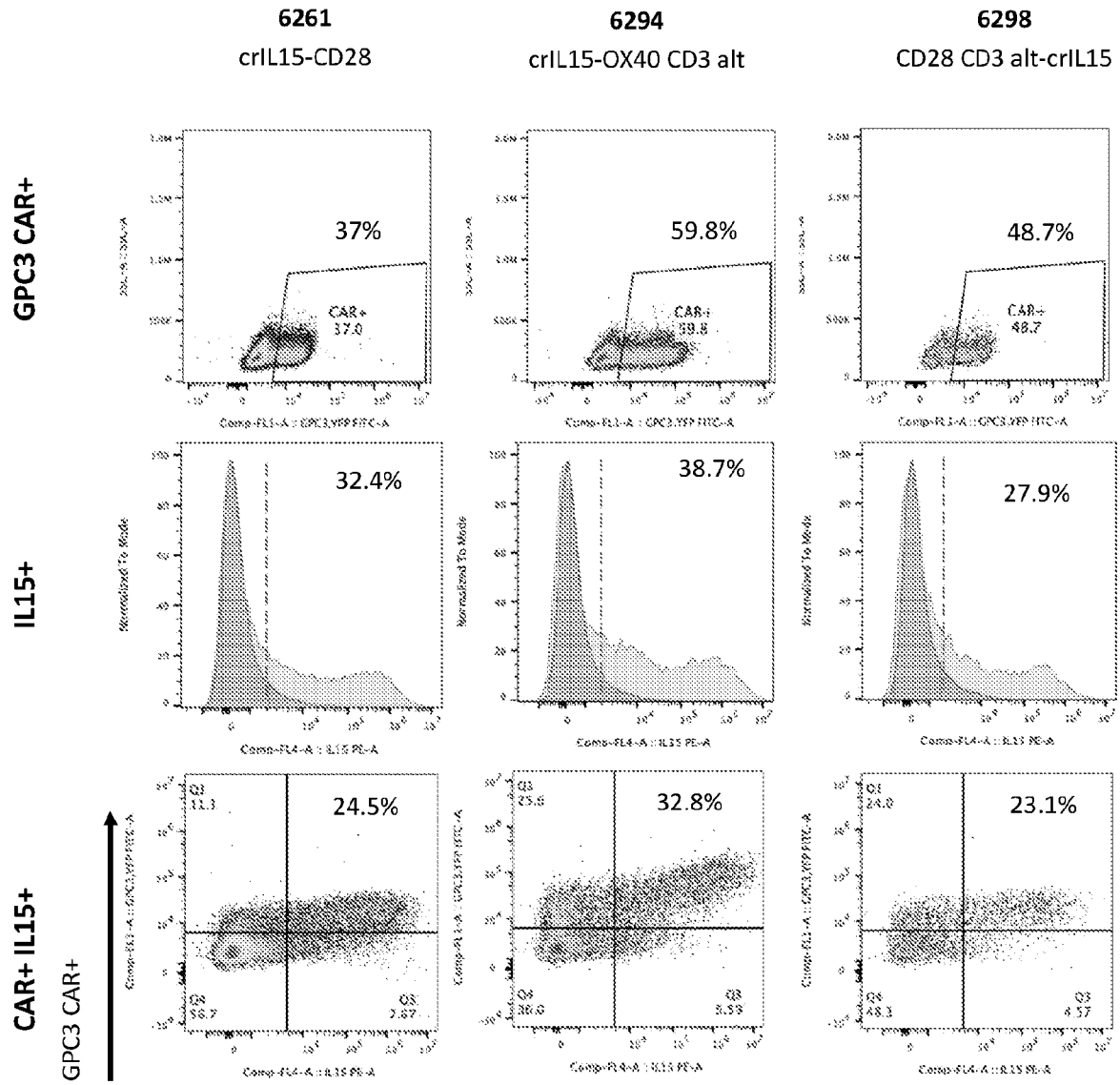

6261
crIL15-CD28
6294
crIL15-OX40 CD3 alt
6298
CD28 CD3 alt-crIL15
GPC3 CAR+
IL15+
CAR+ IL15+
GPC3 CAR+
37%
59.8%
48.7%
32.4%
38.7%
27.9%
24.5%
32.8%
23.1%

FIG. 21A

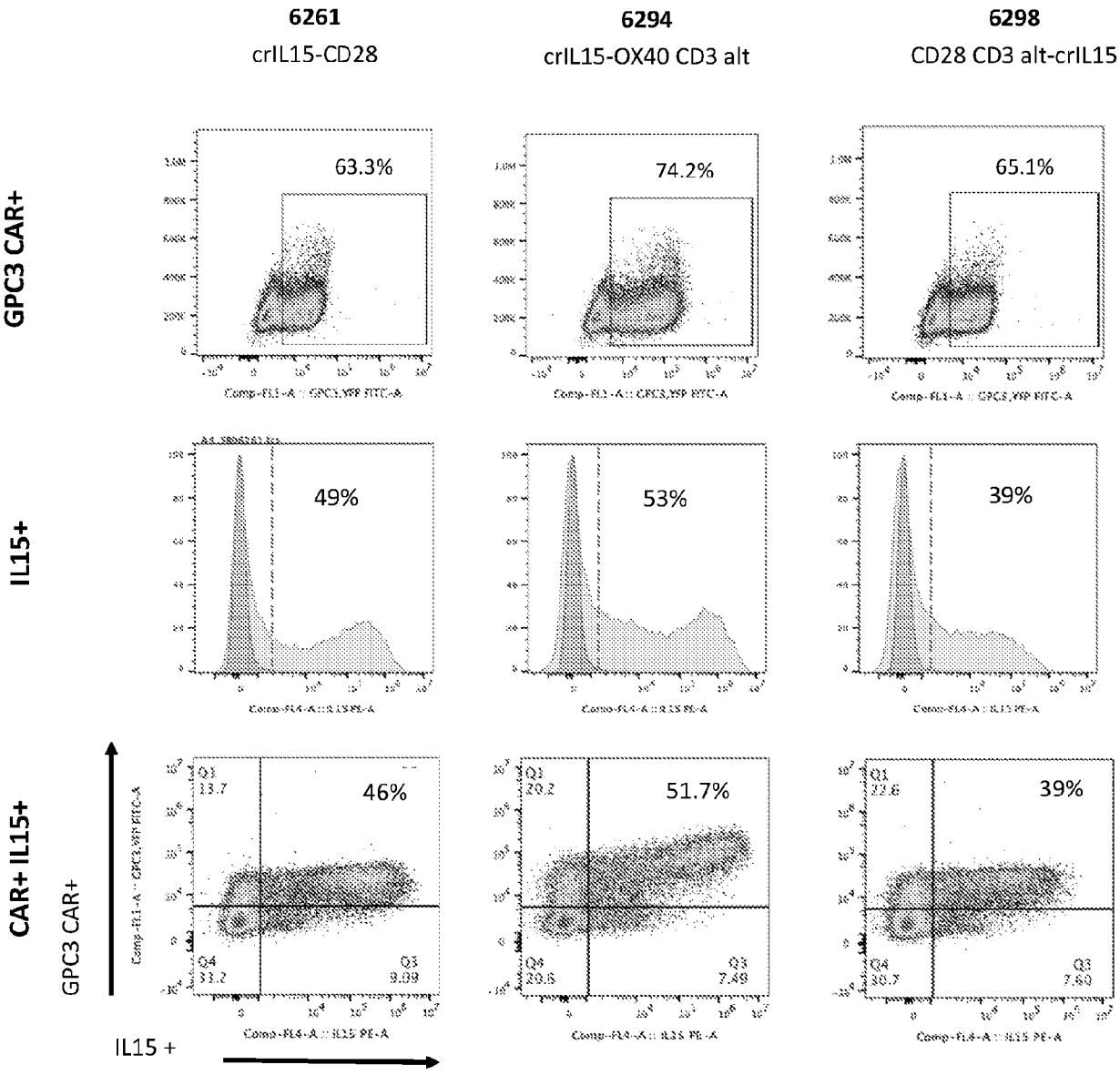

6261
crIL15-CD28
6294
crIL15-OX40 CD3 alt
6298
CD28 CD3 alt-crIL15
GPC3 CAR+
IL15+
CAR+ IL15+
63.3%
74.2%
65.1%
49%
53%
39%
46%
51.7%
39%
GPC3 CAR+
IL15 +

FIG. 21B

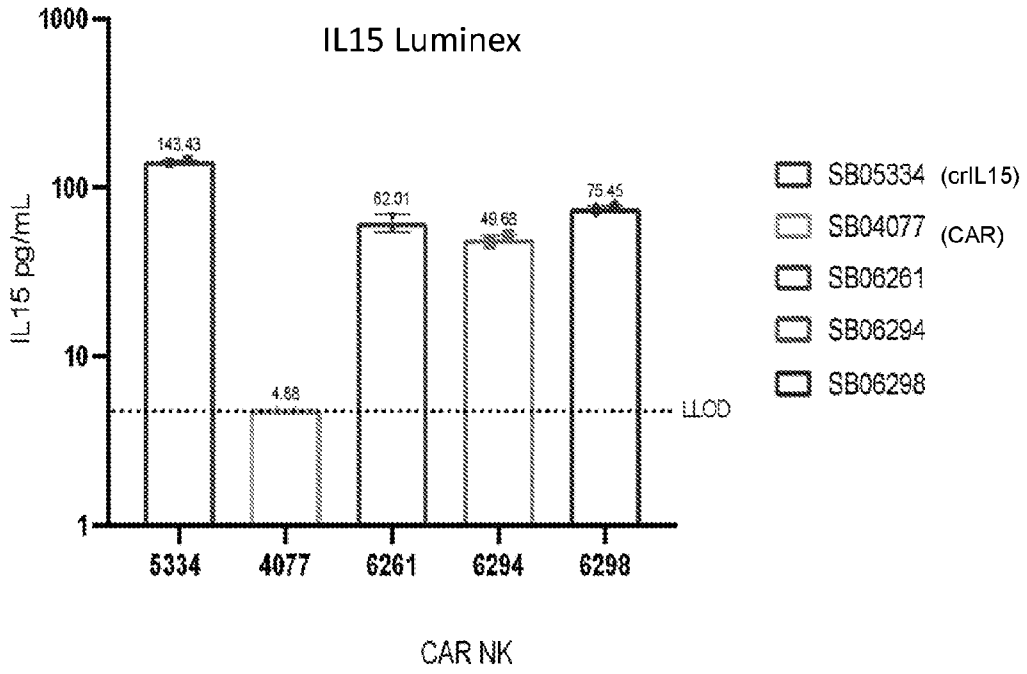


FIG. 22A

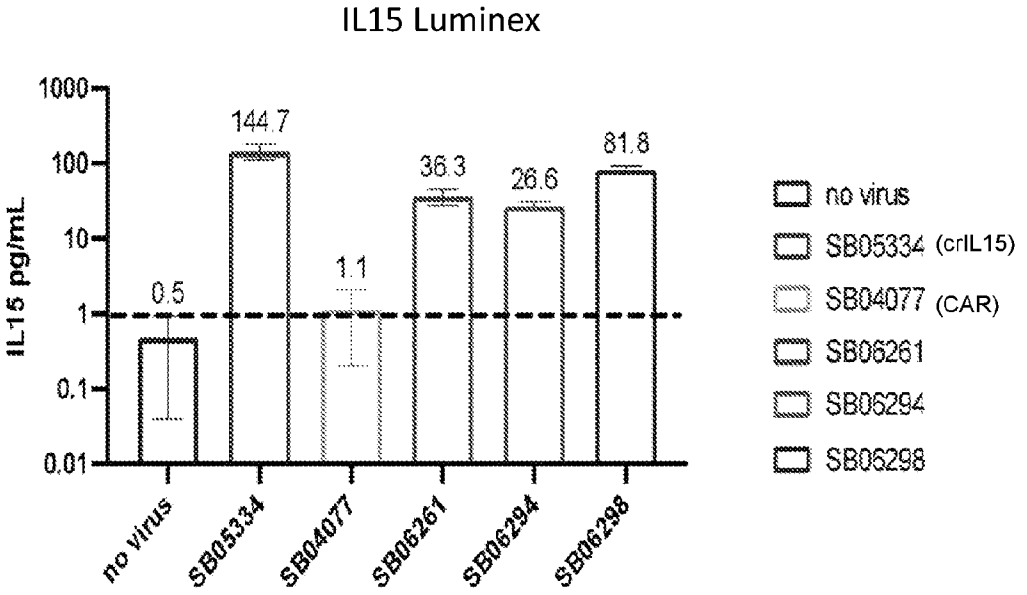


FIG. 22B

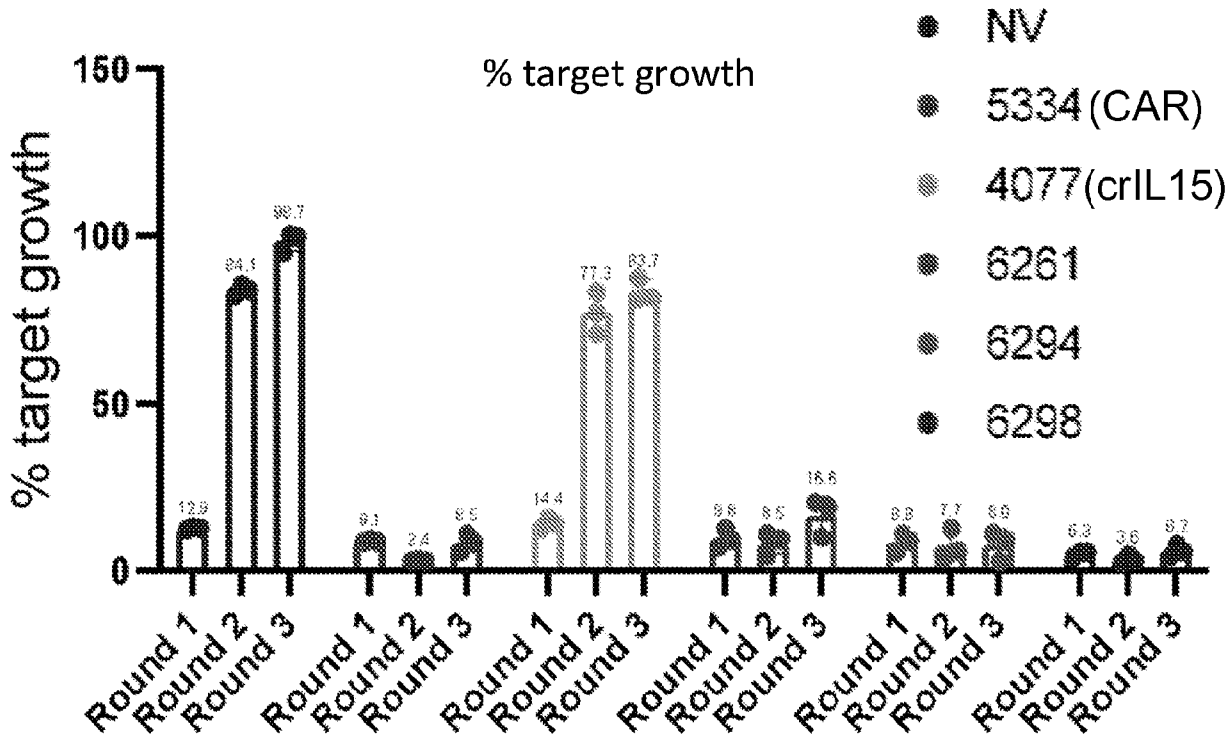


FIG. 23A

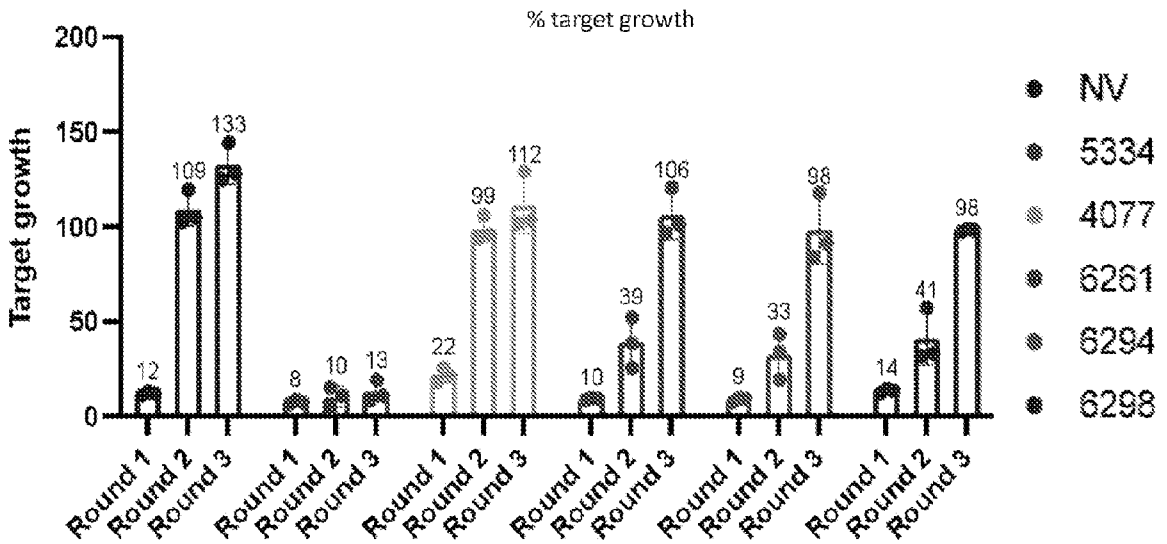


FIG. 23B

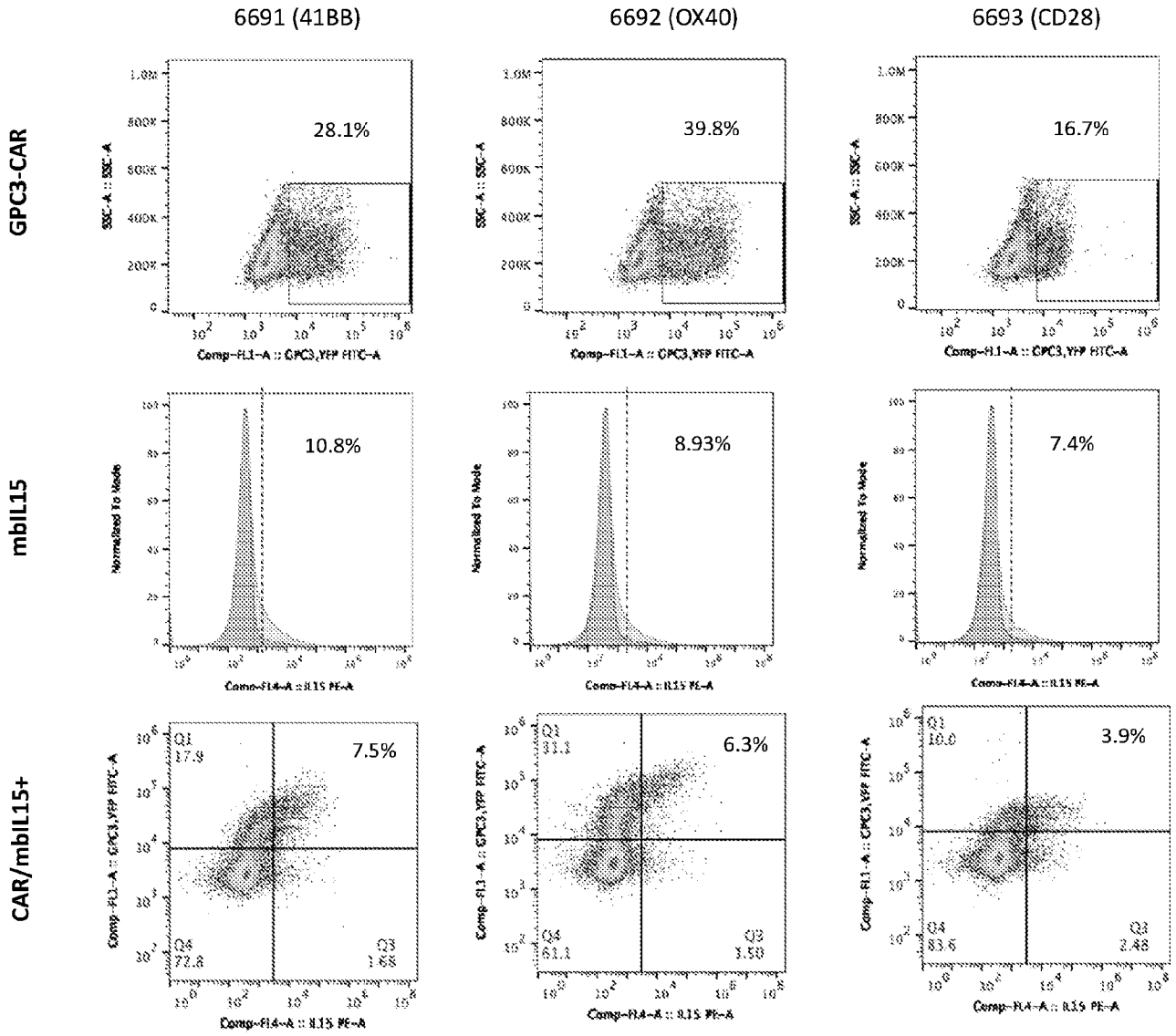

6691 (41BB)
6692 (OX40)
6693 (CD28)
GPC3-CAR
mbIL15
CAR/mbIL15+
28.1%
39.8%
16.7%
10.8%
8.93%
7.4%
7.5%
6.3%
3.9%

FIG. 24A

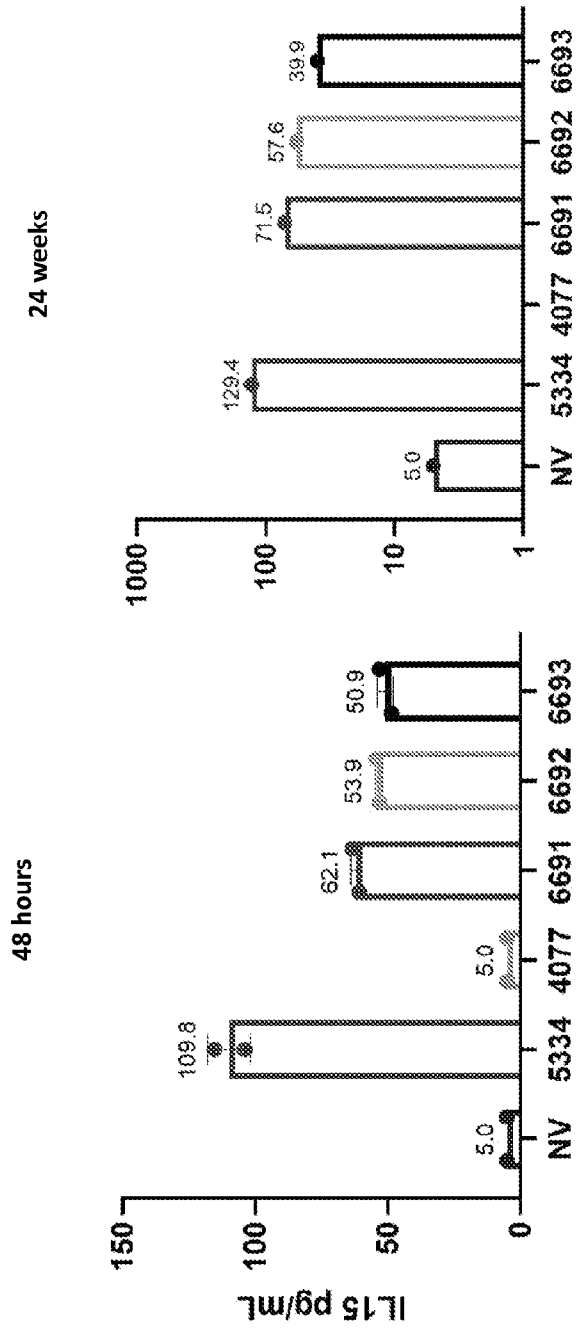

48 hours
24 weeks
IL15 pg/mL
150
100
50
0
1000
100
10
1
5.0
109.8
5.0
62.1
53.9
50.9
5.0
129.4
71.5
57.6
39.9
NV
5334
4077
6691
6692
6693

FIG. 24B

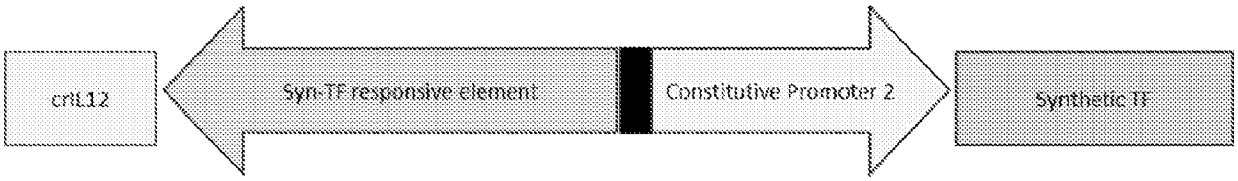
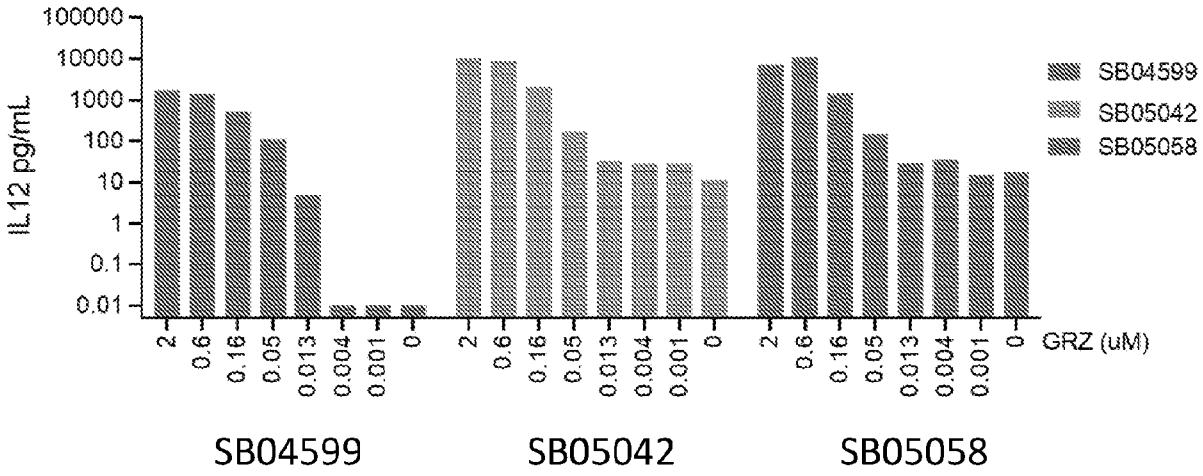


FIG. 25



Fold-change at 0.16 uM*	-	~180	~84

FIG. 26

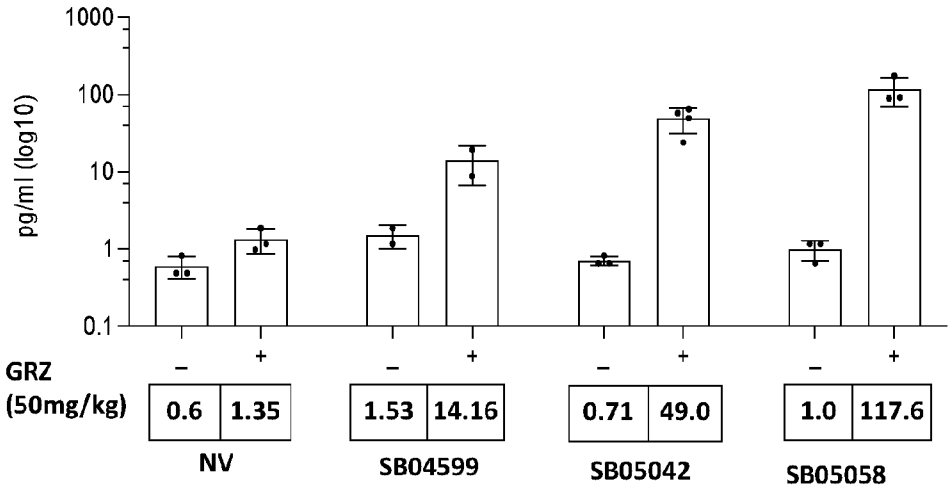


FIG. 27A

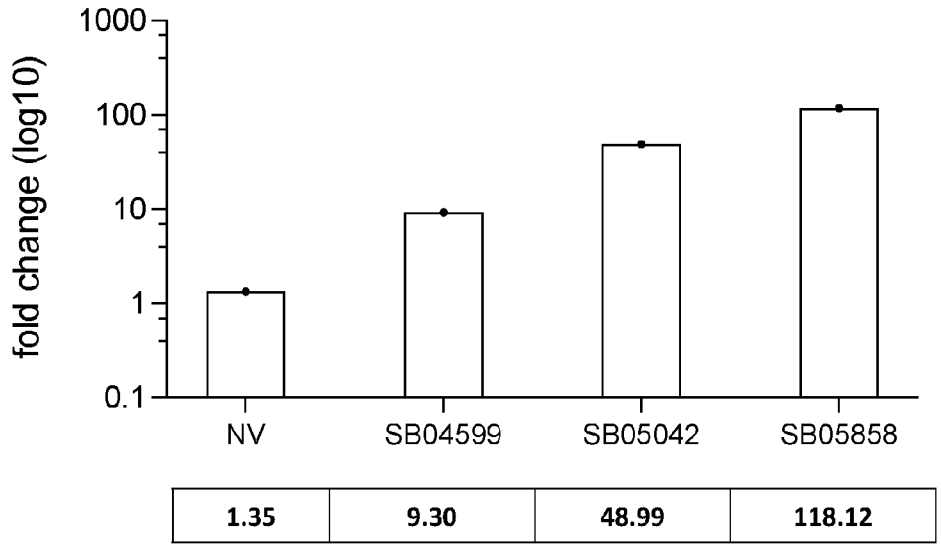


FIG. 27B

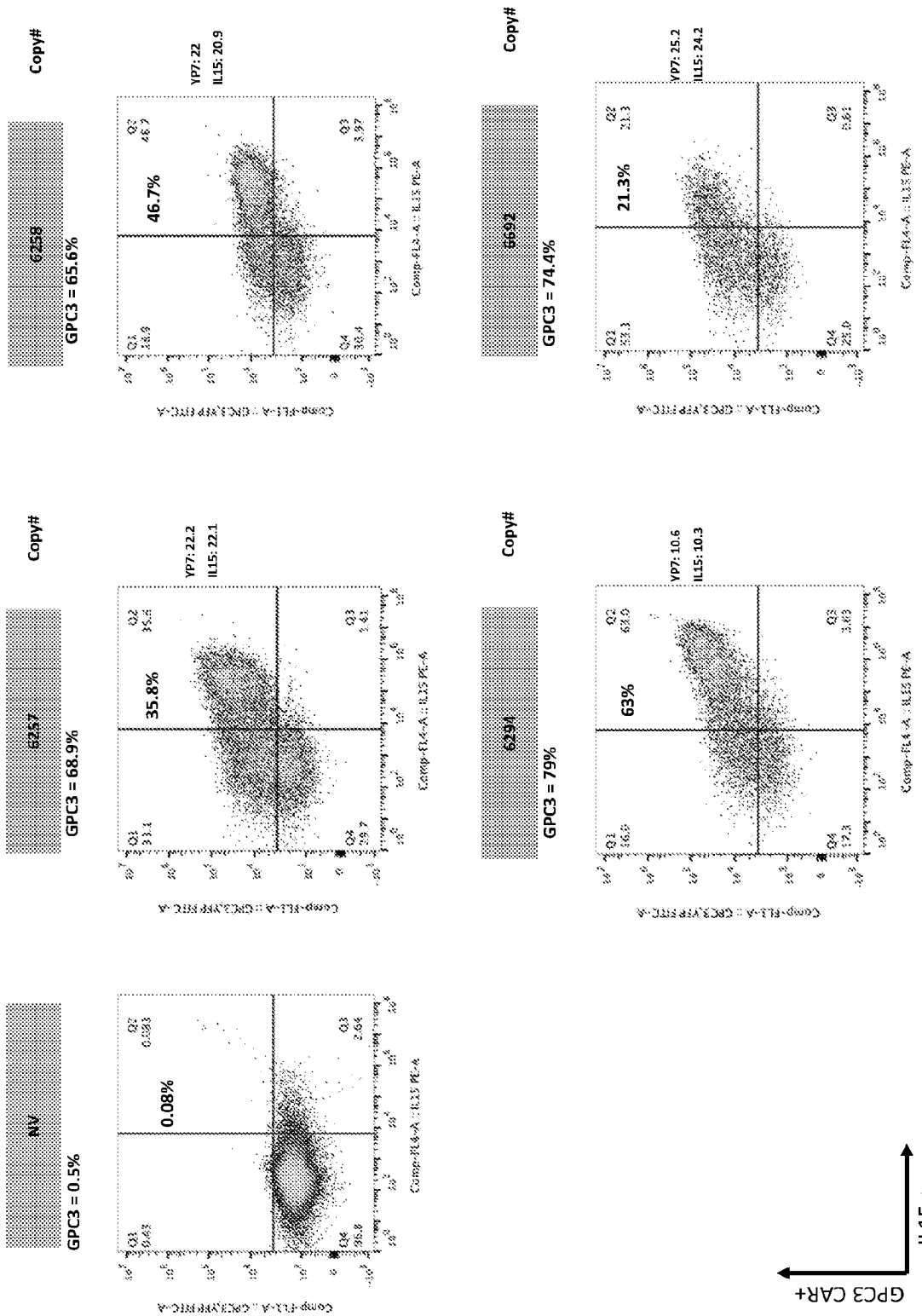

NIV
GPC3 = 0.5%
0.08%
6257
GPC3 = 68.9%
35.8%
YP7: 22.2
IL15: 22.1
6258
GPC3 = 65.6%
46.7%
YP7: 22
IL15: 20.9
6294
GPC3 = 79%
63%
YP7: 10.6
IL15: 10.3
6692
GPC3 = 74.4%
21.3%
YP7: 25.2
IL15: 24.2
Copy#
GPC3 CAR+
IL15 +

FIG. 28A

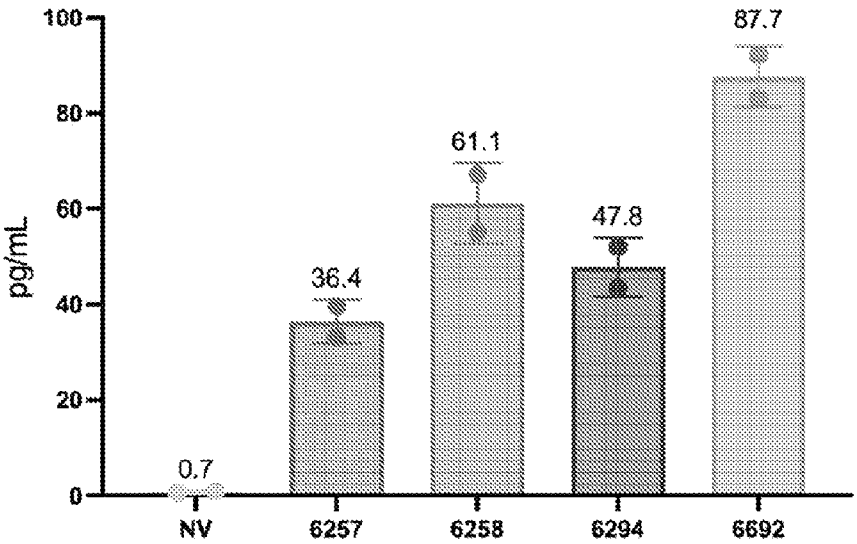


FIG. 28B

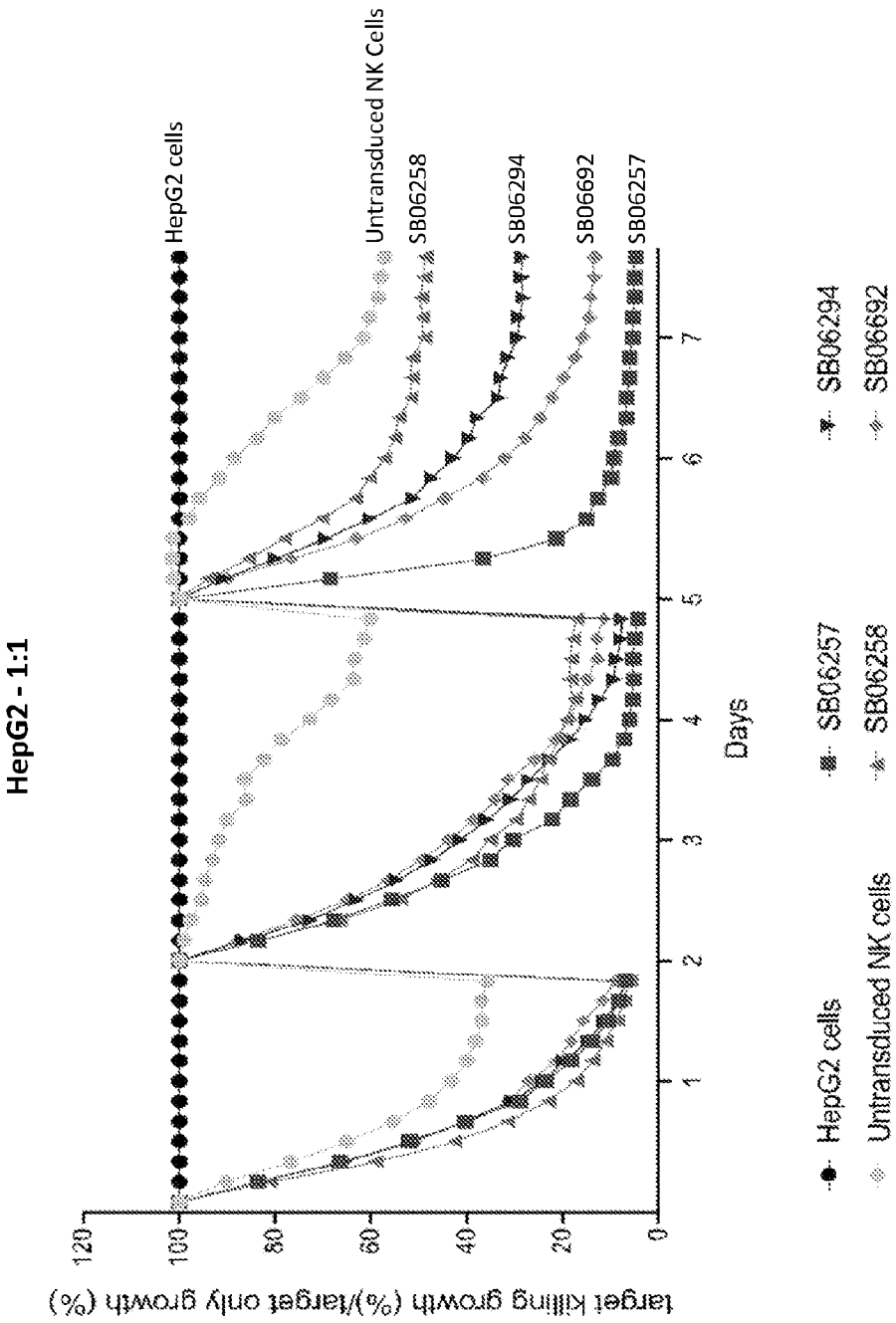

HepG2 - 1:1
target killing growth (%)/target only growth (%)
120
100
80
60
40
20
0
1
2
3
4
5
6
7
Days
HepG2 cells
Untransduced NK Cells
SB06258
SB06294
SB06692
SB06257
HepG2 cells
Untransduced NK cells
SB06257
SB06258
SB06294
SB06692

FIG. 28C

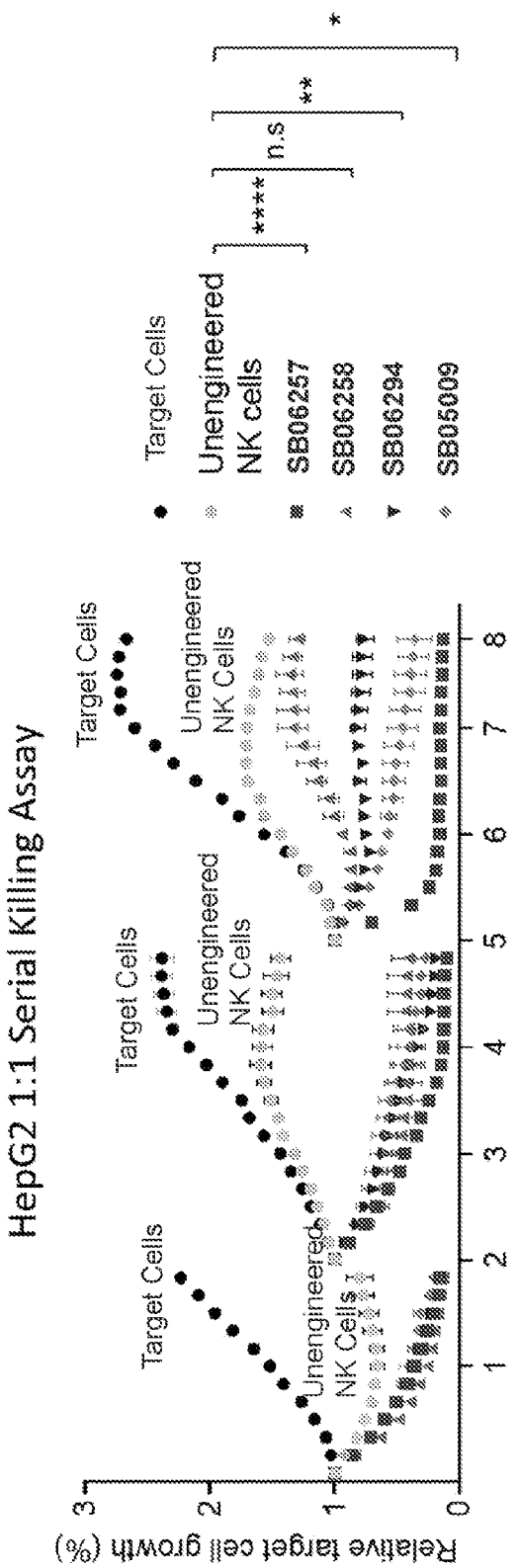

HepG2 1:1 Serial Killing Assay
Relative target cell growth (%)
0
1
2
3
1
2
3
4
5
6
7
8
Target Cells
Unengineered NK Cells
Target Cells
Unengineered NK Cells
Target Cells
Unengineered NK Cells
Target Cells
Unengineered NK Cells
Target Cells
Unengineered NK cells
SB06257
SB06258
SB06294
SB05009
****
n.s
**
*

FIG. 29A

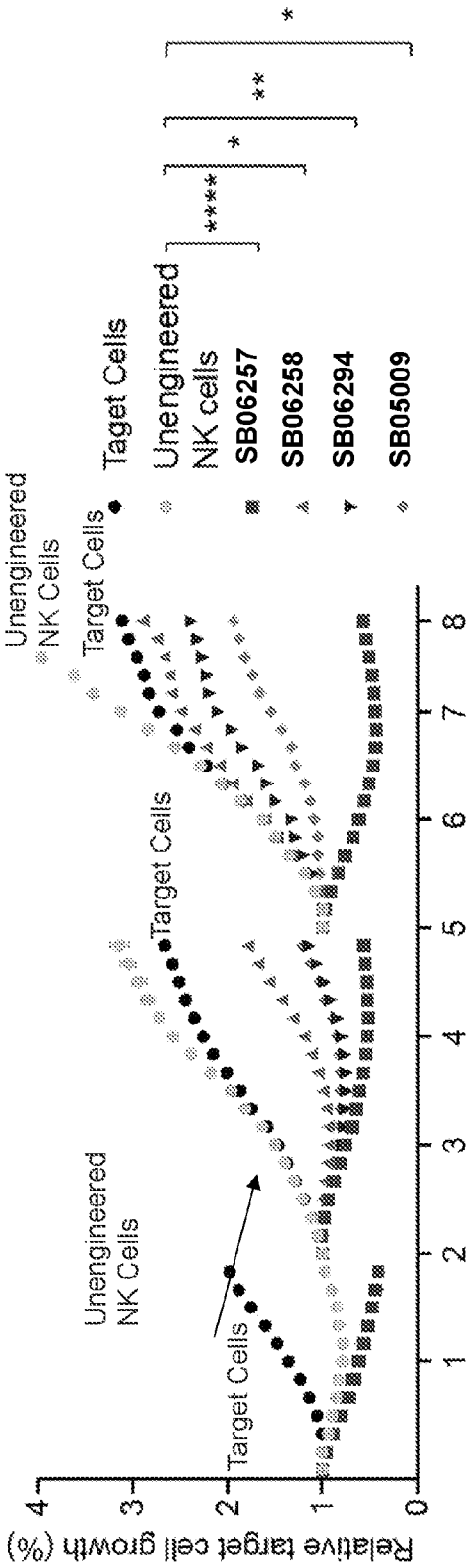

HuH-7 1:1 Serial Killing Assay
Relative target cell growth (%)
0
1
2
3
4
1
2
3
4
5
6
7
8
Unengineered NK Cells
Target Cells
Unengineered NK Cells
Target Cells
Target Cells
Taget Cells
Unengineered NK cells
SB06257
SB06258
SB06294
SB05009
****
*
**
*

FIG. 29B

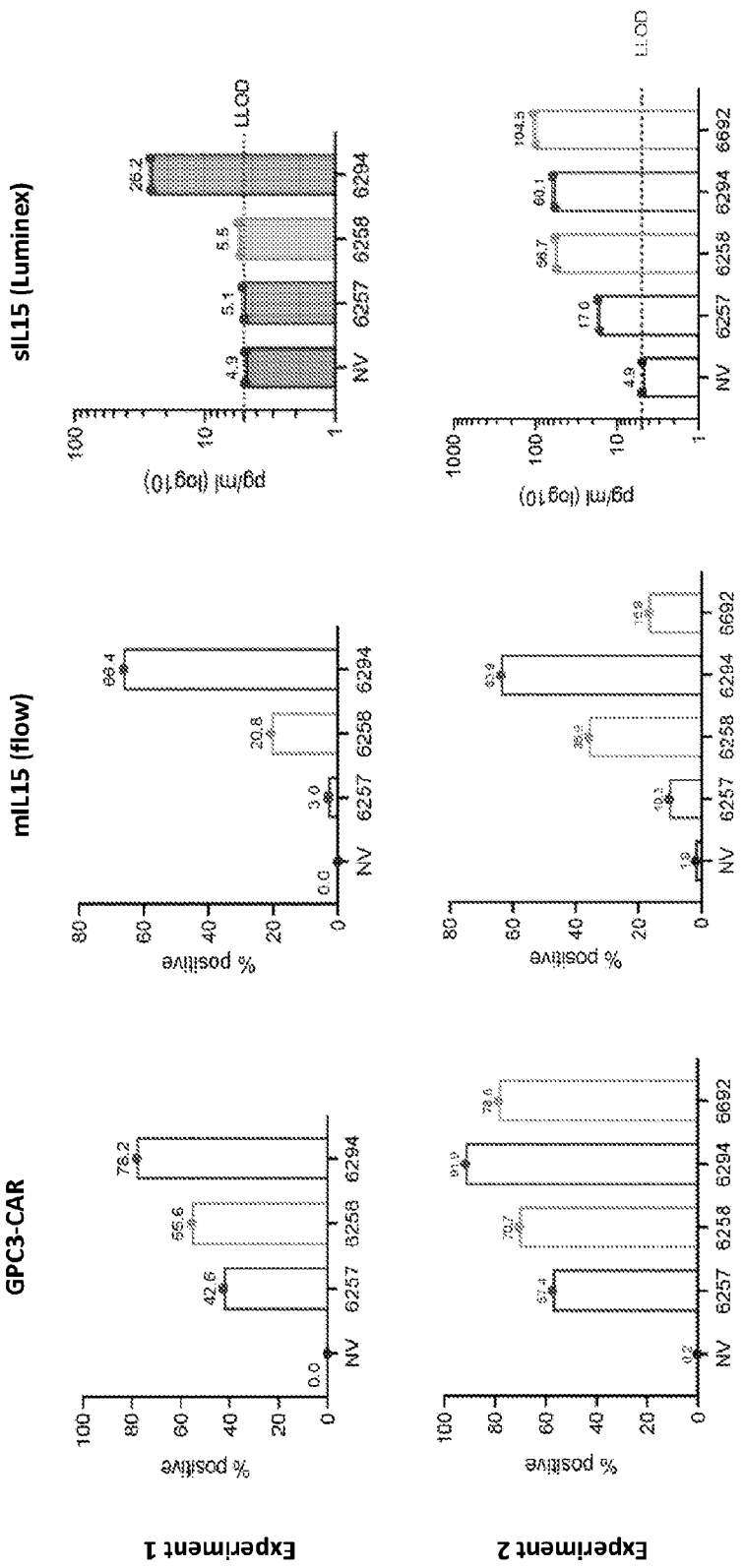

GPC3-CAR
mIL15 (flow)
sIL15 (Luminex)
Experiment 1
Experiment 2
% positive
pg/ml (log10)
NV
6257
6258
6294
6692
LLOD

FIG. 30A

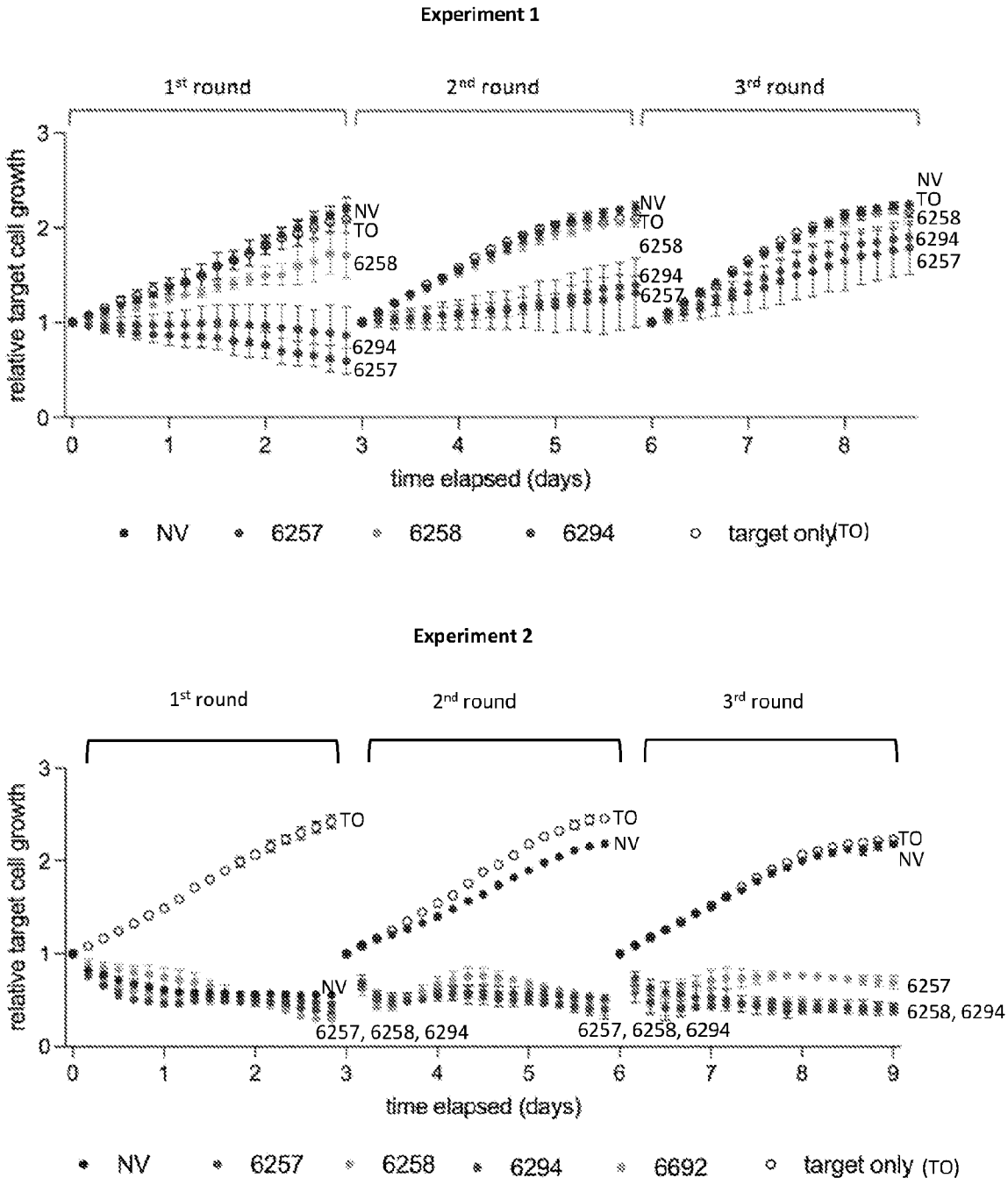

Experiment 1
1st round
2nd round
3rd round
relative target cell growth
time elapsed (days)
NV
TO
6258
6294
6257
NV
6257
6258
6294
target only (TO)
Experiment 2
1st round
2nd round
3rd round
relative target cell growth
time elapsed (days)
TO
NV
6257, 6258, 6294
6257
6258, 6294
NV
6257
6258
6294
6692
target only (TO)

FIG. 30B

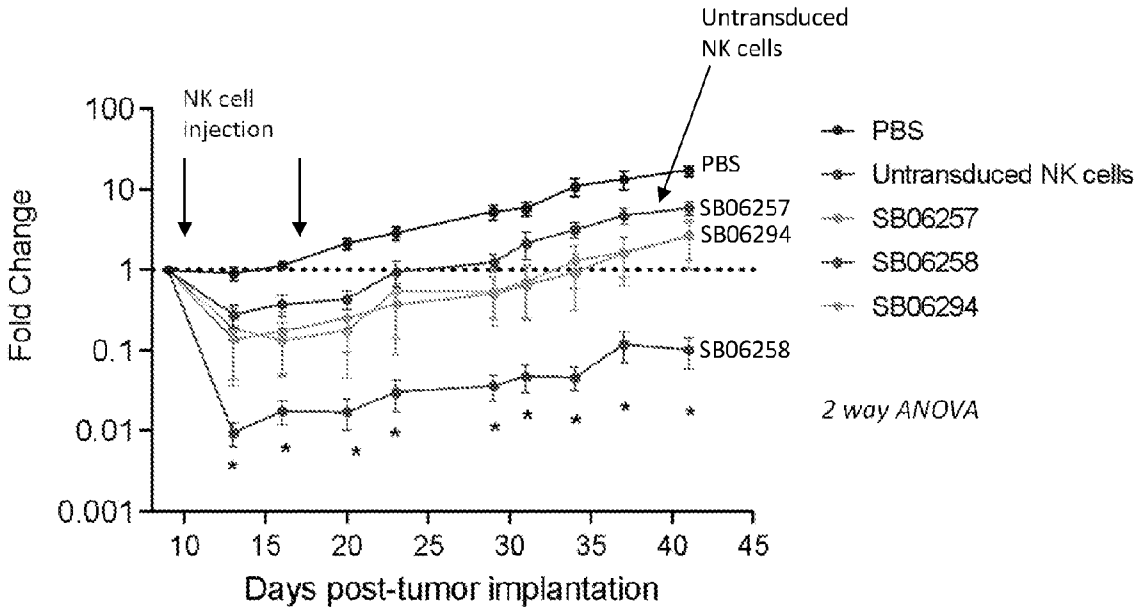

Untransduced
NK cells
NK cell
injection
100
10
1
0.1
0.01
0.001
Fold Change
10
15
20
25
30
35
40
45
Days post-tumor implantation
PBS
SB06257
SB06294
SB06258
PBS
Untransduced NK cells
SB06257
SB06258
SB06294
2 way ANOVA

FIG. 31

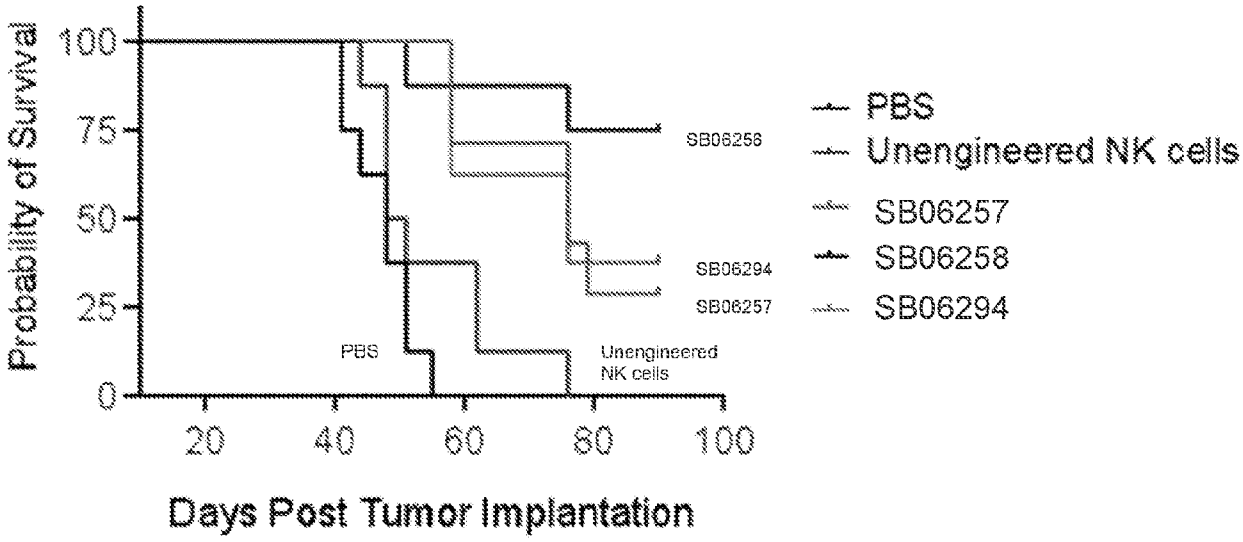
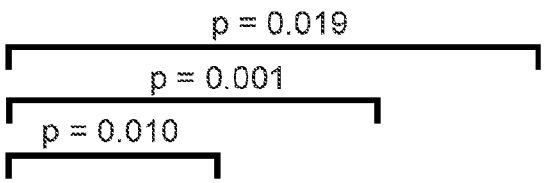


FIG. 32A



	PBS	Unengineered	SB06257	SB06258	SB06294
Median Survival (days)	48	49.5	76	undefined	76

FIG. 32B

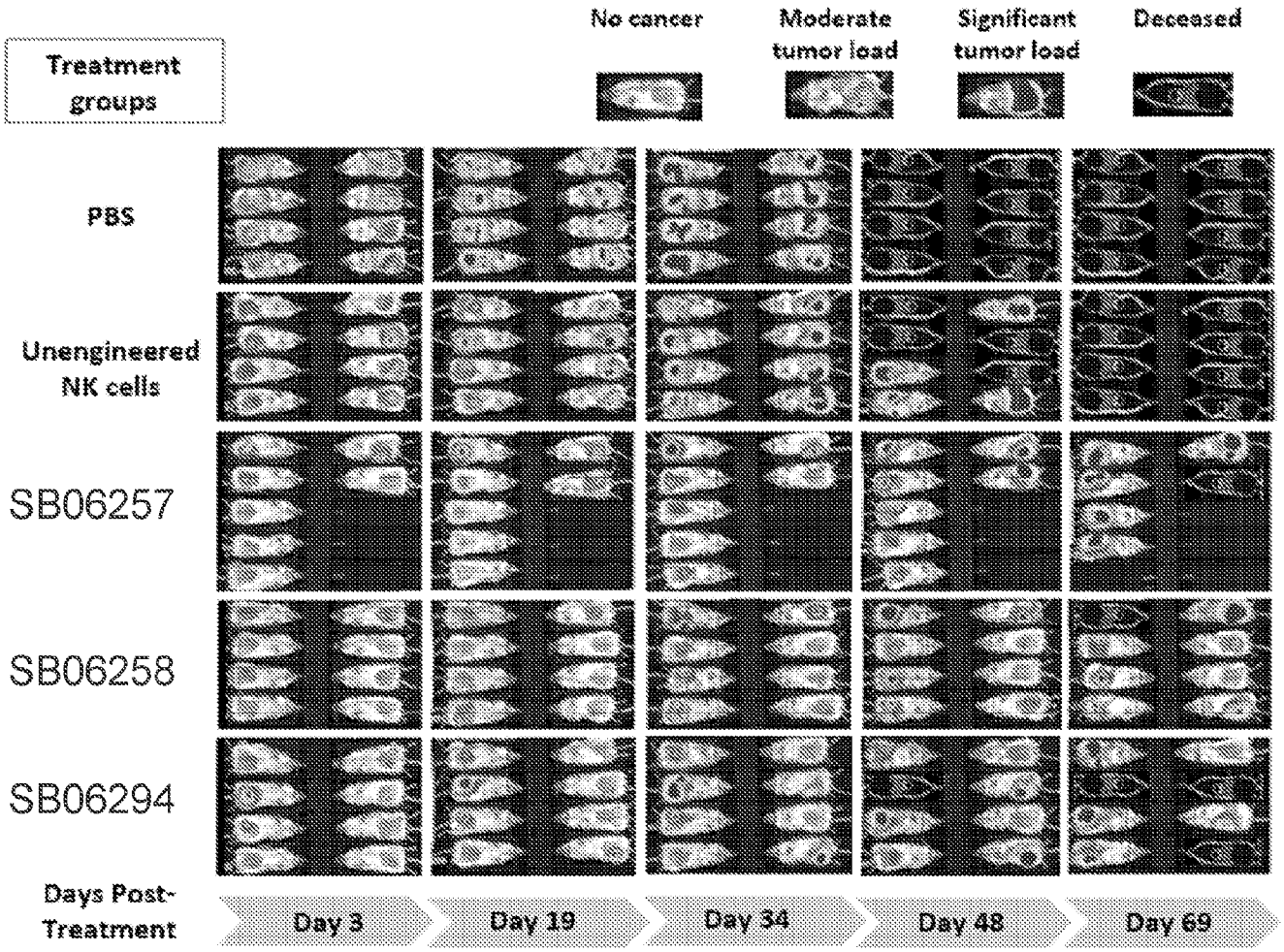

No cancer
Moderate tumor load
Significant tumor load
Deceased
Treatment groups
PBS
Unengineered NK cells
SB06257
SB06258
SB06294
Days Post-Treatment
Day 3
Day 19
Day 34
Day 48
Day 69

FIG. 33

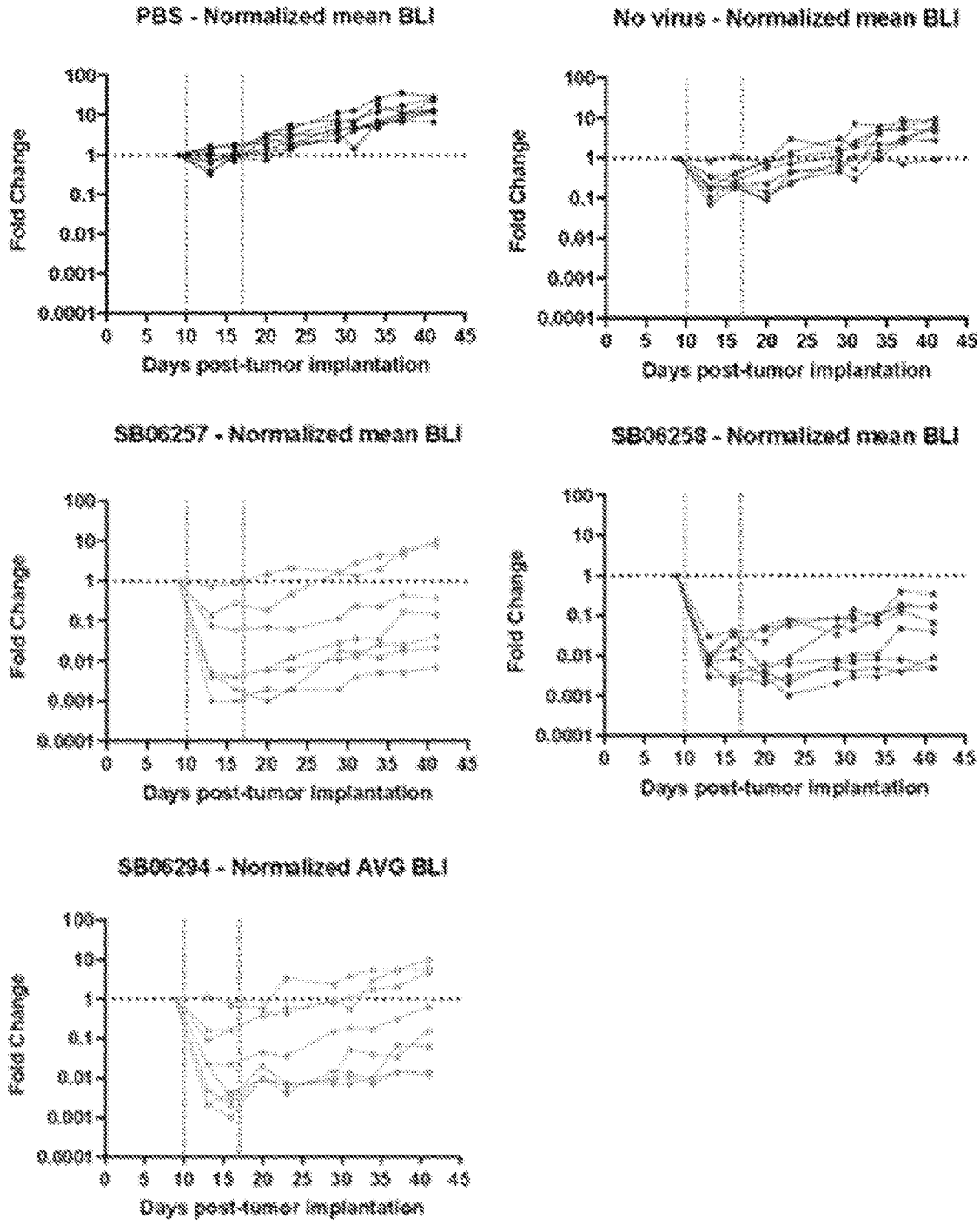

PBS - Normalized mean BLI
No virus - Normalized mean BLI
SB06257 - Normalized mean BLI
SB06258 - Normalized mean BLI
SB06294 - Normalized AVG BLI
Fold Change
Days post-tumor implantation
100
10
1
0.1
0.01
0.001
0.0001
0 5 10 15 20 25 30 35 40 45

FIG. 34

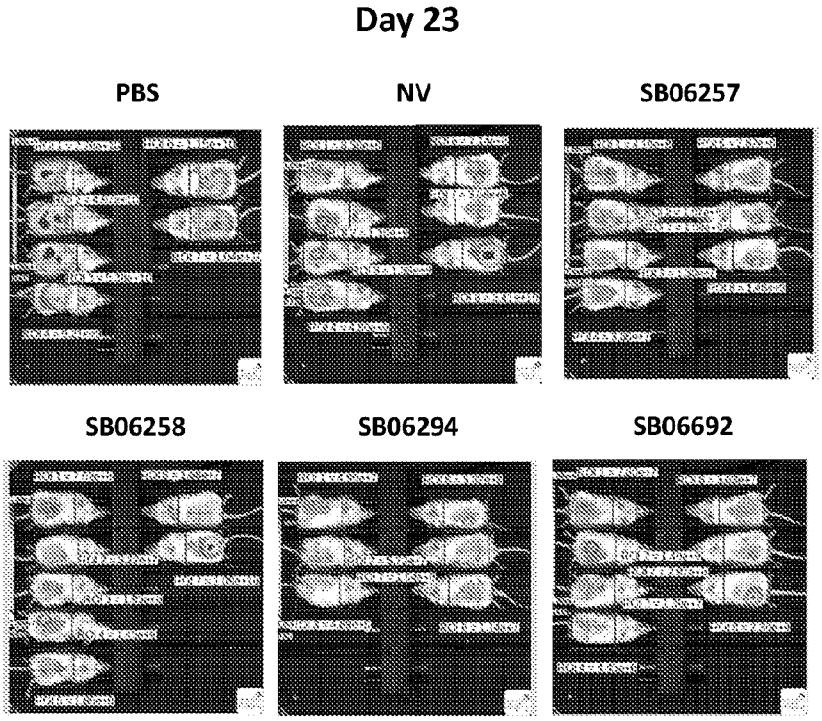


FIG. 35A

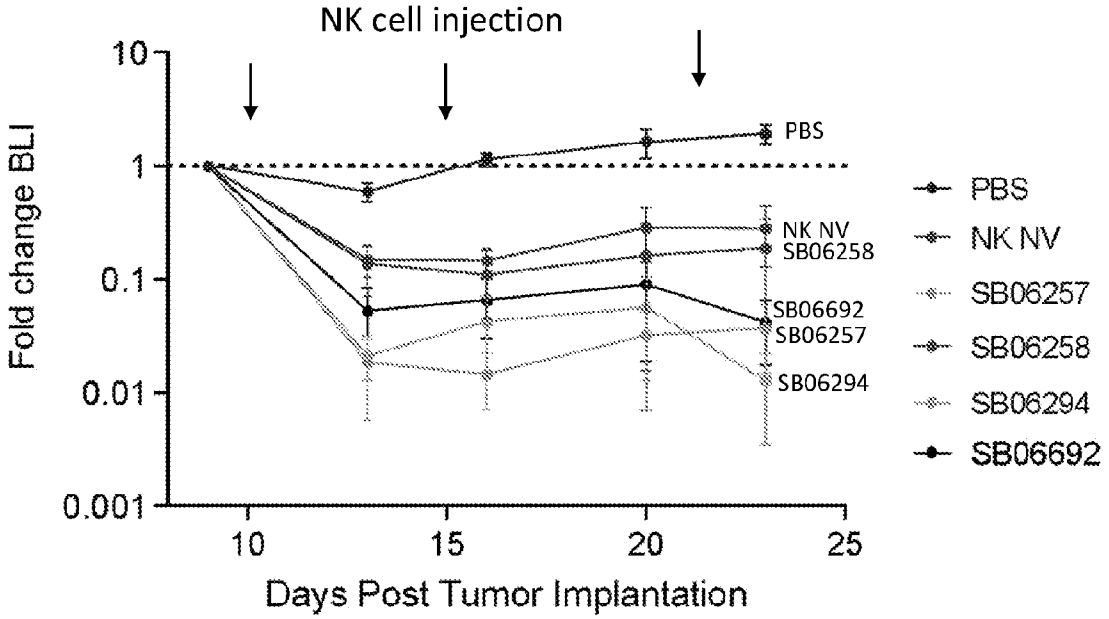


FIG. 35B

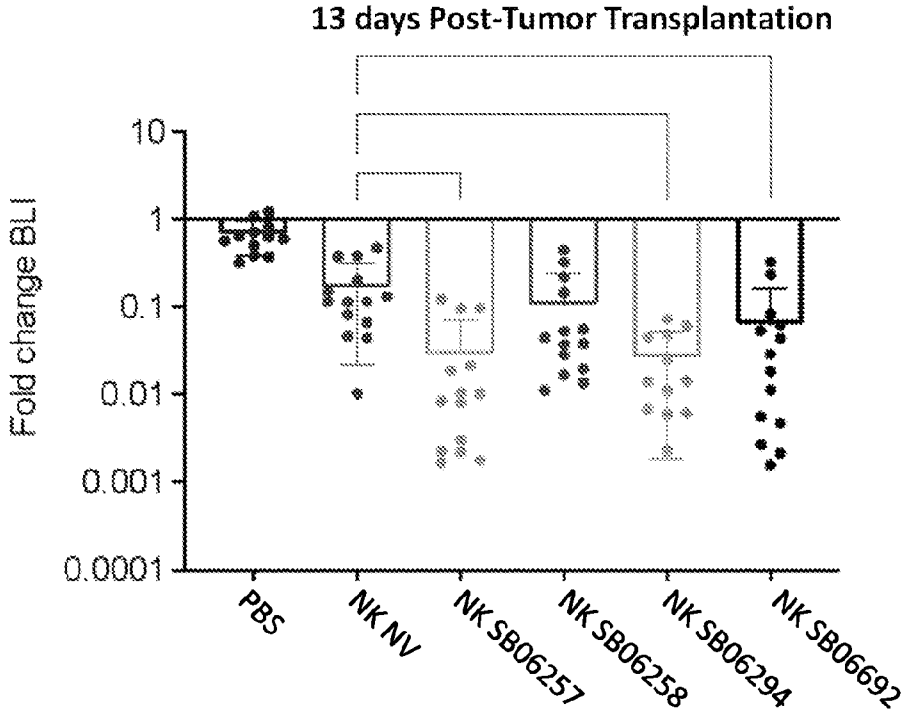


FIG. 36A

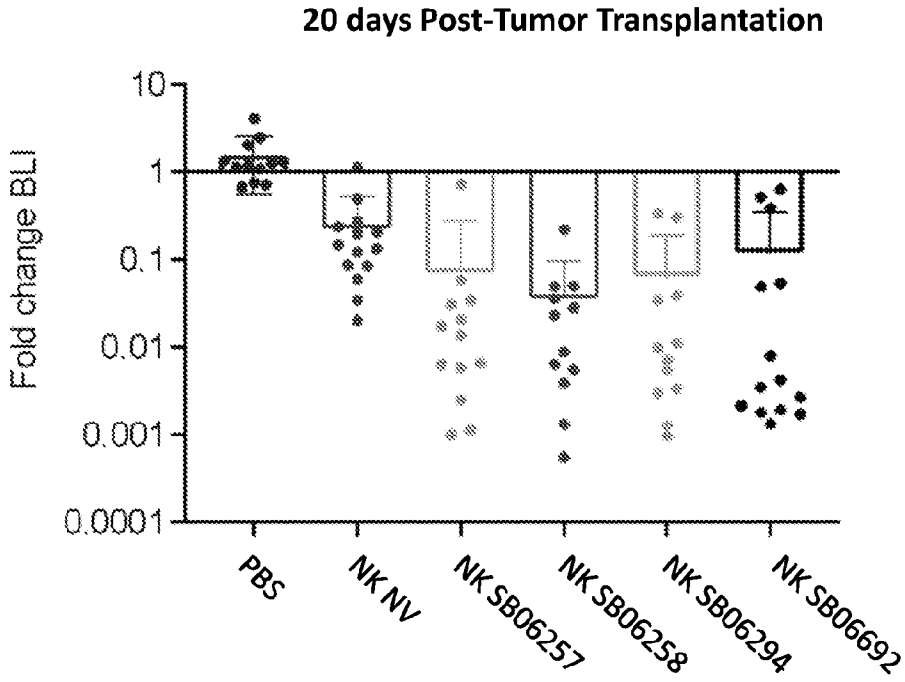


FIG. 36B

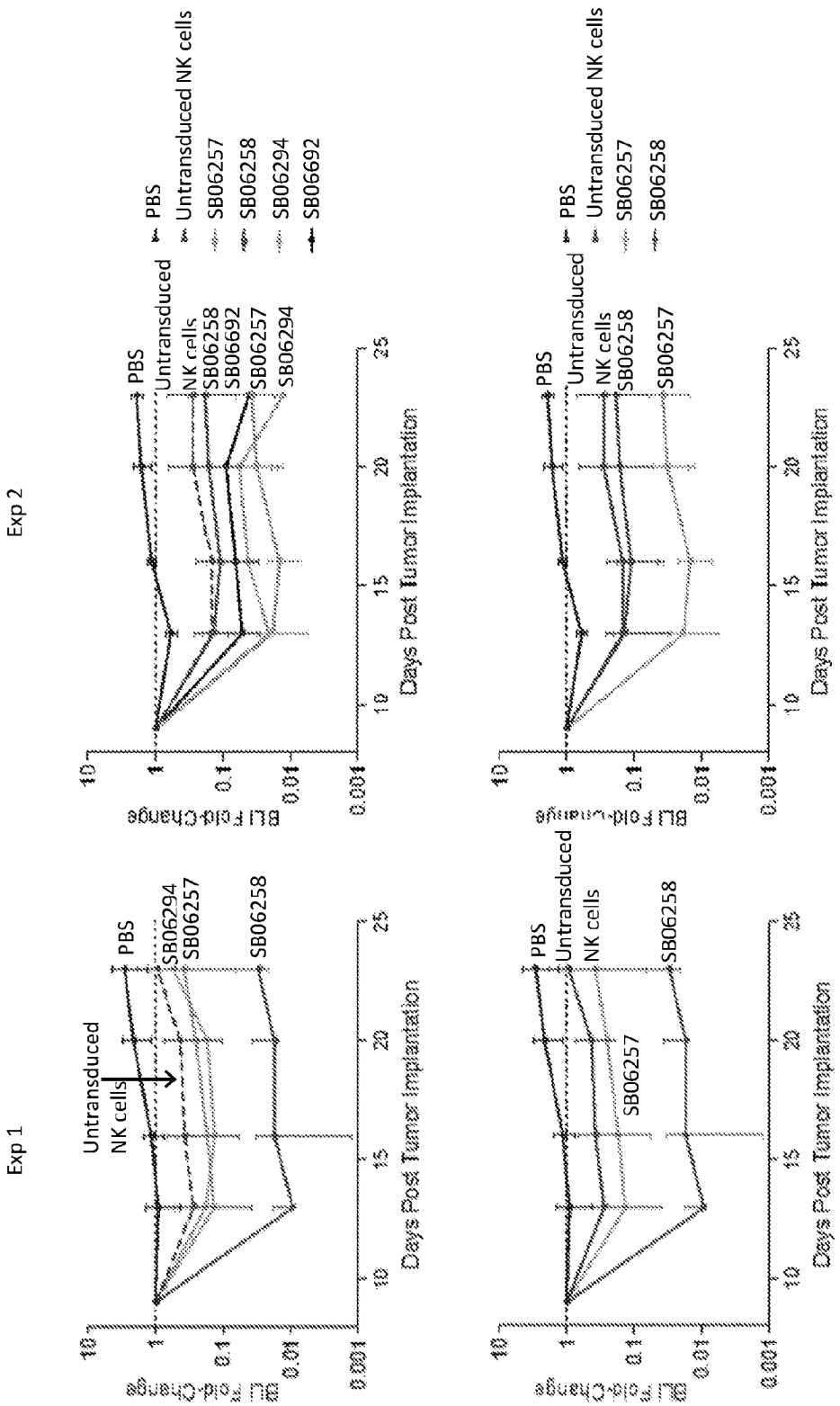

Exp 1
Exp 2
Untransduced NK cells
PBS
SB06294
SB06257
SB06258
Untransduced NK cells
SB06692
BLI Fold-Change
Days Post Tumor Implantation

FIG. 37A

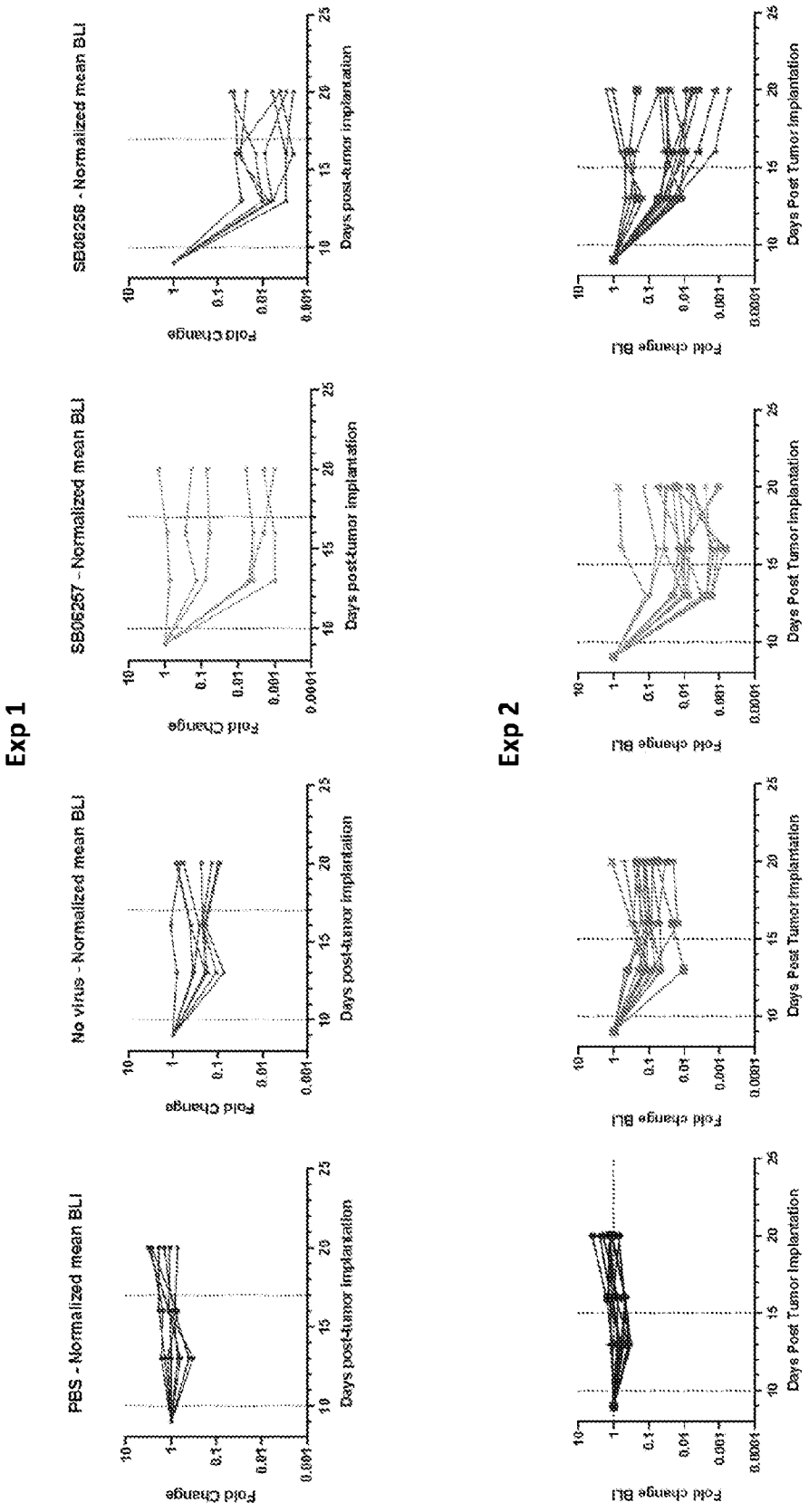

Exp 1
Exp 2
PBS - Normalized mean BLI
No virus - Normalized mean BLI
SB06257 - Normalized mean BLI
SB06258 - Normalized mean BLI
Fold Change
Days post-tumor implantation
Fold change BLI
Days Post Tumor Implantation

FIG. 37B

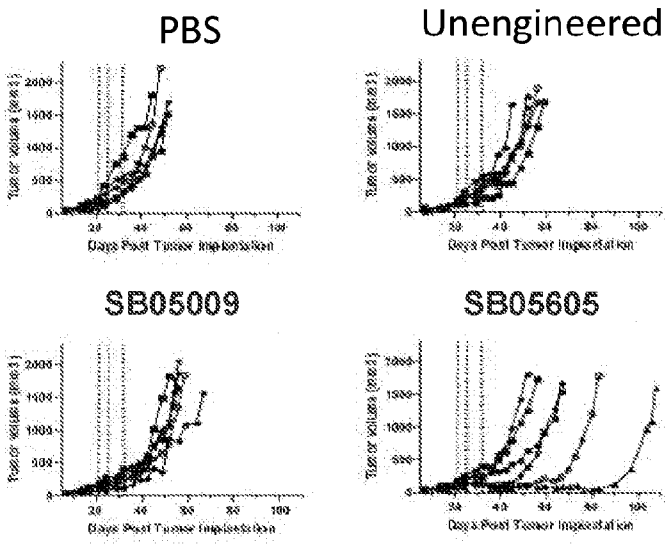
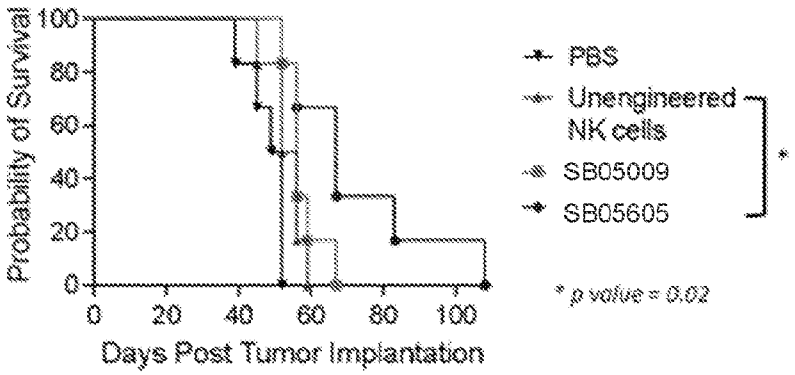


FIG. 38A



	PBS	Unengineered	SB05009	SB05605
Median Survival (days)	50.5	54	56	67

FIG. 38B

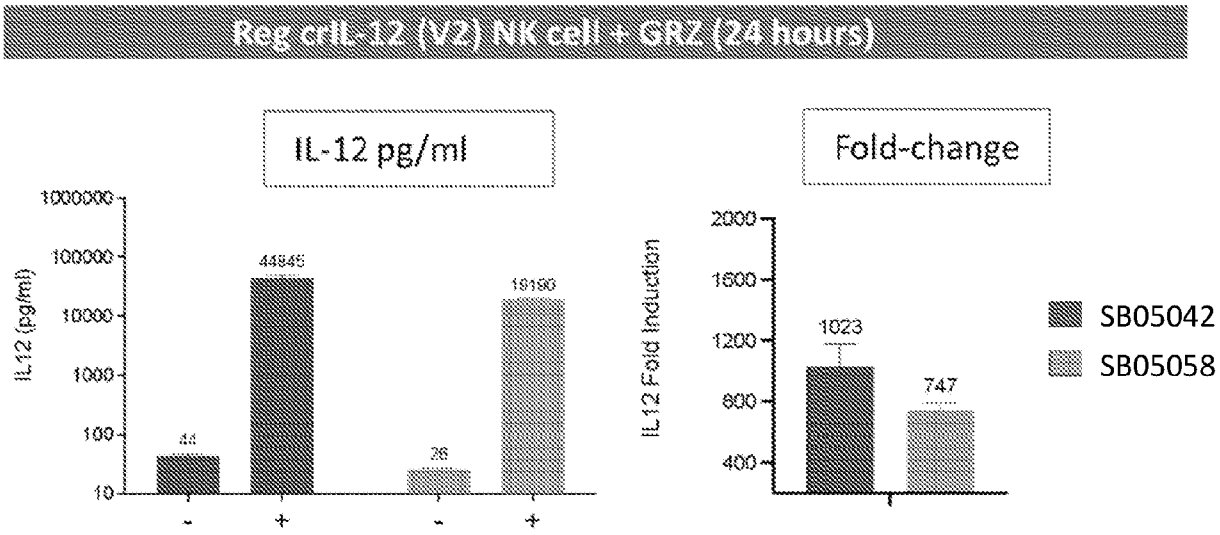


FIG. 39A

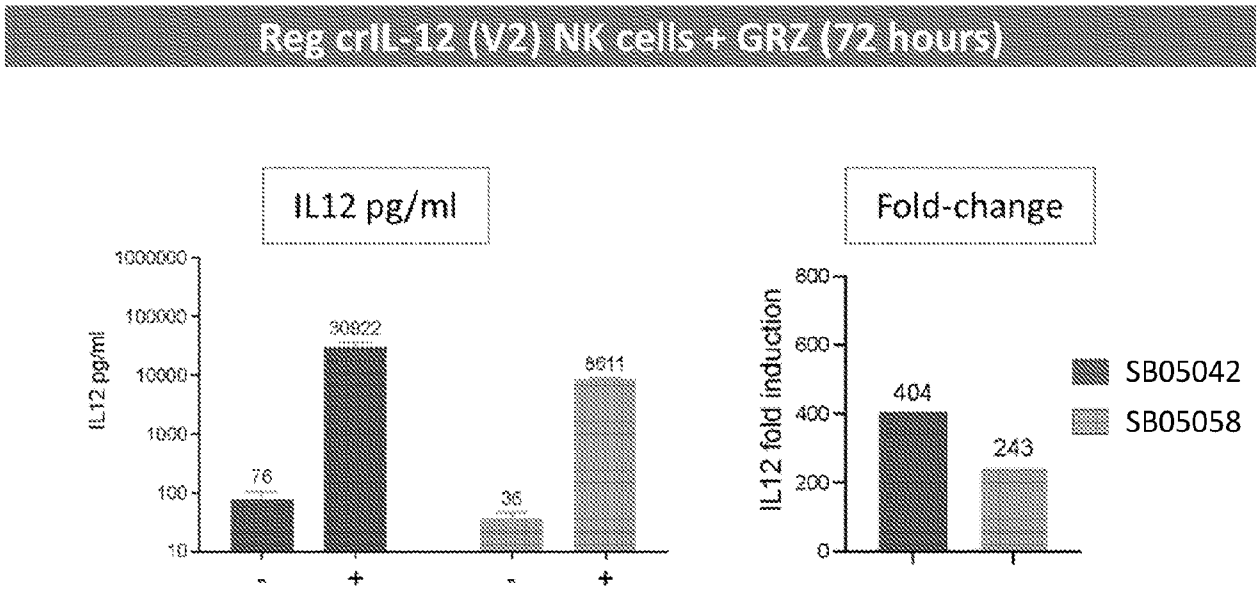
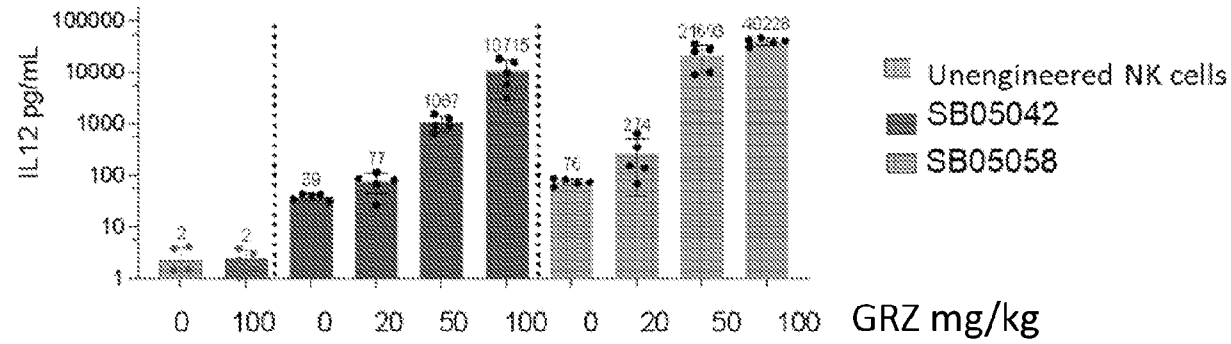
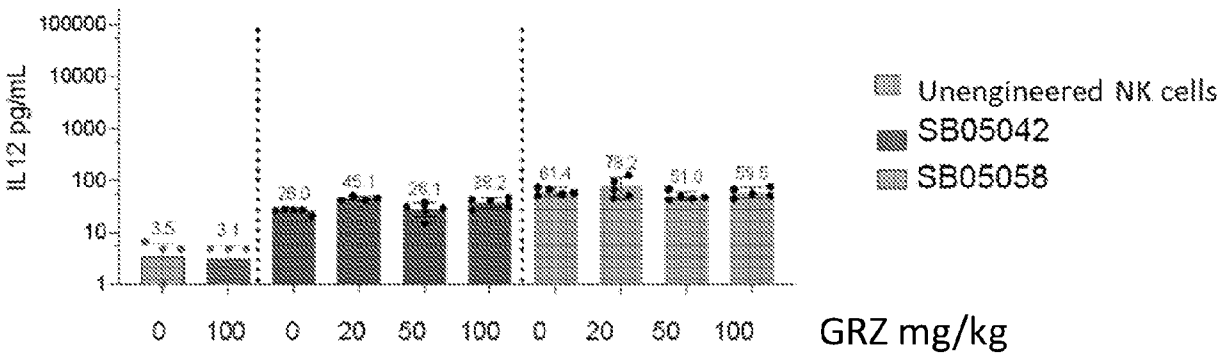


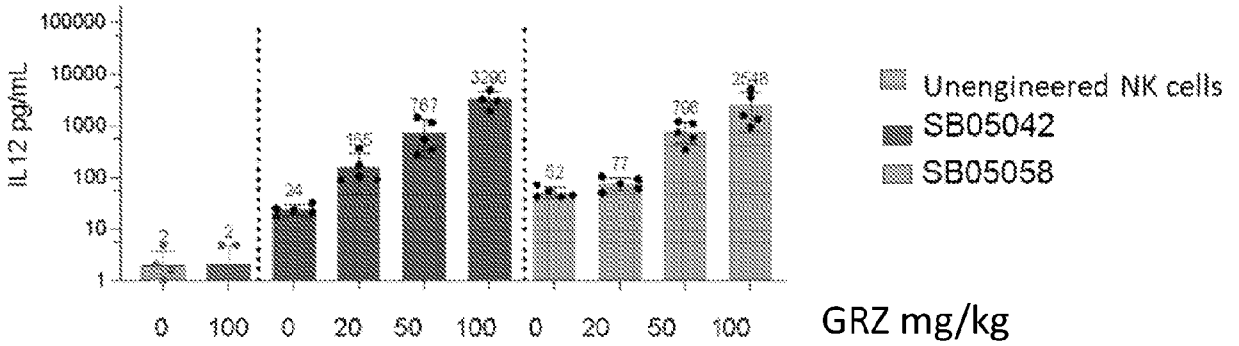
FIG. 39B



Day 2 (ON)



Day 9 (OFF)



Day 11 (ON)

FIG. 40

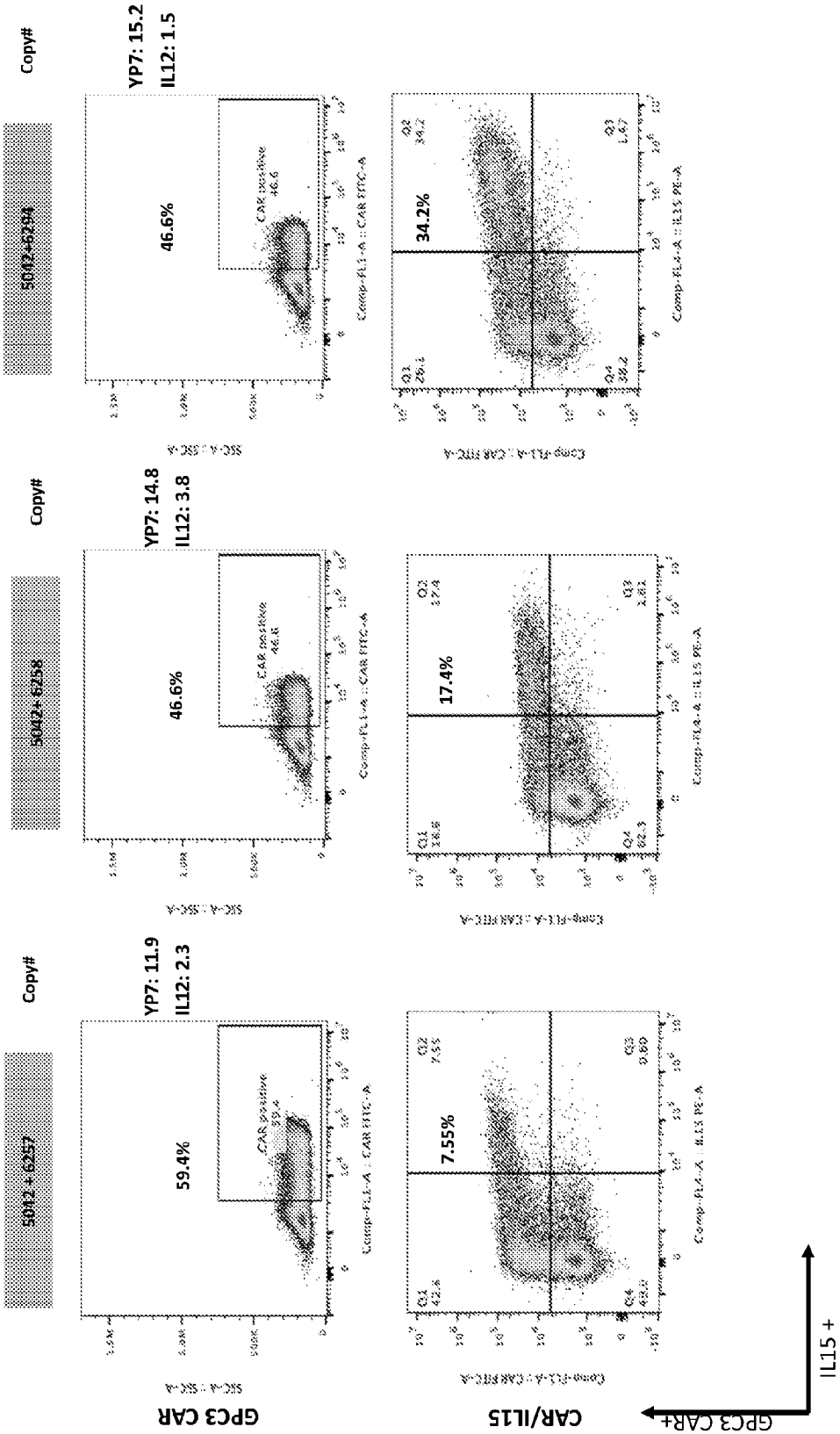

Copy#
5012 + 6257
YP7: 11.9
IL12: 2.3
59.4%
7.55%
Copy#
5042+ 6258
YP7: 14.8
IL12: 3.8
46.6%
17.4%
Copy#
5042+6294
YP7: 15.2
IL12: 1.5
46.6%
34.2%
GPC3 CAR
CAR/IL15
GPC3 CAR+
IL15 +

FIG. 41

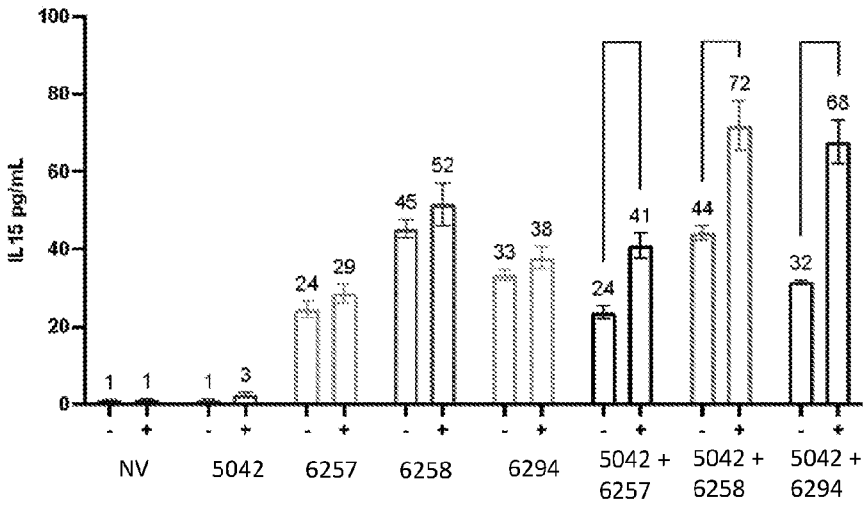


FIG. 42A

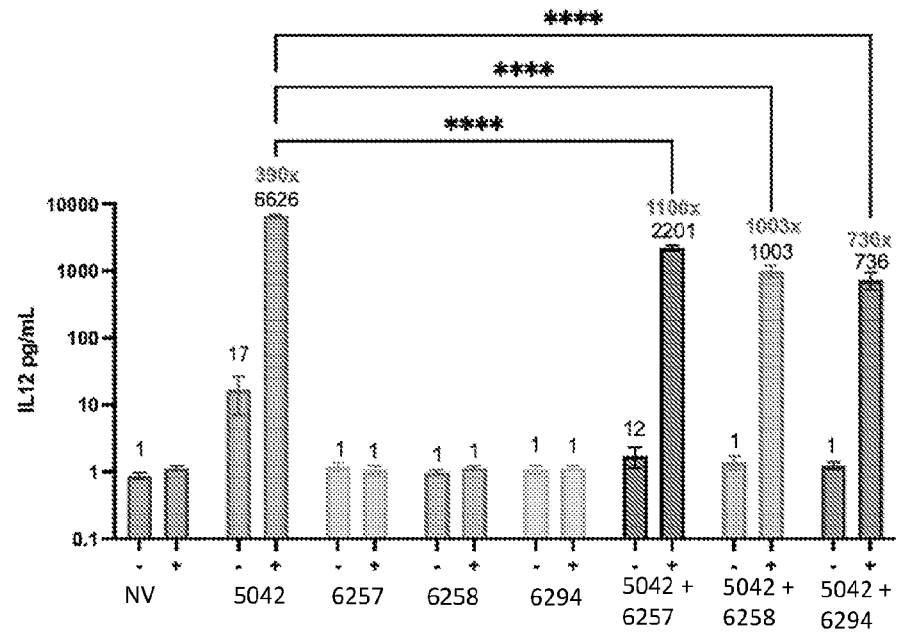


FIG. 42B

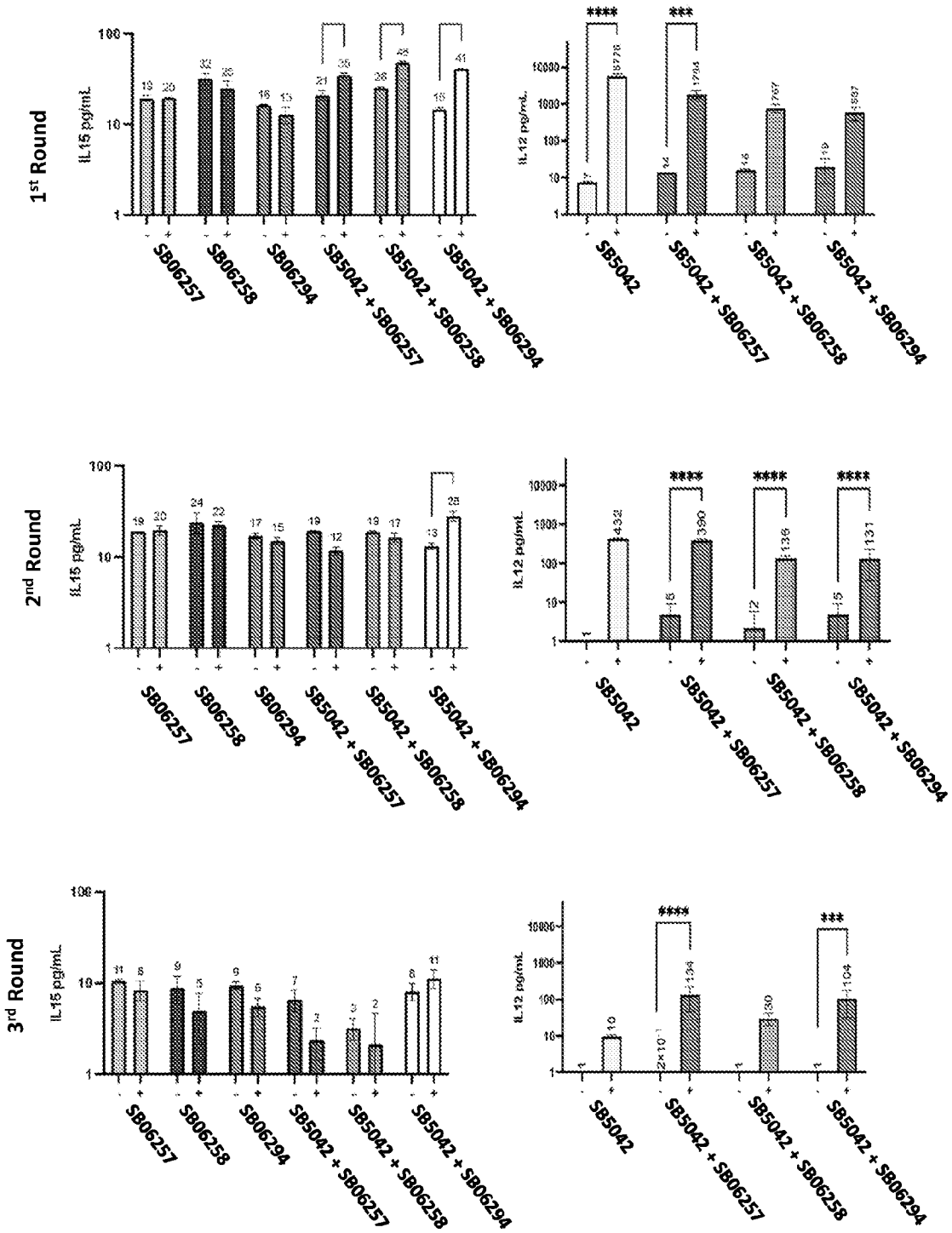

1st Round
2nd Round
3rd Round
IL15 pg/mL
IL12 pg/mL
SB06257
SB06258
SB06294
SB5042 + SB06257
SB5042 + SB06258
SB5042 + SB06294
SB5042
****
***

FIG. 43

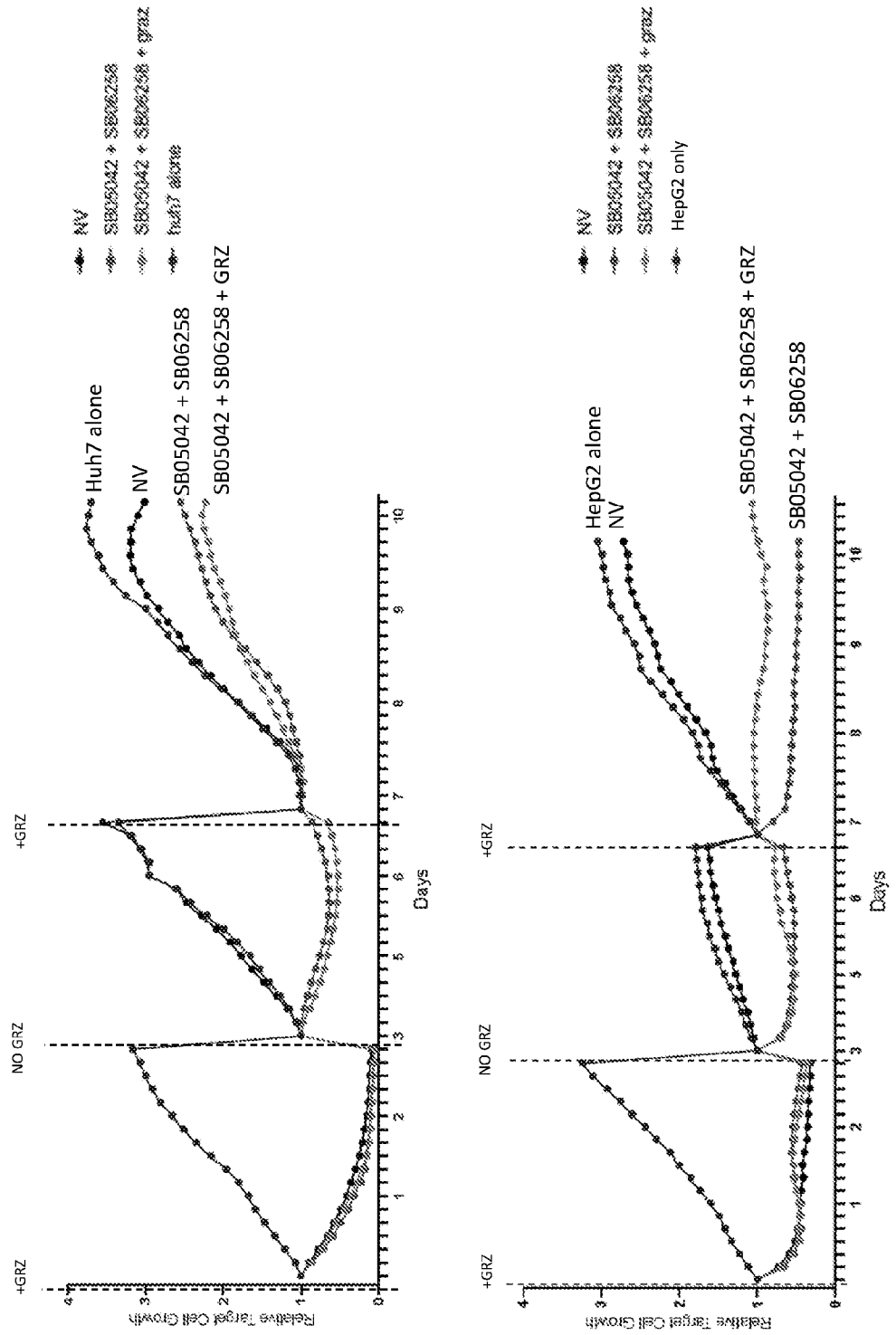

NV
SB05042 + SB06258
SB05042 + SB06258 + grnz
huh7 alone
Huh7 alone
NV
SB05042 + SB06258
SB05042 + SB06258 + GRZ
+GRZ
NO GRZ
+GRZ
Relative Target Cell Growth
Days
NV
SB05042 + SB06258
SB05042 + SB06258 + grnz
HepG2 only
HepG2 alone
NV
SB05042 + SB06258 + GRZ
SB05042 + SB06258
+GRZ
NO GRZ
+GRZ
Relative Target Cell Growth
Days

FIG. 44A

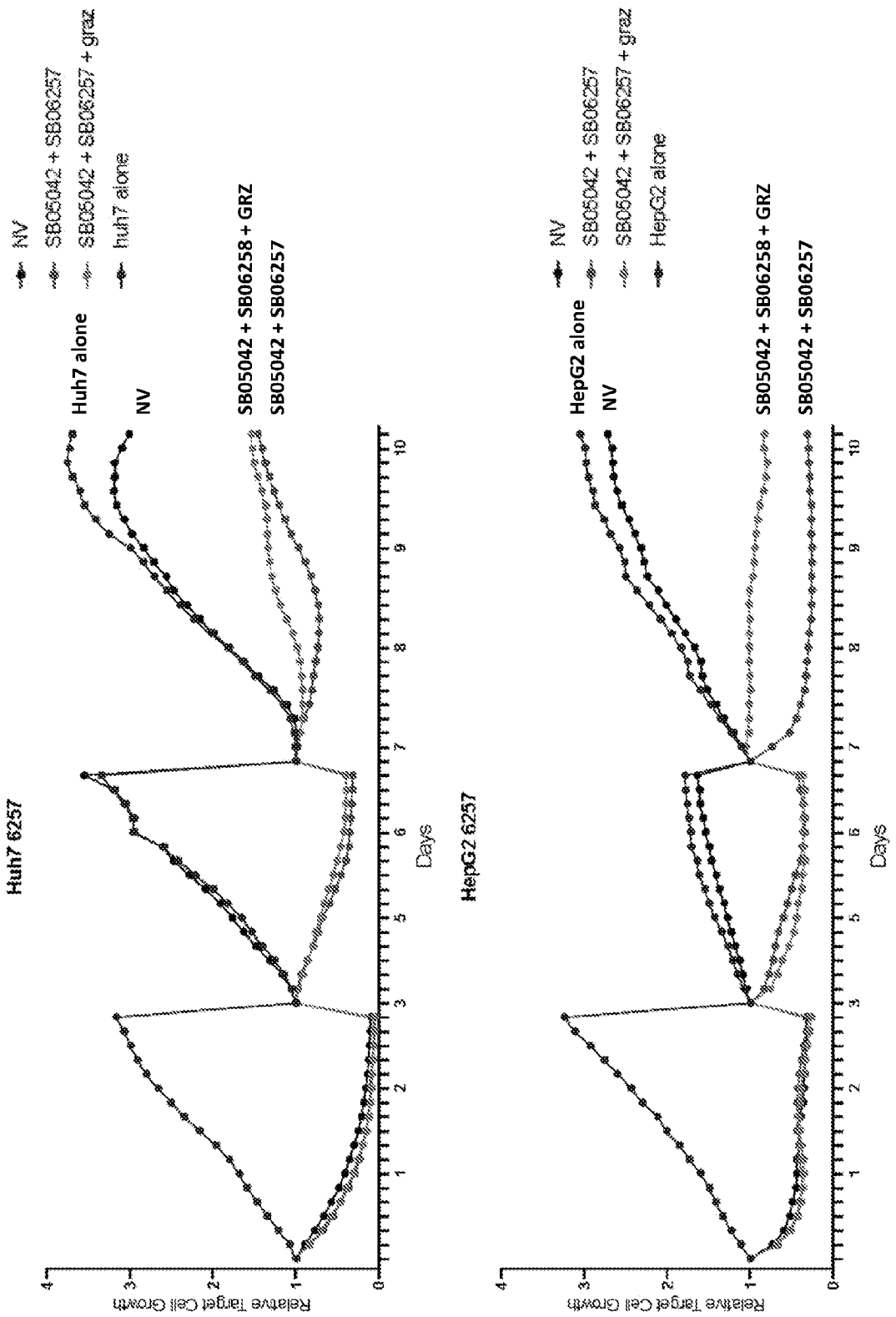

Huh7 6257
HepG2 6257
NV
SB05042 + SB06257
SB05042 + SB06257 + graz
huh7 alone
HepG2 alone
Huh7 alone
NV
SB05042 + SB06258 + GRZ
SB05042 + SB06257
Relative Target Cell Growth
Days

FIG. 44B

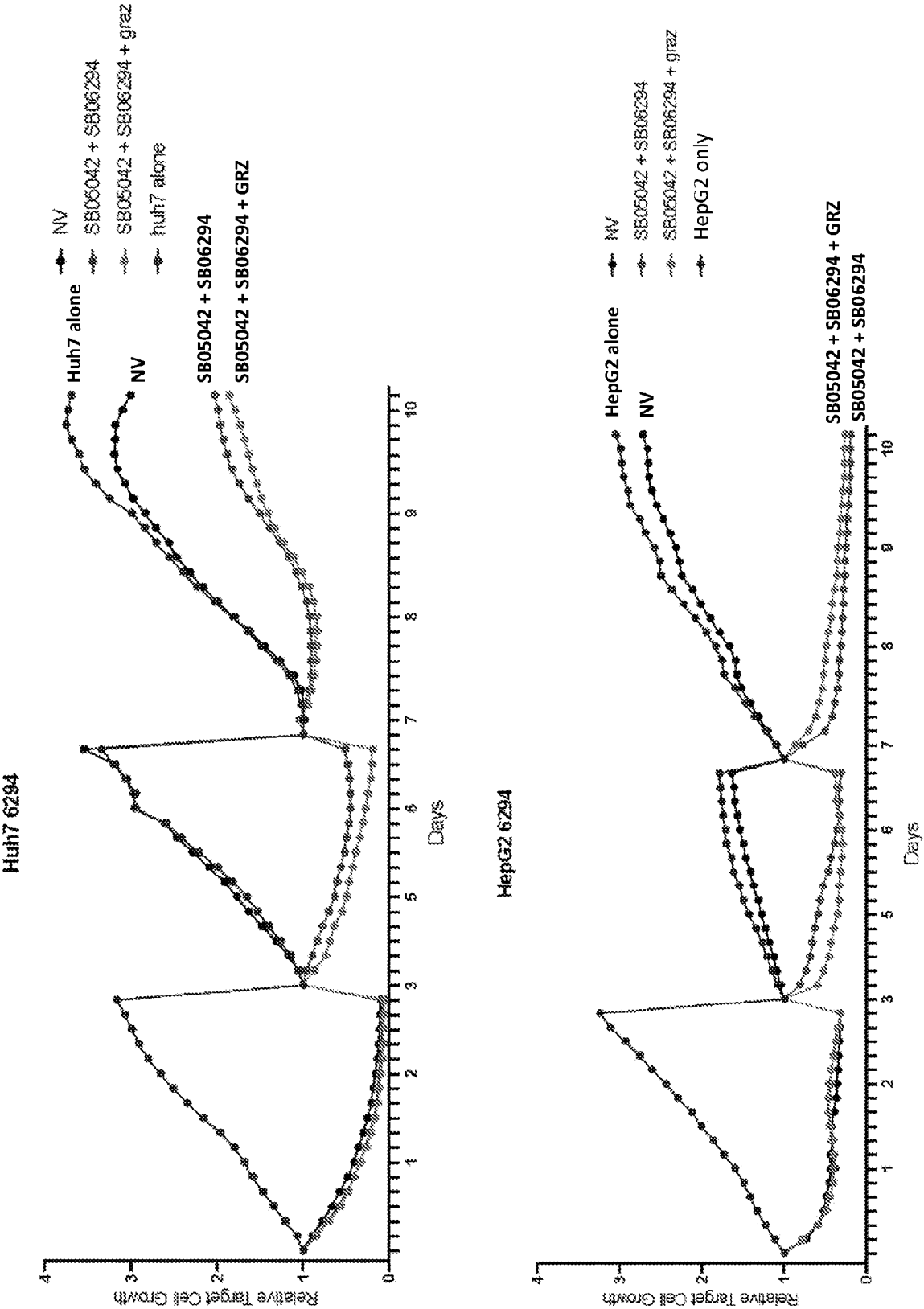

Huh7 6294
Relative Target Cell Growth
Days
NV
SB05042 + SB06294
SB05042 + SB06294 + graz
huh7 alone
Huh7 alone
NV
SB05042 + SB06294
SB05042 + SB06294 + GRZ
HepG2 6294
Relative Target Cell Growth
Days
NV
SB05042 + SB06294
SB05042 + SB06294 + graz
HepG2 only
HepG2 alone
NV
SB05042 + SB06294 + GRZ
SB05042 + SB06294

FIG. 44C

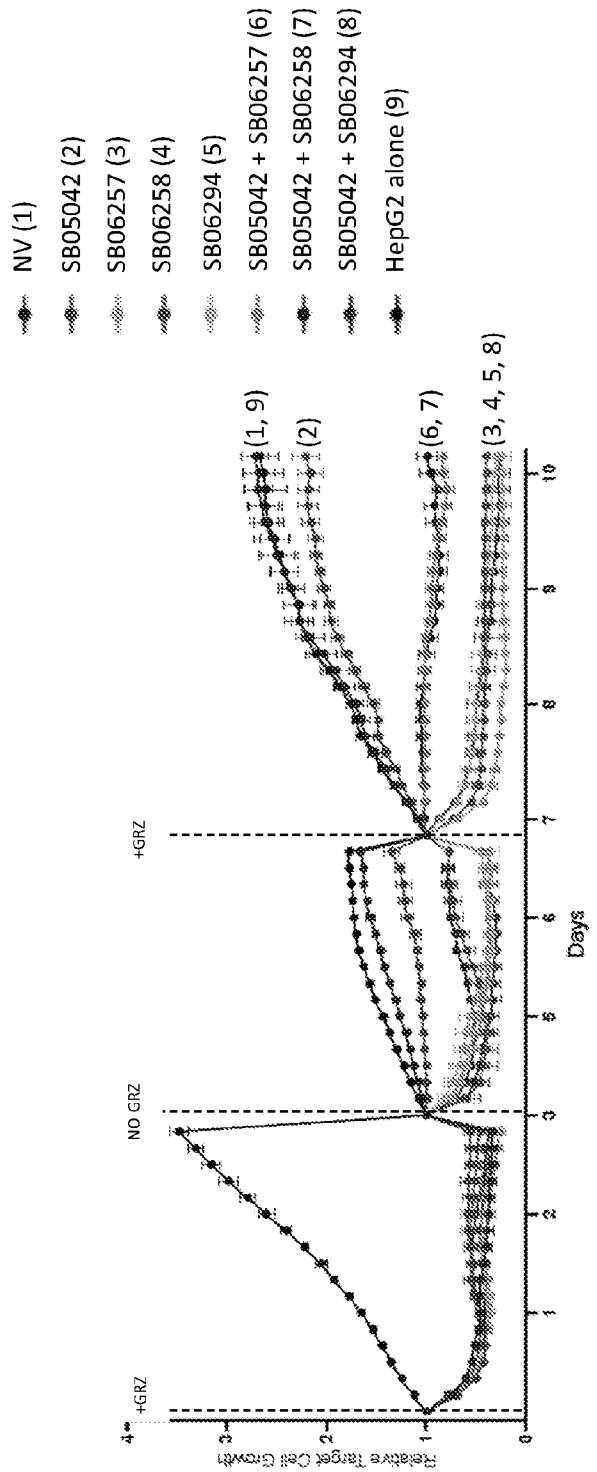

NV (1)
SB05042 (2)
SB06257 (3)
SB06258 (4)
SB06294 (5)
SB05042 + SB06257 (6)
SB05042 + SB06258 (7)
SB05042 + SB06294 (8)
HepG2 alone (9)
+GRZ
NO GRZ
+GRZ
(1, 9)
(2)
(6, 7)
(3, 4, 5, 8)
Days
Relative Target Cell Growth

FIG. 44D

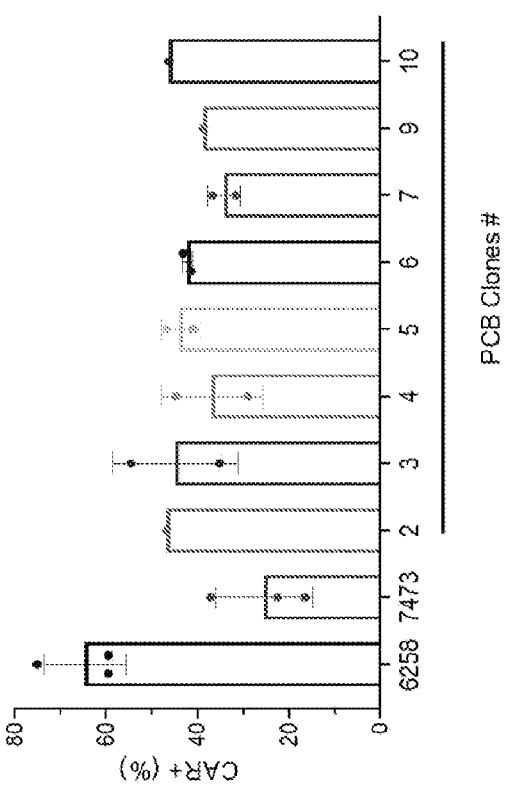


FIG. 45B

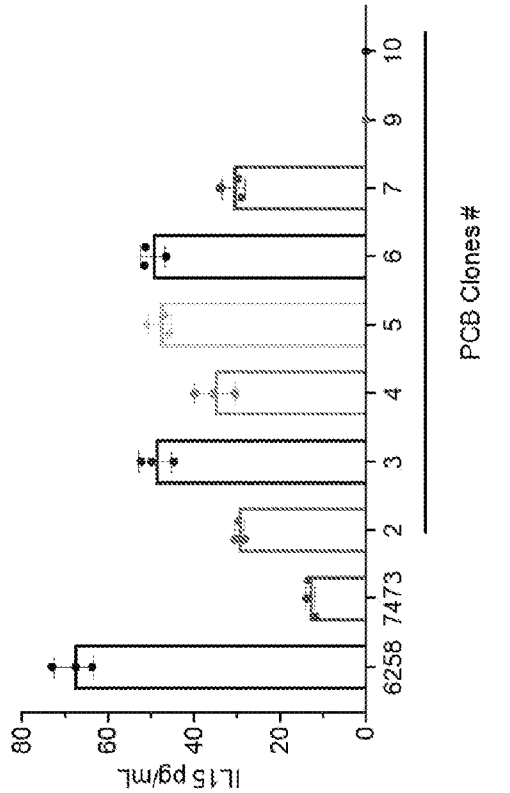


FIG. 45D

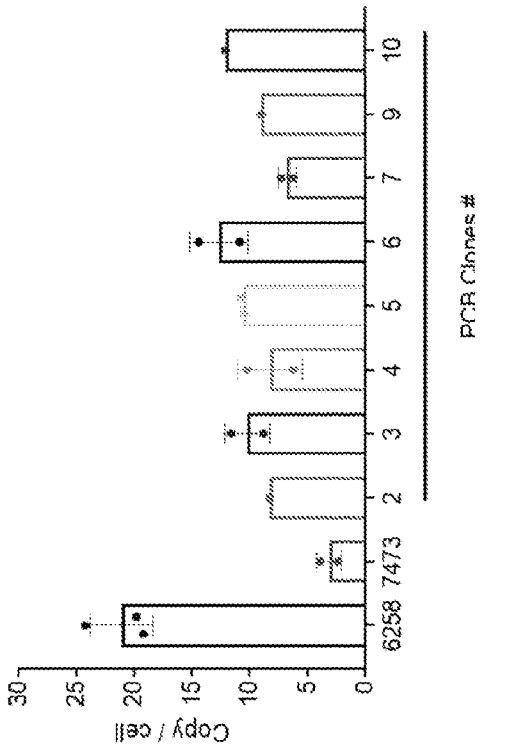


FIG. 45A

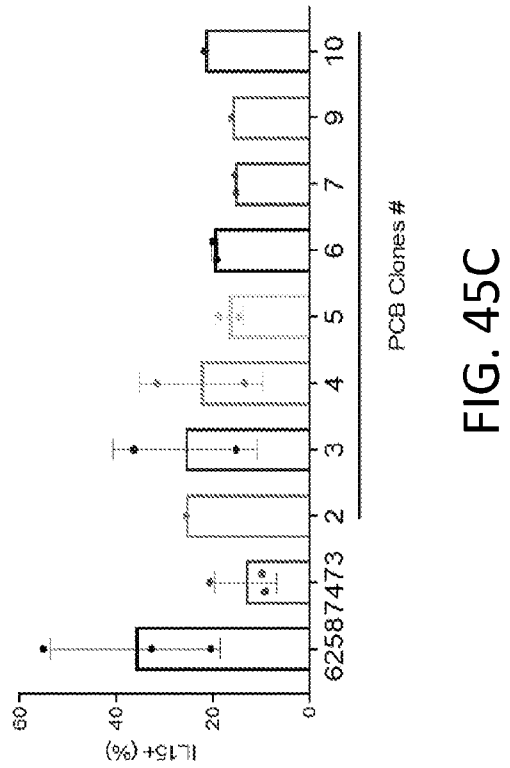


FIG. 45C

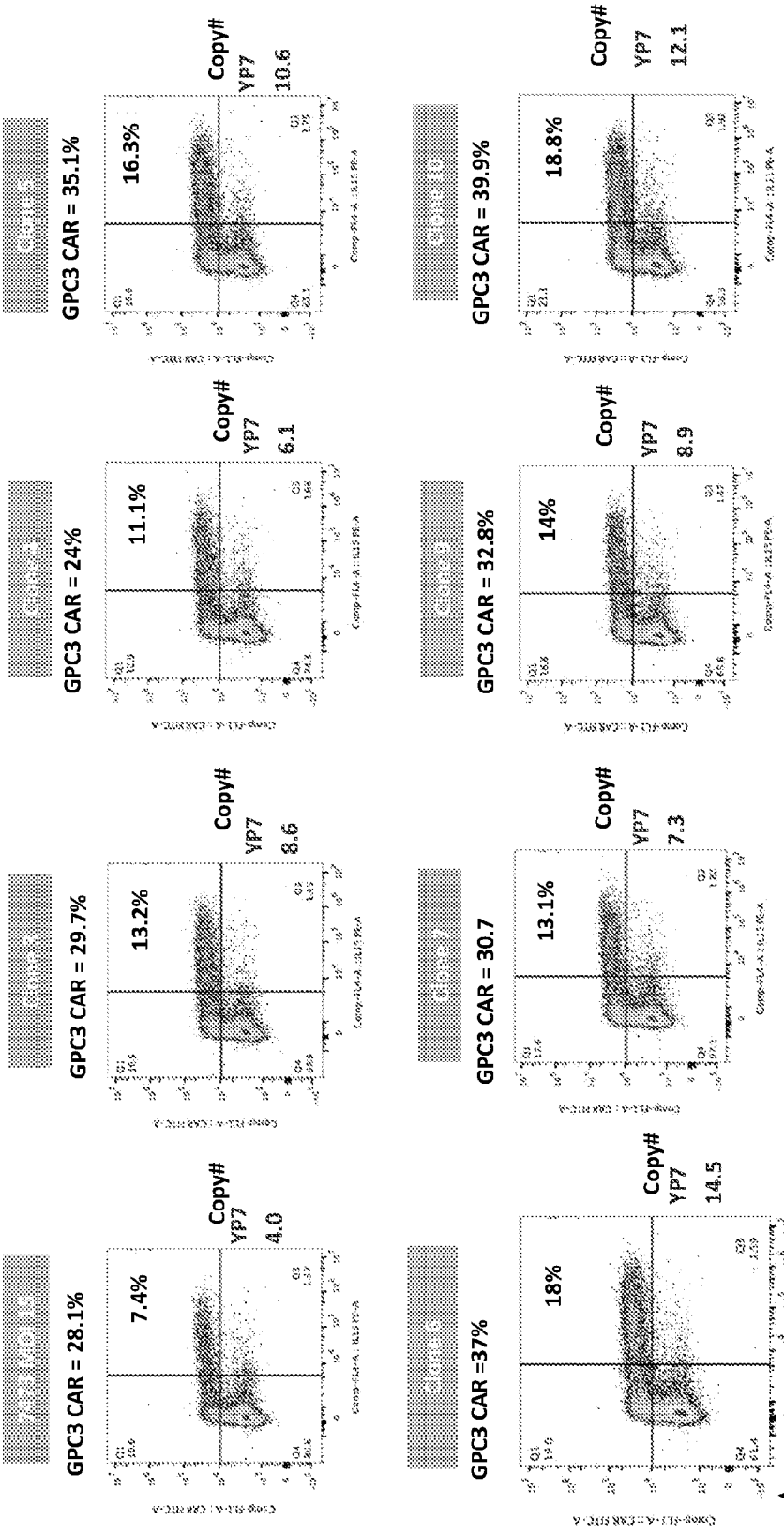

GPC3 CAR = 28.1%
7.4%
Copy# YP7 4.0
GPC3 CAR = 29.7%
13.2%
Copy# YP7 8.6
GPC3 CAR = 24%
11.1%
Copy# YP7 6.1
GPC3 CAR = 35.1%
16.3%
Copy# YP7 10.6
GPC3 CAR =37%
18%
Copy# YP7 14.5
GPC3 CAR = 30.7
13.1%
Copy# YP7 7.3
GPC3 CAR = 32.8%
14%
Copy# YP7 8.9
GPC3 CAR = 39.9%
18.8%
Copy# YP7 12.1
IL15 +
GPC3 CAR+

FIG. 46A

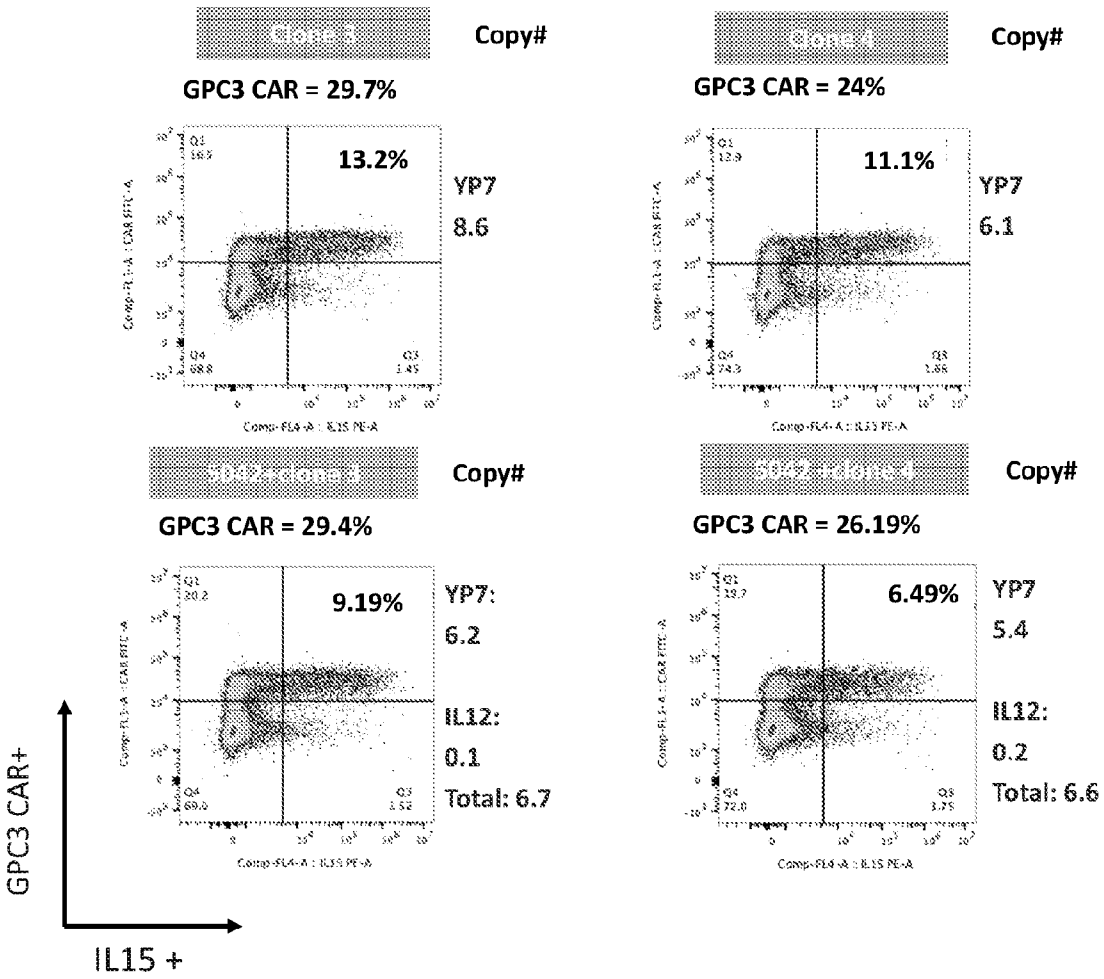

clone 3
Copy#
GPC3 CAR = 29.7%
13.2%
YP7
8.6
clone 4
Copy#
GPC3 CAR = 24%
11.1%
YP7
6.1
sb2-clone 3
Copy#
GPC3 CAR = 29.4%
9.19%
YP7:
6.2
IL12:
0.1
Total: 6.7
sb2-clone 4
Copy#
GPC3 CAR = 26.19%
6.49%
YP7
5.4
IL12:
0.2
Total: 6.6
GPC3 CAR+
IL15 +

FIG. 46B

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2022/033893

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - INV. - A61K 35/17; C12N 5/078 (2022.01)
ADD. - C07K 19/00; C12N 5/10; C12N 15/11; C12N 15/12; C12N 15/85 (2022.01)

CPC - INV. - A61K 35/17; C12N 5/0634 (2022.08)

ADD. - C07K 19/00; C12N 15/11; C12N 15/85; C12N 5/10 (2022.08)

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2020/0171093 A1 (SENTI BIOSCIENCES INC.) 04 June 2020 (04.06.2020) entire document	9, 11
---		---
Y		1-3, 10
Y	WO 2019/014390 A1 (REFUGE BIOTECHNOLOGIES INC.) 17 January 2019 (17.01.2019) entire document	1-3, 10
A	US 2021/0094994 A1 (BAYLOR COLLEGE OF MEDICINE) 01 April 2021 (01.04.2021) entire document	1-3, 9-11
A	WO 2020/118634 A1 (CARSGEN THERAPEUTICS CO. LTD. et al) 18 June 2020 (18.06.2020) entire document	1-3, 9-11
A	US 2018/0002719 A1 (INTREXON CORPORATION) 04 January 2018 (04.01.2018) entire document	1-3, 9-11
A	LIU et al. Armored Inducible Expression of IL-12 Enhances Antitumor Activity of Glypican-3-Targeted Chimeric Antigen Receptor-Engineered T Cells in Hepatocellular Carcinoma, J. Immunol., 29 May 2019, Vol. 203, Pgs. 198-207. entire document	1-3, 9-11
A	WO 2021/087205 A1 (SENTI BIOSCIENCES INC.) 06 May 2021 (06.05.2021) entire document	1-3, 9-11
P, X	WO 2022/098922 A1 (SENTI BIOSCIENCES INC.) 12 May 2022 (12.05.2022) entire document	1-3, 9-11

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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

Date of the actual completion of the international search	Date of mailing of the international search report
19 September 2022	OCT 13 2022
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, VA 22313-1450 Facsimile No. 571-273-8300	Authorized officer Taina Matos Telephone No. PCT Helpdesk: 571-272-4300

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2022/033893

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☐ forming part of the international application as filed:

      ☐ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☒ furnished subsequent to the international filing date for the purposes of international search only:

      ☒ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☒ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US2022/033893

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

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

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

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

3. ☒ Claims Nos.: 4-8, 12-15
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

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

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

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

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

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

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

Remark on Protest

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

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

☐ No protest accompanied the payment of additional search fees.