# COMPOSITIONS AND METHODS FOR ENHANCING ADOPTIVE T CELL THERAPEUTICS

## CROSS-REFERENCE TO RELATED APPLICATIONS

**[0001]** This application claims the benefit of U.S. Provisional Application No. 63/550,520, filed on February 6, 2024, the contents of which are herein incorporated by reference in its entirety.

## STATEMENT REGARDING SEQUENCE LISTING

**[0002]** The Sequence Listing XML associated with this application is provided in XML file format and is hereby incorporated by reference into the specification. The name of the XML file containing the Sequence Listing XML is MNBI_002_01WO_SeqList_ST26.xml. The XML file is 538,224 bytes, and created on February 5, 2025, and is being submitted electronically via USPTO Patent Center.

## FIELD

**[0003]** The present disclosure relates generally to compositions and methods for enhancing T cell therapeutics. The disclosure provides recombinant nucleic acid constructs encoding polypeptides that promote T cell signaling, efficacy and/or *in vivo* persistence, cells that comprise such recombinant nucleic acids, methods for preparing T cells for use in cell therapies, as well as methods for identifying mutations useful for improving T cell therapy.

## BACKGROUND

**[0004]** Adoptive T cell therapies, including chimeric antigen receptor (CAR) T cells and engineered T cell receptor (TCR) T cells, have revolutionized cancer therapy. To date, five CD19 targeted CAR-T cell therapies have been approved by the FDA for use against hematological B cell cancers. Despite this success, meaningful responses have not been achieved for patients with solid tumors, which represent ~90% of adult cancers. In solid tumor cancers, the effectiveness of T cell therapies is limited by a complex combination of factors including: fitness of engineered T cells in tumors, T cell exhaustion, poor T cell persistence *in vivo*, and immunosuppressive environmental factors. Despite recent advances, there is still a need to develop alternative approaches to effectively treat solid tumor cancers.

**[0005]** One approach that has been explored for improving CAR T cells is to genetically modify the cells to improve their functionality in treating solid tumors. However, while there are examples of CAR T cells with improved functionality there remains a large unmet need.

As such, there remains a need in the art for alternative solutions to address the significant unmet need for effective adoptive T cell therapies and for enhancing engineered T cell fitness.

## SUMMARY

**[0006]** This section provides a general summary of the disclosure, and is not comprehensive of its full scope or all of its features. The disclosure provides recombinant nucleic acid constructs and methods for enhancing adoptive T cell therapies. The recombinant nucleic acid constructs of the disclosure encode polypeptides with mutations wherein the mutations enhance the therapeutic efficacy of T cells by altering T cell signaling, decreasing T cell exhaustion and/or by enhancing *in vivo* persistence and fitness of engineered T cells.

**[0007]** In one aspect, the disclosure provides recombinant polypeptides comprising a caspase-associated recruitment domain (CARD) containing protein or a functional fragment thereof, wherein the recombinant polypeptide comprises no more than 724 amino acids.

**[0008]** In one aspect, the disclosure provides recombinant polypeptides comprising: (a) a CARD domain, and (b) a recruitment domain or a functional fragment thereof, wherein the recruitment domain is capable of binding to (i) a substrate localized to the intracellular side of the plasma membrane of a cell and/or (ii) a target polypeptide comprising a phosphorylated tyrosine, wherein the recombinant polypeptide comprises no more than 724 amino acids.

**[0009]** In one aspect, the disclosure provides recombinant polypeptides comprising: (a) a first polypeptide capable of binding to a CARD domain on BCL10, and (b) a recruitment domain or a functional fragment thereof, wherein the recruitment domain is capable of binding to (i) a substrate localized to the intracellular side of the plasma membrane of a cell and/or (ii) a target polypeptide comprising a phosphorylated tyrosine, wherein the recombinant polypeptide comprises no more than 724 amino acids.

**[0010]** In one aspect, the disclosure provides a recombinant polypeptide described herein, wherein when the recombinant polypeptide is expressed in an immune cell and the immune cell binds a target antigen, the immune cell exhibits decreased Interferon gamma (IFNg) and/or Interleukin-2 (IL-2) production compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1. In some embodiments, the recombinant polypeptide is expressed in an immune cell and the immune cell binds a target antigen, the immune cell exhibits equivalent or enhanced: (a) BCL10 binding, (b) BCL10 and MALT1 complex formation, (c) NF-κB and/or AP-1 signaling, (d) MALT1 paracaspase activity, (e)

cytokine production, (f) peak tumor expansion, (g) peak peripheral blood expansion, (h) contraction following peak tumor expansion, and/or (i) contraction following peak peripheral blood expansion, compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1. In some embodiments, the recombinant polypeptide is expressed in an immune cell and the immune cell binds a target antigen, the immune cell exhibits equivalent or higher IFNg and/or IL-2 production compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1. In some embodiments, the recombinant polypeptide comprises no more than 700, no more than 650, no more than 600, no more than 550, no more than 500, no more than 450, no more than 400, no more than 350, no more than 300, no more than 250, no more than 200, or no more than 150 amino acids.

**[0011]** In some embodiments, the CARD domain-containing protein or the functional fragment thereof, the CARD domain, or the recombinant polypeptide, is capable of binding to a CARD domain on BCL10. In some embodiments, the CARD domain-containing protein or the functional fragment thereof, the CARD domain, or the recombinant polypeptide, is capable of forming a complex with BCL10 and MALT1.

**[0012]** In some embodiments, the CARD domain-containing protein or the functional fragment thereof, or the CARD domain, comprises or consists of a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to any one of SEQ ID NO: 293-323. In some embodiments, the CARD domain-containing protein is selected from, or wherein the CARD domain is derived from a protein selected from, CARD9, CARD10, CARD11, and CARD14. In some embodiments, the CARD domain-containing protein is, or wherein the CARD domain is derived from, CARD11.

**[0013]** In some embodiments, the recombinant polypeptide comprises a full coiled-coil domain or a truncated portion thereof, wherein the start of the full coiled-coil domain corresponds to amino acid position 123 of SEQ ID NO: 1, and wherein the end of the full coiled-coil domain corresponds to amino acid position 442 of SEQ ID NO: 1; optionally, wherein the full coiled-coil domain comprises a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to amino acids 123-442 of SEQ ID NO: 1. In some embodiments, the full coiled-coil domain comprises at least 310 amino acids. In some embodiments, the recombinant polypeptide comprises the full coiled-coil domain. In some embodiments, the recombinant polypeptide comprises a truncated portion of the coiled-coil domain having no more than 300, no more than 290, no more than 280, no more than 270, no more than 260, no more than 250, no more than 240, no more than 230, no

more than 220, no more than 210, no more than 200, no more than 190, no more than 180, no more than 170, no more than 160, no more than 150, no more than 140, no more than 130, no mor than 120, no more than 110, no more than 100, no more than 95, no more than 90, no more than 85, no more than 80, no more than 75, no more than 70, no more than 60, no more than 50, or no more than 40 amino acids; preferably, wherein the truncated portion of the coiled-coil domain has no more than 260 amino acids. In some embodiments, the coiled-coil domain corresponding to amino acids 253-324 of SEQ ID NO: 1 is removed or replaced by a polypeptide comprising no more than 20, 15, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acids. In some embodiments, the region within the coiled-coil domain is replaced by a polypeptide comprising a sequence of SGGGGSGGGGS (SEQ ID NO:431) or QAGKRSLPD (SEQ ID NO: 432), or a sequence having at most 1, 2, or 3 mutations thereto.

**[0014]** In some embodiments, the recombinant polypeptide comprises an inhibitory domain or a truncated portion thereof, wherein the start of the inhibitory domain corresponds to amino acid position 443 of SEQ ID NO: 1, and wherein the end of the inhibitory domain corresponds to amino acid position 577 of SEQ ID NO: 1; optionally, wherein the inhibitory domain comprises a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to amino acids 443-577 of SEQ ID NO: 1. In some embodiments, the recombinant polypeptide comprises an inhibitory domain or a truncated portion thereof, wherein the inhibitory domain comprises a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 441. In some embodiments, the inhibitory domain comprises at least 130, or at least 125, amino acids. In some embodiments, the recombinant polypeptide comprises the full inhibitory domain. In some embodiments, the recombinant polypeptide comprises a truncated portion of the inhibitory domain having no more than 120, no more than 110, no more than 100, no more than 95, no more than 90, no more than 85, no more than 80, no more than 75, no more than 70, no more than 60, no more than 50, or no more than 40 amino acids. In some embodiments, the inhibitory domain comprises N-terminal and/or C-terminal truncation(s). In some embodiments, the inhibitory domain comprises a truncation of at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, or at least 90 amino acids at the N-terminus. In some embodiments, the inhibitory domain comprises a truncation of at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, or at least 90 amino acids at the C-terminus. In some embodiments, the inhibitory domain comprises a truncation of at least 5, at least 10, at

least 15, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, or at least 90 amino acids in between the N-terminus and the C-terminus.

**[0015]** In some embodiments, the inhibitory domain or the truncated portion thereof comprises at least 1, at least 2, at least 3, at least 4, at least 5, or at least 6 serines that are capable of being phosphorylated; optionally wherein the serines are capable of being phosphorylated by a protein kinase C (PKC); optionally, wherein the PKC comprises PKCβ and/or PKCθ. In some embodiments, the inhibitory domain or the truncated portion thereof comprises a sequence of DA**S**PRT (SEQ ID NO: 425), RAK**S**PI (SEQ ID NO: 426), DA**S**PSS (SEQ ID NO: 427), SRS**S**IMS (SEQ ID NO: 428), RKF**S**LER (SEQ ID NO: 429), and/or FRP**S**VTS (SEQ ID NO: 430), or a sequence having at most 1, 2, or 3 mutations thereto; optionally, where the inhibitory domain or the truncated portion thereof comprises the sequence of RAK**S**PI (SEQ ID NO: 426), DA**S**PSS (SEQ ID NO: 427), and/or SRS**S**IMS (SEQ ID NO: 428).

**[0016]** In some embodiments, the recombinant polypeptide comprises, from N-term to C-term: the coiled-coil domain or the truncated portion thereof, and the inhibitory domain or the truncated portion thereof, without any additional amino acid in between.

**[0017]** In some embodiments, the recombinant polypeptide comprises (b) a recruitment domain or a functional fragment thereof, wherein the recruitment domain is capable of binding to (i) a substrate localized to the intracellular side of the plasma membrane of a cell and/or (ii) a target polypeptide comprising a phosphorylated tyrosine. In some embodiments, the recruitment domain is capable of binding to a substrate indirectly localized to the intracellular side of the plasma membrane, and wherein the recruitment domain binds a polypeptide or lipid that is directly localized to the intracellular side of the plasma membrane of a cell. In some embodiments, the recruitment domain is a Src Homology region 2 (SH2) domain, a Src Homology region 3 (SH3) domain, a phosphotyrosine-binding (PTB) domain, or a pleckstrin homology (PH) domain. In some embodiments, the recruitment domain is a Src Homology region 2 (SH2) domain. In some embodiments, the recruitment domain or the functional fragment thereof comprises a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a segment of at least 30, 40, 50, 60, 70, 80, 90, or 97 amino acids within the region of amino acids 622-716 of SEQ ID NO: 1.

**[0018]** In some embodiments, the recombinant polypeptide comprises, from N-term to C-term: the inhibitory domain or the truncated portion thereof, and the recruitment domain or the functional fragment thereof, with no more than 5, no more than 10, no more than 15, no

more than 20, or no more than 25, amino acids in between. In some embodiments, the recombinant polypeptide comprises an amino acid sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to any one of SEQ ID NO: 433-438 in between the inhibitory domain or the truncated portion thereof and the recruitment domain or the functional fragment thereof. In some embodiments, the recombinant polypeptide comprises, from N-terminus to C-terminus, the CARD domain, optionally the coiled-coil domain, optionally the inhibitory domain, and the recruitment domain.

**[0019]** In one aspect, the disclosure provides a nucleic acid construct comprising a polynucleotide sequence encoding a recombinant polypeptide described herein. In some embodiments, the nucleic acid construct further comprises a polynucleotide sequence encoding one or more additional polypeptides and/or a non-coding RNA. In some embodiments, the one or more additional polypeptides comprise a chimeric antigen receptor (CAR), a recombinant T cell receptor (TCR), an additional potency enhancement polypeptide, a cytokine, a chemokine, a growth factor, a safety switch, or any combination thereof. In some embodiments, the one or more additional polypeptides comprise a CAR or a recombinant TCR.

**[0020]** In some embodiments, the nucleic acid construct comprises a first homology arm before the 5' end of the nucleic acid sequence encoding the recombinant polypeptide and a second homology arm after the 3' end of the nucleic acid sequence encoding the recombinant polypeptide; optionally, wherein the lengths of the two homology arms are between about 150 bp and about 1500 bp. In some embodiments, the length of at least one of the homology arms is about 200bp, 225 bp, about 250 bp, about 275 bp, about 300 bp, about 325 bp, about 350 bp, about 375 bp, about 400 bp, about 425 bp, about 450 bp, about 475 bp, or about 500 bp; optionally, wherein the lengths of both of the homology arms are about 225 bp, about 250 bp, about 275 bp, about 300 bp, about 325 bp, about 350 bp, about 375 bp, about 400 bp, about 425 bp, about 450 bp, about 475 bp, about 500 bp, about 525 bp, about 550 bp, about 575 bp, or about 600 bp. In some embodiments, the total length of the homology arms is between about 400 bp and about 500 bp, about 500 bp and about 600 bp, about 600 bp and about 700 bp, about 700 bp and about 800 bp, about 800 bp and about 900 bp, about 900 bp and about 1000 bp, about 1000 bp and about 1100 bp, or about 1100 bp and about 1200 bp. In some embodiments, the nucleic acid construct is less than 10 kb, less than 9 kb, less than 8 kb, less than 7 kb, less than 6 kb, less than 5 kb, less than 4 kb, or less than 3 kb in length. In some embodiments, the nucleic acid construct is less than about 5.0

kb in length. In some embodiments, the nucleic acid construct is less than about 4.7 kb in length.

**[0021]** In one aspect, the disclosure provides a vector comprising a nucleic acid construct described herein. In some embodiments, the vector is a viral vector. In some embodiments, the viral vector is selected from a retrovirus vector, an adenovirus vector, and an adeno-associated virus (AAV) vector; optionally, wherein the viral vector is an AAV vector.

**[0022]** In one aspect, the disclosure provides an engineered cell comprising a recombinant polypeptide or a nucleic acid construct described herein. In some embodiments, activating the engineered cell results in the secretion of IFNg by the cell at a level that is at least 10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold higher than the IFNg secretion level of a control cell lacking the recombinant polypeptide. In some embodiments, activating the engineered cell results in the secretion of IL-2 by the cell at a level that is at least 10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold higher than the IL-2 secretion level of a control cell lacking the recombinant polypeptide.

**[0023]** In some embodiments, activation of the engineered cell and subsequent removal of the target antigen results in CARD11-PIK3R3 signaling by the cell at a level that is at least 10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold lower than the CARD11-PIK3R3 signaling of a control cell comprising a CARD11-PIK3R3 fusion protein lacking the inhibitory domain. In some embodiments, activation of the engineered cell and subsequent removal of the target antigen results in CARD11-PIK3R3 signaling by the cell at a level that is at least 10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold lower than the CARD11-PIK3R3 signaling of a control cell comprising a CARD11-PIK3R3 fusion protein lacking one of more of S466, S512, S535, S559, S644, and S652 according to SEQ ID NO: 518 in the inhibitory domain.

**[0024]** In some embodiments, CARD11-PIK3R3 signaling in the cell in the absence of a target antigen is at a level that is at least 10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold lower than the CARD11-PIK3R3 signaling of a control cell comprising a control CARD11-PIK3R3 fusion protein lacking the inhibitory domain. In some embodiments, CARD11-PIK3R3 signaling in the cell in the absence of a target antigen is at a level that is at least

10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold lower than the CARD11-PIK3R3 signaling of a control cell comprising a control CARD11-PIK3R3 fusion protein lacking one of more of S466, S512, S535, S559, S644, and S652 according to SEQ ID NO: 518 in the inhibitory domain. In some embodiments, the cell is selected from the group consisting of a T cell, a macrophage, a monocyte, and a natural killer (NK) cell. In some embodiments, the cell is a T cell. In some embodiments, the cell further comprises: (i) a chimeric antigen receptor (CAR) having specificity for a target antigen; or (ii) a recombinant T cell receptor (TCR) having specificity for a target antigen.

**[0025]** In one aspect, the disclosure provides a composition comprising an engineered cell or a plurality of an engineered cell described herein.

**[0026]** In one aspect, the disclosure provides a method of preparing an engineered cell, the method comprising expressing in the cell a recombinant polypeptide described herein, thereby making the engineered cell. In some embodiments, the method comprises introducing into the cell a nucleic acid construct described herein. In some embodiments, the method comprises introducing into the cell a nucleic acid construct described herein, thereby making the engineered cell. In some embodiments, the step of introducing comprises homology-directed repair (HDR)-mediated insertion using the gene editing machinery.

**[0027]** In some embodiments, the HDR occurs in 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 95%, at least 98%, or at least 99%, of a population of the cells; optionally, wherein the HDR occurs in between about 20%-30%, about 30%-40%, about 40%-50%, about 50%-60%, about 60%-70%, about 70%-80%, about 80%-90%, or about 90%-100%, of the cells. In some embodiments, the step of introducing comprises HDR-mediated insertion in the absence of an HDR enhancer; optionally, wherein the HDR enhancer is M3814 or AZD7648.

**[0028]** In some embodiments, the nucleic acid construct comprises a first homology arm before the 5' end of the nucleic acid sequence encoding the recombinant polypeptide and a second homology arm after the 3' end of the nucleic acid sequence encoding the recombinant polypeptide; optionally, wherein the lengths of the two homology arms are between about 150 bp and about 1500 bp. In some embodiments, the length of each of the homology arms is at least 200 bp. In some embodiments, the length of at least one of the homology arms is about 200bp, 225 bp, about 250 bp, about 275 bp, about 300 bp, about 325 bp, about 350 bp, about 375 bp, about 400 bp, about 425 bp, about 450 bp, about 475 bp, or about 500 bp; optionally, wherein the lengths of both of the homology arms are about

225 bp, about 250 bp, about 275 bp, about 300 bp, about 325 bp, about 350 bp, about 375 bp, about 400 bp, about 425 bp, about 450 bp, about 475 bp, about 500 bp, about 525 bp, about 550 bp, about 575 bp, or about 600 bp. In some embodiments, the total length of the homology arms is between about 400 bp and about 500 bp, about 500 bp and about 600 bp, about 600 bp and about 700 bp, about 700 bp and about 800 bp, about 800 bp and about 900 bp, about 900 bp and about 1000 bp, about 1000 bp and about 1100 bp, or about 1100 bp and about 1200 bp.

**[0029]** In some embodiments of the method, the nucleic acid construct comprises, from 5' to 3', the first homology arm, a promoter, a CAR encoding sequence, a polynucleotide sequence encoding the recombinant polypeptide, and the second homology arm. In some embodiments of the method, the nucleic acid construct is introduced via AAV. In some embodiments, the method thereby produces an engineered cell comprising: (i) the recombinant polypeptide; (ii) the immune receptor (e.g., CAR); and (iii) the modified TRAC gene locus. In some embodiments, the method thereby produces an engineered cell comprising: (i) the recombinant polypeptide; (ii) the immune receptor (e.g., CAR); and (iii) the modified TRAJ intron splice acceptor locus.

**[0030]** In one aspect, the disclosure provides a method of treating a subject, comprising administering to the subject an engineered cell described herein or an engineered cell prepared by a method described herein. In some embodiments, the subject has a cancer or an autoimmune disease.

**[0031]** In one aspect, the disclosure provides recombinant polypeptides comprising a caspase-associated recruitment domain (CARD) containing protein or a functional fragment thereof, wherein the recombinant polypeptide comprises no more than 724 amino acids.

**[0032]** In one aspect, the disclosure provides recombinant polypeptides comprising a caspase-associated recruitment domain (CARD) containing protein or a functional fragment thereof, wherein the recombinant polypeptide does not comprise a full length CARD containing protein.

**[0033]** In one aspect, the disclosure provides recombinant polypeptides comprising a recruitment domain or a functional fragment thereof, wherein the recruitment domain is capable of binding to (i) a substrate localized to the intracellular side of the plasma membrane of a cell and/or (ii) a target polypeptide comprising a phosphorylated tyrosine, wherein the recombinant polypeptide comprises no more than 724 amino acids.

**[0034]** In one aspect, the disclosure provides recombinant polypeptides comprising a recruitment domain or a functional fragment thereof, wherein the recruitment domain is

capable of binding to (i) a substrate localized to the intracellular side of the plasma membrane of a cell and/or (ii) a target polypeptide comprising a phosphorylated tyrosine, wherein the recombinant polypeptide does not comprise a full length sequence of an endogenous protein comprising the recruitment domain.

**[0035]** In one aspect, the disclosure provides recombinant polypeptides comprising: (a) a caspase-associated recruitment domain (CARD), and (b) a recruitment domain or a functional fragment thereof, wherein the recruitment domain is capable of binding to (i) a substrate localized to the intracellular side of the plasma membrane of a cell and/or (ii) a target polypeptide comprising a phosphorylated tyrosine.

**[0036]** In one aspect, the disclosure provides recombinant polypeptides comprising: (a) a caspase-associated recruitment domain (CARD), and (b) a recruitment domain or a functional fragment thereof, wherein the recruitment domain is capable of binding to (i) a substrate localized to the intracellular side of the plasma membrane of a cell and/or (ii) a target polypeptide comprising a phosphorylated tyrosine, wherein the recombinant polypeptide comprises no more than 724 amino acids.

**[0037]** In one aspect, the disclosure provides recombinant polypeptides comprising: (a) a caspase-associated recruitment domain (CARD), and (b) a recruitment domain or a functional fragment thereof, wherein the recruitment domain is capable of binding to (i) a substrate localized to the intracellular side of the plasma membrane of a cell and/or (ii) a target polypeptide comprising a phosphorylated tyrosine, wherein the recombinant polypeptide does not comprise a full length sequence of an endogenous protein comprising the CARD and/or a full length sequence of an endogenous protein comprising the recruitment domain.

**[0038]** In one aspect, the disclosure provides recombinant polypeptides comprising: (a) a first polypeptide capable of binding to a caspase-associated recruitment domain (CARD) on BCL10, and (b) a recruitment domain or a functional fragment thereof, wherein the recruitment domain is capable of binding to (i) a substrate localized to the intracellular side of the plasma membrane of a cell and/or (ii) a target polypeptide comprising a phosphorylated tyrosine, wherein the recombinant polypeptide comprises no more than 724 amino acids.

**[0039]** In one aspect, the disclosure provides recombinant polypeptides comprising: (a) a first polypeptide capable of binding to a caspase-associated recruitment domain (CARD) on BCL10, and (b) a recruitment domain or a functional fragment thereof, wherein the recruitment domain is capable of binding to (i) a substrate localized to the intracellular side

of the plasma membrane of a cell and/or (ii) a target polypeptide comprising a phosphorylated tyrosine, wherein the recombinant polypeptide does not comprise a full length sequence of an endogenous protein capable of binding the CARD recruitment domain of BCL10.

**[0040]** In some embodiments, when the recombinant polypeptide is expressed in an immune cell and the immune cell binds a target antigen, the immune cell exhibits decreased: (a) BCL10 binding, (b) BCL10 and MALT1 complex formation, (c) NFAT, NF-κB and/or AP-1 signaling, (d) cytokine production, (e) JAK/STAT signaling, (f) co-stimulatory molecule signaling, (g) phospholipase gamma signaling, and/or (h) transcription factor activity, compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1.

**[0041]** In some embodiments, when the recombinant polypeptide is expressed in an immune cell and the immune cell binds a target antigen, the immune cell exhibits decreased Interferon gamma (IFNg) and/or Interleukin-2 (IL-2) production compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1.

**[0042]** In some embodiments, when the recombinant polypeptide is expressed in an immune cell and the immune cell binds a target antigen, the immune cell exhibits equivalent or enhanced: (a) BCL10 binding, (b) BCL10 and MALT1 complex formation, (c) NF-κB and/or AP-1 signaling, (d) MALT1 paracaspase activity, (e) cytokine production, (f) peak tumor expansion, (g) peak peripheral blood expansion, (h) contraction following peak tumor expansion, and/or (i) contraction following peak peripheral blood expansion, compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1.

**[0043]** In some embodiments, when the recombinant polypeptide is expressed in an immune cell and the immune cell binds a target antigen, the immune cell exhibits equivalent or higher IFNg and/or IL-2 production compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1.

**[0044]** In some embodiments, the recombinant polypeptide comprises no more than 700, no more than 650, no more than 600, no more than 550, no more than 500, no more than 450, no more than 400, no more than 350, no more than 300, no more than 250, no more than 200, or no more than 150 amino acids.

**[0045]** In some embodiments, the CARD containing protein or the functional fragment thereof, the CARD, or the recombinant polypeptide, is capable of binding to a CARD domain on BCL10.

**[0046]** In some embodiments, the CARD containing protein or the functional fragment thereof, the CARD, or the recombinant polypeptide, is capable of forming a complex with BCL10 and MALT1.

**[0047]** In some embodiments, the CARD containing protein or the functional fragment thereof, or the CARD, comprises no more than 40, no more than 50, no more than 60, no more than 70, no more than 80, no more than 90, no more than 100, no more than 150, no more than 200, no more than 300, no more than 400, or no more than 500 amino acids.

**[0048]** In some embodiments, the CARD containing protein or the functional fragment thereof, or the CARD, comprises or consists of a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to any one of SEQ ID NO: 293-323.

**[0049]** In some embodiments, the CARD containing protein is selected from, or wherein the CARD is derived from a protein selected from,CARD9, CARD10, CARD11, and CARD14. In some embodiments, the CARD containing protein is, or wherein the CARD is derived from, CARD11.

**[0050]** In some embodiments, the recombinant polypeptide comprises a full CARD domain or a truncated portion thereof, wherein the start of the full CARD domain corresponds to amino acid position 11 of SEQ ID NO: 1, and wherein the end of the full CARD domain corresponds to amino acid position 103 of SEQ ID NO: 1. In some embodiments, the full CARD domain comprises a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to one of SEQ ID NO: 293-323. In some embodiments, the full CARD domain comprises at least 90 amino acids. In some embodiments, the recombinant polypeptide comprises the full CARD domain.

**[0051]** In some embodiments, the CARD domain comprises N-terminal and/or C-terminal truncation(s). In some embodiments, the CARD domain comprises a truncation of at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, or at least 50 amino acids at the N-terminus. In some embodiments, the CARD domain comprises a truncation of at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, or at least 50 amino acids at the C-terminus. In some embodiments, the CARD domain comprises a truncation of at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, or at least 50 amino acids in between the N-terminus and the C-terminus.

**[0052]** In some embodiments, the recombinant polypeptide does not comprise any full or truncated portion of CARD domain having at least 30 amino acids in length.

**[0053]** In some embodiments, the recombinant polypeptide comprises or consists of a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to any one of SEQ ID NO: 225-245.

**[0054]** In some embodiments, the recombinant polypeptide does not comprise the polypeptide sequence "MDDY" within 10 amino acids before the N-term of the start of the CARD domain.

**[0055]** In some embodiments, when the recombinant polypeptide is expressed in an immune cell and the immune cell binds a target antigen, the immune cell exhibits decreased: (a) BCL10 binding, (b) BCL10 and MALT1 complex formation, (c) NFAT, NF-κB and/or AP-1 signaling, (d) cytokine production, (e) JAK/STAT signaling, (f) co-stimulatory molecule signaling, (g) phospholipase gamma signaling, and/or (h) transcription factor activity, compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1.

**[0056]** In some embodiments, when the recombinant polypeptide is expressed in an immune cell and the immune cell binds a target antigen, the immune cell exhibits decreased IFNg and/or IL-2 production compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1.

**[0057]** In some embodiments, when the recombinant polypeptide is expressed in an immune cell and the immune cell binds a target antigen, the immune cell exhibits equivalent or enhanced: (a) BCL10 binding, (b) BCL10 and MALT1 complex formation, (c) NF-κB and/or AP-1 signaling, (d) MALT1 paracaspase activity, (e) cytokine production, (f) peak tumor expansion, (g) peak peripheral blood expansion, (h) contraction following peak tumor expansion, and/or (i) contraction following peak peripheral blood expansion, compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1.

**[0058]** In some embodiments, when the recombinant polypeptide is expressed in an immune cell and the immune cell binds a target antigen, the immune cell exhibits equivalent or higher IFNg and/or IL-2 production compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1.

**[0059]** In some embodiments, the recombinant polypeptide comprises a full coiled-coil domain or a truncated portion thereof, wherein the start of the full coiled-coil domain corresponds to amino acid position 123 of SEQ ID NO: 1, and wherein the end of the full coiled-coil domain corresponds to amino acid position 442 of SEQ ID NO: 1; optionally, wherein the full coiled-coil domain comprises a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to amino acids 123-442 of SEQ ID NO:

1. In some embodiments, the full coiled-coil domain comprises at least 310 amino acids. In some embodiments, the recombinant polypeptide comprises the full coiled-coil domain.

**[0060]** In some embodiments, the recombinant polypeptide comprises a truncated portion of the coiled-coil domain having no more than 300, no more than 290, no more than 280, no more than 270, no more than 260, no more than 250, no more than 240, no more than 230, no more than 220, no more than 210, no more than 200, no more than 190, no more than 180, no more than 170, no more than 160, no more than 150, no more than 140, no more than 130, no mor than 120, no more than 110, no more than 100, no more than 95, no more than 90, no more than 85, no more than 80, no more than 75, no more than 70, no more than 60, no more than 50, or no more than 40 amino acids; preferably, wherein the truncated portion of the coiled-coil domain has no more than 260 amino acids.

**[0061]** In some embodiments, the coiled-coil domain comprises N-terminal and/or C-terminal truncation(s). In some embodiments, the coiled-coil domain comprises a truncation of at least 5, at least 10, at least 15, 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, at least 110, at least 120, at least 130, at least 140, at least 150, at least 160, at least 170, at least 180, at least 190, at least 200, at least 210, at least 220, at least 230, at least 240, at least 250, at least 260, or at least 270 amino acids at the N-terminus. In some embodiments, the coiled-coil domain comprises a truncation of at least 5, at least 10, at least 15, 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, at least 110, at least 120, at least 130, at least 140, at least 150, at least 160, at least 170, at least 180, at least 190, at least 200, at least 210, at least 220, at least 230, at least 240, at least 250, at least 260, or at least 270 amino acids at the C-terminus.

**[0062]** In some embodiments, the coiled-coil domain comprises a truncation of at least 5, at least 10, at least 15, 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, at least 110, at least 120, at least 130, at least 140, at least 150, at least 160, at least 170, at least 180, at least 190, at least 200, at least 210, at least 220, at least 230, at least 240, at least 250, at least 260, or at least 270 amino acids in between the N-terminus and the C-terminus; optionally, the coiled-coil domain comprises a truncation of about 60 to about 80 amino acids in between the N-terminus and the C-terminus.

**[0063]** In some embodiments, a region within the coiled-coil domain corresponding to amino acids 253-324 of SEQ ID NO: 1 is removed or replaced by a polypeptide comprising no more than 20, 15, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acids.

**[0064]** In some embodiments, the region within the coiled-coil domain is replaced by a polypeptide comprising a sequence of SGGGGSGGGGS (SEQ ID NO: 431) or QAGKRSLPD (SEQ ID NO: 432), or a sequence having at most 1, 2, or 3 mutations thereto.

**[0065]** In some embodiments, the recombinant polypeptide does not comprise any full or truncated portion of coiled-coil domain having at least 30 amino acids in length.

**[0066]** In some embodiments, the recombinant polypeptide comprises or consists of a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to any one of SEQ ID NO: 193-224 and 246-276.

**[0067]** In some embodiments, when the recombinant polypeptide is expressed in an immune cell and the immune cell binds a target antigen, the immune cell exhibits decreased: (a) BCL10 binding, (b) BCL10 and MALT1 complex formation, (c) NFAT, NF-κB and/or AP-1 signaling, (d) cytokine production, (e) JAK/STAT signaling, (f) co-stimulatory molecule signaling, (g) phospholipase gamma signaling, and/or (h) transcription factor activity, compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1.

**[0068]** In some embodiments, when the recombinant polypeptide is expressed in an immune cell and the immune cell binds a target antigen, the immune cell exhibits decreased IFNg and/or IL-2 production compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1.

**[0069]** In some embodiments, when the recombinant polypeptide is expressed in an immune cell and the immune cell binds a target antigen, the immune cell exhibits equivalent or enhanced: (a) BCL10 binding, (b) BCL10 and MALT1 complex formation, (c) NF-κB and/or AP-1 signaling, (d) MALT1 paracaspase activity, (e) cytokine production, (f) peak tumor expansion, (g) peak peripheral blood expansion, (h) contraction following peak tumor expansion, and/or (i) contraction following peak peripheral blood expansion, compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1.

**[0070]** In some embodiments, when the recombinant polypeptide is expressed in an immune cell and the immune cell binds a target antigen, the immune cell exhibits equivalent or enhanced IFNg and/or IL-2 production compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1.

**[0071]** In some embodiments, the recombinant polypeptide comprises an inhibitory domain or a truncated portion thereof, wherein the start of the inhibitory domain corresponds to amino acid position 443 of SEQ ID NO: 1, and wherein the end of the inhibitory domain corresponds to amino acid position 577 of SEQ ID NO: 1; optionally, wherein the inhibitory

domain comprises a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to amino acids 443-577 of SEQ ID NO: 1.

**[0072]** In some embodiments, the recombinant polypeptide comprises an inhibitory domain or a truncated portion thereof, wherein the inhibitory domain comprises a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 441.

**[0073]** In some embodiments, the inhibitory domain comprises at least 130, or at least 125, amino acids. In some embodiments, the recombinant polypeptide comprises the full inhibitory domain.

**[0074]** In some embodiments, the recombinant polypeptide comprises a truncated portion of the inhibitory domain having no more than 120, no more than 110, no more than 100, no more than 95, no more than 90, no more than 85, no more than 80, no more than 75, no more than 70, no more than 60, no more than 50, or no more than 40 amino acids.

**[0075]** In some embodiments, the inhibitory domain comprises N-terminal and/or C-terminal truncation(s). In some embodiments, the inhibitory domain comprises a truncation of at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, or at least 90 amino acids at the N-terminus. In some embodiments, the inhibitory domain comprises a truncation of at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, or at least 90 amino acids at the C-terminus.

**[0076]** In some embodiments, the inhibitory domain comprises a truncation of at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, or at least 90 amino acids in between the N-terminus and the C-terminus.

**[0077]** In some embodiments, the recombinant polypeptide does not comprise any full or truncated portion of inhibitory domain having at least 30 amino acids in length.

**[0078]** In some embodiments, the inhibitory domain or the truncated portion thereof comprises at least 1, at least 2, at least 3, at least 4, at least 5, or at least 6 serines that are capable of being phosphorylated; optionally wherein the series are capable of being phosphorylated by a protein kinase C (PKC); optionally, wherein the PKC comprises PKCβ and/or PKCθ.

**[0079]** In some embodiments, the inhibitory domain or the truncated portion thereof comprises a sequence of DASPRT (SEQ ID NO:425), RAKSPI (SEQ ID NO:426), DASPSS (SEQ ID NO:427), SRSSIMS (SEQ ID NO: 428), RKFSLER (SEQ ID NO: 429), and/or FRPSVTS (SEQ ID NO: 430), or a sequence having at most 1, 2, or 3 mutations thereto;

optionally, where the inhibitory domain or the truncated portion thereof comprises the sequence of RAKSPI (SEQ ID NO:426), DASPSS (SEQ ID NO:427), and/or SRSSIMS (SEQ ID NO: 428).

**[0080]** In some embodiments, the recombinant polypeptide comprises, from N-term to C-term: the coiled-coil domain or the truncated portion thereof, and the inhibitory domain or the truncated portion thereof, without any additional amino acid in between.

**[0081]** In some embodiments, the recombinant polypeptide comprises or consists of a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to any one of SEQ ID NO: 179-192, 277-288, and 289-292.

**[0082]** In some embodiments, when the recombinant polypeptide is expressed in an immune cell and the immune cell binds a target antigen, the immune cell exhibits equivalent or enhanced: (a) BCL10 binding, (b) BCL10 and MALT1 complex formation, (c) NF-κB and/or AP-1 signaling, and/or (d) MALT1 paracaspase activity, (e) cytokine production, (f) peak tumor expansion, (g) peak peripheral blood expansion, (h) contraction following peak tumor expansion, and/or (i) contraction following peak peripheral blood expansion, compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1.

**[0083]** In some embodiments, when the recombinant polypeptide is expressed in an immune cell, the immune cell exhibits equivalent or enhanced IFNg and/or IL-2 production compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1.

**[0084]** In some embodiments, the recombinant polypeptide comprises (b) a recruitment domain or a functional fragment thereof, wherein the recruitment domain is capable of binding to (i) a substrate localized to the intracellular side of the plasma membrane of a cell and/or (ii) a target polypeptide comprising a phosphorylated tyrosine.

**[0085]** In some embodiments, the substrate localized to the intracellular side of the plasma membrane of a cell is a phosphoinositide; optionally, wherein the phosphoinositide is selected from phosphatidylinositol (3,4,5)-trisphosphate (PIP3) and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2).

**[0086]** In some embodiments, the recombinant polypeptide binds to the phosphoinositide with a Kd of less than 50 μM, 10 μM, 5 μM, 1μM, 0.5 μM, 0.1 μM, 0.05 μM, or 0.01 μM, and wherein the Kd is analyzed using SPR.

**[0087]** In some embodiments, the target polypeptide is derived from IGF-1R, CTLA-4, or CD28.

**[0088]** In some embodiments, the phosphorylated tyrosine is located at the position corresponding to pY1221 of SEQ ID NO: 324, or to pY1346 of SEQ ID NO: 325.

**[0089]** In some embodiments, the polypeptide binds to the target polypeptide with a Kd of less than 10 μM, 5 μM, 1μM, 0.5 μM, 0.1 μM, 0.05 μM, or 0.01 μM, and wherein the Kd is analyzed by fluorescence polarization assay.

**[0090]** In some embodiments, the recruitment domain is capable of binding to a substrate indirectly localized to the intracellular side of the plasma membrane, and wherein the recruitment domain binds a polypeptide or lipid that is directly localized to the intracellular side of the plasma membrane of a cell.

**[0091]** In some embodiments, the recruitment domain is a Src Homology region 2 (SH2) domain, a Src Homology region 3 (SH3) domain, a phosphotyrosine-binding (PTB) domain, or a pleckstrin homology (PH) domain. In some embodiments, the recruitment domain is a Src Homology region 2 (SH2) domain. In some embodiments, the start of the recruitment domain corresponds to amino acid position 622 of SEQ ID NO: 1 and wherein the end of the recruitment domain corresponds to amino acid position 716 of SEQ ID NO: 1.

**[0092]** In some embodiments, the recruitment domain comprises at least 90 amino acids.

**[0093]** In some embodiments, the recombinant polypeptide comprises the functional fragment of the recruitment domain.

**[0094]** In some embodiments, the recruitment domain comprises N-terminal and/or C-terminal truncation(s). In some embodiments, the recruitment domain comprises a truncation of at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, or at least 50 amino acids at the N-terminus. In some embodiments, the recruitment domain comprises a truncation of at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, or at least 50 amino acids at the C-terminus.

**[0095]** In some embodiments, the recruitment domain comprises a truncation of at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, or at least 50 amino acids in between the N-terminus and the C-terminus.

**[0096]** In some embodiments, the recruitment domain or the functional fragment thereof comprises a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a segment of at least 30, 40, 50, 60, 70, 80, 90, or 97 amino acids within the region of amino acids 622-716 of SEQ ID NO: 1.

**[0097]** In some embodiments, the recombinant polypeptide does not comprise any recruitment domain having at least 30 amino acids in length.

**[0098]** In some embodiments, the recombinant polypeptide comprises, from N-term to C-term: the inhibitory domain or the truncated portion thereof, and the recruitment domain or

the functional fragment thereof, with no more than 5, no more than 10, no more than 15, no more than 20, or no more than 25, amino acids in between.

**[0099]** In some embodiments, the recombinant polypeptide comprises an amino acid sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to any one of SEQ ID NO: 433-438in between the inhibitory domain or the truncated portion thereof and the recruitment domain or the functional fragment thereof.

**[0100]** In some embodiments, the recombinant polypeptide comprises, from N-terminus to C-terminus, the CARD domain, optionally the coiled-coil domain, optionally the inhibitory domain, and the recruitment domain.

**[0101]** In some embodiments, the recombinant polypeptide comprises no more than 40, no more than 35, no more than 30, no more than 25, no more than 20, no more than 15, no more than 10, or no more than 5, amino acids, in between the CARD containing protein or functional fragment thereof and the recruitment domain.

**[0102]** In some embodiments, the recombinant polypeptide comprises or consists of a sequence having at least 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to any one of SEQ ID NO: 3-150.

**[0103]** In some embodiments, the recombinant polypeptide comprises or consists of a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to any one of SEQ ID NO: 151-178.

**[0104]** In some embodiments, the recombinant polypeptide comprises an aspartatic acid (D) at the position corresponding to amino acid 602 of SEQ ID NO: 1.

**[0105]** In one aspect, the disclosure provides nucleic acid constructs comprising a polynucleotide sequence encoding the recombinant polypeptide of the disclosure.

**[0106]** In some embodiments, the polynucleotide sequence encoding the recombinant polypeptide is operably linked to a promoter. In some embodiments, the promoter is a constitutive promoter or an inducible promoter. In some embodiments, the promoter is or comprises a minimal TATA promoter, pGK promoter, actin promoter, CD4 promoter, CD8a promoter, CD8b promoter, TCRa promoter, TCRb promoter, CD3d promoter, CD3g promoter, CD3e promoter, CD3z promoter, CARD9 promoter, CARD10 promoter, CARD11 promoter, CARD14 promoter, PIK3R3 promoter, CD25 promoter, IL2 promoter, IL7 promoter, IL15 promoter, KLRG-1 promoter, HLA-DR promoter, CD38 promoter, CD69 promoter, Ki-67 promoter, CD11a promoter, CD58 promoter, CD99 promoter, CD62L promoter, CD103 promoter, CCR4 promoter, CCR5 promoter, CCR6 promoter, CCR9 promoter, CCR10 promoter, CXCR3 promoter, CXCR4 promoter, CLA promoter,

Granzyme A promoter, Granzyme B promoter, Perforin promoter, CD57 promoter, CD161 promoter, IL-18Ra promoter, CD69 promoter, GzmB promoter, T-bet promoter, IFNgamma promoter, TIM3 promoter, IL4 promoter, GATA3 promoter, IL1 promoter, IL5 promoter, IL6 promoter, IL13 promoter, IL10 promoter, IL17A promoter, IL6 promoter, IL21 promoter, IL23R promoter, FoxP3 promoter, CTLA4 promoter, CD25 promoter, PD1 promoter, CD45RO promoter, CCR7 promoter, CD28 promoter, CD95 promoter, CD28 promoter, CD27 promoter, CD127 promoter, PD-1 promoter, CD122 promoter, CD132 promoter, c-Kit promoter, nuclear factor of activated T cells (NFAT) promoter, programmed death 1 (PD-1) promoter, T cell immunoglobulin mucin-3 (TIM-3) promoter, cytotoxic T lymphocyte antigen-4 (CTLA4) promoter, lymphocyte-activation protein 3 (LAG-3) promoter, tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) promoter, B- and T-lymphocyte attenuator (BTLA) promoter, CD25 promoter, CD69 promoter, Fas ligand (FasL) promoter, TIGIT promoter, TGF-beta promoter, T-bet promoter, Eomes promoter, GATA3 promoter, CD45RA promoter, 2B4 promoter, Type I interferon (IFN) alpha, Type I IFN beta promoter, IFN gamma promoter, IRF3 promoter, IRF7 promoter, NFkB promoter, AP-1 promoter, TNF-alpha promoter, CD130 promoter, NR4A1 promoter, NR4A2, NR4A3 promoter, MND promoter, EF-1alpha promoter, short EF-1alpha promoter, CAG promoter, ubiquitin/S27a promoter, SV40 promoter, SV40 early promoter, adenovirus major late promoter, mouse metallothionein-I promoter, Moloney murine leukemia virus (MMLV) long terminal repeat (LTR) region, CMV promoter, immunoglobulin promoter, heat shock promoter, polyoma virus promoter, fowlpox virus promoter, bovine papilloma virus promoter, avian sarcoma virus promoter, retrovirus promoter, hepatitis-B virus promoter, PGK promoter, vaccinia virus 7.5K promoter, TK promoter of HSV, mouse mammary tumor virus (MMTV) promoter, LTR promoter of HIV, promoter of moloney virus, Epstein Barr virus (EBV) promoter, Rous sarcoma virus (RSV) promoter, U6 promoter, or UBC promoter.

**[0107]** In some embodiments, the promoter is a constitutive promoter. In some embodiments, the constitutive promoter is or comprises a CD4 promoter, CD8a promoter, CD8b promoter, TCRa promoter, TCRb promoter, CD3d promoter, CD3g promoter, CD3e promoter, CD3z promoter, CARD9 promoter, CARD10 promoter, CARD11 promoter, CARD14 promoter, or PIK3R3 promoter.

**[0108]** In some embodiments, the promoter is or comprises an MND promoter or a short EF1a promoter.

**[0109]** In some embodiments, the polynucleotide sequence encoding the recombinant polypeptide is not operably linked to a promoter.

**[0110]** In some embodiments, the one or more cleavable linkers comprise a P2A, E2A, F2A, or T2A self-cleaving peptide.

**[0111]** In some embodiments, the nucleic acid construct further comprises one or more nucleic acid coding or regulatory sequence. In some embodiments, the one or more nucleic acid coding or regulatory sequence is or comprises ribosomal binding sites, enhancer elements, activator elements, translational start sequences, translational termination sequences, transcription start sequences, transcription termination sequences, polyadenylation signal sequences, a 70 bp poly(A) tract, a 100 bp poly(A) tract, a 172 bp poly(A) tract, a 200 bp poly(A) tract, a 300 bp poly(A) tract, a 325 bp poly(A) tract, replication elements, RNA processing and export elements, transposon sequences, transposase sequences, insulator sequences, internal ribosome entry sites (IRES), 5'UTRs, 3'UTRs, mRNA 3' end processing sequences, boundary elements, locus control regions (LCR), matrix attachment regions (MAR), recombination or cassette exchange sequences, linker sequences, cleavable linker sequences, secretion signals, resistance markers, anchoring peptides, localization signals, fusion tags, affinity tags, chaperonins, proteases, or any combination thereof.

**[0112]** In some embodiments, the nucleic acid construct further comprises a polynucleotide sequence encoding one or more additional polypeptides and/or a non-coding RNA. In some embodiments, the non-coding RNA comprises a shRNA or a microRNA. In some embodiments, the one or more additional polypeptides comprise a chimeric antigen receptor (CAR), a recombinant T cell receptor (TCR), an additional potency enhancement polypeptide, a cytokine, a chemokine, a growth factor, a safety switch, or any combination thereof. In some embodiments, the one or more additional polypeptides comprise a CAR or a recombinant TCR.

**[0113]** In some embodiments, the CAR or recombinant TCR has specificity for a target antigen selected from the group consisting of CD1, CD1a, CD1b, CD1c, CD1d, CD1e, CD2, CD3d, CD3e, CD3g, CD3ε, CD4, CD5, CD7, CD8a, CD8b, CD19, CD20, CD21, CD22, CD23, CD24, CD25, CD27, CD28, CD30, CD33, CD34, CD38, CD40, CD44v6, CD45, CD46, CD47 CD48, CD52, CD59, CD66, CD70, CD71, CD72, CD73, CD79A, CD79B, CD80 (B7.1), CD86 (B7.2), CD94, CD95, CD97, CD123, CD134, CD140 (PDGFR4), CD152, CD154, CD158, CD171, CD178, CD179, CD179a, CD181 (CXCR1), CD182 (CXCR2), CD183 (CXCR3), CD210, CD213A2, CD246, CD252, CD253, CD261, CD262,

CD272, CD273 (PD-L2), CD274 (PD-L1), CD276 (B7H3), CD279, CD295, CD339 (JAG1), CD340 (HER2), CDH17, CEA, CLECL1, CLL-1, CLDN6, CLDN18.2, CS1, DLL3, LY6G6D, GCC, p53R175H, PRAME, EGFR, EGFRvIII, FGFR2, AFP, CA125, MUC-1, MAGE, ALPI, alkaline phosphatase placental-like 2 (ALPPL2), B-cell maturation antigen (BCMA), green fluorescent protein (GFP), enhanced green fluorescent protein (eGFP), KLK2, KLK3, Mesothelin, IL13Ra2, ggcc, signal regulatory protein α (SIRPα), TCRalpha, TCRbeta, TSHR, GD2, GD3, Tn Ag, cMET, Axl, ROR1, ROR2, GPC1, GPC2, GPC3, FLT3, TAG72, CEA, EPCAM, KIT (CD117), IL-13Ra2, IL-11Ra, PSCA, PRSS21, VEGFR2, LewisY, PDGFRβ, SSEA-4, folate receptor alpha, ERBB2 (Her2/neu), MUC1, MUC16, NCAM, prostase, PAP, ELF2M, Ephrin B2, IGF-I receptor, CAIX, LMP2, gp100, bcr-abl, tyrosinase, EphA2, STEAP1, STEAP2, fucosyl GM1, sLe, GM3, TGS5, HMWMAA, o-acetyl-GD2, folate receptor beta, TEM1/CD248, TEM7R, ROPN1, GPRC5D, GPA33, CXORF61, ALK, Polysialic acid, PLAC1, GloboH, NY-BR-1, UPK2, HAVCR1, ADRB3, PANX3, GPR20, LY6K, OR51E2, TARP, WT1, NY-ESO-1, LAGE-la, MAGE-Al, legumain, HPV E6,E7, MAGE-A4, ETV6-AML, sperm protein 17, XAGE1, Tie 2, MAD-CT-1, MAD-CT-2, Fos-related antigen 1, p53, p53 mutant, p53R175H, KRAS, mutant KRAS, KRAS G12D, prostein, surviving, telomerase, PCTA-1/Galectin 8, MelanA/MART1, Ras mutant, hTERT, sarcoma translocation breakpoints, ML-IAP, ERG (TMPRSS2 ETS fusion gene), NA17, PAX3, androgen receptor, cyclin Bl, MYCN, RhoC, TRP-2, CYP1B1, BORIS, SART3, PAX5, OY-TES1, LCK, AKAP-4, SSX2, RAGE-1, human telomerase reverse transcriptase, RU1, RU2, intestinal carboxyl esterase, mut hsp70-2, LAIR1, FCAR, LILRA2, CD300LF, CLEC12A, BST2, EMR2, LY75, FCRL5, IGLL1, PSMA, TROP2, citrullinated vimentin, the extracellular portion of the APRIL protein, and any combinations thereof.

**[0114]** In some embodiments, the CAR or recombinant TCR has specificity for a target antigen selected from the group consisting of B7H3, BCMA, CD19, CD20, CD22, CD70, CD79a, CD79b, CDH17, CLDN6, CLDN18.2, DLL3, GCC, GD2, GD3, GPC3, GPRC5D, GPA33, KLK3, LY6G6D, p53R175H, PRAME, ROPN1, STEAP1, and STEAP2.

**[0115]** In some embodiments, the CAR comprises one or more costimulatory domains. In some embodiments, the one or more costimulatory domains are derived from a protein selected from the group comprising CD3zeta, CD28, 4-1BB, OX40, IL2Rb turbodomains, MYD88/CD40, MYD88/CD40 inducible constimulatory domain, CD2, B7-1/CD80; B7-2/CD86; B7-H1/PD-L1; B7-H2; B7-H3; B7-H4; B7-H6; B7-H7; BTLA/CD272; CD28; CTLA-4; Gi24/VISTA/B7-H5; ICOS/CD278; PD-1; PD-L2/B7-DC; PDCD6); 4-

1BB/TNFSF9/CD137; 4-1BB Ligand/TNFSF9; BAFF/BLyS/TNFSF13B; BAFF R/TNFRSF13C; CD27/TNFRSF7; CD27 Ligand/TNFSF7; CD30/TNFRSF8; CD30 Ligand/TNFSF8; CD40/TNFRSF5; CD40/TNFSF5; CD40 Ligand/TNFSF5; DR3/TNFRSF25; GITR/TNFRSF18; GITR Ligand/TNFSF18; HVEM/TNFRSF14; LIGHT/TNFSF14; Lymphotoxin-alpha/TNF-beta; OX40/TNFRSF4; 0X40 Ligand/TNFSF4; RELT/TNFRSF19L; TACI/TNFRSF13B; TL1A/TNFSF15; TNF-alpha; TNF RII/TNFRSF1B); 2B4/CD244/SLAMF4; BLAME/SLAMF8; CD2; CD2F-10/SLAMF9; CD48/SLAMF2; CD58/LFA-3; CD84/SLAMF5; CD229/SLAMF3; CRACC/SLAMF7; NTB- A/SLAMF6; SLAM/CD150); CD2; CD7; CD53; CD82/Kai-1; CD90/Thyl; CD96; CD160; CD200; CD300a/LMIRl; HLA Class I; HLA-DR; Ikaros; Integrin alpha 4/CD49d; Integrin alpha 4 beta 1; Integrin alpha 4 beta 7/LPAM-l; LAG-3; TCL1A; TCL1B; CRTAM; DAP12; Dectin- 1/CLEC7A; DPPIV/CD26; EphB6; TIM-1/KIM-1/HAVCR; TIM-4; TSLP; TSLP R; lymphocyte function associated antigen-1 (LFA-1); NKG2C, an immunoreceptor tyrosinebased activation motif (ITAM), CD27, CD134/OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD7, LIGHT, NKG2C, and any combinations thereof.

**[0116]** In some embodiments, the additional potency enhancement polypeptide is or comprises a dominant negative form of an inhibitor of a cell-mediated immune response of the immune cell (e.g., TGFβR2 DNR), c-Jun, CCL19, CCL21, IL2R, IL7, IL7Ralpha, IL15, IL15RA, IL18, decoy-resistant IL18 (DR-18), MyD88/CD40, PD1-CD28 switch receptor, PD1-41BB switch receptor, CD40L-CD28 switch receptor, CTBR12 switch receptor, or CD8alpha/beta.

**[0117]** In some embodiments, the polynucleotide sequence encoding the recombinant polypeptide and the polynucleotide sequence encoding the one or more additional polypeptides are separated by a polynucleotide sequence encoding one or more cleavable linkers, and wherein all the polynucleotide sequences form a continuous open reading frame.

**[0118]** In some embodiments, the polynucleotide sequence encoding the recombinant polypeptide and the polynucleotide sequence encoding the one or more additional polypeptides are separated by an internal ribozyme entry site (IRES).

**[0119]** In some embodiments, the nucleic acid construct is a DNA construct. In some embodiments, the nucleic acid construct is an RNA construct. In some embodiments, the RNA construct is an mRNA construct.

**[0120]** In some embodiments, the nucleic acid construct comprises one or more modified nucleotides. In some embodiments, the one or more modified nucleotides are or comprise

pseudouridine, N1-methylpseudouridine, 4'-thiouridine, 5-methylcytosine, 2-thio-l-methyl-1-deaza-pseudouridine, 2-thio-l-methyl-pseudouridine, 2-thio-5-aza-uridine, 2-thio-dihydropseudouridine, 2-thio-dihydrouridine, 2-thio-pseudouridine, 4-methoxy-2-thio-pseudouridine, 4-methoxy-pseudouridine, 4-thio-l-methyl- pseudouridine, 4-thio-pseudouridine, 5-aza-uridine, dihydropseudouridine, 5-methyluridine, 5-methyluridine, 5-methoxyuridine, 2'-O-methyl uridine, or any combination thereof. In some embodiments, one or more modified nucleotides are selected from the group consisting of pseudouridine, N1-methylpseudouridine, 5-methylcytosine, 5- methoxyuridine, and a combination thereof. In some embodiments, one or more modified nucleotides are N1- methylpseudouridines.

**[0121]** In some embodiments, the nucleic acid construct comprises a first homology arm before the 5' end of the nucleic acid sequence encoding the recombinant polypeptide and a second homology arm after the 3' end of the nucleic acid sequence encoding the recombinant polypeptide; optionally, wherein the lengths of the two homology arms are between about 150 bp and about 1500 bp. In some embodiments, the length of at least one of the homology arms is about 200bp, 225 bp, about 250 bp, about 275 bp, about 300 bp, about 325 bp, about 350 bp, about 375 bp, about 400 bp, about 425 bp, about 450 bp, about 475 bp, or about 500 bp; optionally, wherein the lengths of both of the homology arms are about 225 bp, about 250 bp, about 275 bp, about 300 bp, about 325 bp, about 350 bp, about 375 bp, about 400 bp, about 425 bp, about 450 bp, about 475 bp, about 500 bp, about 525 bp, about 550 bp, about 575 bp, or about 600 bp. In some embodiments, the total length of the homology arms is between about 400 bp and about 500 bp, about 500 bp and about 600 bp, about 600 bp and about 700 bp, about 700 bp and about 800 bp, about 800 bp and about 900 bp, about 900 bp and about 1000 bp, about 1000 bp and about 1100 bp, or about 1100 bp and about 1200 bp.

**[0122]** In some embodiments, the nucleic acid construct is less than 10 kb, less than 9 kb, less than 8 kb, less than 7 kb, less than 6 kb, less than 5 kb, less than 4 kb, or less than 3 kb in length. In some embodiments, the nucleic acid construct is less than about 5.0 kb in length. In some embodiments, the nucleic acid construct is less than about 4.7 kb in length.

**[0123]** In some embodiments, the length of the polynucleotide sequence encoding the one or more additional polypeptides is less than about 2100 bp, less than about 2150 bp, less than about 2200 bp, less than about 2250 bp, less than about 2300 bp, less than about 2350 bp, or less than about 2400 bp.

**[0124]** In some embodiments, the length of the one or more additional polypeptides is less than about 700 amino acids, less than about 725 amino acids, less than about 750 amino

acids, less than about 775 amino acids, less than about 800 amino acids, less than about 825 amino acids, less than about 850 amino acids, less than about 875 amino acids, or less than about 900 amino acids.

**[0125]** In one aspect, the disclosure provides nucleic acid constructs comprising, from 5' to 3': (1) a promoter, (2) a CAR encoding sequence, (3) a cleavable linker encoding sequence, and (4) a polynucleotide encoding the recombinant polypeptide of the disclosure.

**[0126]** In one aspect, the disclosure provides nucleic acid constructs comprising, from 5' to 3': (1) a promoter, (2) a polynucleotide encoding the recombinant polypeptide of the disclosure, (3) a cleavable linker encoding sequence, and (4) a CAR encoding sequence.

**[0127]** In one aspect, the disclosure provides nucleic acid constructs comprising, from 5' to 3': (1) a promoter, (2) a CAR encoding sequence, (3) a cleavable linker encoding sequence, (4) a polynucleotide encoding the recombinant polypeptide of the disclosure, (5) a cleavable linker encoding sequence, and (6) a polynucleotide encoding an additional potency enhancement polypeptide, a cytokine, a chemokine, or a growth factor.

**[0128]** In one aspect, the disclosure provides nucleic acid constructs comprising, from 5' to 3': (1) a promoter, (2) a polynucleotide sequence encoding the recombinant polypeptide of the disclosure, (3) a cleavable linker encoding sequence, (4) a CAR encoding sequence, (5) a cleavable linker encoding sequence, and (6) a polynucleotide encoding an additional potency enhancement polypeptide, a cytokine, a chemokine, or a growth factor.

**[0129]** In one aspect, the disclosure provides nucleic acid constructs comprising, from 5' to 3': (1) a promoter, (2) a polynucleotide sequence encoding the recombinant polypeptide of the disclosure, (3) a cleavable linker encoding sequence, (4) a polynucleotide encoding an additional potency enhancement polypeptide, a cytokine, a chemokine, or a growth factor (5) a cleavable linker encoding sequence, and (6) a CAR encoding sequence.

**[0130]** In one aspect, the disclosure provides nucleic acid constructs comprising, from 5' to 3': (1) a promoter, (2) a polynucleotide encoding an additional potency enhancement polypeptide, a cytokine, a chemokine, or a growth factor, (3) a cleavable linker encoding sequence, (4) a polynucleotide sequence encoding the recombinant polypeptide of the disclosure, (5) a cleavable linker encoding sequence, and (6) a CAR encoding sequence.

**[0131]** In one aspect, the disclosure provides nucleic acid constructs comprising, from 5' to 3': (1) a promoter, (2) a polynucleotide encoding an additional potency enhancement polypeptide, a cytokine, a chemokine, or a growth factor, (3) a cleavable linker encoding sequence, (4) a CAR encoding sequence, (5) a cleavable linker encoding sequence, and (6) a polynucleotide sequence encoding the recombinant polypeptide of the disclosure.

**[0132]** In one aspect, the disclosure provides nucleic acid constructs comprising, from 5' to 3': (1) a cleavable linker encoding sequence, (2) a CAR encoding sequence, (3) a cleavable linker encoding sequence, (4) a polynucleotide sequence encoding the recombinant polypeptide of the disclosure, and (5) a cleavable linker encoding sequence.

**[0133]** In one aspect, the disclosure provides nucleic acid constructs comprising, from 5' to 3': (1) a cleavable linker encoding sequence, (2) a polynucleotide sequence encoding the recombinant polypeptide of the disclosure, (3) a cleavable linker encoding sequence, (4) a CAR encoding sequence, and (5) a cleavable linker encoding sequence.

**[0134]** In one aspect, the disclosure provides nucleic acid constructs comprising, from 5' to 3': (1) a cleavable linker encoding sequence, (2) a CAR encoding sequence, (3) a cleavable linker encoding sequence, (4) a polynucleotide sequence encoding the recombinant polypeptide of the disclosure, (5) a cleavable linker encoding sequence, (6) a polynucleotide encoding an additional potency enhancement polypeptide, a cytokine, a chemokine, or a growth factor, (7) a cleavable linker encoding sequence.

**[0135]** In one aspect, the disclosure provides nucleic acid constructs comprising, from 5' to 3': (1) a cleavable linker encoding sequence, (2) a CAR encoding sequence, (3) a cleavable linker encoding sequence, (4) a polynucleotide encoding an additional potency enhancement polypeptide, a cytokine, a chemokine, or a growth factor, (5) a cleavable linker encoding sequence, (6) a polynucleotide sequence encoding the recombinant polypeptide of the disclosure, (7) a cleavable linker encoding sequence.

**[0136]** In one aspect, the disclosure provides nucleic acid constructs comprising, from 5' to 3': (1) a cleavable linker encoding sequence, (2) a polynucleotide sequence encoding the recombinant polypeptide of the disclosure, (3) a cleavable linker encoding sequence, (4) a CAR encoding sequence, (5) a cleavable linker encoding sequence, (6) a polynucleotide encoding an additional potency enhancement polypeptide, a cytokine, a chemokine, or a growth factor, (7) a cleavable linker encoding sequence.

**[0137]** In one aspect, the disclosure provides nucleic acid constructs comprising, from 5' to 3': (1) a cleavable linker encoding sequence, (2) a polynucleotide sequence encoding the recombinant polypeptide of the disclosure, (3) a cleavable linker encoding sequence, (4) a polynucleotide encoding an additional potency enhancement polypeptide, a cytokine, a chemokine, or a growth factor, (5) a cleavable linker encoding sequence, (6) a CAR encoding sequence, (7) a cleavable linker encoding sequence.

**[0138]** In one aspect, the disclosure provides nucleic acid constructs comprising, from 5' to 3': (1) a cleavable linker encoding sequence, (2) a polynucleotide encoding an additional

potency enhancement polypeptide, a cytokine, a chemokine, or a growth factor, (3) a cleavable linker encoding sequence, (4) a CAR encoding sequence, (5) a cleavable linker encoding sequence, (6) a polynucleotide sequence encoding the recombinant polypeptide of the disclosure, (7) a cleavable linker encoding sequence.

**[0139]** In one aspect, the disclosure provides nucleic acid constructs comprising, from 5' to 3': (1) a cleavable linker encoding sequence, (2) a polynucleotide encoding an additional potency enhancement polypeptide, a cytokine, a chemokine, or a growth factor, (3) a cleavable linker encoding sequence, (4) a polynucleotide sequence encoding the recombinant polypeptide of the disclosure, (5) a cleavable linker encoding sequence, (6) a CAR encoding sequence, (7) a cleavable linker encoding sequence.

**[0140]** In one aspect, the disclosure provides nucleic acid constructs comprising: (1) a CAR encoding sequence, and (2) a polynucleotide sequence encoding the recombinant polypeptide of the disclosure, wherein the nucleic acid construct is an RNA construct.

**[0141]** In one aspect, the disclosure provides compositions comprising: (1) a first nucleic acid construct encoding a CAR, and (2) a second nucleic acid construct encoding the recombinant polypeptide of the disclosure, wherein the first and the second nucleic acid constructs are both RNA constructs.

**[0142]** In one aspect, the disclosure provides compositions comprising: (1) a CAR encoding sequence, (2) a polynucleotide sequence encoding the recombinant polypeptide of the disclosure, and (3) a polynucleotide encoding an additional potency enhancement polypeptide, a cytokine, a chemokine, or a growth factor, wherein the nucleic acid construct is an RNA construct.

**[0143]** In one aspect, the disclosure provides compositions comprising: (1) a first nucleic acid construct encoding a CAR, (2) a second nucleic acid construct encoding the recombinant polypeptide of the disclosure, and (3) a third nucleic acid construct encoding a polynucleotide encoding an additional potency enhancement polypeptide, a cytokine, a chemokine, or a growth factor, and wherein the first, second, and third nucleic acid constructs are each RNA constructs.

**[0144]** In one aspect, the disclosure provides vectors comprising the nucleic acid construct of the disclosure. In some embodiments, the vector is a plasmid. In some embodiments, the vector is a synthetic DNA vector. In some embodiments, the vector is a linear DNA vector. In some embodiments, the vector is a closed linear DNA vector. In some embodiments, the vector is a synthetic RNA vector. In some embodiments, the vector is a phagemid vector. In some embodiments, the vector is a viral vector. In some embodiments, the viral vector is

selected from a retrovirus vector, an adenovirus vector, and an adeno-associated virus (AAV) vector; optionally, wherein the viral vector is an AAV vector. In some embodiments, the AAV vector is AAV6 vector. In some embodiments, the AAV vector is AAV9 vector. In some embodiments, the AAV vector is a split-intein dual AAV vector. In some embodiments, the retrovirus is a lentivirus. In some embodiments, the lentivirus is a VSV-G pseudotyped lentivirus. In some embodiments, the vector is a redirected lentiviral vector. In some embodiments, the vector is a fusosome. In some embodiments, the vector is a lentiviral particle engineered with the anti-CD3 Cocal glycoprotein. In some embodiments, the vector is an enveloped delivery vehicle. In some embodiments, the vector is self-replicating RNA virus. In some embodiments, the vector is mRNA-packaging virus-like particle. In some embodiments, the vector is RNP-packaging virus-like particle.

**[0145]** In some embodiments, activating the engineered cell results in the secretion of IL-2 by the cell at a level that is at least 10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold higher than the IL-2 secretion level of a control cell lacking the recombinant polypeptide.

**[0146]** In some embodiments, activation of the engineered cell and subsequent removal of the target antigen results in CARD11-PIK3R3 signaling by the cell at a level that is at least 10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold lower than the CARD11-PIK3R3 signaling of a control cell comprising a CARD11-PIK3R3 fusion protein lacking the inhibitory domain.

**[0147]** In some embodiments, activation of the engineered cell and subsequent removal of the target antigen results in CARD11-PIK3R3 signaling by the cell at a level that is at least 10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold lower than the CARD11-PIK3R3 signaling of a control cell comprising a CARD11-PIK3R3 fusion protein lacking one of more of S466, S512, S535, S559, S644, and S652 according to SEQ ID NO: 518 in the inhibitory domain.

**[0148]** In some embodiments, CARD11-PIK3R3 signaling in the cell in the absence of a target antigen is at a level that is at least 10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold lower than the CARD11-PIK3R3 signaling of a control cell comprising a control CARD11-PIK3R3 fusion protein lacking the inhibitory domain.

**[0149]** In some embodiments, CARD11-PIK3R3 signaling in the cell in the absence of a target antigen is at a level that is at least 10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold lower than the CARD11-PIK3R3 signaling of a control cell comprising a control CARD11-PIK3R3 fusion protein lacking one of more of S466, S512, S535, S559, S644, and S652 according to SEQ ID NO: 518in the inhibitory domain.

**[0150]** In some embodiments, the CARD11-PIK3R3 signaling is measured by: (a) BCL10 binding, (b) BCL10 and MALT1 complex formation, (c) NFAT, NF-κB and/or AP-1 signaling, (d) cytokine production, (e) JAK/STAT signaling, (f) co-stimulatory molecule signaling, (g) phospholipase gamma signaling, and/or (h) transcription factor activity.

**[0151]** In some embodiments, expression of the recombinant polypeptide is under the control of an endogenous promoter. In some embodiments, the endogenous promoter is a TCRa promoter, a TCRb promoter, a CD3d promoter, a CD3g promoter, a CD3e promoter, a CD3z promoter, a CARD9 promoter, a CARD10 promoter, a CARD11 promoter, a CARD14 promoter, or a PIK3R3 promoter.

**[0152]** In some embodiments, expression of the recombinant polypeptide is under the control of an exogenous promoter. In some embodiments, the exogenous promoter is or comprises a minimal TATA promoter, pGK promoter, actin promoter, CD4 promoter, CD8a promoter, CD8b promoter, TCRa promoter, TCRb promoter, CD3d promoter, CD3g promoter, CD3e promoter, CD3z promoter, CARD9 promoter, CARD10 promoter, CARD11 promoter, CARD14 promoter, PIK3R3 promoter, CD25 promoter, IL2 promoter, IL7 promoter, IL15 promoter, KLRG-1 promoter, HLA-DR promoter, CD38 promoter, CD69 promoter, Ki-67 promoter, CD11a promoter, CD58 promoter, CD99 promoter, CD62L promoter, CD103 promoter, CCR4 promoter, CCR5 promoter, CCR6 promoter, CCR9 promoter, CCR10 promoter, CXCR3 promoter, CXCR4 promoter, CLA promoter, Granzyme A promoter, Granzyme B promoter, Perforin promoter, CD57 promoter, CD161 promoter, IL-18Ra promoter, CD69 promoter, GzmB promoter, T-bet promoter, IFNgamma promoter, TIM3 promoter, IL4 promoter, GATA3 promoter, IL1 promoter, IL5 promoter, IL6 promoter, IL13 promoter, IL10 promoter, IL17A promoter, IL6 promoter, IL21 promoter, IL23R promoter, FoxP3 promoter, CTLA4 promoter, CD25 promoter, PD1 promoter, CD45RO promoter, CCR7 promoter, CD28 promoter, CD95 promoter, CD28 promoter, CD27 promoter, CD127 promoter, PD-1 promoter, CD122 promoter, CD132 promoter, c-Kit promoter, nuclear factor of activated T cells (NFAT) promoter, programmed death 1 (PD-1) promoter, T cell immunoglobulin mucin-3 (TIM-3) promoter, cytotoxic T

lymphocyte antigen-4 (CTLA4) promoter, lymphocyte-activation protein 3 (LAG-3) promoter, tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) promoter, B- and T-lymphocyte attenuator (BTLA) promoter, CD25 promoter, CD69 promoter, Fas ligand (FasL) promoter, TIGIT promoter, TGF-beta promoter, T-bet promoter, Eomes promoter, GATA3 promoter, CD45RA promoter, 2B4 promoter, Type I interferon (IFN) alpha, Type I IFN beta promoter, IFN gamma promoter, IRF3 promoter, IRF7 promoter, NFkB promoter, AP-1 promoter, TNF-alpha promoter, CD130 promoter, NR4A1 promoter, NR4A2, NR4A3 promoter, MND promoter, EF-1alpha promoter, short EF-1alpha promoter, CAG promoter, ubiquitin/S27a promoter, SV40 promoter, SV40 early promoter, adenovirus major late promoter, mouse metallothionein- I promoter, Moloney murine leukemia virus (MMLV) long terminal repeat (LTR) region, CMV promoter, immunoglobulin promoter, heat shock promoter, polyoma virus promoter, fowlpox virus promoter, bovine papilloma virus promoter, avian sarcoma virus promoter, retrovirus promoter, hepatitis-B virus promoter, PGK promoter, vaccinia virus 7.5K promoter, TK promoter of HSV, mouse mammary tumor virus (MMTV) promoter, LTR promoter of HIV, promoter of oloney virus, Epstein Barr virus (EBV) promoter, Rous sarcoma virus (RSV) promoter, U6 promoter, or UBC promoter.

**[0153]** In some embodiments, the nucleic acid construct is inserted in a T-cell receptor (TCR) locus, a CD3 locus, a B2 microglobulin (B2M) locus, a class II transactivator (CIITA) locus, a CARD9 locus, a CARD10 locus, a CARD11 locus, a CARD14 locus, a PIK3R3 locus, or a safe harbor locus. In some embodiments, the TCR locus is or comprises a TRAC locus, a TRBC1 locus, a TRBC2 locus, or a TRAJ locus.

**[0154]** In some embodiments, the cell does not express a gene product of an endogenous TRAC locus. In some embodiments, the cell does not express a gene product of an endogenous TRBC1 locus. In some embodiments, the cell does not express a gene product of an endogenous TRBC2 locus.

**[0155]** In some embodiments, the CD3 locus is or comprises: a CD3d locus, a CD3g locus, a CD3e locus, or CD3z locus. In some embodiments, the cell does not express a gene product of an endogenous CD3d locus. In some embodiments, the cell does not express a gene product of an endogenous CD3g locus. In some embodiments, the cell does not express a gene product of an endogenous CD3e locus. In some embodiments, the cell does not express a gene product of an endogenous CD3z locus.

**[0156]** In some embodiments, the safe harbor locus is or comprises an AAVS1, ABO, CCR5, CLYBL, CXCR4, F3, FUT1, HMGB1, KDM5D, LRP1, MICA, MICB, RHD, ROSA26, or SHS231 locus.

**[0157]** In some embodiments, the nucleic acid construct is inserted is an exon, an intron, between an intron and an exon, or a regulatory region.

**[0158]** In some embodiments, the cell is selected from the group of consisting of a T cell, a CD4+ T cell, a CD8+ T cell, a regulatory T cell (Treg), a gamma delta T cell (γδT), an invariant natural killer T (iNKT) cell, a mucosal associated invariant T (MAIT) cell, a macrophage, a monocyte, a natural killer (NK) cell, a tumor infiltrating lymphocyte (TIL), a cytotoxic T cell, a T helper cell, a memory T cell, a central memory T (TCM) cell, a stem memory T (TSCM) cell, a stem-cell-like memory T cell (or stem-like memory T cells), an effector memory T (TEM) cell, a TEMRA (CD45RA+) cell, an effector T cell, a Th1 cell, a Th2 cell, a Th9 cell, a Th17 cell, a Th22 cell, a Tfh (follicular helper) cell, a natural killer T (NKT) cell, a transitional memory T (TTM) cell, a terminal effector T (TTE) cell, a naïve T (TN) cell, a hematopoietic stem cell, and a progenitor cell of the lymphoid lineage. In some embodiments, the cell is selected from the group consisting of a T cell, a macrophage, a monocyte, and a natural killer (NK) cell. In some embodiments, the cell is a T cell. In some embodiments, the T cell is selected from the group consisting of a regulatory T cell (Treg), a gamma delta T cell, a CD8+ T cell, an invariant iNKT cell, a MAIT cell, a CAR T cell, a tumor-infiltrating lymphocyte, and an engineered T cell comprising a transcriptional receptor.

**[0159]** In some embodiments, the cell is an autologous cell. In some embodiments, the cell is an allogeneic cell. In some embodiments, the cell is a primary cell. In some embodiments, the cell is derived from a stem cell. In some embodiments, the cell is genetically modified.

**[0160]** In some embodiments, the cell has reduced or eliminated expression of an endogenous T cell receptor.

**[0161]** In some embodiments, the cell is more prone to display central memory phenotype than a control cell lacking the recombinant polypeptide.

**[0162]** In some embodiments, the cell further comprises: (i) a chimeric antigen receptor (CAR) having specificity for a target antigen; or (ii) a recombinant T cell receptor (TCR) having specificity for a target antigen.

**[0163]** In some embodiments, the engineered cell comprises a nucleic acid construct expressing the CAR or recombinant TCR; optionally, wherein the nucleic acid construct is an RNA construct.

**[0164]** In some embodiments, the target antigen is CD1, CD1a, CD1b, CD1c, CD1d, CD1e, CD2, CD3d, CD3e, CD3g, CD3ε, CD4, CD5, CD7, CD8a, CD8b, CD19, CD20, CD21, CD22, CD23, CD24, CD25, CD27, CD28, CD30, CD33, CD34, CD38, CD40, CD44v6, CD45, CD46, CD47 CD48, CD52, CD59, CD66, CD70, CD71, CD72, CD73, CD79A, CD79B, CD80 (B7.1), CD86 (B7.2), CD94, CD95, CD97, CD123, CD134, CD140 (PDGFR4), CD152, CD154, CD158, CD171, CD178, CD179, CD179a, CD181 (CXCR1), CD182 (CXCR2), CD183 (CXCR3), CD210, CD213A2, CD246, CD252, CD253, CD261, CD262, CD272, CD273 (PD-L2), CD274 (PD-L1), CD276 (B7H3), CD279, CD295, CD339 (JAG1), CD340 (HER2), CDH17, CEA, CLECL1, CLL-1, CLDN6, CLDN18.2, CS1, DLL3, LY6G6D, GCC, p53R175H, PRAME, EGFR, EGFRvIII, FGFR2, AFP, CA125, MUC-1, MAGE, ALPI, alkaline phosphatase placental-like 2 (ALPPL2), B-cell maturation antigen (BCMA), green fluorescent protein (GFP), enhanced green fluorescent protein (eGFP), KLK2, KLK3, Mesothelin, IL13Ra2, ggcc, signal regulatory protein α (SIRPα), TCRalpha, TCRbeta, TSHR, GD2, GD3, Tn Ag, cMET, Axl, ROR1, ROR2, GPC1, GPC2, GPC3, FLT3, TAG72, CEA, EPCAM, KIT (CD117), IL-13Ra2, IL-11Ra, PSCA, PRSS21, VEGFR2, LewisY, PDGFRβ, SSEA-4, folate receptor alpha, ERBB2 (Her2/neu), MUC1, MUC16, NCAM, prostase, PAP, ELF2M, Ephrin B2, IGF-I receptor, CAIX, LMP2, gp100, bcr-abl, tyrosinase, EphA2, STEAP1, STEAP2, fucosyl GM1, sLe, GM3, TGS5, HMWMAA, o-acetyl-GD2, folate receptor beta, TEM1/CD248, TEM7R, ROPN1, GPRC5D, GPA33, CXORF61, ALK, Polysialic acid, PLAC1, GloboH, NY-BR-1, UPK2, HAVCR1, ADRB3, PANX3, GPR20, LY6K, OR51E2, TARP, WT1, NY-ESO-1, LAGE-la, MAGE-Al, legumain, HPV E6,E7, MAGE-A4, ETV6-AML, sperm protein 17, XAGE1, Tie 2, MAD-CT-1, MAD-CT-2, Fos-related antigen 1, p53, p53 mutant, p53R175H, KRAS, mutant KRAS, KRAS G12D, prostein, surviving, telomerase, PCTA-1/Galectin 8, MelanA/MART1, Ras mutant, hTERT, sarcoma translocation breakpoints, ML-IAP, ERG (TMPRSS2 ETS fusion gene), NA17, PAX3, androgen receptor, cyclin Bl, MYCN, RhoC, TRP-2, CYP1B1, BORIS, SART3, PAX5, OY-TES1, LCK, AKAP-4, SSX2, RAGE-1, human telomerase reverse transcriptase, RU1, RU2, intestinal carboxyl esterase, mut hsp70-2,LAIR1, FCAR, LILRA2, CD300LF, CLEC12A, BST2, EMR2, LY75, FCRL5, IGLL1, PSMA, TROP2, citrullinated vimentin, the extracellular portion of the APRIL protein, and any combinations thereof. In some embodiments, the target antigen is B7H3, BCMA, CD19, CD20, CD22, CD70, CD79a, CD79b, CDH17, CLDN6, CLDN18.2, DLL3, GCC, GD2, GD3, GPC3, GPRC5D, GPA33, KLK3, LY6G6D, p53R175H, PRAME, ROPN1, STEAP1, or STEAP2.

**[0165]** In some embodiments, the engineered cell has cell surface expression of the CAR.

**[0166]** In some embodiments, the cell further comprises a chimeric auto antigen receptor (CAAR) having specificity for a target antigen. In some embodiments, the engineered cell has cell surface expression of the CAAR.

**[0167]** In some embodiments, the cell further comprises a B-cell autoantibody receptor (BAR) having specificity for a target antigen. In some embodiments, the engineered cell has cell surface expression of the BAR.

**[0168]** In some embodiments, the cell further comprises a non-coding RNA. In some embodiments, the non-coding RNA comprises a shRNA or a microRNA.

**[0169]** In some embodiments, the cell further comprises an additional potency enhancement polypeptide, a cytokine, a chemokine, a growth factor, a tolerogenic factor, a safety switch, or any combination thereof. In some embodiments, the additional potency enhancement polypeptide is or comprises a dominant negative form of an inhibitor of a cell-mediated immune response of the immune cell (e.g., TGFβR2 DNR), c-Jun, CCL19, CCL21, IL2R, IL7, IL7Ralpha, IL15, IL15RA, IL18, decoy-resistant IL18 (DR-18), MyD88/CD40, PD1-CD28 switch receptor, PD1-41BB switch receptor, CD40L-CD28 switch receptor, CTBR12 switch receptor, or CD8alpha/beta.

**[0170]** In some embodiments, the safety switch is or comprises a herpesvirus thymidine kinase (HSV-tk) gene, an Escherichia coli cytosine deaminase (EC-CD) gene, or an inducible Caspase9 (iCasp9) protein.

**[0171]** In some embodiments, the cell further comprises one or more modifications that inactivate or disrupt one or more alleles of TGFbeta, Regnase-1, FAS, PTPN2, NR4A3, CD52, PD-1, B2M, CIITA, SOCS1, CBLB, DGKalpha, and/or DGKζ.

**[0172]** In some embodiments, the cell is a mammalian cell. In some embodiments, the cell is selected from the group consisting of a human cell, a mouse cell, and a canine cell.

**[0173]** In some embodiments, the cell further comprises a sequence specific nuclease, a nucleic acid programmable DNA binding protein, an RNA guided nuclease, an RNA-guided nuclease comprising a Cas nuclease and a guide RNA (CRISPR-Cas combination), a ribonucleoprotein (RNP) complex comprising a gRNA and a Cas nuclease, a homing endonuclease, a zinc finger nuclease (ZF) nucleic acid binding entity, a transcription activator-like effector (TALE) nucleic acid binding entity, a meganuclease, a Cas nuclease, a core Cas protein, a homing endonuclease, an endonuclease-deficient-Cas protein, an enzymatically inactive Cas protein, a CRISPR- associated transposase (CAST), a Type II or Type V Cas protein, or a functional portion thereof.

**[0174]** In some embodiments, the cell further comprises Casl, Cas2, Cas3, Cas4, Cas5, Cas6, Cas7, Cas8a, Cas8b, Cas8c, Cas9, CaslO, Casl2, Casl2a (Cpfl), Casl2b (C2cl), Casl2c (C2c3), Casl 2d (CasY), Casl2e (CasX), Casl2f (C2cl0), Cas 12g, Casl2h, Casl2i, Cas 12k (C2c5), Casl3, Casl3a (C2c2), Casl3b, Casl3c, Casl3d, C2c4, C2c8, C2c9, Cmrl, Cmr2, Cmr3, Cmr4, Cmr5, Cmr6, Csdl, Csd2, Cas5d, Csel, Cse2, Cse3, Cse4, Cas5e, Csfl, Csml, Csm2, Csm3, Csm4, Csm5, Csnl, Csn2, Cstl, Cst2, Cas5t, Cshl, Csh2, Cas5h, Csal, Csa2, Csa3, Csa4, Csa5, Cas5a, CsxlO, Csxl l, Csyl, Csy2, Csy3, Csy4, Mad7, SpCas9, eSpCas9, SpCas9-HFl, HypaSpCas9, HeFSpCas9, evoSpCas9 high-fidelity variants of SpCas9, SaCas9, NmeCas9, CjCas9, StCas9, TdCas9, LbCasl2a, AsCasl2a, AacCasl2b, BhCasl2b v4, TnpB, dCas (D10A), dCas (H840A), dCasl3a, dCasl3b, or a functional fragment thereof.

**[0175]** In some embodiments, the recombinant nucleic acid sequence is integrated at exon 8 of an endogenous PIK3R3 locus. In some embodiments, the recombinant nucleic acid sequence is integrated at intron 7 of an endogenous PIK3R3 locus. In some embodiments, the recombinant nucleic acid sequence is integrated at exon 13 of an endogenous CARD11 locus. In some embodiments, the recombinant nucleic acid sequence is integrated at intron 12 of an endogenous CARD11 locus.

**[0176]** In some embodiments, the cell has reduced exhaustion, increased proliferative capacity, enhanced replicative lifespan, decreased replicative senescence, enhanced anti-tumor effect, reduced dysfunction, enhanced persistence, and/or increase intratumoral presence in vivo.

**[0177]** In some embodiments, the cell has increased or decreased signaling through the CARD11-BCL10-MALT1 complex, NF-κB, AP-1, NFAT, JAK/STAT, and/or MEK/ERK pathways.

**[0178]** In one aspect, the disclosure provides compositions comprising the engineered cell of the disclosure, or a plurality of the engineered cell of the disclosure. In some embodiments, the composition further comprises a pharmaceutically acceptable excipient.

**[0179]** In some embodiments, at least at or about 70%, 75%, 80%, 85%, 90%, or 95% of the engineered cells, or of the total cells, in the composition express the recombinant polypeptide of the disclosure.

**[0180]** In some embodiments, at least at or about 70%, 75%, 80%, 85%, 90%, or 95% of the engineered cells, or of the total cells, in the composition express the CAR or recombinant TCR. In some embodiments, at least at or about 70%, 75%, 80%, 85%, 90%, or 95% of the engineered cells, or of the total cells, in the composition do not express a gene product of an endogenous TRAC locus. In some embodiments, at least at or about 70%, 75%, 80%, 85%,

90%, or 95% of the engineered cells, or of the total cells, in the composition express the recombinant polypeptide of the disclosure; at least at or about 70%, 75%, 80%, 85%, 90%, or 95% of the engineered cells, or of the total cells, in the composition express the CAR or recombinant TCR; and/or at least at or about 70%, 75%, 80%, 85%, 90%, or 95% of the engineered cells, or of the total cells, in the composition do not express a gene product of an endogenous TRAC locus.

**[0181]** In some embodiments, the composition comprises CD4+ T cells and CD8+ T cells, and the percentage of CD4+ T cells in the composition is between at or about 20% and at or about 80%, or at or about 20%, 30%, 40%, 50%, 60%, 70%, or 80% of the total cells in the composition; and/or the percentage of CD8+ T cells in the composition is between at or about 20% and at or about 80%, or at or about 20%, 30%, 40%, 50%, 60%, 70%, or 80% of the total cells in the composition; and/or the ratio of CD4+ T cells to CD8+ T cells is from at or about 1:3 to at or about 3:1, optionally at or about 1:1.

**[0182]** In one aspect, the disclosure provides methods of preparing the engineered cell the disclosure, the method comprising expressing the recombinant polypeptide in the cell, thereby making the engineered cell.

**[0183]** In one aspect, the disclosure provides methods of preparing an engineered cell, the method comprising expressing in the cell the recombinant polypeptide of the disclosure, thereby making the engineered cell.

**[0184]** In some embodiments, the method comprises introducing into the cell the nucleic acid construct of the disclosure.

**[0185]** In one aspect, the disclosure provides methods of preparing an engineered cell, the method comprising introducing into the cell the nucleic acid construct of the disclosure, thereby making the engineered cell.

**[0186]** In some embodiments, the cell is in vivo. In some embodiments, the cell is prepared in vivo. In some embodiments, the cell is prepared in the body of a subject in need of treatment.

**[0187]** In some embodiments, the recombinant polypeptide is expressed in the cell, and/or the nucleic acid construct is introduced into the cell, by contacting the cell with a vector or a particle.

**[0188]** In some embodiments, the vector is a plasmid, a synthetic DNA vector, a linear DNA vector, a closed linear DNA vector, a phagemid vector, an RNA vector, an mRNA vector, or a viral vector. In some embodiments, the viral vector is selected from a retrovirus vector, an adenovirus vector, and an adeno-associated virus (AAV) vector. In some embodiments, the

retrovirus is a lentivirus, optionally a VSV-G pseudotyped lentivirus. In some embodiments, the AAV vector is an AAV6 vector or an AAV9 vector.

**[0189]** In some embodiments, the vector is a fusosome, a lentiviral particle engineered with the anti-CD3 Cocal glycoprotein, or an enveloped delivery vehicle. In some embodiments, the vector is a self-replicating RNA virus, an mRNA-packaging virus-like particle, or a ribonucleoprotein (RNP)-packaging virus-like particle. In some embodiments, the particle is a lipid nanoparticle (LNP), a selective organ targeting (SORT) LNP, or an antibody targeted LNP. In some embodiments, the LNP comprises: (i) an ionizable lipid (e.g., an amino lipid), (ii) a sterol or other structural lipid, (iii) a non-cationic helper lipid or phospholipid, and (iv) a PEG-lipid (e.g., a PEG-modified lipid). In some embodiments, the particle is a polymer nanoparticle or a protein nanoparticle.

**[0190]** In some embodiments, the cell is ex vivo. In some embodiments, the cell is prepared ex vivo. In some embodiments, the cell is prepared outside of the body of a subject in need of treatment.

**[0191]** In some embodiments, the nucleic acid construct is introduced into the cell by electroporation or transfection. In some embodiments, the transfection is lipofection.

**[0192]** In some embodiments, the nucleic acid construct is a plasmid, a synthetic DNA vector, a linear DNA vector, a closed linear DNA vector, a phagemid vector, an RNA vector, or an mRNA vector. In some embodiments, the nucleic acid construct is introduced via a viral vector. In some embodiments, the viral vector is selected from a retrovirus vector, an adenovirus vector, and an adeno-associated virus (AAV) vector. In some embodiments, the retrovirus is a lentivirus, optionally a VSV-G pseudotyped lentivirus. In some embodiments, the AAV vector is an AAV6 vector or an AAV9 vector.

**[0193]** In some embodiments, the method comprises genetically modifying the cell for expression of the recombinant polypeptide.

**[0194]** In some embodiments, the method comprises introducing into the cell one or more nucleic acids encoding a sequence specific nuclease or a nucleic acid programmable DNA binding protein.

**[0195]** In some embodiments, the method additionally comprises introducing into the cell one or more guide RNAs. In some embodiments, the sequence-specific nuclease is an RNA guided nuclease. In some embodiments, the sequence-specific nuclease or nucleic acid programmable DNA binding domain is a homing endonuclease, a zinc finger nuclease (ZF) nucleic acid binding entity, a transcription activator-like effector (TALE) nucleic acid binding entity, a meganuclease, a Cas nuclease, a core Cas protein, a homing endonuclease,

an endonuclease-deficient-Cas protein, an enzymatically inactive Cas protein, a CRISPR-associated transposase (CAST), a Type II or Type V Cas protein, or a functional portion thereof. In some embodiments, the Cas nuclease is Casl, Cas2, Cas3, Cas4, Cas5, Cas6, Cas7, Cas8a, Cas8b, Cas8c, Cas9, CaslO, Casl2, Casl2a (Cpfl), Casl2b (C2cl), Casl2c (C2c3), Casl 2d (CasY), Casl2e (CasX), Casl2f (C2cl0), Cas 12g, Casl2h, Casl2i, Cas 12k (C2c5), Casl3, Casl3a (C2c2), Casl3b, Casl3c, Casl3d, C2c4, C2c8, C2c9, Cmrl, Cmr2, Cmr3, Cmr4, Cmr5, Cmr6, Csdl, Csd2, Cas5d, Csel, Cse2, Cse3, Cse4, Cas5e, Csfl, Csml, Csm2, Csm3, Csm4, Csm5, Csnl, Csn2, Cstl, Cst2, Cas5t, Cshl, Csh2, Cas5h, Csal, Csa2, Csa3, Csa4, Csa5, Cas5a, CsxlO, Csxl l, Csyl, Csy2, Csy3, Csy4, Mad7, SpCas9, eSpCas9, SpCas9-HFl, HypaSpCas9, HeFSpCas9, and evoSpCas9 high-fidelity variants of SpCas9, SaCas9, NmeCas9, CjCas9, StCas9, TdCas9, LbCasl2a, AsCasl2a, AacCasl2b, BhCasl2b v4, TnpB, dCas (D10A), dCas (H840A), dCasl3a, dCasl3b, or a functional portion thereof. In some embodiments, the RNA-guided nuclease is a Life Edit nuclease (LEG), optionally a LEG14 nuclease.

**[0196]** In some embodiments, the method comprises introducing into the cell a ribonucleoprotein (RNP) complex comprising the sequence-specific nuclease or nucleic acid programmable DNA binding.

**[0197]** In some embodiments, introducing the nucleic acid construct comprises contacting the cell with the particle of the disclosure or the vector of the disclosure.

**[0198]** In some embodiments, the nucleic acid construct is introduced via electroporation. In some embodiments, the nucleic acid construct is introduced via AAV. In some embodiments, the nucleic acid construct is introduced via lentivirus. In some embodiments, the nucleic acid construct is introduced via LNP.

**[0199]** In some embodiments, the method further comprises expressing in the cell an immune receptor that binds to an antigen target in a target cell. In some embodiments, expressing the immune receptor comprises administering a nucleic acid construct encoding the immune receptor to the cell; optionally, wherein the nucleic construct is an RNA construct.

**[0200]** In some embodiments, the immune receptor and the recombinant polypeptide are encoded by the same nucleic acid construct. In some embodiments, the immune receptor and the recombinant polypeptide are encoded by different nucleic acid constructs.

**[0201]** In some embodiments, expressing the immune receptor comprises introducing to the cell a nucleic acid construct encoding the immune receptor. In some embodiments, the nucleic acid construct encoding the immune receptor is introduced via a viral vector.

**[0202]** In some embodiments, activating the immune receptor results in the secretion of IFNg by the cell at a level that is at least 10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold higher than the IFNg secretion level of a control cell lacking the recombinant polypeptide.

**[0203]** In some embodiments, activating the immune receptor results in the secretion of IL-2 by the cell at a level that is at least 10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold higher than the IL-2 secretion level of a control cell lacking the recombinant polypeptide.

**[0204]** In some embodiments, the nucleic acid construct is inserted into an endogenous locus of the cell.

**[0205]** In some embodiments, the endogenous locus is or comprises a TRAC gene locus, a TRBC1 gene locus, a TRBC2 gene locus, a TRAJ locus, a CARD9 locus, a CARD10 locus, a CARD11 locus, a CARD14 locus, or a PIK3R3 locus. In some embodiments, the endogenous locus is or comprises a TRAC gene locus. In some embodiments, the endogenous locus is or comprises a TRAJ locus. In some embodiments, the TRAJ locus is or comprises a TRAJ intron splice acceptor locus.

**[0206]** In some embodiments, the endogenous locus is or comprises a safe harbor locus. In some embodiments, the safe harbor locus is an AAVS1, ABO, CCR5, CLYBL, CXCR4, F3, FUT1, HMGB1, KDM5D, LRP1, MICA, MICB, RHD, ROSA26, or SHS231 locus.

**[0207]** In some embodiments, the first insertion site is an exon. In some embodiments, the first insertion site is an intron. In some embodiments, the first insertion site is between an intron and an exon. In some embodiments, the first insertion site is in a regulatory region. In some embodiments, the first insertion site is 25 nucleotides or less from a protospacer adjacent motif (PAM) sequence, wherein the PAM sequence is ngg, nag, ngrrt, ngrm, nnnngatt, nnnnryac, nnagaaw, naaaac, tttv, ttn, attn, tttn, gttn, or yttn and wherein: (i) r = a or g, (ii) y = c or t, (iii) w = a or t, (iv) v = a or c or g, and (v) n = a, c, t, or g.

**[0208]** In some embodiments, the cell comprises a modified TRAC gene locus. In some embodiments, the cell does not express an endogenous TCR.

**[0209]** In some embodiments, the method further comprises introducing a gene editing machinery into the cell. In some embodiments, the step of introducing comprises homology-directed repair (HDR)-mediated insertion using the gene editing machinery. In some embodiments, the gene editing machinery is CRISPR/Cas9. In some embodiments, the gene editing machinery is introduced via electroporation. In some embodiments, the nucleic acid

construct is introduced via AAV. In some embodiments, the nucleic acid construct is introduced via LNP.

**[0210]** In some embodiments, the method thereby produces an engineered cell comprising: (i) the recombinant polypeptide; (ii) the immune receptor (e.g., CAR); and (iii) the modified TRAC gene locus.

**[0211]** In some embodiments, the cell is for use in a cell therapy.

**[0212]** In one aspect, the disclosure provides methods of treating a subject, comprising administering to the subject the cell of the disclosure, or the cell prepared by the method of the disclosure. In some embodiments, the subject has a cancer or an autoimmune disease.

**[0213]** In one aspect, the disclosure provides uses of the cell of the disclsoure, or the cell prepared by the method of the disclsoure, for the manufacture of a medicament for the treatment of a disease. In some embodiments, the disease is a cancer or an autoimmune disease.

**[0214]** In some embodiments, the autoimmune disease is lupus, systemic lupus erythematosus, rheumatoid arthritis, psoriasis, psoriatic arthritis, multiple sclerosis, Crohn's disease, ulcerative colitis, Addison's disease, Graves' disease, Sjogren's syndrome, Hashimoto's thyroiditis, or celiac disease.

**[0215]** In some embodiments, the cancer is a hematological cancer. In some embodiments, the cancer is myeloid neoplasm, myelodysplastic syndromes (MDS), myeloproliferative/myelodysplastic syndromes, acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), chronic myelogenous leukemia (CML), blast crisis chronic myelogenous leukemia (bcCML), B-cell acute lymphoid leukemia (B-ALL), T-cell acute lymphoid leukemia (T-ALL), T-cell lymphoma, or B-cell lymphoma.

**[0216]** In some embodiments, the cancer is a solid tumor. In some embodiments, the cancer is small cell lung cancer, colorectal cancer, testicular cancer, ovarian cancer, melanoma, lymphoma, leukemia, multiple myeloma, prostate cancer, breast cancer, non-small cell lung cancer, gastric cancer, esophageal cancer, liver cancer, kidney cancer, head & neck cancer, glioblastoma, neuroblastoma, soft tissue sarcoma, uterine cancer, brain cancer, skin cancer, renal cancer, bladder cancer, pancreatic cancer, thyroid cancer, eye cancer, gastrointestinal cancer, carcinoma, or sarcoma.

**[0217]** In some embodiments, the cancer expresses CD1, CD1a, CD1b, CD1c, CD1d, CD1e, CD2, CD3d, CD3e, CD3g, CD3ε, CD4, CD5, CD7, CD8a, CD8b, CD19, CD20, CD21, CD22, CD23, CD24, CD25, CD27, CD28, CD30, CD33, CD34, CD38, CD40, CD44v6,

CD45, CD46, CD47 CD48, CD52, CD59, CD66, CD70, CD71, CD72, CD73, CD79A, CD79B, CD80 (B7.1), CD86 (B7.2), CD94, CD95, CD97, CD123, CD134, CD140 (PDGFR4), CD152, CD154, CD158, CD171, CD178, CD179, CD179a, CD181 (CXCR1), CD182 (CXCR2), CD183 (CXCR3), CD210, CD213A2, CD246, CD252, CD253, CD261, CD262, CD272, CD273 (PD-L2), CD274 (PD-L1), CD276 (B7H3), CD279, CD295, CD339 (JAG1), CD340 (HER2), CDH17, CEA, CLECL1, CLL-1, CLDN6, CLDN18.2, CS1, DLL3, LY6G6D, GCC, p53R175H, PRAME, EGFR, EGFRvIII, FGFR2, AFP, CA125, MUC-1, MAGE, ALPI, alkaline phosphatase placental-like 2 (ALPPL2), B-cell maturation antigen (BCMA), green fluorescent protein (GFP), enhanced green fluorescent protein (eGFP), KLK2, KLK3, Mesothelin, IL13Ra2, ggcc, signal regulatory protein α (SIRPα), TCRalpha, TCRbeta, TSHR, GD2, GD3, Tn Ag, cMET, Axl, ROR1, ROR2, GPC1, GPC2, GPC3, FLT3, TAG72, CEA, EPCAM, KIT (CD117), IL-13Ra2, IL-11Ra, PSCA, PRSS21, VEGFR2, LewisY, PDGFRβ, SSEA-4, folate receptor alpha, ERBB2 (Her2/neu), MUC1, MUC16, NCAM, prostase, PAP, ELF2M, Ephrin B2, IGF-I receptor, CAIX, LMP2, gp100, bcr-abl, tyrosinase, EphA2, STEAP1, STEAP2, fucosyl GM1, sLe, GM3, TGS5, HMWMAA, o-acetyl-GD2, folate receptor beta, TEM1/CD248, TEM7R, ROPN1, GPRC5D, GPA33, CXORF61, ALK, Polysialic acid, PLAC1, GloboH, NY-BR-1, UPK2, HAVCR1, ADRB3, PANX3, GPR20, LY6K, OR51E2, TARP, WT1, NY-ESO-1, LAGE-la, MAGE-Al, legumain, HPV E6,E7, MAGE-A4, ETV6-AML, sperm protein 17, XAGE1, Tie 2, MAD-CT-1, MAD-CT-2, Fos-related antigen 1, p53, p53 mutant, p53R175H, KRAS, mutant KRAS, KRAS G12D, prostein, surviving, telomerase, PCTA-1/Galectin 8, MelanA/MART1, Ras mutant, hTERT, sarcoma translocation breakpoints, ML-IAP, ERG (TMPRSS2 ETS fusion gene), NA17, PAX3, androgen receptor, cyclin Bl, MYCN, RhoC, TRP-2, CYP1B1, BORIS, SART3, PAX5, OY-TES1, LCK, AKAP-4, SSX2, RAGE-1, human telomerase reverse transcriptase, RU1, RU2, intestinal carboxyl esterase, mut hsp70-2,LAIR1, FCAR, LILRA2, CD300LF, CLEC12A, BST2, EMR2, LY75, FCRL5, IGLL1, PSMA, TROP2, citrullinated vimentin, the extracellular portion of the APRIL protein, or any combination thereof. In some embodiments, the cancer expresses B7H3, BCMA, CD19, CD20, CD22, CD70, CD79a, CD79b, CDH17, CLDN6, CLDN18.2, DLL3, GCC, GD2, GD3, GPC3, GPRC5D, GPA33, KLK3, LY6G6D, p53R175H, PRAME, ROPN1, STEAP1, or STEAP2.

**[0218]** In some embodiments, the subject is not administered a lymphodepletive agent within 7 days prior to administration of the cell. In some embodiments, the subject is not administered cyclophosphamide, fludarabine, or bendamustine within 7 days prior to

administration of the cell. In some embodiments, the subject is not administered at least 600,000 IU/kg of IL-2 every 8 hours.

**[0219]** In some embodiments, the method does not comprise a checkpoint therapy which blocks PD-1 or CTLA-4 signaling.

**[0220]** In some embodiments, the cell has reduced exhaustion, increased proliferative capacity, enhanced replicative lifespan, decreased replicative senescence, enhanced anti-tumor effect, reduced dysfunction, enhanced persistence, and/or increase intratumoral presence in vivo. In some embodiments, the cell has increased or decreased signaling through the CARD11-BCL10-MALT1 complex, NF-κB, AP-1, NFAT, JAK/STAT, and/or MEK/ERK pathways.

**[0221]** In some embodiments, the subject is administered one or more additional therapeutic agents. In some embodiments, the one or more additional therapeutic agents are selected from the group consisting of a small molecule inhibitor, a chemotherapeutic agent, a cancer immunotherapy, an antibody, a cellular therapy, a nucleic acid, a surgery, a radiotherapy, an anti-angiogenic therapy, an anti-DNA repair therapy, an anti-inflammatory therapy, an anti-neoplastic agent, a growth inhibitory agent, a cytotoxic agent, a vaccine, a small molecule agonist, a virus-based therapy, an antibody-drug conjugate, a recombinant protein, a fusion protein, a natural compound, a peptide, a PROteolysis-TArgeting Chimera (PROTAC), and any combination thereof.

## BRIEF DESCRIPTION OF THE DRAWINGS

**[0222]** **FIGS. 1A-1F** provide an overview of CARD11-PIK3R3 signaling as well as schematics of the patient derived CARD11-PIK3R3 fusion protein and a selection of the engineered CARD11-PIK3R3 fusion proteins described herein. **FIG. 1A** shows a schematic of the CARD11-PIK3R3 fusion protein and identifies the portions of the fusion derived from CARD11 (i.e., the CARD domain, coiled-coil domain, and inhibitory domain with representative phosphorylation sites) and the portion of the fusion derived from PIK3R3 (i.e., the SH2 domain). **FIGS. 1B-1D** depict CAR signaling and CARD11-PIK3R3 signaling in different cell states. **FIG. 1B** depicts that, in the absence of tumor antigen, there is minimal CAR signaling and CARD11-PIK3R3 signaling. **FIG. 1C** depicts that, upon initial binding of tumor antigen, CAR signaling is initiated and results in phosphorylated T cell signaling adapters that become available as substrates for CARD11-PIK3R3 interaction via the PIK3R3 SH2 domain. **FIG. 1D** depicts that, upon further CAR signaling, phosphorylation of the inhibitory domain causes conformational changes in the CARD11-

PIK3R3 fusion protein that lead to robust CARD11, Bcl10, Malt1 (CBM) complex signaling (culminating in, for example and without limitation, NF-kB activation, JnK activation, and paracaspase signaling). **FIG. 1E** depicts that there is minimal CAR signaling and CARD11-PIK3R3 signaling in the absence of tumor antigen (upon elimination of the tumor cell) due to, without wishing to be bound by theory, the resumption of autoinhibition of the CARD11-PIK3R3 fusion protein. **FIG. 1F** is a schematic depicting the patient derived CARD11-PIK3R3 fusion protein and two optimized CARD11-PIK3R3 fusion proteins. The schematic depicts the locations of the corresponding CARD domains, coiled-coil domains, inhibitory domains (ID), SH2 domains, and fusion breakpoints.

**[0223] FIGS. 2A-2B** show the T cell phenotype of primary human T cells isolated from human donors transduced with TCR alone, a TCR and a patient-derived CARD11-PIK3R3 fusion (TCR+C11P3), a TCR and an a first optimized CARD11-PIK3R3 fusion (TCR+C11P3tr1), a TCR and an a second optimized CARD11-PIK3R3 fusion (TCR+C11P3tr2), the patient-derived CARD11-PIK3R3 fusion alone (C11P3), the first optimized CARD11-PIK3R3 fusion alone (C11P3tr1), or the second optimized CARD11-PIK3R3 fusion alone (C11P3tr2). **FIG. 2A** shows the percentage of cells with a particular phenotype, and **FIG. 2B** shows flow cytometry data showing the percentage of cells expressing CCR7 and CD45RA. **FIGS. 2A and 2B** demonstrate that T cells transduced with the patient derived and optimized fusions (alone or in combination with a TCR) induce a greater central memory phenotype as compared T cells transduced with a TCR alone

**[0224] FIGS. 3A-3B** show the level of IFNgamma (**FIG. 3A)** and IL-2 secretion (**FIG. 3B)** for T cells transduced with TCR alone, a TCR and a patient-derived CARD11-PIK3R3 fusion (TCR+C11P3), a TCR and an a first optimized CARD11-PIK3R3 fusion (TCR+C11P3tr1), a TCR and an a second optimized CARD11-PIK3R3 fusion (TCR+C11P3tr2), the patient-derived CARD11-PIK3R3 fusion alone (C11P3), the first optimized CARD11-PIK3R3 fusion alone (C11P3tr1), or the second optimized CARD11-PIK3R3 fusion alone (C11P3tr2) after co-culture with two different antigen positive cell lines and one antigen negative cell line. **FIG. 3A** shows that T cells expressing a TCR and the patient derived or optimized fusions secreted higher levels of IFNgamma compared to T cells express a TCR alone. **FIG. 3B** shows that T cells expressing a TCR and the patient derived or optimized fusions secreted higher levels of IL-2 compared to T cells express a TCR alone.

**[0225] FIGS. 4A-4D** show the percentage of primary T cells that are positive for a CAR and negative for CD3 (as a marker for TCR expression) after transduction with lentiviral vectors

encoding the CAR or CAR+fusion constructs and editing the TRAJ intron splice acceptor locus to knockout TCR expression. **FIG. 4A** shows the percentage of cells that are CAR+, **FIG. 4B** shows the MFI, **FIG. 4C** shows the percentage of cells that are CD3 negative, and **FIG. 4D** shows flow cytometry data showing the percentage of cells expressing CAR and CD3.

**[0226] FIGS. 5A-5B** show the T cell phenotype of primary human T cells isolated from human donors that were untransduced with no TCR knockout (UTD no KO), untransduced with TCR knockout (UTD KO), transduced with CAR alone (CAR), a CAR and a patient-derived fusion (CAR P2A C11P3), or a CAR and an optimized fusion (CAR P2A C11P3tr1). **FIG. 5A** shows the percentage of cells with a particular phenotype, and **FIG. 5B** shows flow cytometry data showing the percentage of cells expressing CCR7 and CD45RA. **FIGS. 5A and 5B** show that cells transduced with CAR+C11P3 or CAR+C11P3tr1 had greater central memory phenotype than cells transduced with CAR alone.

**[0227] FIGS. 6A-6B** show the level of IL-2 secretion (**FIG. 6A) and** IFNgamma (**FIG. 6B)** secretion for untransduced T cells or T cells transduced with CAR alone (CAR), a CAR and a patient-derived fusion (CAR+C11P3), or a CAR and an optimized fusion (CAR+C11P3tr1) after co-culture with an antigen positive cell line and an antigen negative cell line. **FIG. 6A** shows that T cells expressing a CAR and the patient derived or optimized fusions secreted higher levels of IL-2 compared to T cells express a CAR alone. **FIG. 6B** shows that T cells expressing a CAR and the patient derived or optimized fusions secreted higher levels of IFNgamma compared to T cells express a CAR alone.

**[0228] FIGS. 7A-7B** show primary T cells edited with a CAR or a CAR plus an optimized fusion (CAR+C11P3tr1) knocked-into the TRAJ intron splice acceptor locus with expression under control of the endogenous TRAC promoter. Editing was done via AAV and CRISPR/Cas9 mediated homology directed repair at two AAV MOI doses. **FIG. 7A** shows the percentage of cells that are CAR+, **FIG. 7B** shows the MFI, **FIG. 7A** shows that the engineered CARD11-PIK3R3 fusion proteins, which are shorter than the patient-derived fusions can be delivered with a CAR via a single AAV6 vector. **FIG. 7B** shows that there is no significant difference in CAR MFI between the different constructs.

**[0229] FIG. 8** shows the T cell phenotype of primary human T cells isolated from human donors that were untransduced but had the TCR knockout (UTD KO), transduced with CAR alone (CAR) at two different MOI doses (1e5 or 5e5), or a CAR and an optimized fusion (CAR C11P3tr1) at two different MOI doses (1e5 or 5e5). **FIG. 8** shows there are not

significant differences in T cell phenotype when comparing AAV6 delivered CAR T cells and AAV6 delivered CAR+C11P3tr1 cells.

**[0230] FIGS. 9A-9B** show the level of IFNgamma (**FIG. 9A)** and IL-2 secretion (**FIG. 9B)** for untransduced but had the TCR knockout (UTD KO), transduced with CAR alone (CAR) at two different MOI doses (1e5 or 5e5), or a CAR and an optimized fusion (CAR C11P3tr1) at two different MOI doses (1e5 or 5e5) after co-culture with an antigen high cell line, and antigen low cell line, and an antigen negative cell line. **FIG. 9A** shows that T cells expressing a CAR and an optimized fusion secreted IFNgamma in an antigen dependent manner. **FIG. 9B** shows that T cells expressing a CAR and an optimized fusion secreted IL-2 in an antigen dependent manner.

**[0231] FIGS. 10A-10D** show the results of an in vivo experiment where mice treated with CAR T cells harboring the patient derived CARD11-PIK3R3 fusion protein or CAR T cells harboring an engineered CARD11-PIK3R3 fusion protein were able to clear tumors upon multiple tumor challenges. **FIG. 10A** shows measurements of tumor volumes over time for mice bearing SHP77 tumors treated with untransduced T cells. **FIG. 10B** shows measurements of tumor volumes over time for mice bearing SHP77 tumors treated with CAR only T cells (dark grey line) compared to untransduced T cells (light grey line). Finally, **FIGS. 10C and 10D** show measurements of tumor volumes over time for mice bearing SHP77 tumors treated with CAR plus the patient derived CARD11-PIK3R3 fusion protein (**FIG. 10C**; dark grey line) or treated with CAR plus an engineered CARD11-PIK3R3 fusion protein (**FIG. 10D**; dark grey line).

**[0232] FIGS. 11A-11G** show the results of an in vivo experiment where mice treated with OT-I T cells harboring the patient derived CARD11-PIK3R3 fusion protein or OT-I T cells harboring an engineered CARD11-PIK3R3 fusion protein were able to clear tumors at low doses. **FIG. 11A** shows measurements of tumor volumes over time for mice bearing B16-Ova cells treated with untransduced OT-I T cells. **FIGS. 11B-11D** show measurements of tumor volumes over time for mice bearing B16-Ova tumors treated with 50,000 (**FIG. 11B**), 10,000 (**FIG. 11C**), or 2,000 (**FIG. 11D**) OT-I T cells harboring the patient derived CARD11-PIK3R3 fusion protein. **FIGS. 11E-11G** show measurements of tumor volumes over time for mice bearing B16-Ova tumors treated with 50,000 (**FIG. 11E**), 10,000 (**FIG. 11F**), or 2,000 (**FIG. 11G**) OT-I T cells harboring an engineered CARD11-PIK3R3 fusion protein.

**[0233] FIGS. 12A-12H** show the results of in vitro and in vivo assays comparing the performance of CAR T cells co-expressing a TGFbR2-DNR protein and CAR T cells co-

expressing an engingeered CARD11-PIK3R3 fusion protein. **FIG. 12A** shows interferon gamma (IFNγ) expression for untransduced T cells, CAR T cells co-expressing a TGFbR2-DNR protein, or CAR T cells co-expressing an engineered CARD11-PIK3R3 fusion protein co-cultured with two different SCLC tumor cell lines (SHP77 and DMS273). **FIG. 12B** shows interleukin-2 (IL-2) expression for untransduced T cells, CAR T cells co-expressing a TGFbR2-DNR protein, or CAR T cells co-expressing an engineered CARD11-PIK3R3 fusion protein co-cultured with two different SCLC tumor cell lines (SHP77 and DMS273). **FIGS. 12C and 12D** show in vitro growth of SHP77 (**FIG. 12C**) tumor cells or DMS273 tumor cells (**FIG. 12D**) co-cultured with untransduced T cells, CAR T cells co-expressing a TGFbR2-DNR protein, or CAR T cells co-expressing an engineered CARD11-PIK3R3 fusion protein. **FIGS. 12E and 12G** show measurements of tumor volumes over time for mice bearing SHP77 tumors treated with either $2x10^5$ (**FIG. 12E**) or $1x10^6$ (**FIG. 12G**) untransduced T cells (light grey line) or CAR T cells co-expressing a TGFbR2-DNR protein (dark grey line). **FIGS. 12F and 12H** show measurements of tumor volumes over time for mice bearing SHP77 tumors treated with either $2x10^5$ (**FIG. 12F**) or $1x10^6$ (**FIG. 12H**) untransduced T cells (light grey line) or CAR T cells co-expressing an engineered CARD11-PIK3R3 fusion protein (dark grey line).

**[0234] FIGS. 13A-13D** show schematics for both non-targeted and targeted methods of expressing a CARD11-PIK3R3 fusion protein using, for example, LVV delivery (**FIG. 13A**), targeted insertion of a CARD11-PIK3R3 fusion protein using, for example, an RNA targeted nuclease and AAV (**FIG. 13B**), and re-creation of a CARD11-PIK3R3 fusion protein using, for example, dual/twin prime editing or PASSIGE to insert an SH2 gene fragment into exon 13 or exon 14 (or intron 12 or intron 13) of the CARD11 gene locus (**FIGS. 13C and 13D**).

**[0235]** In the following detailed description, reference is made to the Figures, which form a part hereof. In the Figures, similar symbols generally identify similar components, unless context dictates otherwise. The illustrative alternatives described in the detailed description, drawings, and claims are not meant to be limiting. Other alternatives may be used and other changes may be made without departing from the spirit or scope of the subject matter presented here. It will be readily understood that the aspects, as generally described herein, and illustrated in the Figures, can be arranged, substituted, combined, and designed in a wide variety of different configurations, all of which are explicitly contemplated and make part of this application.

**[0236]** In the following detailed description, reference is made to the Figures, which form a part hereof. In the Figures, similar symbols generally identify similar components, unless context dictates otherwise. The illustrative alternatives described in the detailed description, drawings, and claims are not meant to be limiting. Other alternatives may be used and other changes may be made without departing from the spirit or scope of the subject matter presented here. It will be readily understood that the aspects and embodiments, as generally described herein, and illustrated in the Figures, can be arranged, substituted, combined, and designed in a wide variety of different configurations (for example, combined with other aspects and/or embodiments described herein), all of which are explicitly contemplated and make part of this application.

## DETAILED DESCRIPTION OF THE DISCLOSURE

**[0237]** In general, the present disclosure relates, *inter alia*, to compositions and methods for improving adoptive T cell therapy. The disclosers have discovered ways to improve the therapeutic efficacy of T cells by altering T cell signaling, decreasing T cell exhaustion and/or by enhancing *in vivo* persistence and fitness of engineered T cells. T cell cancers can undergo positive selection of beneficial genetic alterations (e.g., mutations). In some embodiments, the present disclosure relates to exploiting such beneficial mutations to improve the effectiveness of T cell therapeutics.

### I. DEFINITIONS

**[0238]** Unless otherwise defined, all terms of art, notations, and other scientific terms or terminology used herein are intended to have the meanings commonly understood by those of skill in the art to which this application pertains. In some cases, terms with commonly understood meanings are defined herein for clarity and/or for ready reference, and the inclusion of such definitions herein should not necessarily be construed to represent a substantial difference over what is generally understood in the art. Many of the techniques and procedures described or referenced herein are well understood and commonly employed using conventional methodology by those skilled in the art. All publications, patent applications, patents, GenBank or other accession numbers and other references mentioned herein are incorporated by reference in their entirety for all purposes.

**[0239]** The singular form “a”, “an”, and “the” include plural references unless the context clearly dictates otherwise. For example, the term “a cell” includes one or more cells,

including mixtures thereof. “A and/or B” is used herein to include all of the following alternatives: “A”, “B”, “A or B”, and “A and B.”

**[0240]** Where a range of values is provided, it is understood that each intervening value, to the tenth of the unit of the lower limit unless the context clearly dictates otherwise, between the upper and lower limit of that range and any other stated or intervening value in that stated range, is encompassed within the disclosure. The upper and lower limits of these smaller ranges may independently be included in the smaller ranges, and are also encompassed within the disclosure, subject to any specifically excluded limit in the stated range. Where the stated range includes one or both of the limits, ranges excluding either or both of those included limits are also included in the disclosure.

**[0241]** Certain ranges are presented herein with numerical values being preceded by the term “approximately” or “about.” The terms “approximately” and “about” are used interchangeably and mean a quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length that varies by up to 20%, preferably up to 10%, more preferably up to 5%, and more preferably still up to 1%, of a reference quantity, level, value, number, frequency, percentage, dimension, size, amount, weight or length, inclusive of the endpoints. When the term "about" is used in conjunction with a numerical range, it modifies that range by extending the boundaries above and below the numerical values set forth, unless otherwise apparent from context that it is impossible to extend the boundary beyond certain points (*e.g.*, below 0% or above 100% in some cases). Further, the terms “approximately” and “about’ are used herein to provide literal support for the exact number that it precedes, as well as a number that is near to or approximately the number that the term precedes. In determining whether a number is near to or approximately a specifically recited number, the near or approximating unrecited number may be a number which, in the context in which it is presented, provides the substantial equivalent of the specifically recited number. For example, “about” can mean within 1 or more than 1 standard deviation, per the practice in the art. Alternatively, “about” can mean a range of up to 20%, preferably up to 10%, more preferably up to 5%, and more preferably still up to 1% of a given value. Alternatively, particularly with respect to biological systems or processes, the term can mean within an order of magnitude, preferably within 5-fold, and more preferably within 2-fold, of a value.

**[0242]** As used herein, the term “comparable level” refers to a level that is in between 50% and 200%, inclusive of end points, of the reference level in the control set of conditions or circumstances, unless otherwise apparent from context that it is impossible to extend the

boundary beyond certain points. In some embodiments, a comparable level may be preferably between 60% and 170%, more preferably between 70% and 140%, even more preferably between 80% and 125%, or more preferably still between 90% and 110%, of the reference level. Those of ordinary skill in the art will appreciate the appropriate control set in obtaining the reference level. In general, the appropriate control set shares identifical features with the test set except the feature(s) of interest, so that the observed results warrant a reasonable conclusion that any changes in the level observed are caused by or indicative of the variation in the feature(s) that are varied.

**[0243]** As will be understood by one having ordinary skill in the art, for any and all purposes, such as in terms of providing a written description, all ranges disclosed herein also encompass any and all possible sub-ranges and combinations of sub-ranges thereof. Any listed range can be easily recognized as sufficiently describing and enabling the same range being broken down into at least equal halves, thirds, quarters, fifths, tenths, etc. As a non-limiting example, each range discussed herein can be readily broken down into a lower third, middle third and upper third, etc. As will also be understood by one skilled in the art all language such as "up to," "at least," "greater than," "less than," and the like include the number recited and refer to ranges which can be subsequently broken down into sub-ranges as discussed above. Finally, as will be understood by one skilled in the art, a range includes each individual member. Thus, for example, a group having 1-3 articles refers to groups having 1, 2, or 3 articles. Similarly, a group having 1-5 articles refers to groups having 1, 2, 3, 4, or 5 articles, and so forth.

**[0244]** It is understood that aspects and embodiments of the disclosure described herein include "comprising," "consisting," and "consisting essentially of" aspects and embodiments. As used herein, "comprising" is synonymous with "including," "containing," or "characterized by," and is inclusive or open-ended and does not exclude additional, unrecited elements or method steps. As used herein, "consisting of" excludes any elements, steps, or ingredients not specified in the claimed composition or method. As used herein, "consisting essentially of" does not exclude materials or steps that do not materially affect the basic and novel characteristics of the claimed composition or method. Any recitation herein of the term "comprising", particularly in a description of components of a composition or in a description of steps of a method, is understood to encompass those compositions and methods consisting essentially of and consisting of the recited components or step.

**[0245]** The term "percent identity," as used herein in the context of two or more nucleic acids or proteins, refers to two or more sequences or subsequences that are the same or have a specified percentage of nucleotides or amino acids that are the same (e.g., about 60% sequence identity, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or higher identity over a specified region, when compared and aligned for maximum correspondence over a comparison window or designated region) as measured using a BLAST or BLAST 2.0 sequence comparison algorithms with default parameters described below, or by manual alignment and visual inspection. See e.g., the NCBI web site at ncbi.nlm.nih.gov/BLAST. Such sequences are then the to be "substantially identical." This definition also refers to, or may be applied to, the complement of a test sequence. This definition also includes sequences that have deletions and/or additions, as well as those that have substitutions. Generally, sequence identity can exist over a region that is at least about 20 amino acids or nucleotides in length, or over a region that is 10-100 amino acids or nucleotides in length, or over the entire length of a given sequence.

**[0246]** If necessary, sequence identity can be calculated using published techniques and widely available computer programs, such as the GCS program package (Devereux et al, Nucleic Acids Res. 12:387, 1984), BLASTP, BLASTN, FASTA (Atschul et al., J. Molecular Biol. 215:403, 1990). Sequence identity can be measured using sequence analysis software such as the Sequence Analysis Software Package of the Genetics Computer Group at the University of Wisconsin Biotechnology Center (1710 University Avenue, Madison, Wis. 53705), with the default parameters thereof.

**[0247]** As used herein, the term "mutation" refers to a point mutation, a gene fusion, a substitution, a gain-of-function mutation, a stop-gain mutation, an insertion mutation, a deletion mutation, a duplication mutation and/or a translocation. The mutation may be in one or more genes. The mutation may be naturally occurring. Alternatively, the mutation may be induced or engineered. As used herein, the term "vector" refers to a nucleic acid construct designed for transfer between host cells, and that may be used for the purpose of transformation, e.g., the introduction of heterologous DNA into a host cell. As such, in some embodiments, the vector can be a replicon, such as a plasmid, phage, or cosmid, into which another DNA segment may be inserted so as to bring about the replication of the inserted segment. In some embodiments, the expression vector can be an integrating vector.

**[0248]** As used herein, the term "viral vector" refers either to a nucleic acid molecule (e.g., a transfer plasmid) that includes virus-derived nucleic acid elements that generally facilitate transfer of the nucleic acid molecule or integration into the genome of a cell or to a viral

particle that mediates nucleic acid transfer. Viral particles will generally include various viral components and sometimes also host cell components in addition to nucleic acid(s). The term viral vector may refer either to a virus or viral particle capable of transferring a nucleic acid into a cell or to the transferred nucleic acid itself. Viral vectors and transfer plasmids contain structural and/or functional genetic elements that are primarily derived from a virus. Viral vectors that can be used in the disclosure include, for example, retrovirus vectors, adenovirus vectors, and adeno-associated virus vectors, lentivirus vectors, herpes virus, simian virus 40 (SV40), and bovine papilloma virus vectors (see, for example, Gluzman (Ed.), Eukaryotic Viral Vectors, CSH Laboratory Press, Cold Spring Harbor, N.Y.). For example, a recombinant polypeptide as disclosed herein can be produced in a eukaryotic host, such as a mammalian cells (e.g., COS cells, NIH 3T3 cells, or HeLa cells). These cells are available from many sources, including the American Type Culture Collection (Manassas, VA). In selecting an expression system, care should be taken to ensure that the components are compatible with one another. Artisans or ordinary skill are able to make such a determination. Furthermore, if guidance is required in selecting an expression system, skilled artisans may consult P. Jones, "Vectors: Cloning Applications", John Wiley and Sons, New York, N.Y., 2009).

**[0249]** As used herein, the term "retroviral vector" refers to a viral vector or plasmid containing structural and functional genetic elements, or portions thereof, that are primarily derived from a retrovirus. The retroviral vector can be a lentiviral vector. As used herein, the term "lentiviral vector" refers to a viral vector or plasmid containing structural and functional genetic elements, or portions thereof, including LTRs that are primarily derived from a lentivirus, which is a genus of retrovirus. Lentiviral vectors offer several attractive properties as gene-delivery vehicles, including: (i) sustained gene delivery through stable vector integration into host genome; (ii) the capability of infecting both dividing and non-dividing cells; (iii) broad tissue tropisms, including important gene- and cell-therapy-target cell types; (iv) no expression of viral proteins after vector transduction; (v) the ability to deliver complex genetic elements, such as polycistronic or intron-containing sequences; (vi) a potentially safer integration site profile; and (vii) a relatively easy system for vector manipulation and production.

**[0250]** As used herein, the term "pharmaceutically acceptable carrier" as used herein means any suitable carriers, diluents or excipients. These include all aqueous and non-aqueous isotonic sterile injection solutions, which may contain anti-oxidants, buffers and solutes, which render the composition isotonic with the blood of the intended recipient; aqueous and

non-aqueous sterile suspensions, which may include suspending agents and thickening agents, dispersion media, antifungal and antibacterial agents, isotonic and absorption agents and the like. It will be understood that compositions of the present disclosure may also include other supplementary physiologically active agents. The carrier must be pharmaceutically "acceptable" in the sense of being compatible with the other ingredients of the composition and not injurious to the subject.

**[0251]** As used herein, the term "PEGylation" refers to modifying a protein by covalently attaching polyethylene glycol (PEG) to the protein, with "PEGylated" referring to a protein having a PEG attached. A range of PEG, or PEG derivative sizes with optional ranges of from about 10,000 Daltons to about 40,000 Daltons may be attached to the recombinant polypeptides of the disclosure using a variety of chemistries. In some embodiments, the average molecular weight of the PEG, or PEG derivative, is about 1 kD to about 200 kD such as, e.g., about 10 kD to about 150 kD, about 50 kD to about 100 kD, about 5 kD to about 100 kD, about 20 kD to about 80 kD, about 30 kD to about 70 kD, about 40 kD to about 60 kD, about 50 kD to about 100 kD, about 100 kD to about 200 kD, or about 150 kD to about 200 kD. In some embodiments, the average molecular weight of the PEG, or PEG derivative, is about 5 kD, about 10 kD, about 20 kD, about 30 kD, about 40 kD, about 50 kD, about 60 kD, about 70 kD, or about 80 kD. In some embodiments, the average molecular weight of the PEG, or PEG derivative, is about 40 kD.

**[0252]** As used herein, the terms "administration" and "administering" refer to the delivery of a bioactive composition or formulation by an administration route including, but not limited to, oral, intravenous, intra-arterial, intramuscular, intraperitoneal, subcutaneous, intramuscular, and topical administration, or combinations thereof. The term includes, but is not limited to, administering by a medical professional and self-administering.

**[0253]** As used herein, the term "injection" includes intravenous, intramuscular, intra-arterial, intrathecal, intraventricular, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, intracerebrospinal, and intrasternal injection and infusion.

**[0254]** The term "cancer" generally refers to the presence of cells possessing characteristics typical of cancer-causing cells, such as uncontrolled proliferation, immortality, metastatic potential, rapid growth and proliferation rate, and certain characteristic morphological features. Cancer cells can be in the form of a tumor, but such cells can exist alone within an animal subject, or can be a non-tumorigenic cancer cell, such as a leukemia cell. These terms include a solid tumor, a soft tissue tumor, or a metastatic lesion. As used herein, the term

"cancer" includes premalignant, as well as malignant cancers. In some embodiments, the cancer is a solid tumor, a soft tissue tumor, or a metastatic lesion.

**[0255]** As used herein, and unless otherwise specified, a "therapeutically effective" or "pharmaceutically effective" amount or number of a subject construct, nucleic acid, cell, or composition of the disclosure generally refer to an amount or number sufficient for a construct, nucleic acid, cell, or composition to accomplish a stated purpose relative to the absence of the composition, e.g., to provide a therapeutic benefit in the treatment or management of the cancer, or to delay or minimize one or more symptoms associated with the cancer. A therapeutically effective amount of a compound means an amount of therapeutic agent, alone or in combination with other therapeutic agents, which provides a therapeutic benefit in the treatment or management of the cancer. The term "therapeutically effective amount" can encompass an amount that improves overall therapy, reduces or avoids symptoms or causes of the cancer, or enhances the therapeutic efficacy of another therapeutic agent. An example of an "effective amount" is an amount sufficient to contribute to the treatment, prevention, or reduction of a symptom or symptoms of a disease, which could also be referred to as a "therapeutically effective amount." A "reduction" of a symptom means decreasing of the severity or frequency of the symptom(s), or elimination of the symptom(s). The exact amount of a composition including a "therapeutically effective amount" will depend on the purpose of the treatment, and will be ascertainable by one skilled in the art using known techniques (see, e.g., Lieberman, Pharmaceutical Dosage Forms (vols. 1-3, 1992); Lloyd, The Art, Science and Technology of Pharmaceutical Compounding (1999); Pickar, Dosage Calculations (1999); and Remington: The Science and Practice of Pharmacy, 20th Edition, 2003, Gennaro, Ed., Lippincott, Williams & Wilkins).

**[0256]** As used herein, a "subject" or an "individual" includes animals, such as human (e.g., human subjects) and non-human animals. In some embodiments, a "subject" or "individual" is a patient under the care of a physician. Thus, the subject can be a human patient or an individual who has, is at risk of having, or is suspected of having a disease of interest (e.g., cancer) and/or one or more symptoms of the disease. The subject can also be an individual who is diagnosed with a risk of the condition of interest at the time of diagnosis or later. The term "non-human animals" includes all vertebrates, e.g., mammals, e.g., rodents, e.g., mice, e.g. non-human primates, and non- mammals, e.g., sheep, dogs, cows, chickens, amphibians, reptiles, etc.

**[0257]** The term "engineered cell" as used herein refers to a cell that has been altered in at least some way by human intervention, including, for example, by genetic alterations or modifications such that the engineered cell differs from a wild-type cell.

**[0258]** The terms "decrease," "reduced," "reduction," and "decreased" are all used herein generally to mean a lowering by a statistically significant amount. However, for avoidance of doubt, "decrease," "reduced," "reduction," "decreased" means a lowering by at least 10% as compared to a reference level, for example a lowering by at least about 20%, or at least about 30%, or at least about 40%, or at least about 50%, or at least about 60%, or at least about 70%, or at least about 80%, or at least about 90%, or up to and including a 100% lowering (i.e. absent level as compared to a reference sample), or any lowering between 10-100% as compared to a reference level. In some embodiments, the cells are engineered to have reduced expression of one or more targets relative to an unaltered or unmodified wild-type cell.

**[0259]** The terms "increase" and "increased" are all used herein generally to mean an increase by a statistically significant amount. However, for avoidance of doubt, "increase" and "increased" mean an increase by at least 10% as compared to a reference level, for example an increase by at least about 20%, at least about 30%, at least about 40%, at least about 50%, at least about 60%, at least about 70%, at least about 80%, at least about 90%, at least 1-fold, at least 2-fold, at least 3-fold, at least 5-fold, at least 10-fold, at least 20-fold, at least 30-fold, at least 50-fold, at least 100-fold, at least 200-fold, at least 500-fold, or at least 1000-fold, or any increase between 10% and 1000-fold as compared to a reference level. In some embodiments, the cells are engineered to have increased expression of one or more targets relative to an unaltered or unmodified wild-type cell.

**[0260]** As used herein, the terms "conservative amino acid substitution" and "conservative substitution" refer to amino acid substitutions in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), beta-branched side chains (e.g., threonine, valine, isoleucine) and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine).

**[0261]** Headings, e.g., (a), (b), (i) etc., are presented merely for ease of reading the specification and claims. The use of headings in the specification or claims does not require the steps or elements be performed in alphabetical or numerical order or the order in which they are presented. It is appreciated that certain features of the disclosure, which are, for clarity, described in the context of separate embodiments, may also be provided in combination in a single embodiment. Conversely, various features of the disclosure, which are, for brevity, described in the context of a single embodiment, may also be provided separately or in any suitable sub-combination. All combinations of the embodiments pertaining to the disclosure are specifically embraced by the present disclosure and are disclosed herein just as if each and every combination was individually and explicitly disclosed. In addition, all sub-combinations of the various embodiments and elements thereof are also specifically embraced by the present disclosure and are disclosed herein just as if each and every such sub- combination was individually and explicitly disclosed herein.

## II. T CELL SIGNALING

### *a. TCR Signaling*

**[0262]** Upon engagement of a peptide-MHC complex, the immunoreceptor tyrosine-based activation motifs (ITAMs) contained within the intracellular tails of the TCR complex become phosphorylated and recruit Zap70. Zap70 then recruits and activates the downstream LAT signalosome containing LAT, PLCy, Slp76. This signalosome causes the activation of the Ras/MAPK/ERK signaling leading to AP-1 transcriptional activity, the activation of PKCθ leading to NF-κB transcriptional activity and finally the influx of calcium which is responsible for NFAT transcriptional activity. Additionally, signaling from costimulatory receptors, such as 41BB, and cytokine support from IL-2 enhances these signaling pathways, and is required for T cells to undergo full activation and avoid anergic cell death. When fully activated, T cells rapidly proliferate and differentiate, secrete inflammatory cytokines, and cytotoxic cells begin performing target killing through release of cytotoxic granules. After resolution of this acute response, activated T cells undergo contraction to a form a small population of long-lived memory T cells that surveil the body, ready to respond to future encounters with the antigen.

### *b. T Cell Signaling*

**[0263]** T cells become fully activated through T cell receptor (TCR) engagement, costimulatory signaling and cytokine support. TCR activation induces NFAT, NF-κB and AP-1 transcription factors signaling, IL-2 and other inflammatory cytokine secretion.

*c. T Cell Exhaustion*

**[0264]** T cell exhaustion is a state of hypo-responsiveness induced in effector T cell populations after chronic exposure to antigen. Exhaustion is marked by the upregulation of surface inhibitory receptors such as PD-1, TIM-3, LAG-3 and CTLA-4 among others, and the inability to proliferate long term, secrete inflammatory cytokines and kill target cells effectively. Exhausted T cells exist in a distinct transcriptional and epigenetic landscape. While exhaustion has been well characterized in chronic viral infections, it is also implicated in cancer, in which tumor infiltrating T cells, and engineered T cell therapies exhibit signs of exhaustion and fail to control tumor growth. To better treat chronic infections or cancer, T cell exhaustion must be prevented or overcome. In fact, checkpoint therapies which block signaling from the PD-1 or CTLA-4 axis of inhibition have proven somewhat effective in reinvigorating T cell responses in certain cancers leading to limited but effective tumor control.

*d. NFAT Signaling*

**[0265]** The transcription factor nuclear factor of activated T cells (NFAT) is involved in T cell activation as well as programs of T cell exhaustion. At resting state, NFAT transcription factors are found in the cytoplasm in a phosphorylated state, unable to translocate to the nucleus to induce signaling. The extracellular influx of $Ca^{2+}$ that occurs during T cell activation activates the calcium dependent phosphatase calcineurin, resulting in dephosphorylation of NFAT, and its subsequent translocation and signaling in the nucleus. NFAT binds DNA in multiple ways, as a monomer, dimer or as a complex with other transcription factors. In particular NFAT is known to cooperatively bind with the transcription factor AP-1, inducing transcription of activation associated genes critical for effector T cell function. In contrast, when NFAT is not bound by AP-1, but is instead partnerless, it has been found to induce the expression of exhaustion associated genes, such as the inhibitory receptor PD-1. Additionally, studies have determined that exhaustion induced by partnerless NFAT can be remedied in CAR T cells by over-expressing the AP-1 family member, cJun. NFAT induces the expression of transcription factors TOX and NR4A1/2/3, which have been associated with T cell exhaustion and deletion or knockdown of these transcription factors has improved T cell phenotype and *in vivo* tumor control.

**[0266]** As NFAT signaling is key to successful T cell activation, but also plays a role in exhaustion, particularly in the absence of AP-1, it can be used to determine how genetic

modifications in T cells influences the dynamics of NFAT signaling. Targeting the NFAT transcriptional pathway can be beneficial for T cell therapeutics.

***e. NF-κB Signaling***

**[0267]** NF-κB (nuclear factor kappa light chain enhancer of B cells) is a family of transcription factors that induces transcriptional programs critical for T cell activation and effector function. NF-κB signaling occurs in many cell types. In T cells, NF-κB signaling induces a wide range of transcriptional programs responsible for proliferation and memory formation, resistance to apoptosis, cytokine secretion, and the production of a robust effector T cell response. NF-κB signaling is induced through two pathways, the canonical and noncanonical pathways.

**[0268]** In the canonical pathway NFKB1 is bound in the cytoplasm by IkBa and IkB-like molecule p105, forming a complex that prevents the nuclear translocation of NKFB1. When activated, TCR signaling induces activation of PKCθ, which in turn phosphorylates and activates CARD11. Activated CARD11 forms a complex with BCL10 and MALT1, and ultimately causes the phosphorylation and degradation of IkBa and IkB-like molecule p105, releasing NF-κB to translocate to the nucleus.

**[0269]** The non-canonical pathway is triggered by signaling through tumor necrosis factor receptor (TNFR) family members, which includes the costimulatory domain 41BB. At rest, the NFKB2 is bound by p100, during activation NF-κB -inducing kinase (NIK) activates IKKa, which in turn phosphorylates p100 and leads to the release and translocation of NKFB2 to the nucleus.

**[0270]** In syngeneic models of solid tumors, endogenous T cells that can respond to the tumors required NF-κB signaling to mediate tumor clearance. While NF-κB is induced through TCR signaling, it is also stimulated through the costimulatory domain 41BB (a TNFRS superfamily member), which is used clinically in FDA approved chimeric antigen receptor (CARs) therapies. CAR therapies using 41BB costimulatory domains have been found to persist longer in patients as compared to CAR therapies using other costimulatory domains, such as CD28. The persistence of 41BB CARs is directly linked to NF-κB signaling, which improves CAR T resistance to apoptosis through suppression of apoptotic proteins such as Bim.

***f. AP-1 Signaling***

**[0271]** AP-1 transcription factors are a family of homo or hetero-dimeric proteins formed from complexes of JUN, FOS, ATF or MAF proteins. AP-1 signaling is induced by the

phosphorylation cascade called the Mitogen Activated Protein Kinase (MAPK) pathway, triggered by TCR, cytokine/chemokine, or growth factor signaling. AP-1 often signals in complex with NFAT, and as indicated in the NFAT section, the loss of AP-1 causes partnerless NFAT to induce exhaustion programs in T cells, however this exhaustion can be remedied by overexpression of the AP-1 family member c-Jun. Related to this, loss of AP-1 is also known to induce an anergic cell state, where the T cell has partially activated through the TCR, but does not have enough costimulatory and cytokine signaling to induce full activation.

**[0272]** AP-1 dimers are activated by a plethora of physiological and pathological stimuli. Studies have reported that AP-1 proteins, mostly those that belong to the Jun group, control cell life and death through their ability to regulate the expression and function of cell cycle regulators such as Cyclin D1, p53, p21(cip1/waf1), p19(ARF) and p16. Amongst the Jun proteins, c-Jun is unique in its ability to positively regulate cell proliferation through the repression of tumor suppressor gene expression and function, and induction of cyclin D1 transcription. These actions are antagonized by JunB, which upregulates tumor suppressor genes and represses cyclin D1. An important target for AP-1 effects on cell life and death is the tumor suppressor p53, whose expression as well as transcriptional activity, are modulated by AP-1 proteins.

**[0273]** The concomitant induction of NFAT and AP-1 takes concerted activation of two different signaling pathways: calcium/calcineurin, which promotes NFAT dephosphorylation, nuclear translocation and activation; and protein kinase C (PKC)/Ras, which promotes the synthesis, phosphorylation, and activation of members of the Fos and Jun families of transcription factors. (Shaulian E, Karin M. AP-1 as a regulator of cell life and death. Nat Cell Biol 2002 45. 2002;4(5):E131-E136.).

***g. IL-2 Signaling***

**[0274]** IL-2 is a pleiotropic cytokine required for activation, proliferation differentiation, and maintenance of T cells. Naïve T cells express a low affinity IL-2 receptor requiring high volumes of IL-2 to initiate activation, while memory and regulatory T cells express a high affinity IL-2 receptor requiring much lower amounts of IL-2 for effective signaling. The IL-2 receptor makes use of the JAK/STAT signaling cascade, resulting in wide ranging transcriptional changes. Though CD8+ T cells are responsible for the cytotoxic effector response to foreign antigens, CD8+ T cells themselves cannot effectively produce IL-2 and rely instead on helper CD4+ T cells to produce IL-2 and other cytokine support.

**[0275]** IL-2 is so critical for T cell persistence and proliferation that high dose IL-2 therapy has been FDA approved for certain cancers, with the hopes of expanding endogenous cytotoxic T cell populations to induce tumor rejection. In metastatic melanoma and renal cell carcinoma trials, high dose IL-2 therapy induces limited (7%) long term response rates.

**[0276]** However, this therapy is not well tolerated by patients, causing multiple deaths, and likely contributes to the expansion of CD4+CD25+FoxP3+ regulatory T cells. Regulatory T cells are known to be pro-tumorigenic and high numbers of regulatory T cells (Tregs) are correlated with poor prognosis in solid tumor settings. Toxicity and the expansion of pro-tumor Treg populations present so much of a challenge to IL-2 therapy that synthetic orthogonal IL-2 cytokine therapies have recently been developed to target the effects of IL-2 cytokine therapy to cytotoxic T cells and avoid expansion of Tregs.

**[0277]** IL-2 secretion is highly relevant for the persistence and proliferation of T cell therapeutic products, therefore the inventors/disclosers assessed IL-2 secretion capacity of each mutation when expressed in CAR Jurkat cell lines and after co-culture with target cells. As a follow up for relevant hits, the inventors/disclosers assessed the capacity for mutations to improve CAR T cell *in vitro* target killing when cultured without supplemental IL-2. This setting, where IL-2 is withheld, better reflects the challenges of the tumor microenvironment, where IL-2 is scarce and autocrine secretion of IL-2 would be beneficial for maintenance of proliferation and long-term killing capabilities.

***h. CARD11-BCL10-MALT Signalosome***

**[0278]** Formation of a CARD11-BCL10-MALT (CBM) signaling complex is a key event in T- and B cell receptor-induced gene expression. After exposure to distinct immune triggers, these molecules form self-organizing filaments with MALT1 protease activity to regulate canonical nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) signalling pathways and the degradation of mRNA-binding proteins, which provides two layers of control of inflammatory gene expression. Deregulation of CARD11, BCL10 or MALT1 expression or CBM signaling have been associated with cancer, immunodeficiency, and autoimmunity (J. Ruland, L. Hartjes, CARD-BCL-10-MALT1 signalling in protective and pathological immunity. Nat Rev Immunol 19, 118-134 (2019).

**[0279]** In normal T cells, T cell receptor (TCR) signaling activates PKCθ, which in turn promotes the assembly of the CARD11-BCL10-MALT1 (CBM) signalosome. The CBM complex subsequently has three major outputs: NF-κB transcriptional activity, AP-1 transcriptional activity and MALT1 proteolytic activity (FIG. 5). The assembly of the

CARD11-BCL10-MALT1 signalosome complex is an essential step in regulating NF-κB in lymphoid immune cells.

**[0280]** An inhibitory domain present in CARD11 may allow for intramolecular autoinhibition which can prevent CARD11 binding to BCL10 in the absence of upstream signals. Upon phosphorylation of the inhibitory domain in normal T cells, CARD11 autoinhibition can be relieved and the CARD11 protein can oligomerize and promote prion-like assembly of the CBM complex with recruitment of BCL10-MALT1 filaments, which then can allow for CBM complex signaling.

**[0281]** Genes involved in CBM signaling include but are not limited to caspase recruitment domain family member 11 (CARD11), capping protein regulator and myosin 1 linker 2 (CARMIL2), mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1), B-cell lymphoma 10 (BCL10). CARD11 can be involved in both the innate and adaptive immune systems. CARD11 is implicated in the activation of NF-kB by the TCR complex.

**[0282]** T-cell signaling pathways, including the CARD11-BCL10-MALT signalasome and NF-κB signaling, are key to the activation of chimeric antigen receptor (CAR) T-cells. CAR T-cell therapy has demonstrated significant efficacy for treatment of hematologic cancers; however, some of these treatments are associated with severe side effects such as cytokine release syndrome (CRS) and immune effector cell-associated neurotoxicity syndrome (ICANS) (see, for example, Chohan et al. *Curr Hematol. Malig. Rep.* 2023; 18(2):9-18 and Hay et al. *Blood.* 2017; 130(21):2295-2306, which are incorporated by reference herein in their entirety). Without wishing to be bound by theory, modulating the intensity of certain signaling pathways in CAR-T cells is a potential strategy to limit excessive CAR T-cell activation, and could reduce harmful side effects while maintaining CAR T-cell efficacy. Signaling molecules (*e.g.*, CARD11-PIK3R3 fusions and/or CARD domain-containing polypeptides) with reduced signaling intensity could provide this effect. For example, signaling molecules with substitutions at certain amino acid positions and/or truncations of certain domains could provide reduced intensity of particular signaling pathways and thereby reduce side effects of CAR T-cell therapy. In some embodiments, a CARD11-PIK3R3 fusion or a CARD domain-containing polypeptide could provide reduced signaling intensity through the CARD11-BCL10-MALT signalasome and/or through NF-κB.

***i. JAK/STAT signaling***

**[0283]** The biochemistry of JAK/STAT signaling is well known to a person of skill in the art. Briefly, signaling begins with extracellular association of cytokines or growth factors

with their corresponding transmembrane receptors. This facilitates trans-activation of receptor-bound Janus kinases (JAKS) by putting them in spatial proximity and by prompting conformational changes that distance their kinase domains from inhibitory pseudokinase domains. Activated JAKS then phosphorylate latent STAT monomers, leading to dimerization, nuclear translocation, and DNA binding. In mammals, 4 JAKS (JAK1, JAK2, JAK3, TYK2) and 7 STATs (STAT1, STAT2, STAT3, STAT4, STAT5a, STAT5b, and STAT6) are employed by more than 50 cytokines and growth factors. (Villarino A V., Kanno Y, O'Shea JJ. Mechanisms and consequences of JAK–STAT signaling in the immune system. Nat Immunol 2017 184. 2017;18(4):374-384).

***j. Co-Stimulatory Molecule Signaling***

**[0284]** Co-stimulatory and co-inhibitory molecules are cell surface receptors and ligands that are classified into various families on the basis of their structure and functions.

**[0285]** Co-stimulatory and co-inhibitory receptors determine the functional outcome of T cell receptor (TCR) signaling. The specific recognition of cognate antigenic peptides presented by MHC molecules triggers T cell receptor signaling, but it is co-stimulatory and co-inhibitory receptors on T cells that direct T cell function and determine T cell fate. T cell co-signaling receptors have been broadly defined as cell-surface molecules that can transduce signals into T cells to positively (co-stimulatory receptors) or negatively (co-inhibitory receptors) modulate TCR signaling. Examples of co-stimulatory or co-inhibitory receptors includes CD28 and CTLA-4, both of which bind to ligands B7-1 and 7-2. Other genes involved in co-stimulatory molecule signaling include TNFR2, TNFRS1B, and ICOS (Chen L, Flies DB. Molecular mechanisms of T cell co-stimulation and co-inhibition. Nat Rev Immunol 2013 134. 2013;13(4):227-242.)

***k. RAS/MEK/ERK Signaling***

**[0286]** As is known by the skilled in the art, the sarcoma/mitogen-activated protein kinase kinase/extracellular receptor kinase (RAS/MEK/ERK) is a conserved signaling pathway that plays pivotal roles in cell proliferation, survival and differentiation. The aberrant activation of the RAS/MEK/ERK signaling pathway induces tumors. Efforts have been dedicated to targeting this signaling pathway for cancer treatment. The aberrant activation of the signaling pathway contributes to tumorigenesis and tumor development.

**[0287]** It is also known that triggering of a T-cell receptor by its cognate antigen results in nearly immediate activation of downstream signaling cascades, including the RAS/MEK/ERK pathway. Studies have also shown that RAS/MEK/ERK signaling is

memory stage-dependent in human T cells, conferring susceptibility to alloreactive T-cell selective inhibition.

**[0288]** As an important upstream molecular marker of the RAS–RAF–MEK–ERK pathway, RAS serves as a molecular switch by binding GTP/GDP, and it includes four isoforms:HRAS, KRAS4A, KRAS4B, and NRAS. KRAS is the most frequent isoform in all human cancers. KRAS4A and KRAS4B are the different splicing isoforms of the same gene. When the transmembrane receptors (receptor tyrosine kinase, RTKs) are activated, the complexes of growth-factor-receptor bound protein 2 (GRB2) and son of sevenless (SOS) in the cytoplasm are recruited to the inner surface of cell membrane. (McCubrey JA, Steelman LS, Bäsecke J, Martelli AM. Raf/mek/erk signaling. Target Ther Acute Myeloid Leuk. January 2015:275-305.)

**[0289]** Several mutations identified by the inventors/disclosers are in genes having various roles in this signaling pathway.

***l. Phospholipase C Gamma Signaling***

**[0290]** Phospholipase C (PLC) is an essential mediator of cellular signaling. PLC regulates multiple cellular processes by generating bioactive molecules such as inositol-1,4,5-triphosphate (IP3) and diacylglycerol (DAG). These products propagate and regulate cellular signaling via calcium (Ca2+) mobilization and activation of protein kinase C (PKC), other kinases, and ion channels. PLCγ1, one of the primary subtypes of PLC, is directly activated by membrane receptors, including receptor tyrosine kinases (RTKs), and adhesion receptors such as integrin. PLCγ1 mediates signaling through direct interactions with other signaling molecules via SH domains, as well as its lipase activity. PLCγ1 is frequently enriched and mutated in various cancers, and is involved in the processes of tumorigenesis, including proliferation, migration, and invasion. (Jang HJ, Suh PG, Lee YJ, Shin KJ, Cocco L, Chae YC. PLCγ1: Potential arbitrator of cancer progression. Adv Biol Regul. 2018;67:179-189, and Patterson RL, Van Rossum DB, Nikolaidis N, Gill DL, Snyder SH. Phospholipase C-γ: diverse roles in receptor-mediated calcium signaling. Trends Biochem Sci. 2005;30(12):688-697.

**[0291]** The phospholipase C gamma signaling pathway has been implicated in T cell lymphomas and primarily in cutaneous T cell lymphomas (CTCL). Nine *PLCG1* mutations (p.R48W, p.S312L, p.D342N, p.S345F, p.S520F, p.R1158H, p.E1163K, p.D1165H, and the in-frame indel p.VYEEDM1161V) have been identified in Sézary Syndrome, the leukemic variant of CTCL. (V. M. Patel et al., Frequent and Persistent PLCG1 Mutations in Sézary

Cells Directly Enhance PLCγ1 Activity and Stimulate NFκB, AP-1, and NFAT Signaling. J Invest Dermatol 140, 380-389.e384 (2020).

## III. CARD11-PIK3R3 FUSION PROTEINS

### *CARD11*

**[0292]** Caspase-associated recruitment domain (CARD) is a conserved homology domain comprising a 6-helix bundle or 5-helix bundle. CARDs can mediate protein-protein interactions between key apoptotic signaling molecules. Non-limiting examples of CARDs include CARD6, CARD8, CARD9, CARD10, CARD11, CARD14, CARD16, CARD18, and CARD19. Non-limiting examples of CARD-containing proteins include those described in Boyle and Monie bioRxiv 087908; doi: https://doi.org/10.1101/087908; and/or Park. In J Mol Med. 2019 Mar; 43(3):1119-1127, such as human caspase-1, -2, -4, and -5, mouse caspase-1, -2, -11, and -12, ASC, NOD1, NOD2, Apaf-1, BCL-10, and RIG-I.

**[0293]** As used herein, the term "CARD domain" refers to a caspase-associaed recruitment domain. In some embodiments, the CARD containing protein, or functional fragment thereof, is derived from a CARD protein selected from CARD9 (UniProt # Q9H257), CARD10 (UniProt # Q9BWT7) CARD11 (UniProt # Q9BXL7), or CARD14 (UniProt # Q9H257) (Wang et al. J Biol Chem. 2001 Jun 15;276(24):21405-9; Bertin et al. J Biol Chem. 2001 Apr 13;276(15):11877-82.). In some embodiments, the CARD containing protein, or functional fragment thereof, is derived from a CARD11 protein (UniProt #Q9BXL7), or a functional fragment thereof. In some embodiments, the CARD containing protein comprises or consists of a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to any one of SEQ ID NOs: 293-323. In some embodiments, the functional fragment of the CARD containing protein is derived from a protein selected from, or wherein the CARD is derived from a protein selected from, CARD9, CARD10, CARD11, and CARD14. In some embodiments, the functional fragment of the CARD containing protein is derived from CARD11 and comprises or consists of a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 295.

**[0294]** In some embodiments, the function of the CARD containing protein or the functional fragment thereof is to bind to a CARD domain on BCL10 (Bertin et al. J. Biol Chem. 201 Apr; 276(15):11877-11882). In some embodiments, the functional fragment thereof comprises at least 40, at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 150, at least 200, at least 300, at least 400, or at least 500 amino acids. Non-limiting

examples of a cell include a T cell, a macrophage, a monocyte, and a natural killer (NK) cell. In some embodiments, activation of the cell produces the substrate localized to the intracellular side of the plasma membrane. In some embodiments, the substrate localized to the intracellular side of the plasma membrane of a cell is a phosphoinositide. Non-limiting examples of phosphoinosities include those described in Posor et al. Nat Rev Mol Cell Biol. 2022 Dec;23(12):797-816. In some embodiments, the phosphoinositide is selected from phosphatidylinositol (3,4,5)-trisphosphate (PIP3), phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) (Hawse and Cattley J. Bio. Chem. 2019 Mar; 294(13):4793-4805; Sun et al. PLoS ONE. 2011 Nov; 6(11): e27227). In some embodiments, the polypeptide binds to the phosphoinositide with a Kd of less than 100 μM, 50 μM, 10 μM, 5 μM, 1μM, 0.5 μM, 0.1 μM, 0.05 μM, or 0.01 μM, and wherein the Kd is analyzed using SPR as described in Yu et al. Molecular Cell. 2004 March; 13(5):p677-688.

**[0295]** CARD11, also known as Caspase Recruitment Domain Family Member 11, CARMA1, Carma 1, BIMP3, Caspase Recruitment Domain-Containing Protein 11, BCL10-Interacting Maguk Protein 3, CARD-Containing MAGUK Protein 1, Card-Maguk Protein 1, IMD11A, BENTA, IMD11, or PPBL, is encoded by the human CARD11 gene. CARD11 is a membrane-associated guanylate kinase (MAGUK) family protein, a class of proteins that functions as molecular scaffolds for the assembly of multiprotein complexes at specialized regions of the plasma membrane. CARD11 is also a member of the CARD protein family, defined by the presence of a caspase-associated recruitment domain (CARD). CARD11 plays a role in various immune responses including, amoung others, lymphocyte activation, proliferation, and differentiation, and plays a role as a positive regulator of NF-kB activation.

**[0296]** CARD11 is ~130 kDa and exhibits a modular structure comprising a CARD domain, a LATCH domain, a coiled-coil domain, an inhibitory domain, and a MAGUK domain (which itself is made up of a PDZ domain, SH3 domain, and GUK domain) CARD11 is expressed in immune cells, with variations in expression levels observed across different cell types and states (Lu et al. The CBM-opathies-A Rapidly Expanding Spectrum of Human Inborn Errors of Immunity Caused by Mutations in the CARD11-BCL10-MALT1 Complex. Front Immunol. 2018 Sep 19;9:2078).

**[0297]** CARD11 is a member of the CARD11-BCL10-MALT1 (CBM) signalosome, which is essential for T cell activation and function upon antigen stimulation (Ruland et al. 2019. Nat. Rev. Immunol. 19,118-134). The CBM complex is known to have three important outputs: NF-kB transcriptional activity, AP-1 transcriptional activity, and MALT1

proteolytic activity (Kutzner et al. 2022. Sci Signaling. 15: eabk3083). In the absence of signaling from the TCR, the CARD11 inhibitory domain blocks CARD11 from forming a complex with BCL10 and MALT1. However, after TCR signaling, the inhibitory domain is phosphorylated and the CARD11 protein is able to oligimerize and recruite BCL10 and MALT1 to form the CBM complex.

**[0298]** Human CARD11 (isoform 203) has a CARD domain (SEQ ID NO: 295), a LATCH domain (SEQ ID NO: 342), a coiled-coil domain (SEQ ID NO: 343), an inhibitory domain (SEQ ID NO: 344), and a MAGUK domain (SEQ ID NO: 345). The MAGUK domain is made up of a PDZ domain (SEQ ID NO: 346), SH3 domain (SEQ ID NO: 347), and GUK domain (SEQ ID NO: 348).As used herein, a human CARD11 CARD domain refers to the CARD domain near the N-terminus of the human CARD11 protein. In some embodiments, the human CARD11 CARD domain has an amino acid sequence that corresponds to amino acids 11-103 of SEQ ID NO: 1, or an amino acid sequence that has at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with amino acids 11-103 of SEQ ID NO: 1. In some embodiments, the human CARD11 CARD domain has an amino acid sequence corresponding to amino acids 11-103 of SEQ ID NO:1, or an amino acid sequence that has at least about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with amino acids 11-103 of SEQ ID NO: 1.

**[0299]** As used herein, a human CARD11 LATCH domain refers to the LATCH domain between the N-terminal CARD domain and the C-terminal coiled-coil domain of the human CARD11 protein. In some embodiments, the human CARD11 LATCH domain has an amino acid sequence that corresponds to amino acids 104-122 of SEQ ID NO: 1, or an amino acid sequence that has at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with amino acids 104-122 of SEQ ID NO: 1. In some embodiments, the human CARD11 LATCH domain has an amino acid sequence corresponding to amino acids 104-122 of SEQ ID NO: 1, or an amino acid sequence that has at least about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with amino acids 104-122 of SEQ ID NO: 1.

**[0300]** As used herein, a human CARD11 coiled-coil domain refers to the coiled-coil domain between the N-terminal LATCH domain and the C-terminal inhibitory domain of the human CARD11 protein. In some embodiments, the human CARD11 coiled-coil domain has an amino acid sequence that corresponds to amino acids 123-442 of SEQ ID NO: 1, or an amino acid sequence that has at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with amino acids 123-442 of SEQ ID NO: 1. In some

embodiments, the human CARD11 coiled-coil domain has an amino acid sequence corresponding to amino acids 123-442 of SEQ ID NO: 1, or an amino acid sequence that has at least about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with amino acids 123-442 of SEQ ID NO: 1.

**[0301]** As used herein, a human CARD11 inhibitory domain refers to the inhibitory domain (ID) between the N-terminal coiled-coil domain and the C-terminal MAGUK domain of the human CARD11 protein. . In some embodiments, the human CARD11 inhibitory domain comprises a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 344. In some embodiments, the human CARD11 inhibitory domain has an amino acid sequence that corresponds to amino acids 443-577 of SEQ ID NO: 1, or an amino acid sequence that has at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with amino acids 443-577 of SEQ ID NO: 1. In some embodiments, the human CARD11 inhibitory domain has an amino acid sequence corresponding to amino acids 443-577 of SEQ ID NO: 1, or an amino acid sequence that has at least about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with amino acids 443-577 of SEQ ID NO: 1. In some embodiments, the human CARD11 inhibitory domain comprises a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 441.

**[0302]** As used herein, a human CARD11 MAGUK domain refers to the MAGUK domain near the C-terminus of the human CARD11 protein. In some embodiments, the human CARD11 MAGUK domain has an amino acid sequence that corresponds to amino acids 667-1140 of SEQ ID NO: 518, or an amino acid sequence that has at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with amino acids 667-1140 of SEQ ID NO: 518. In some embodiments, the human CARD11 MAGUK domain has an amino acid sequence corresponding to amino acids 667-1140 of SEQ ID NO: 518, or an amino acid sequence that has at least about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with amino acids 667-1140 of SEQ ID NO: 518.

**[0303]** The human CARD11 gene has eight transcripts, three of which encode a protein isoform of CARD11 (Ensemble, Gene: CARD11). The eight transcripts are named CARD11-201, CARD11-202, CARD11-203, CARD11-204, CARD11-205, CARD11-206, CARD11-201, and CARD11-208 (Ensembl, Gene: CARD11, ENSG00000198286). The coding DNA sequence (CDS) of the six transcripts are as set forth in SEQ ID NO: 349-354, respectively. The amino acid sequences of the three protein isoforms are as set forth in SEQ ID NO: 516-518, respectively (**Table 1**).

**[0304]** Transcript CARD11-203 (SEQ ID NO: 351; Ensembl Transcript ID: ENST00000396946.9; NCBI Reference Sequence: NM_001324281.3) encodes isoform CARD11-203 (SEQ ID NO: 518; Uniprot Accession No. Q9BXL7; NCBI Reference Sequence: NP_001311210.1), which has 1154 amino acids. CARD11-203 is the longest transcript of the human CARD11 gene. It is designated as the representative transcript in the Ensembl database. In identifying the representative transcript, Ensembl aims to identity the transcript that, on balance, has the highest coverage of conserved exons, highest expression, longest coding sequence and is represented in other key resources, such as NCBI and UniProt. All splice junctions of the CARD11-203 transcript are supported by at least one non-suspect mRNA.

**[0305]** Transcript CARD11-201 (SEQ ID NO: 349; Ensembl Transcript ID: ENST00000355508.3; NCBI Reference Sequence: NM_001324281.3) encodes isoform CARD11-201 (SEQ ID NO: 516; Uniprot Accession No. H7BY05), which has 271 amino acids.

**[0306]** Transcript CARD11-202 (Ensembl Transcript ID: ENST00000356408.3:) encodes isoform CARD11-202 (SEQ ID NO: 517; Uniprot Accession No. E2QRC0), which has 73 amino acids. The amino acid sequences of the three isoforms are listed in **Table 1**. The amino acids corresponding to the various domains in the human CARD11 protein are also identified in **Table 1**. "Beginning Sequence" refers to the region N-terminal to the CARD domain. "End Sequeunce" refers to the region C-terminal to the MAGUK domain.

**Table 1. Amino Acid SEQ ID NOs for CARD11 Isoforms**

| | CARD11-201 | CARD11-202 | CARD11-203 | SEQ ID NO |
|---|---|---|---|---|
| Beginning Sequence | N/A | 1-10 | 1-10 | 395 |
| CARD Domain | N/A | 11-73 | 11-103 | 295 |
| LATCH Domain | N/A | N/A | 104-122 | 342 |
| Coiled-Coil Domain | N/A | N/A | 123-442 | 343 |
| Inhibitory Domain | 1-49 | N/A | 443-577 | 344 |
| First Connection | 50-136 | N/A | 578-666 | 396 |

| | | | | |
|---|---|---|---|---|
| MAGUK Domain | 137-271 | N/A | 667-1140 | 345 |
| End Sequence | N/A | N/A | 1141-1154 | 397 |

**[0307]** As used herein, a human CARD11 PDZ domain refers to the PDZ domain near the N-terminus of the human CARD11 MAGUK domain. In some embodiments, the human CARD11 PDZ domain has an amino acid sequence that corresponds to amino acids 667-755 of SEQ ID NO: 518, or an amino acid sequence that has at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with amino acids 667-755 of SEQ ID NO: 518. In some embodiments, the human CARD11 PDZ domain has an amino acid sequence corresponding to amino acids 667-755 of SEQ ID NO: 518, or an amino acid sequence that has at least about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with amino acids 667-755 of SEQ ID NO: 518.

**[0308]** As used herein, a human CARD11 SH3 domain refers to the SH3 domain between the N-terminal PDZ domain and the C-terminal GUK domain of the human CARD11 MAGUK domain. In some embodiments, the human CARD11 SH3 domain has an amino acid sequence that corresponds to amino acids 773-806 of SEQ ID NO: 518, or an amino acid sequence that has at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with amino acids 773-806 of SEQ ID NO: 518. In some embodiments, the human CARD11 SH3 domain has an amino acid sequence corresponding to amino acids 773-806 of SEQ ID NO: 518, or an amino acid sequence that has at least about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with amino acids 773-806 of SEQ ID NO: 518.

**[0309]** As used herein, a human CARD11 GUK domain refers to the GUK domain near the C-terminus of the human CARD11 MAGUK domain. In some embodiments, the human CARD11 GUK domain has an amino acid sequence that corresponds to amino acids 973-1140 of SEQ ID NO: 518, or an amino acid sequence that has at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with amino acids 973-1140 of SEQ ID NO: 518. In some embodiments, the human CARD11 GUK domain has an amino acid sequence corresponding to amino acids 973-1140 of SEQ ID NO: 518, or an amino acid sequence that has at least about 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identity with amino acids 973-1140 of SEQ ID NO: 518.

**[0310]** The amino acid sequences of the CARD11-203 PDZ, SH3, and GUK domains in **Table 2**. "PDZ to SH3 Connection" refers to the region connecting the PDZ domain and the SH3 domain. "SH3 to GUK Connection" refers to the region connecting the SH3 domain and the GUK domain.

**Table 2. Amino Acid SEQ ID NOs for CARD11 Domains**

| | CARD11-203 | SEQ ID NO |
|---|---|---|
| PDZ Domain | 667-755 | 346 |
| PDZ to SH3 Connection | 756-772 | 398 |
| SH3 Domain | 773-806 | 347 |
| SH3 to GUK Connection | 807-972 | 399 |
| GUK Domain | 973-1140 | 348 |

***PIK3R3***

**[0311]** PIK3R3, also known as phosphatidylinositol 3-kinase regulatory subunit gamma (UniProt Q92569), is encoded by the human PIK3R3 gene (NCBI gene ID 8503).

**[0312]** PIK3R3, also known as phosphatidylinositol 3-kinase regulatory subunit gamma, p55, p55-GAMMA, p55PIK, phosphoinositide-3-kinase regulatory subunit 3, is encoded by the human PIK3R3 gene. PIK3R3 is a regulatory subunit of PI3K and phosphorylates phosphatidylinositol (and similar compounds).

**[0313]** PIK3R3 is ~62kDa and comprises both a catalytic and regulatory subunit including. PIK3R3 comprises two SH2 domains through which it binds activated (phosphorylated) protein-tyrosine kinases to regulate their activity.

The human PIK3R3 gene has seven transcripts, five of which encode a protein isoform of PIK3R3 (Ensemble, Gene: PIK3R3, ENSG00000117461). The seven transcripts are named PIK3R3-201, PIK3R3-202, PIK3R3-203, PIK3R3-204, PIK3R3-205, PIK3R3-206, and PIK3R3-207 (Ensemble, Gene: PIK3R3, ENSG00000117461).

***CARD11-PIK3R3 Fusion Protein***

**[0314]** A CARD11-PIK3R3 fusion protein has previously been derived from a patient with CD4+ T cell lymphoma (Wang et al., Genomic profiling of Sézary syndrome identifies alterations of key T cell signaling and differentiation genes. *Nature Genetics* 47, 1426-1434

(2015). Thereapeutic T cells expressing this CARD11-PIK3R3 fusion protein demonstrate augmented CARD11–BCL10–MALT1 complex signalling and anti-tumour efficacy in several immunotherapy-refractory models in an antigen-dependent manner (Garcia et al., Naturally occurring T cell mutations enhance engineered T cell therapies. *Nature*. 626, 626-634 (2024)). Therapeutic T cells expressing the CARD11-PIK3R3 fusion protein showed no evidence of malignant transformation up to 418 days after T-cell transfer, underscoring the safety of such an approach. These references are herein incorporated by reference in their entirety.

**[0315]** The present disclosure provides recombinant nucleic acids encoding a recombinant polypeptide as described herein, wherein the recombinant nucleic acid additionally encodes a CARD-containing protein (e.g., a CARD11-PIK3R3 fusion protein) or a functional fragment thereof, for example those described in PCT/US2023/075738, which is herein incorporated by reference in its entirety.

***Engineered CARD11-PIK3R3 Fusion Proteins***

**[0316]** The present disclosure provides recombinant polypeptides comprising a caspase-associated recruitment domain (CARD) or a functional fragment thereof. A functional fragment of a recombinant polypeptide comprising a CARD can be, for example, a fragment that provides NF-κB transcriptional activity, AP-1 transcriptional activity, and/or MALT1 proteolytic activity activity at a level at least 70%, 75%, 80%, 85%, 90%, or 95% of that of a full-length protein, as determined by the *in vitro* CAR Jurkat assay.

**[0317]** The disclosure further relates to recombinant polypeptides comprising a CARD-containing protein or a functional fragment thereof, or to recombinant nucleic acids encoding such polypeptides, comprising a domain capable of binding to (i) a substrate localized to the intracellular side of the plasma membrane of a cell and/or (ii) a target polypeptide comprising a phosphorylated tyrosine (pTyr). In some embodiments, the substrate localized to the intracellular side of the plasma membrane of a cell is a phosphoinositide. Non-limiting examples of phosphoinosities include those described in Posor et al. Nat Rev Mol Cell Biol. 2022 Dec;23(12):797-816. In some embodiments, the phosphoinositide is selected from phosphatidylinositol (3,4,5)-trisphosphate (PIP3), phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2) (Hawse and Cattley J. Bio. Chem. 2019 Mar; 294(13):4793-4805; Sun et al. PLoS ONE. 2011 Nov; 6(11): e27227). In some embodiments, the polypeptide binds to the phosphoinositide with a Kd of less than 100 μM,

50 μM, 10 μM, 5 μM, 1μM, 0.5 μM, 0.1 μM, 0.05 μM, or 0.01 μM, and wherein the Kd is analyzed using SPR as described in Yu et al. Molecular Cell. 2004 March; 13(5):p 677-688.

**[0318]** In some embodiments, the domain is capable of binding to a substrate indirectly localized to the intracellular side of the plasma membrane. Indirect localization can refer to localization of the substrate to the intracellular side of the plasma membrane through, for example, binding of the substrate to another polypeptide or lipid that is directly localized to the intracellular side of the plasma membrane. Indirect localization can refer to localization of the substrate to the intracellular side of the plasma membrane through, for example, interaction of the substrate with another polypeptide or lipid that is directly localized to the intracellular side of the plasma membrane. In some embodiments, the domain is capable of binding to a substrate directly localized to the intracellular side of the plasma membrane. Direct localization can refer to localization of the substrate to the intracellular side of the plasma membrane through, for example, binding of the substrate to the intracellular side of the plasma membrane itself. Direct localization can refer to localization of the substrate to the intracellular side of the plasma membrane through, for example, interaction of the substrate with the intracellular side of the plasma membrane itself.

**[0319]** The disclosure further relates to recombinant polypeptides comprising a recruitment domain or a functional fragment thereof. The disclosure further relates to recombinant polypeptides comprising (a) a caspase-associated recruitment domain (CARD), and (b) a recruitment domain or a functional fragment thereof. The disclosure further relates to recombinant polypeptides comprising: (a) a first polypeptide capable of binding to a caspase-associated recruitment domain (CARD) on BCL10, and (b) a recruitment domain or a functional fragment thereof. In some embodiments, the recruitment domain is capable of binding to (i) a substrate localized to the intracellular side of the plasma membrane of a cell and/or (ii) a target polypeptide comprising a phosphorylated tyrosine (pTyr). In some embodiments, the recombinant polypeptide does not comprise a full length sequene of an endogenous protein comprising the recruitment domain. In some embodiments, the recombinant polypeptide comprises no more than 724 amino acids. In some embodiments, the recombinant polypeptide does not comprise a full length sequence of an endogenous protein comprising the CARD and/or a full length sequence of an endogenous protein comprising the recruitment domain. In some embodiments, the recombinant polypeptide does not comprise a full length sequence of an endogenous protein capable of binding the CARD recruitment domain of BCL10.

**[0320]** Non-limiting examples of recruitment domains capable of binding to (i) and/or (ii) include Src Homology region 2 (SH2) domains, Src Homology region 3 (SH3) domains, pleckstrin homology (PH) domains, and phosphytyrosine-binding (PTB) domains. SH2 and PTB domains mediate protein-protein interactions involved in many signal transduction pathways. SH2, SH3, and PTB domains that can be used in the disclosure include, for example, those disclosed in Schlessinger et al. Sci STKE. 2003 Jul 15;2003(191):RE12. SH2 containing proteins can bind to plasma membrane lipids through a different binding pocket than the pTyr binding pocket; most SH2 domains bind plasma membrane lipids and many have high phosphoinositide specificity (Park et al., Cell. 2016 Apr 7;62(1):7-20). In some embodiments, the recruitment domain is, or comprises, an SH3 domain. In some embodiments, the recruitment domain is, or comprises, a PTB domain. In some embodiments, the recruitment domain is, or comprises, a PH domain. In some embodiments, the recruitment domain is, or comprises, an SH2 domain.

**[0321]** In some embodiments, the start of the recruitment domain corresponds to amino acid position 622 of SEQ ID NO: 1 and the end of the recruitment domain corresponds to amino acid position 716 of SEQ ID NO: 1. In some embodiments, the recruitment domain comprises at least 90 amino acids.

**[0322]** In some embodiments, the recombinant polypeptide comprises a functional fragment of the recruitment domain. In some embodiments, the recombinant polypeptide comprises a truncated portion of the recruitment domain having no more than 85, no more than 80, no more than 75, no more than 70, no more than 60, no more than 50, or no more than 40, amino acids.

**[0323]** The recruitment domain may comprise a truncation at the N-terminus, at the C-terminus, or both. In some embodiments, the recruitment domain comprises a truncation of at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, or at least 50 amino acids at the N-terminus. In some embodiments, the recruitment domain comprises a truncation of at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, or at least 50 amino acids at the C-terminus. In some embodiments, the recruitment domain comprises a truncation of at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, or at least 50 amino acids in between the N-terminus and the C-terminus. In some embodiments, the recruitment domain or the functional fragment thereof comprises a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a segment of at least 30, 40, 50, 60, 70, 80, 90, or 97 amino acids within the region of amino acids 622-716 of

SEQ ID NO: 1. In some embodiments, the recombinant polypeptide does not comprise any recruitment domain having at least 30 amino acids in length.

**[0324]** In some embodiments, the SH2 domain comprises or consists of a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to any one of SEQ ID NOs: 305 and 307-437. In some embodiments, the SH2 domain is from a PIK3R3 protein (UniProt # Q92569). In some embodiments, the SH2 domain comprises the motif of a conserved arginine residue in the FLVR motif (Arg βB5 or Arg175 in the v-Src SH2 domain). Most conserved resides are clustered on the βB strand, and the conserved arginine residue in the FLVR motif plays the central role in forming a double hydrogen bond with the phosphate group of pTyr. Additional residues that are key for phosphopeptide binding are His βD4, Lys βD6, and Arg αA2, which coordinate and anchor the aromatic ring of the phospho-tyrosine (Diop et al. Int J Mol Sci. 2022 Dec 15;23(24):15944). In some embodiments, the SH2 domain is an engineered SH2 domain with an enhanced affinity for phosphotyrosine (e.g., Veggiani et al. Protein Sci. 2019 Feb;28(2):403-413).

**[0325]** Non-limiting examples of target polypeptides comprising a pTyr include P110α/PIK3CA, P110β/PIK3CB, P110δ/PIK3CD, IGF-1R, ErbB2, CTLA-4, and CD28. In some embodiments, the target polypeptide is derived from IGF-1R, CTLA-4, or CD28. In some embodiments, the target polypeptide comprises or consists of a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to any one of SEQ ID NOs: 298-304. In some embodiments, the pTyr is located at the position corresponding to pY1346 of SEQ ID NO: 325. In some embodiments, the pTyr is located at the position corresponding to pY1221 of SEQ ID NO: 324. In some embodiments, the polypeptide binds to the target polypeptide with a Kd of less than 100 μM, 50 μM, 10 μM, 5 μM, 1μM, 0.5 μM, 0.1 μM, 0.05 μM, or 0.01 μM, wherein the Kd is analyzed by flourescence polarization assay as described in Hause et al. PLoS One. 2012;7(9):e44471. In some embodiments, the polypeptide has a higher affinity for the target polypeptide comprising the pTyr than a control polypeptide without phosphorylation at the corresponding tyrosine position. In some embodiments, the polypeptide has at least 2-fold, at least 5-fold, at least 10-fold, at least 50-fold, or at least 100-fold, higher affinity (lower Kd) for the target polypeptide comprising the phosphorylated tyrosine than a control polypeptide without phosphorylation at the corresponding tyrosine position.

**[0326]** In some embodiments, the domain is capable of binding to a substrate indirectly localized to the intracellular side of the plasma membrane. Indirect localization can refer to localization of the substrate to the intracellular side of the plasma membrane through, for

example, binding of the substrate to another polypeptide or lipid that is directly localized to the intracellular side of the plasma membrane. Indirect localization can refer to localization of the substrate to the intracellular side of the plasma membrane through, for example, interaction of the substrate with another polypeptide or lipid that is directly localized to the intracellular side of the plasma membrane. In some embodiments, the domain is capable of binding to a substrate directly localized to the intracellular side of the plasma membrane. Direct localization can refer to localization of the substrate to the intracellular side of the plasma membrane through, for example, binding of the substrate to the intracellular side of the plasma membrane itself. Direct localization can refer to localization of the substrate to the intracellular side of the plasma membrane through, for example, interaction of the substrate with the intracellular side of the plasma membrane itself.

**[0327]** Furthermore, the disclosure relates to recombinant polypeptides comprising a CARD containing protein or a functional fragment thereof, and a domain capable of binding to (i) a substrate localized to the intracellular side of the plasma membrane of a cell and/or (ii) a target polypeptide comprising a phosphorylated tyrosine (also called a CARD fusion polypeptide), as disclosed herein. In some embodiments, the polypeptide comprises a CARD containing protein, or a functional fragment thereof, and an SH2 domain (also called a CARD-SH2 fusion polypeptide). In some embodiments, the polypeptide is a CARD11-PIK3R3 fusion polypeptide. In some embodiments, the recombinant nucleic acid constructs encode a CARD11-PIK3R3 fusion polypeptide or any truncations thereof.

**[0328]** In some embodiments, the disclosure relates to a polypeptide comprising a functional fragment of a CARD containing protein derived from a CARD11 protein and an SH2 domain from a PIK3R3 protein (also called a CARD11-PIK3R3 fusion polypeptide).

**[0329]** The present disclosure further relates to polypeptides wherein the domain capable of binding to the target polypeptide comprising the phosphorylated tyrosine, or the SH2 domain, or the second polypeptide portion, is located at the N-terminus of the CARD containing protein or functional fragment thereof, between the CARD containing protein and the Coiled-coil domain, or at the C-terminus of the CARD containing protein and/or the Coiled-coil domain. In some embodiments, the polypeptide comprises the CARD domain derived from a CARD11 protein followed by the Coiled-coil domain derived from the CARD11 protein. The present disclosure further relates to polypeptides wherein the domain capable of binding to the target polypeptide comprising the phosphorylated tyrosine, or the SH2 domain, or the second polypeptide portion, is located close to the C-terminus of the polypeptide, wherein the polypeptide has no more than 50, 40, 30, 20, 15, 10, or 5 amino

acids at the C-terminus of the domain in (b), or the SH2 domain, or the second polypeptide portion.

**[0330]** In some embodiments, the polypeptide does not comprise an inhibitory domain (ID) or a portion thereof. In other embodiments, the polypeptide comprises an ID or a portion thereof.

**[0331]** In some embodiments, the CARD11-PIK3R3 fusion polypeptide includes a CARD domain (i.e., containing protein or functional fragment thereof), a CARD inhibitory domain (ID), a coiled-coil domain, and an SH2 domain from PIK3R3. In some embodiments, the CARD11-PIK3R3 fusion polypeptide includes a functional fragment of a CARD containing protein derived from CARD11domain, a coiled-coil domain, and an SH2 domain from PIK3R3.

**[0332]** In some embodiments, the polypeptide of the disclosure has a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to SEQ ID NO: 3-292 and functional variants thereof, optionally comprising at least one mutation listed in **Table 3**.

**[0333]** In some embodiments, the polypeptide of the disclosure has a sequence selected from the group consisting of SEQ ID NO: 3-292 and functional variants thereof, optionally comprising at least one mutation listed in **Table 3**. In some embodiments, the polynucleotide of the disclosure has a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to SEQ ID NO: 3-292 and functional variants thereof, optionally comprising at least one mutation listed in **Table 3**. In some embodiments, the polynucleotide of the disclosure has a sequence selected from the group consisting of SEQ ID NO: 3-292 and functional variants thereof, optionally comprising at least one mutation listed in **Table 3**.

**[0334]** The present disclosure provides engineered CARD11-PIK3R3 proteins that have fewer amino acids than the patient derived full-length human CARD11-PIK3R3 fusion. In some embodiments, the engineered CARD11-PIK3R3 protein is a recombinant polypeptide comprising a caspase-associated recruitment domain (CARD) containing protein or a functional fragment thereof, wherein the recombinant polypeptide comprises no more than 724 amino acids. In some embodiments, the recombinant polypeptide does not comprise a full length CARD containing protein.

**[0335]** In some embodiments, the recombinant polypeptide comprises no more than 700, no more than 650, no more than 600, no more than 550, no more than 500, no more than 450, no more than 400, no more than 350, no more than 300, no more than 250, no more than

200, or no more than 150 amino acids. In some embodiments, the CARD containing protein or the functional fragment thereof, the CARD, comprises no more than 40, no more than 50, no more than 60, no more than 70, no more than 80, no more than 90, no more than 100, no more than 150, no more than 200, no more than 300, no more than 400, or no more than 500 amino acids.

**[0336]** In some embodiments, the recombinant polypeptide comprises a full CARD domain. In some embodiments, the full CARD domain comprises at least 90 amino acids. In some embodiments, the recombinant polypeptide comprises a truncated portion of a full CARD domain. In some embodiments, the recombinant polypeptide comprises a truncated portion of the CARD domain having no more than 85, no more than 80, no more than 75, no more than 70, no more than 60, no more than 50, or no more than 40, amino acids.

**[0337]** The CARD domain may comprise a truncation at the N-terminus, the C-terminus, or both. In some embodiments, the CARD domain comprises a truncation of at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, or at least 50 amino acids at the N-terminus. In some embodiments, the CARD domain comprises a truncation of at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, or at least 50 amino acids at the C-terminus. In some embodiments, the CARD domain comprises a truncation of at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, or at least 50 amino acids in between the N-terminus and the C-terminus. In some embodiments, the recombinant polypeptide does not comprise any full or truncated portion of CARD domain having at least 30 amino acids in length.

**[0338]** In some embodiments, the recombinant polypeptide comprises or consists of a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to any one of SEQ ID NO: 225-245. In some embodiments, the recombinant polypeptide does not comprise the polypeptide sequence “MDDY” within 10 amino acids before the N-term of the start of the CARD domain.

**[0339]** In some embodiments, the recombinant polypeptide comprises a full coiled-coil domain (*e.g.,* a full CARD11 coiled-coil domain). In some embodiments, the start of the full coiled-coil domain corresponds to amino acid position 123 of SEQ ID NO: 1, and the end of the full coiled-coil domain corresponds to amino acid position 442 of SEQ ID NO: 1. In some embodiments, the full coiled-coil domain comprises a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to amino acids 123-442 of SEQ ID NO: 1. In some embodiments, the full coiled-coil domain comprises at least 310 amino acids.

**[0340]** In some embodiments, the recombinant polypeptide comprises a truncated portion of a coiled-coil domain (*e.g.,* a truncated portion of a CARD11 coiled-coil domain). In some embodiments, the recombinant polypeptide comprises a truncated portion of the coiled-coil domain having no more than 300, no more than 290, no more than 280, no more than 270, no more than 260, no more than 250, no more than 240, no more than 230, no more than 220, no more than 210, no more than 200, no more than 190, no more than 180, no more than 170, no more than 160, no more than 150, no more than 140, no more than 130, no mor than 120, no more than 110, no more than 100, no more than 95, no more than 90, no more than 85, no more than 80, no more than 75, no more than 70, no more than 60, no more than 50, or no more than 40, amino acids; preferably, wherein the truncated portion of the coiled-coil domain has no more than 260 amino acids.

**[0341]** The coiled-coil domain may comprise a truncation at the N-terminus, the C-terminus, or both. In some embodiments, the coiled-coil domain comprises a truncation of at least 5, at least 10, at least 15, 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, at least 110, at least 120, at least 130, at least 140, at least 150, at least 160, at least 170, at least 180, at least 190, at least 200, at least 210, at least 220, at least 230, at least 240, at least 250, at least 260, or at least 270 amino acids at the N-terminus. In some embodiments, the coiled-coil domain comprises a truncation of at least 5, at least 10, at least 15, 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, at least 110, at least 120, at least 130, at least 140, at least 150, at least 160, at least 170, at least 180, at least 190, at least 200, at least 210, at least 220, at least 230, at least 240, at least 250, at least 260, or at least 270 amino acids at the C-terminus. In some embodiments, the coiled-coil domain comprises a truncation of at least 5, at least 10, at least 15, 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, at least 110, at least 120, at least 130, at least 140, at least 150, at least 160, at least 170, at least 180, at least 190, at least 200, at least 210, at least 220, at least 230, at least 240, at least 250, at least 260, or at least 2700270 amino acids in between the N-terminus and the C-terminus; optionally, the coiled-coil domain comprises a truncation of about 60 to about 80 amino acids in between the N-terminus and the C-terminus.

**[0342]** A region within the coil-coil domain may be removed or replaced. In some embodiments, a region within the coiled-coil domain corresponding to amino acids 253-324 of SEQ ID NO: 1 is removed or replaced by a polypeptide comprising no more than 20, 15, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acids. In some embodiments, the region within

the coiled-coil domain is replaced by a polypeptide comprising a sequence of SGGGGSGGGGS (SEQ ID NO: 431) or QAGKRSLPD (SEQ ID NO: 432), or a sequence having at most 1, 2, or 3 mutations thereto. In some embodiments, the recombinant polypeptide does not comprise any full or truncated portion of coiled-coil domain having at least 30 amino acids in length. In some embodiments, the recombinant polypeptide comprises or consists of a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to any one of SEQ ID NO: 193-224 and 246-276.

**[0343]** In some embodiments, the recombinant polypeptide comprises a full inhibitory domain (*e.g.,* a full CARD11 inhibitory domain). In some embodiments, the inhibitory domain comprises a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 344. In some embodiments, the start of the inhibitory domain corresponds to amino acid position 443 of SEQ ID NO: 1, and the end of the inhibitory domain corresponds to amino acid position 577 of SEQ ID NO: 1. In some embodiments, the inhibitory domain comprises a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to amino acids 443-577 of SEQ ID NO: 1. In some embodiments, the human CARD11 inhibitory domain comprises a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 441. In some embodiments, the inhibitory domain comprises at least 130, or at least 125, amino acids.

**[0344]** In some embodiments, the recombinant polypeptide comprises a truncated portion of an inhibitory domain (*e.g.,* a truncated portion of a CARD11 inhibitory domain). In some embodiments, the recombinant polypeptide comprises a truncated portion of the inhibitory domain having no more than 120, no more than 110, no more than 100, no more than 95, no more than 90, no more than 85, no more than 80, no more than 75, no more than 70, no more than 60, no more than 50, or no more than 40, amino acids.

**[0345]** The inhibitory domain may comprise a truncation at the N-terminus, the C-terminus, or both. In some embodiments, the inhibitory domain comprises a truncation of at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, or at least 90 amino acids at the C-terminus. In some embodiments, the inhibitory domain comprises a truncation of at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, or at least 90 amino acids in between the N-terminus and the C-terminus. In some embodiments, the recombinant polypeptide does not comprise any full or truncated portion of inhibitory domain having at least 30 amino acids in length.

**[0346]** The inhibitory domain may comprise a certain number of serines capable of being phosphorylated. In some embodiments, the inhibitory domain or the truncated portion thereof comprises at least 1, at least 2, at least 3, at least 4, at least 5, or at least 6 serines that are capable of being phosphorylated. In some embodiments, the serines are capable of being phosphorylated by a protein kinase C (PKC). Non-limiting examples of PKC include PKCβ and PKCθ. In some embodiments, the native serine phosphorylation site(s) in the full-length wildtype inhibitory domain, which would otherwise be removed due to truncation, are inserted into the truncated inhibitory domain sequence. Thus, in some embodiments, the truncated inhibitory domain may comprise more serine phosphorylation sites than the corresponding region of the wildtype inhibitory domain. Such design may provide additional regulation of the activity of the polypeptides of the disclosure. Additional descriptions of serine phosphorylation in the inhibitory domain can be found, for example, in Sommer et al., Immunity. 2005 Dec;23(6):561-74, the content of which is incorporated by reference in its entirety.

**[0347]** In some embodiments, the inhibitory domain or the truncated portion thereof comprises a sequence of DASPRT (SEQ ID NO: 425), RAKSPI (SEQ ID NO: 426), DASPSS (SEQ ID NO: 427), SRSSIMS (SEQ ID NO: 428), RKFSLER (SEQ ID NO: 429), and/or FRPSVTS (SEQ ID NO: 430), or a sequence having at most 1, 2, or 3 mutations thereto. In some embodiments, the inhibitory domain or the truncated portion thereof comprises DASPRT (SEQ ID NO: 425), RAKSPI (SEQ ID NO: 426), DASPSS (SEQ ID NO: 427), and/or SRSSIMS (SEQ ID NO: 428), or a sequence having at most 1, 2, or 3 mutations thereto. In some embodiments, the inhibitory domain or the truncated portion thereof comprises RAKSPI (SEQ ID NO: 426), DASPSS (SEQ ID NO: 427), and/or SRSSIMS (SEQ ID NO: 428), or a sequence having at most 1, 2, or 3 mutations thereto. In some embodiments, the inhibitory domain or the truncated portion thereof comprises SRSSIMS (SEQ ID NO: 428), RKFSLER (SEQ ID NO: 429), and/or FRPSVTS (SEQ ID NO: 430), or a sequence having at most 1, 2, or 3 mutations thereto. In some embodiments, the inhibitory domain or the truncated portion thereof comprises DASPRT (SEQ ID NO: 425), RAKSPI (SEQ ID NO: 426), and DASPSS (SEQ ID NO: 427). In some embodiments, the inhibitory domain or the truncated portion thereof comprises RAKSPI (SEQ ID NO: 426), DASPSS (SEQ ID NO: 427) and SRSSIMS (SEQ ID NO: 428). In some embodiments, the inhibitory domain or the truncated portion thereof comprises DASPSS (SEQ ID NO: 427), SRSSIMS (SEQ ID NO: 428) and RKFSLER (SEQ ID NO: 429). In some embodiments, the inhibitory domain or the truncated portion thereof comprises

SRSSIMS (SEQ ID NO: 428), RKFSLER (SEQ ID NO: 429), and FRPSVTS (SEQ ID NO: 430). In some embodiments, the inhibitory domain or the truncated portion thereof comprises DASPRT (SEQ ID NO: 425) and RAKSPI (SEQ ID NO: 426). In some embodiments, the inhibitory domain or the truncated portion thereof comprises RAKSPI (SEQ ID NO: 426), and DASPSS (SEQ ID NO: 427). In some embodiments, the inhibitory domain or the truncated portion thereof comprises DASPSS (SEQ ID NO: 427) and SRSSIMS (SEQ ID NO: 428). In some embodiments, the inhibitory domain or the truncated portion thereof comprises SRSSIMS (SEQ ID NO: 428) and RKFSLER (SEQ ID NO: 429). In some embodiments, the inhibitory domain or the truncated portion thereof comprises RKFSLER (SEQ ID NO: 429), and FRPSVTS (SEQ ID NO: 430).

**[0348]** In some embodiments, the serine capable of being phosphorylated in DASPRT (SEQ ID NO: 425), or a sequence having at most 1, 2, or 3 mutations thereto, is located at a position corresponding to S466 according to SEQ ID NO: 518 in the inhibitory domain. In some embodiments, the serine capable of being phosphorylated in RAKSPI (SEQ ID NO: 426), or a sequence having at most 1, 2, or 3 mutations thereto, is located at a position corresponding to S512 according to SEQ ID NO: 518 in the inhibitory domain. In some embodiments, the serine capable of being phosphorylated in DASPSS (SEQ ID NO: 427), or a sequence having at most 1, 2, or 3 mutations thereto, is located at a position corresponding to S535 according to SEQ ID NO: 518 in the inhibitory domain. In some embodiments, the serine capable of being phosphorylated in SRSSIMS (SEQ ID NO: 428), or a sequence having at most 1, 2, or 3 mutations thereto, is located at a position corresponding to S559 according to SEQ ID NO: 518 in the inhibitory domain. In some embodiments, the serine capable of being phosphorylated in RKFSLER (SEQ ID NO: 429), or a sequence having at most 1, 2, or 3 mutations thereto, is located at a position corresponding to S644 according to SEQ ID NO: 518 in the inhibitory domain. In some embodiments, the serine capable of being phosphorylated in FRPSVTS (SEQ ID NO: 430), or a sequence having at most 1, 2, or 3 mutations thereto, is located at a position corresponding to S652 according to SEQ ID NO: 518 in the inhibitory domain.

**[0349]** In some embodiments, the recombinant polypeptide comprises, from N-term to C-term: the coiled-coil domain or the truncated portion thereof, and the inhibitory domain or the truncated portion thereof, without any additional amino acid in between. In some embodiments, the recombinant polypeptide comprises or consists of a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to any one of SEQ ID NO: 179-192, 277-288, and 289-292.

**[0350]** The recombinant polypeptide may comprise the domains described herein in a particular order, from N-terminus to C-terminus, optionally with no more than a particular number of amino acids between any two domains. In some embodiments, the recombinant polypeptide comprises from N-terminus to C-terminus: the inhibitory domain or the truncated portion thereof, and the recruitment domain or the functional fragment thereof, with no more than 5, no more than 10, no more than 15, no more than 20, or no more than 25, amino acids in between. In some embodiments, the recombinant polypeptide comprises an amino acid sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to any one of SEQ ID NO: 433-438 in between the inhibitory domain or the truncated portion thereof and the recruitment domain or the functional fragment thereof. In some embodiments, the recombinant polypeptide comprises, from N-terminus to C-terminus, the CARD domain, optionally the coiled-coil domain, optionally the inhibitory domain, and the recruitment domain.

**[0351]** In some embodiments, the recombinant polypeptide comprises no more than 40, no more than 35, no more than 30, no more than 25, no more than 20, no more than 15, no more than 10, or no more than 5, amino acids, in between the CARD containing protein or functional fragment thereof and the recruitment domain. In some embodiments, the recombinant polypeptide comprises or consists of a sequence having at least 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to any one of SEQ ID NO: 3-150. In some embodiments, the recombinant polypeptide comprises or consists of a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to any one of SEQ ID NO: 151-178. In some embodiments, the recombinant polypeptide comprises an aspartatic acid acid (D) at the position corresponding to amino acid 602 of SEQ ID NO: 1.

**[0352]** In some embodiments, the CARD containing protein or functional fragment thereof possesses certain binding capabilities and/or functional abilities.

**[0353]** In some embodiments, the CARD containing protein or functional fragment thereof the CARD, or the recombinant polypeptide, is capable of binding to a CARD domain on BCL10. In some embodiments, the CARD containing protein or functional fragment thereof the CARD, or the recombinant polypeptide, is capable of forming a complex with BCL10 and MALT1.

**[0354]** In some embodiments, when the recombinant polypeptide is expressed in an immune cell and when the immune cell binds a target antigen, the immune cell exhibits decreased signaling and/or elicits decreased IFNg and/or IL-2 production compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1. In some embodiments, when

the recombinant polypeptide is expressed in an immune cell and when the immune cell binds a target antigen, the immune cell exhibits equivalent or enhanced signaling and/or elicits equivalent or higher IFNg and/or IL-2 production compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1. In some embodiments, when the is expressed in an immune cell and when the immune cell binds a target antigen, the immune cell exhibits decreased (a) BCL10 binding; (b) BCL10 and MALT1 complex formation, (c) NFAT, NF-κB and/or AP-1 signaling, (d) cytokine production, (e) JAK/STAT signaling, (f) co-stimulatory molecule signaling, (g) phospholipase gamma signaling, and/or (h) transcription factor activity, compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1. In some embodiments, when the is expressed in an immune cell and when the immune cell binds a target antigen, the immune cell exhibits equivalent or enhanced (a) BCL10 binding; (b) BCL10 and MALT1 complex formation, (c) NF-κB and/or AP-1 signaling, (d) MALT1 paracaspase activity; (e) cytokine production, (f) peak tumor expansion, (g) peak peripheral blood expansion; (h) contraction following peak tumor expansion, and/or (i) contraction following peak peripheral blood expansion,, compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1. In some embodiments, the recombinant polypeptide affords certain beneficial technical effects, including delivery via integrating vectors.

**[0355]** In some embodiments, the cell comprises at least one copy, or at least two copies, of an endogenous nucleic acid sequence encoding a CARD11 protein, or a protein comprising a CARD domain from CARD11 without any SH2 domain. In some embodiments, the recombinant nucleic acid of the cell is located at the endogenous CARD11-encoding gene locus, or comprises at least a portion of the endogenous CARD11-encoding gene, of the cell.

**[0356]** In some embodiments, the cell comprises at least one copy, or at least two copies, of an endogenous nucleic acid sequence encoding a PIK3R3 protein, or a protein comprising an SH2 domain from PIK3R3 without any CARD domain. In some embodiments, the recombinant nucleic acid of the cell is located at the endogenous PIK3R3-encoding gene locus, or comprises at least a portion of the endogenous PIK3R3-encoding gene, of the cell.

**TABLE 3. CARD11-PIK3R3 Truncations**

| Description | Sequence | SEQ ID NO |
|---|---|---|

| | | |
|---|---|---|
| C11P3tr1 amino acid sequence | MDDYMETLKDEEDALWENVECNRHMLSRYINPAKLTPYLRQCKVIDEQD<br>EDEVLNAPMLPSKINRAGRLLDILHTKGQRGYVVFLESLEFYYPELYKLVT<br>GKEPTRRFSTIVVEEGHEGLTHFLMNEVIKLQQQMKAKDLQRCELLARLR<br>QLEDEKKQMTLTRVELLTFQERYYKMKEERDSYNDELVKVKDDNYNLA<br>MRYAQLSEEKNMAVMRSRDLQLEIDQLKHRLNKMEEECKLERNQSLKLK<br>NDIENRPKKEQVLELERENEMLKTKNQELQSIIQAGKRSLPDSDKAILDILE<br>HDRKEALEDRQELVNRIYNLQEEARQAEELRDKYLEEKEDLELKCSTLGK<br>DCEMYKHRMNTVMLQLEEVERERDQAFHSRDEAQTQYSQCLIEKDKYRK<br>QIRELEEKNDEMRIEMVRREACIVNLESKLRRLSKDSNNLDQSLPRNLPVTII<br>SQDFGDASPRTNGQEADDSSTSEESPEDSKYFLPYHPPQRRMNLKGIQLQR<br>AKSPISLKRTSDFQAKGHEEEGTDASPSSCGSLPITNSFTKMQPPRSRSSIMSI<br>TAEPPGNDSIVRRYKEDAPHRSTVEEDENLPHYDEKTWFVEDINRVQAEDL<br>LYGKPDGAFLIRESSKKGCYACSVVADGEVKHCVIYSTARGYGFAEPYNL<br>YSSLKELVLHYQQTSLVQHNDSLNVRLAYPVHAQMPSLCR | 3 |
| C11P3tr2 amino acid sequence | MDDYMETLKDEEDALWENVECNRHMLSRYINPAKLTPYLRQCKVIDEQD<br>EDEVLNAPMLPSKINRAGRLLDILHTKGQRGYVVFLESLEFYYPELYKLVT<br>GKEPTRRFSTIVVEEGHEGLTHFLMNEVIKLQQQMKAKDLQRCELLARLR<br>QLEDEKKQMTLTRVELLTFQERYYKMKEERDSYNDELVKVKDDNYNLA<br>MRYAQLSEEKNMAVMRSRDLQLEIDQLKHRLNKMEEECKLERNQSLKLK<br>NDIENRPKKEQVLELERENEMLKTKNQELQSIIQAGKRSLPDSDKAILDILE<br>HDRKEALEDRQELVNRIYNLQEEARQAEELRDKYLEEKEDLELKCSTLGK<br>DCEMYKHRMNTVMLQLEEVERERDQAFHSRDEAQTQYSQCLIEKDKYRK<br>QIRELEEKNDEMRIEMVRREACIVNLESKLRRLSKDSNNLDQSLPRNLPVTII<br>SQDFGDASPRTNGQEADDSSTSEESPEDSKYFLPYHPPQRRMNLKGIQLQR<br>AKSPISLKRTSDFQAKGHEEEGTDASPSSCGSLPITNSFTKMQPPRSRSSIMSI<br>TAEPPGNDSIVRRYKEDADENYFINEEDENLPHYDEKTWFVEDINRVQAED<br>LLYGKPDGAFLIRESSKKGCYACSVVADGEVKHCVIYSTARGYGFAEPYNL<br>YSSLKELVLHYQQTSLVQHNDSLNVRLAYPVHAQMPSLCR | 4 |
| C11P3tr1 nucleic acid sequence | atggacgactacatggagaccctgaaagacgaggaggacgccctgtgggagaacgtggagtgcaacaggcacatg<br>ctgagcagatacatcaaccctgccaagctgacaccctacctgagacagtgcaaggtgatcgacgagcaggacgagg<br>acgaagtgctgaacgctcccatgctgccctctaaaatcaacagagccggcagactgctggacatcctgcacaccaag<br>ggccagaggggctacgtggtgttcctggagagcctggagttctactatcccgagctctacaagctcgtgaccggcaag<br>gagcccaccagaagattcagcaccattgtggtggaggagggacacgaaggcctgacccacttcctgatgaacgaggt<br>gatcaagctgcaacagcaaatgaaggccaaagacctgcaacggtgcgagctgctggccagactgaggcagctgga<br>ggacgagaagaagcagatgaccctgaccagagtggaacttctgaccttccaagagagatactacaagatgaaagag<br>gaacgcgatagctacaacgacgaacttgtcaaagtgaaggacgacaactacaacctggccatgagatacgcccagct<br>gagcgaagagaagaacatggccgtgatgaggagcagggatctgcaactggagatcgaccagctgaagcacaggct<br>gaacaagatggaagaggagtgtaagctggaacggaaccagagcctgaagcttaagaacgacatcgagaacagacc<br>caagaaggagcaagtgctggagctggagcgcgaaaacgagatgctcaagaccaagaatcaggagttgcagagcat<br>catccaagccggaaagagaagcctgcccgacagcgacaaggccatcctggatattctggagcacgataggaaggag<br>gccctggaagacagacaagaactggtgaacagaatctacaatcttcaagaggaagccagacaggcagaagagctca<br>gggacaaatacttgaggaaaaggaggacctggaactgaagtgctccaccctgggaaaggactgcgagatgtacaa<br>gcacagaatgaacaccgtgatgctgcagctggaagaggtcgagagggagcgcgaccaggccttccacagcaggga<br>cgaagcccagacacagtacagccagtgcctgatcgagaaggacaagtacaggaagcagatcagagaactggagga<br>gaagaatgacgagatgaggatcgagatggtcagaagggaggcctgcatcgtgaacctggaaagcaaactgagaag<br>gctttccaaggacagcaacaatctggatcagagtctgcccagaaacctgcccgtgaccatcattagccaggacttcgg<br>cgacgccagtccaaggaccaacggacaggaggccgacgacagctctaccagcgaagagtctcccgaggactctaa<br>atactttctgccctaccatcctccacaaagaaggatgaacctgaagggcattcaactccaaagagccaagagcccaatc<br>agtctgaagagaaccagcgacttccaagccaaaggccacgaggaagaaggaacagacgcctctccaagctcctgcg<br>gctctctgcccatcaccaactccttcaccaagatgcagccacccaggagtagaagcagcatcatgtccatcacagccg<br>aaccacctggcaacgactccatcgtgagaagatacaaggaagacgctccacataggtccaccgtggaagaagacga<br>gaacctgccacactacgacgaaaagacttggttcgtggaggacatcaatagggttcaagctgaggatctgctgtacgg<br>caagcccgacggagcctttctgattcgggagagcagcaagaagggctgctacgcctgctctgtggtggccgacggc<br>gaagtgaagcactgcgtcatctacagcacagctagaggatacggcttcgccgagccatacaacctgtacagcagcctc<br>aaagagctggttcttcactaccagcagaccagcctggtgcagcacaacgattccctcaacgtgagactggcctaccct<br>gtgcacgcccagatgcccagtctgtgcagatga | 328 |
| C11P3tr2 nucleic acid sequence | atggacgactacatggagaccctgaaagacgaggaggacgccctgtgggagaacgtggagtgcaacaggcacatg<br>ctgagcagatacatcaaccctgccaagctgacaccctacctgagacagtgcaaggtgatcgacgagcaggacgagg<br>acgaagtgctgaacgctcccatgctgccctctaaaatcaacagagccggcagactgctggacatcctgcacaccaag<br>ggccagaggggctacgtggtgttcctggagagcctggagttctactatcccgagctctacaagctcgtgaccggcaag | 329 |

| | | |
|---|---|---|
| | gagcccaccagaagattcagcaccattgtggtggaggagggacacgaaggcctgacccacttcctgatgaacgaggt<br>gatcaagctgcaacagcaaatgaaggccaaagacctgcaacggtgcgagctgctggccagactgaggcagctgga<br>ggacgagaagaagcagatgaccctgaccagagtggaacttctgaccttccaagagagatactacaagatgaaagag<br>gaacgcgatagctacaacgacgaacttgtcaaagtgaaggacgacaactacaacctggccatgagatacgcccagct<br>gagcgaagagaagaacatggccgtgatgaggagcagggatctgcaactggagatcgaccagctgaagcacaggct<br>gaacaagatggaagaggagtgtaagctggaacggaaccagagcctgaagcttaagaacgacatcgagaacagacc<br>caagaaggagcaagtgctggagctggagcgcgaaaacgagatgctcaagaccaagaatcaggagttgcagagcat<br>catccaagccggaaagagaagcctgcccgacagcgacaaggccatcctggatattctggagcacgataggaaggag<br>gccctggaagacagacaagaactggtgaacagaatctacaatcttcaagaggaagccagacaggcagaagagctca<br>gggacaaatatacctttgaggaaaaggaggaggacctggaactgaagtgctccaccctgggaaaggactgcgagatgtacaa<br>gcacagaatgaacaccgtgatgctgcagctggaagaggtcgagagggagcgcgaccaggccttccacagcaggga<br>cgaagcccagacacagtacagccagtgcctgatcgagaaggacaagtacaggaagcagatcagagaactggagga<br>gaagaatgacgagatgaggatcgagatggtcagaagggaggcctgcatcgtgaacctggaaagcaaactgagaag<br>gctttccaaggacagcaacaatctggatcagagtctgcccagaaacctgcccgtgaccatcattagccaggacttcgg<br>cgacgccagtccaaggaccaacggacaggaggccgacgacagctctaccagcgaagagtctcccgaggactctaa<br>atactttctgccctaccatcctccacaaagaaggatgaacctgaagggcattcaactccaaagagccaagagcccaatc<br>agtctgaagagaaccagcgacttccaagccaaaggccacgaggaagaaggaacagacgcctctccaagctcctgcg<br>gctctctgcccatcaccaactccttcaccaagatgcagccacccaggagtagaagcagcatcatgtccatcacagccg<br>aaccacctggcaacgactccatcgtgagaagatacaaggaagacgctgacgagaactacttcatcaacgaagaagac<br>gagaacctgccacactacgacgaaaagacttggttcgtggaggacatcaatagggttcaagctgaggatctgctgtac<br>ggcaagcccgacggagcctttctgattcgggagagcagcaagaagggctgctacgcctgctctgtggtggccgacg<br>gcgaagtgaagcactgcgtcatctacagcacagctagaggatacggcttcgccgagccatacaacctgtacagcagc<br>ctcaaagagctggttcttcactaccagcagaccagcctggtgcagcacaacgattccctcaacgtgagactggcctacc<br>ctgtgcacgcccagatgcccagtctgtgcagatga | |
| C11P3-ID truncation (C-99) amino acid sequence | MDDYMETLKDEEDALWENVECNRHMLSRYINPAKLTPYLRQCKVIDEQD<br>EDEVLNAPMLPSKINRAGRLLDILHTKGQRGYVVFLESLEFYYPELYKLVT<br>GKEPTRRFSTIVVEEGHEGLTHFLMNEVIKLQQQMKAKDLQRCELLARLR<br>QLEDEKKQMTLTRVELLTFQERYYKMKEERDSYNDELVKVKDDNYNLA<br>MRYAQLSEEKNMAVMRSRDLQLEIDQLKHRLNKMEEECKLERNQSLKLK<br>NDIENRPKKEQVLELERENEMLKTKNQELQSIIQAGKRSLPDSDKAILDILE<br>HDRKEALEDRQELVNRIYNLQEEARQAEELRDKYLEEKEDLELKCSTLGK<br>DCEMYKHRMNTVMLQLEEVERERDQAFHSRDEAQTQYSQCLIEKDKYRK<br>QIRELEEKNDEMRIEMVRREACIVNLESKLRRLSKDSNNLDQSLSPEDSKYF<br>LPYHPPQRRMNLKGIQLQRAKSPISLKRTSDFQAKGHEEEGTDASPSSCGSL<br>PITNSFTKMQPPRSRSSIMSITAEPPGNDSIVRRYKEDAPHRRWLNHKGVRQ<br>KRLNVWLGIKNEDADENYFINEEDENLPHYDEKTWFVEDINRVQAEDLLY<br>GKPDGAFLIRESSKKGCYACSVVADGEVKHCVIYSTARGYGFAEPYNLYSS<br>LKELVLHYQQTSLVQHNDSLNVRLAYPVHAQMPSLCR | 407 |
| C11P3-ID truncation (C-198) amino acid sequence | MDDYMETLKDEEDALWENVECNRHMLSRYINPAKLTPYLRQCKVIDEQD<br>EDEVLNAPMLPSKINRAGRLLDILHTKGQRGYVVFLESLEFYYPELYKLVT<br>GKEPTRRFSTIVVEEGHEGLTHFLMNEVIKLQQQMKAKDLQRCELLARLR<br>QLEDEKKQMTLTRVELLTFQERYYKMKEERDSYNDELVKVKDDNYNLA<br>MRYAQLSEEKNMAVMRSRDLQLEIDQLKHRLNKMEEECKLERNQSLKLK<br>NDIENRPKKEQVLELERENEMLKTKNQELQSIIQAGKRSLPDSDKAILDILE<br>HDRKEALEDRQELVNRIYNLQEEARQAEELRDKYLEEKEDLELKCSTLGK<br>DCEMYKHRMNTVMLQLEEVERERDQAFHSRDEAQTQYSQCLIEKDKYRK<br>QIRELEEKNDEMRIEMVRREACIVNLESKLRRLSKDSNNLDQSLLKRTSDF<br>QAKGHEEEGTDASPSSCGSLPITNSFTKMQPPRSRSSIMSITAEPPGNDSIVR<br>RYKEDAPHRRWLNHKGVRQKRLNVWLGIKNEDADENYFINEEDENLPHY<br>DEKTWFVEDINRVQAEDLLYGKPDGAFLIRESSKKGCYACSVVADGEVKH<br>CVIYSTARGYGFAEPYNLYSSLKELVLHYQQTSLVQHNDSLNVRLAYPVH<br>AQMPSLCR | 408 |
| C11P3-ID truncation (C-267) amino acid sequence | MDDYMETLKDEEDALWENVECNRHMLSRYINPAKLTPYLRQCKVIDEQD<br>EDEVLNAPMLPSKINRAGRLLDILHTKGQRGYVVFLESLEFYYPELYKLVT<br>GKEPTRRFSTIVVEEGHEGLTHFLMNEVIKLQQQMKAKDLQRCELLARLR<br>QLEDEKKQMTLTRVELLTFQERYYKMKEERDSYNDELVKVKDDNYNLA<br>MRYAQLSEEKNMAVMRSRDLQLEIDQLKHRLNKMEEECKLERNQSLKLK<br>NDIENRPKKEQVLELERENEMLKTKNQELQSIIQAGKRSLPDSDKAILDILE<br>HDRKEALEDRQELVNRIYNLQEEARQAEELRDKYLEEKEDLELKCSTLGK<br>DCEMYKHRMNTVMLQLEEVERERDQAFHSRDEAQTQYSQCLIEKDKYRK | 409 |

| | | |
|---|---|---|
| | QIRELEEKNDEMRIEMVRREACIVNLESKLRRLSKDSNNLDQSLCGSLPITN SFTKMQPPRSRSSIMSITAEPPGNDSIVRRYKEDAPHRRWLNHKGVRQKRL NVWLGIKNEDADENYFINEEDENLPHYDEKTWFVEDINRVQAEDLLYGKP DGAFLIRESSKKGCYACSVVADGEVKHCVIYSTARGYGFAEPYNLYSSLKE LVLHYQQTSLVQHNDSLNVRLAYPVHAQMPSLCR | |
| C11P3-CC truncation (-216)-Gslink amino acid sequence | MDDYMETLKDEEDALWENVECNRHMLSRYINPAKLTPYLRQCKVIDEQD EDEVLNAPMLPSKINRAGRLLDILHTKGQRGYVVFLESLEFYYPELYKLVT GKEPTRRFSTIVVEEGHEGLTHFLMNEVIKLQQQMKAKDLQRCELLARLR QLEDEKKQMTLTRVELLTFQERYYKMKEERDSYNDELVKVKDDNYNLA MRYAQLSEEKNMAVMRSRDLQLEIDQLKHRLNKMEEECKLERNQSLKLK NDIENSGGGGSGGGGSQAEELRDKYLEEKEDLELKCSTLGKDCEMYKHR MNTVMLQLEEVERERDQAFHSRDEAQTQYSQCLIEKDKYRKQIRELEEKN DEMRIEMVRREACIVNLESKLRRLSKDSNNLDQSLPRNLPVTIISQDFGDAS PRTNGQEADDSSTSEESPEDSKYFLPYHPPQRRMNLKGIQLQRAKSPISLKR TSDFQAKGHEEEGTDASPSSCGSLPITNSFTKMQPPRSRSSIMSITAEPPGND SIVRRYKEDAPHRRWLNHKGVRQKRLNVWLGIKNEDADENYFINEEDENL PHYDEKTWFVEDINRVQAEDLLYGKPDGAFLIRESSKKGCYACSVVADGE VKHCVIYSTARGYGFAEPYNLYSSLKELVLHYQQTSLVQHNDSLNVRLAY PVHAQMPSLCR | 410 |
| C11P3-CC truncation (-216)-endogSeq amino acid sequence | MDDYMETLKDEEDALWENVECNRHMLSRYINPAKLTPYLRQCKVIDEQD EDEVLNAPMLPSKINRAGRLLDILHTKGQRGYVVFLESLEFYYPELYKLVT GKEPTRRFSTIVVEEGHEGLTHFLMNEVIKLQQQMKAKDLQRCELLARLR QLEDEKKQMTLTRVELLTFQERYYKMKEERDSYNDELVKVKDDNYNLA MRYAQLSEEKNMAVMRSRDLQLEIDQLKHRLNKMEEECKLERNQSLKLK NDIENQAGKRSLPDQAEELRDKYLEEKEDLELKCSTLGKDCEMYKHRMNT VMLQLEEVERERDQAFHSRDEAQTQYSQCLIEKDKYRKQIRELEEKNDEM RIEMVRREACIVNLESKLRRLSKDSNNLDQSLPRNLPVTIISQDFGDASPRTN GQEADDSSTSEESPEDSKYFLPYHPPQRRMNLKGIQLQRAKSPISLKRTSDF QAKGHEEEGTDASPSSCGSLPITNSFTKMQPPRSRSSIMSITAEPPGNDSIVR RYKEDAPHRRWLNHKGVRQKRLNVWLGIKNEDADENYFINEEDENLPHY DEKTWFVEDINRVQAEDLLYGKPDGAFLIRESSKKGCYACSVVADGEVKH CVIYSTARGYGFAEPYNLYSSLKELVLHYQQTSLVQHNDSLNVRLAYPVH AQMPSLCR | 411 |
| C11P3-ID modification1 amino acid sequence | MDDYMETLKDEEDALWENVECNRHMLSRYINPAKLTPYLRQCKVIDEQD EDEVLNAPMLPSKINRAGRLLDILHTKGQRGYVVFLESLEFYYPELYKLVT GKEPTRRFSTIVVEEGHEGLTHFLMNEVIKLQQQMKAKDLQRCELLARLR QLEDEKKQMTLTRVELLTFQERYYKMKEERDSYNDELVKVKDDNYNLA MRYAQLSEEKNMAVMRSRDLQLEIDQLKHRLNKMEEECKLERNQSLKLK NDIENRPKKEQVLELERENEMLKTKNQELQSIIQAGKRSLPDSDKAILDILE HDRKEALEDRQELVNRIYNLQEEARQAEELRDKYLEEKEDLELKCSTLGK DCEMYKHRMNTVMLQLEEVERERDQAFHSRDEAQTQYSQCLIEKDKYRK QIRELEEKNDEMRIEMVRREACIVNLESKLRRLSKDSNNLDQSLCGSLPITN SFTKMQPPRSRSSIMSITAEPPGNDSIVRRYKEDAPHRSTVEEDNDSGGFDA LDLDDDSHERYSFGPSSIHSSSSSHQSEGLDAYDLEQVNLMFRKFSLERPFR PSVTSVGHVRGPGPSVWLNHKGVRQKRLNVWLGIKNEDADENYFINEEDE NLPHYDEKTWFVEDINRVQAEDLLYGKPDGAFLIRESSKKGCYACSVVAD GEVKHCVIYSTARGYGFAEPYNLYSSLKELVLHYQQTSLVQHNDSLNVRL AYPVHAQMPSLCR | 412 |

***e. Biological Activity of Engineered CARD11-PIK3R3 Fusion Proteins***

**[0357]** In some embodiments the engineered CARD11-PIK3R3 fusion protein is functionally equivalent to a patient derived full length CARD11-PIK3R3 fusion protein. As used herein, "functionally equivalent" refers to having at least one type of biological activity of a patient-derived human CARD11-PIK3R3 fusion protein.

**[0358]** In some embodiments an engineered cell comprising the engineered CARD11-PIK3R3 fusion protein is functionally equivalent to an engineered cell comprising a patient derived full length CARD11-PIK3R3 fusion protein. As used herein, "functionally equivalent" refers to having at least one type of biological activity of an engineered cell comprising a patient-derived human CARD11-PIK3R3 fusion protein.

**[0359]** The polypeptides of the disclosure, or the recombinant nucleic acid constructs of the disclosure, can alter T cell signaling in various ways. In some embodiments, the altering of the T cell signaling can be by enhancing, promoting, improving, reducing, regulating, or modulating the signaling pathway within a T cell. In some embodiments, the altering of the T cell signaling can be by activating, increasing, suppressing, inhibiting, or other means of changing the signaling.

**[0554]** The polypeptides of the disclosure, or the recombinant nucleic acid constructs of the disclosure, can alter T cell signaling through one or more T cell signaling pathways. In some embodiments, the polypeptides or recombinant nucleic acid constructs of the disclosure can alter T cell signaling by increasing one or more pathways and/or reducing one or more pathways, or by a combination of enhancing and/or reducing various pathways, for example as shown in **FIG 2F**. In some embodiments, the mutation can alter cytokine production. The cytokine can be, without limitation, IL-2, IL-4, IL-5, TNF alpha, IFN-gamma, IL-13 and/or other cytokines. In some embodiments, the cytokine is IL-2. In some embodiments, the cytokine is IL-2, and the production of IL-2 is increased.

**[0555]** In some embodiments, the polypeptides or recombinant nucleic acid constructs of the disclosure can alter T cell signaling through one or more T cell signaling pathways. Signaling pathways can be, without limitation, the NFAT pathway, NF-κB pathway, AP-1 pathway, JAK/STAT pathway, RAS/MEK/ERK, and/or phospholipase gamma signaling, or described elsewhere herein.

**[0556]** In some embodiments, the polypeptides or recombinant nucleic acid constructs of the disclosure can alter CARD11-BCL10-MALT1 complex signaling, co-stimulatory molecule signaling, cytokine production and/or transcription factor activity in T cells.

**[0557]** The disclosure also relates to polypeptides having one or more mutations that can reduce T cell exhaustion, increase proliferation, alter effector function, resist T cell dysfunction, increase T cell fitness, enhance *in vivo* persistence, and/or increase intratumoral presence of therapeutic T cells. The disclosure further relates to recombinant nucleic acids encoding polypeptides having one or more mutations, that can reduce T cell exhaustion, increase proliferation, alter effector function, resist T cell dysfunction, increase T cell

fitness, enhance *in vivo* persistence, and/or increase intratumoral presence of therapeutic T cells.

**[0558]** T cell fitness can refer to the ability of a T cell to generate an immune response. The ability of a T cell to perform T cell functions such as signaling, cytokine production, survival, and persistence in a tumor, may contribute to the fitness of a T cell. T cell exhaustion may contribute to reducing its fitness.

**[0559]** In some embodiments, polypeptides of the disclosure, or the recombinant nucleic acid constructs of the disclosure can alter *in vivo* persistence in tumors of therapeutic T cells including the mutation. The *in vivo* persistence of therapeutic T cells can refer to the length of time that the therapeutic T cells exist within the tumor of the host after infusion. In some embodiments, *in vivo* persistence of therapeutic T cells is enhanced. Enhanced *in vivo* persistence of therapeutic T cells can include at least a positive log2 fold change in therapeutic T cells as compared to the input total.

**[0560]** In some embodiments, the polypeptides of the disclosure, or the recombinant nucleic acid constructs of the disclosure can alter the therapeutic efficacy of engineered T cells. Alteration of therapeutic efficacy can include, without limitation, a decrease in T cell exhaustion, increased proliferative capacity, enhanced anti-tumor effect, enhanced replicative lifespan, decreased replicative senescence, enhanced ability to kill, enhanced fitness of engineered T cells and/or other functions or activities of T cells. In some embodiments, the nucleic acid constructs of the disclosure encodes a polypeptide comprising an amino acid sequence having at least about 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% sequence identity to any one of SEQ ID NOs: 3-150 or a variant thereof.

**[0360]** In some embodiments, the nucleic acid constructs of the disclosure include a nucleic acid sequence encoding a polypeptide with at least about 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% identity to any one of SEQ ID NOs: 3-150 or a variant thereof.

## IV. POLYNUCLEOTIDES

**[0361]** In some embodiments, the engineered polypeptides disclosed herein are encoded by a polynucleotide. In some embodiments, the polynucleotide of the disclosure is a recombinant nucleic acid / nucleic acid construct — for example, a plasmid construct or other recombinant constructs. In some embodiments, the polynucleotide is or comprises DNA. In some embodiments, the polynucleotide is or comprises cDNA. In some

embodiments, the polynucleotide is or comprises RNA. In some embodiments, the polynucleotide is or comprises mRNA.

**[0362]** A polynucleotide encoding the engineered polypeptides disclosed herein can be obtained by methods known in the art. For example, the polynucleotide can be obtained from cloned DNA (e.g., from a DNA library), by chemical synthesis, by cDNA cloning, or by the cloning of genomic DNA or fragments thereof, purified from the desired cell. When the polynucleotides are produced by recombinant means, any method known to those skilled in the art for identification of nucleic acids that encode desired genes can be used. Polynucleotides produced by recombinant means may also be referred to as "recombinant nucleic acids" herein. Any method available in the art can be used to obtain a full length (i.e. encompassing the entire coding region) cDNA or genomic DNA encoding a desired human CARD11 protein and PIK3R3 protein, such as from a cell or tissue source. Modified or variant polynucleotides, including truncated forms of CARD11-PIK3R3 fusion proteins such as provided herein, can be engineered from a patient-derived polynucleotide using standard recombinant DNA methods. Polynucleotides can be cloned or isolated using any available methods known in the art for cloning and isolating nucleic acid molecules. Such methods include PCR amplification of nucleic acids and screening of libraries, including nucleic acid hybridization screening, antibody-based screening, and activity-based screening.

**[0363]** Methods for amplification of polynucleotides can be used to isolate polynucleotides encoding a desired protein, including for example, polymerase chain reaction (PCR) methods. PCR can be carried out using any known methods or procedures in the art. Exemplary methods include use of a Perkin-Elmer Cetus thermal cycler and Taq polymerase (Gene Amp). A nucleic acid containing gene of interest can be used as a source material from which a desired polypeptide-encoding nucleic acid molecule can be amplified. For example, DNA and mRNA preparations, cell extracts, tissue extracts from an appropriate source (e.g. testis, prostate, breast), fluid samples (e.g. blood, serum, saliva), samples from healthy and/or diseased subjects can be used in amplification methods. The source can be from any eukaryotic species including, but not limited to, vertebrate, mammalian, human, porcine, bovine, feline, avian, equine, canine, and other primate sources. Nucleic acid libraries also can be used as a source material. Primers can be designed to amplify a desired polynucleotide. For example, primers can be designed based on expressed sequences from which a desired polynucleotide is generated. Primers can be designed based on back-translation of a polypeptide amino acid sequence. If desired, degenerate primers can be used for amplification. Oligonucleotide primers that hybridize to sequences at the 3' and 5'

termini of the desired sequence can be uses as primers to amplify by PCR from a nucleic acid sample. Primers can be used to amplify the entire full-length polynucleotide, or a truncated sequence thereof. Nucleic acid molecules generated by amplification can be sequenced and confirmed to encode a desired polypeptide.

**[0364]** In some embodiments, the polynucleotide encoding the engineered polypeptides disclosed herein additionally encodes at least one additional polypeptide (e.g., a second polypeptide or a second recombinant polypeptide). In some embodiments, the nucleic acid construct further comprises a polynucleotide sequence encoding one or more additional polypeptides and/or a non-coding RNA. In some embodiments, the non-coding RNA comprises a shRNA or a microRNA. In some embodiments, the one or more additional polypeptides comprise a chimeric antigen receptor (CAR), a recombinant T cell receptor (TCR), an additional potency enhancement polypeptide, a cytokine, a chemokine, a growth factor, a safety switch, or any combination thereof. In some embodiments, the one or more additional polypeptides comprise a CAR or a recombinant TCR. In some embodiments, the CAR or TCR is any CAR or TCR described herein.

**[0365]** In some embodiments, the polynucleotide sequence encoding the recombinant polypeptide and the polynucleotide sequence encoding the at least one additional polypeptide are separated by a polynucleotide sequence encoding one or more cleavable linkers. In some embodiments, all these polynucleotide sequences form a continuous open reading frame. In some embodiments, the one or more cleavable linkers comprise a P2A, E2A, F2A, or T2A self-cleaving peptide.

**[0366]** In some embodiments, the polynucleotide sequence encoding the engineered polypeptide and the polynucleotide sequence encoding the at least one additional polypeptide are separated by an internal ribozyme entry site (IRES).

**[0367]** In some embodiments, the polynucleotide is a DNA. In some embodiments, the polynucleotide is an RNA. In some embodiments, the polynucleotide is a mRNA.

**[0368]** In some embodiments, the polynucleotide comprises one or more modified nucleotides. In some embodiments, the one or more modified nucleotides are or comprise pseudouridine, N1-methylpseudouridine, 4'-thiouridine, 5-methylcytosine, 2-thio-l-methyl-1-deaza-pseudouridine, 2-thio-l-methyl-pseudouridine, 2-thio-5-aza-uridine, 2-thio-dihydropseudouridine, 2-thio-dihydrouridine, 2-thio-pseudouridine, 4-methoxy-2-thio-pseudouridine, 4-methoxy-pseudouridine, 4-thio-l-methyl- pseudouridine, 4-thio-pseudouridine, 5-aza-uridine, dihydropseudouridine, 5-methyluridine, 5-methyluridine, 5-methoxyuridine, 2'-O-methyl uridine, or any combination thereof. In some embodiments,

the one or more modified nucleotides are selected from the group consisting of pseudouridine, N1-methylpseudouridine, 5-methylcytosine, 5- methoxyuridine, and a combination thereof. In some embodiments, the one or more modified nucleotides are N1-methylpseudouridines.

**[0369]** The nucleic acid construct may further comprise homology arms before the 5' end and/or after the 3' end of the nucleic acid sequence encoding the recombinant polypeptide. In some embodiments, the nucleic acid construct comprises a first homology arm before the 5' end of the nucleic acid sequence encoding the recombinant polypeptide and a second homology arm after the 3' end of the nucleic acid sequence encoding the recombinant polypeptide. In some embodiments, the lengths of the two homology arms are between about 150 bp and about 1500 bp. In some embodiments, the length of at least one of the homology arms is about 200bp, 225 bp, about 250 bp, about 275 bp, about 300 bp, about 325 bp, about 350 bp, about 375 bp, about 400 bp, about 425 bp, about 450 bp, about 475 bp, or about 500 bp. In some embodiments, the lengths of both of the homology arms are about 225 bp, about 250 bp, about 275 bp, about 300 bp, about 325 bp, about 350 bp, about 375 bp, about 400 bp, about 425 bp, about 450 bp, about 475 bp, about 500 bp, about 525 bp, about 550 bp, about 575 bp, or about 600 bp. In some embodiments, the total length of the homology arms is between about 400 bp and about 500 bp, about 500 bp and about 600 bp, about 600 bp and about 700 bp, about 700 bp and about 800 bp, about 800 bp and about 900 bp, about 900 bp and about 1000 bp, about 1000 bp and about 1100 bp, or about 1100 bp and about 1200 bp.

**[0370]** In some embodiments, the recombinant nucleic acid is less than 10 kb, less than 9 kb, less than 8 kb, less than 7 kb, less than 6 kb, less than 5 kb, less than 4 kb, or less than 3 kb in length. In some embodiments, the recombinant nucleic acid is less than about 5.0kb in length. In some embodiments, the recombinant nucleic acid is less than about 4.7kb in length.

**[0371]** In some embodiments, the length of the polynucleotide sequence encoding the one or more additional polypeptides is less than about 2100 bp, less than about 2150 bp, less than about 2200 bp, less than about 2250 bp, less than about 2300 bp, less than about 2350 bp, or less than about 2400 bp. In some embodiments, the length of the one or more additional polypeptides is less than about 700 amino acids, less than about 725 amino acids, less than about 750 amino acids, less than about 775 amino acids, less than about 800 amino acids, less than about 825 amino acids, less than about 850 amino acids, less than about 875 amino acids, or less than about 900 amino acids.

**[0372]** In some embodiments, the polynucleotide encoding the engineered peptides disclosed herein is comprised with a plasmid. In some embodiments, the plasmid is between about 2 kilobases (kb) and about 10 kb in size, or between about 3 kb and about 8 kb in size. In some embodiments, the plasmid is less than about 8 kb, less than about 7 kb, less than about 6 kb, less than about 5 kb, or less than about 4.5 kb in size. In some embodiments, the plasmid is less than about 8 kb in size. In some embodiments, the plasmid is less than about 7.5 kb in size. In some embodiments, the plasmid is less than about 7 kb in size. In some embodiments, the plasmid is less than about 6.5 kb in size. In some embodiments, the plasmid is less than about 6 kb in size. In some embodiments, the plasmid is less than about 5.5 kb in size. In some embodiments, the plasmid is less than about 5 kb in size. In some embodiments, the plasmid is less than about 4.5 kb in size. In some embodiments, the plasmid is about 4.5 kb in size. In some embodiments, the plasmid is about 5 kb in size. In some embodiments, the plasmid is about 5.5 kb in size. In some embodiments, the plasmid is about 6.5 kb in size. In some embodiments, the plasmid is about 7 kb in size. In some embodiments, the plasmid is about 6 kb in size. In some embodiments, the plasmid is about 7.5 kb in size. In some embodiments, the plasmid is about 8 kb in size.

**[0373]** In some embodiments, the polynucleotide encoding the polynucleotide encoding the engineered CARD11-PIK3R3 fusion protein contains at least one promoter that is operatively linked to control expression of the polynucleotide encoding the engineered CARD11-PIK3R3 fusion protein. In some examples, the polynucleotide contains two, three, or more promoters operatively linked to control expression of the polynucleotide encoding the engineered CARD11-PIK3R3 fusion protein. In some embodiments, polynucleotide can contain regulatory sequences, such as transcription and translation initiation and termination codons, which are specific to the type of host (e.g., bacterium, fungus, plant, or animal) into which the polynucleotide is to be introduced, as appropriate and taking into consideration whether the polynucleotide is DNA- or RNA-based. In some embodiments, the polynucleotide can contain regulatory/control elements, such as a promoter, an enhancer, an intron, a polyadenylation signal, a Kozak consensus sequence, internal ribosome entry sites (IRES), a 2A sequence, and splice acceptor or donor. In some embodiments, the polynucleotide can contain a non-native promoter operably linked to the nucleotide sequence encoding the polynucleotide encoding the engineered CARD11-PIK3R3 fusion protein and/or one or more additional polypeptide(s). In some embodiments, the promoter is selected from among an RNA pol I, pol II or pol III promoter. In some embodiments, the promoter is recognized by RNA polymerase II (e.g., a CMV, SV40 early region or

adenovirus major late promoter). In another embodiment, the promoter is recognized by RNA polymerase III (e.g., a U6 or Hl promoter). In some embodiments, the promoter can be a non-viral promoter or a viral promoter, such as a cytomegalovirus (CMV) promoter, an SV40 promoter, an RSV promoter, and a promoter found in the long-terminal repeat of the murine stem cell virus. Other known promoters also are contemplated.

**[0374]** In some embodiments, the promoter is or comprises a constitutive promoter. Exemplary constitutive promoters include, MND promoter, EF1a promoter, sEF1 promoter, gamma retroviral LTR promoter, CD4 promoter, CD8a promoter, CD8b promoter, TCRa promoter, TCRb promoter, CD3d promoter, CD3g promoter, CD3e promoter, CD3z promoter, CARD9 promoter, CARD10 promoter, CARD11 promoter, CARD14 promoter, PIK3R3 promoter, MND promoter or a short EF1 promoter.

**[0375]** In some embodiments, the promoter is a minimal TATA promoter, a pGK, actin promoter, CD25 promoter, IL2 promoter, IL7 promoter, IL15 promoter, KLRG-1 promoter, HLA-DR promoter, CD38 promoter, CD69 promoter, Ki-67 promoter, CD11a promoter, CD58 promoter, CD99 promoter, CD62L promoter, CD103 promoter, CCR4 promoter, CCR5 promoter, CCR6 promoter, CCR9 promoter, CCR10 promoter, CXCR3 promoter, CXCR4 promoter, CLA promoter, Granzyme A promoter, Granzyme B promoter, Perforin promoter, CD57 promoter, CD161 promoter, IL-18Ra promoter,CD69 promoter, GzmB promoter, T-bet promoter, IFNgamma promoter, TIM3 promoter, IL4 promoter, GATA3 promoter, IL1 promoter, IL5 promoter, IL6 promoter, IL13 promoter, IL10 promoter, IL17A promoter, IL6 promoter, IL21 promoter, IL23R promoter, FoxP3 promoter, CTLA4 promoter, CD25 promoter, PD1 promoter, CD45RO promoter, CCR7 promoter, CD28 promoter, CD95 promoter, CD28 promoter, CD27 promoter, CD127 promoter, PD-1 promoter, CD122 promoter, CD132 promoter, c-Kit promoter, nuclear factor of activated T cells (NFAT) promoter, programmed death 1 (PD-1) promoter, T cell immunoglobulin mucin-3 (TIM-3) promoter, cytotoxic T lymphocyte antigen-4 (CTLA4) promoter, lymphocyte-activation protein 3 (LAG-3) promoter, tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) promoter, B- and T-lymphocyte attenuator (BTLA) promoter, CD25 promoter, CD69 promoter, Fas ligand (FasL) promoter, TIGIT promoter, TGF-beta promoter, T-bet promoter, Eomes promoter, GATA3 promoter, CD45RA promoter, 2B4 promoter, Type I interferon (IFN) alpha, Type I IFN beta promoter, IFN gamma promoter, IRF3 promoter, IRF7 promoter, NF-κB promoter, AP-1 promoter, TNF-alpha promoter, and CD130 promoter, NR4A1 promoter, NR4A2, NR4A3 promoter, MND promoter, EF-1alpha promoter, short EF-1alpha promoter, CAG promoter, ubiquitin/S27a

promoter, SV40 promoter, SV40 early promoter, adenovirus major late promoter, mouse metallothionein-I promoter, Moloney murine leukemia virus (MMLV) long terminal repeat (LTR) region, CMV promoter, immunoglobulin promoter, heat shock promoter, polyoma virus promoter, fowlpox virus promoter, bovine papilloma virus promoter, avian sarcoma virus promoter, retrovirus promoter, hepatitis-B virus promoter, PGK promoter, vaccinia virus 7.5K promoter, TK promoter of HSV, mouse mammary tumor virus (MMTV) promoter, LTR promoter of HIV, promoter of moloney virus, Epstein Barr virus (EBV) promoter, Rous sarcoma virus (RSV) promoter, U6 promoter, or UBC promoter. Exemplary constitutive promoters include, e.g., simian virus 40 early promoter (SV40), cytomegalovirus immediate-early promoter (CMV), human Ubiquitin C promoter (UBC), human elongation factor la promoter (EFla), mouse phosphoglycerate kinase 1 promoter (PGK), and chicken P-Actin promoter coupled with CMV early enhancer (CAG). In some embodiments, the constitutive promoter is a synthetic or modified promoter. In some embodiments, the promoter is or comprises an MND promoter, a synthetic promoter that contains the U3 region of a modified MoMuLV LTR with myeloproliferative sarcoma virus enhancer (see Challita et al. (1995) J. Virol. 69(2):748-755). In some embodiments, the promoter is a tissue-specific promoter. In another embodiment, the promoter is a viral promoter. In another embodiment, the promoter is a non-viral promoter. In some embodiments, exemplary promoters can include, but are not limited to, human elongation factor 1 alpha (EFla) promoter or a modified form thereof or the MND promoter.

**[0376]** In another embodiment, the promoter is a regulated promoter (e.g., inducible promoter). In some embodiments, the promoter is an inducible promoter or a repressible promoter. In some embodiments, the promoter comprises a Lac operator sequence, a tetracycline operator sequence, a galactose operator sequence or a doxycycline operator sequence, or is an analog thereof or is capable of being bound by or recognized by a Lac repressor or a tetracycline repressor, or an analog thereof. In some embodiments, the recombinant nucleic acid does not include a regulatory element, e.g. promoter. In some embodiments, the polynucleotide sequence encoding the recombinant polypeptide is not operably linked to a promoter.

**[0377]** In some embodiments, the polynucleotide sequence further comprises a polynucleotide sequence encoding one or more cleavable linkers. Non-limiting examples of cleavable linkers include P2A, E2A, F2A, and T2A self-cleaving peptides. In some embodiments, the self-cleaving peptide comprises a sequence at least 70%, at least 75%, at

least 80%, at least 85%, at least 90%, at least 95%, at least 99%, or 100% identical to any one of SEQ ID NO: 376-379.

**[0378]** In some embodiments, the recombinant nucleic acid further comprises one or more additional nucleic acid coding or regulatory sequences. Non-limiting examples of control sequences are ribosomal binding sites, enhancer elements, activator elements, translational start sequences, translational termination sequences, transcription start sequences, transcription termination sequences, polyadenylation signal sequences, a 70 bp poly(A) tract, a 100 bp poly(A) tract, a 172 bp poly(A) tract, a 200 bp poly(A) tract, a 300 bp poly(A) tract, a 325 bp poly(A) tract, replication elements, RNA processing and export elements, transposon sequences, transposase sequences, insulator sequences, internal ribosome entry sites (IRES), 5'UTRs, 3'UTRs, mRNA 3' end processing sequences, boundary elements, locus control regions (LCR), matrix attachment regions (MAR), recombination or cassette exchange sequences, linker sequences, cleavable linker sequences, secretion signals, resistance markers, anchoring peptides, localization signals, fusion tags, affinity tags, chaperonins, proteases, or any combination thereof.

**[0379]** In some cases, the nucleic acid sequence encoding the polypeptide disclosed herein contains a signal sequence that encodes a signal peptide. In some aspects, the signal sequence may encode a signal peptide derived from a native polypeptide. In other aspects, the signal sequence may encode a heterologous or non-native signal peptide, such as the exemplary signal peptide of a GMCSFR alpha chain. In some cases, the signal peptide is comprised within the polypeptide.

**[0380]** In some embodiments, the polynucleotide sequence further comprises a polynucleotide sequence encoding one or more additional polypeptides and/or a non-coding RNA, optionally wherein the non-coding RNA comprises a shRNA or a microRNA. In some embodiments, the one or more additional polypeptides comprise an additional potency enhancement polypeptide, a tolerogenic factor, a cytokine, a chemokine, a growth factor, or any combination thereof. Non-limiting examples of additional potency enhancement polypeptides include a dominant negative form of an inhibitor of a cell-mediated immune response of the immune cell (e.g., TGFβR2 DNR), c-Jun, CCL19, CCL21, IL2R, IL7, IL7Ralpha, IL15, IL15RA, IL18, decoy-resistant IL18 (DR-18), MyD88/CD40, PD1-CD28 switch receptor, PD1-41BB switch receptor, CD40L-CD28 switch receptor, CTBR12 switch receptor, or CD8alpha/beta.

**[0381]** In some embodiments, the tolerogenic factor is or comprises A20/TNFAIP3, B2M-HLA-E, CD16, CD16 Fc receptor, CD24, CD27, CD35, CD39, CD46, CD47, CD52, CD55,

CD59, CD64, CD200, CCL21, CCL22, CTLA4-Ig, Cl inhibitor, CR1, DUX4, FASL, HLA-C, HLA-E, HLA-E heavy chain, HLA-F, HLA-G, H2-M3, IDO1, IL-10, IL15-RF, IL-35, IL-39, MANF, Mfge8, PD-L1, Serpinb9, or any combination thereof.

**[0382]** In some embodiments, the polynucleotide contains a nucleic acid sequence encoding one or more additional polypeptides, e.g., one or more marker(s) and/or one or more effector molecules. In some embodiments, the disclosure provides a combination of polynucleotides, in which the engineered polypeptides are encoded by separate polynucleotides. In some embodiments, the one or more marker(s) includes a transduction marker, a surrogate marker and/or a selection marker. Among additional nucleic acid sequences introduced, e.g., encoding for one or more additional polypeptide(s), include nucleic acid sequences that encode a chimeric antigen receptor or heterologous TCR, nucleic acid sequences that can improve the efficacy of therapy, such as by promoting viability and/or function of transferred cells; nucleic acid sequences to provide a genetic marker for selection and/or evaluation of the cells, such as to assess in vivo survival or localization; nucleic acid sequences to improve safety, for example, by making the cell susceptible to negative selection in vivo as described by Lupton S. D. et al., Mol. and Cell Biol., 11:6 (1991); and Riddell et al., Human Gene Therapy 3:319-338 (1992); see also WO 1992008796 and WO 1994028143 describing the use of bifunctional selectable fusion genes derived from fusing a dominant positive selectable marker with a negative selectable marker, and US Patent No. 6,040,177.

**[0383]** Any of the polynucleotide encoding the engineered CARD11-PIK3R3 fusion proteins and/or the additional polypeptide(s) described herein can be encoded by one or more polynucleotides containing one or more nucleic acid sequences encoding polynucleotide encoding the engineered CARD11-PIK3R3 fusion proteins, in any combinations, orientation or arrangements. For example, one, two, three or more polynucleotides can encode one, two, three or more different polypeptides, e.g., polynucleotide encoding the engineered CARD11-PIK3R3 fusion proteins or portions or components thereof, and/or one or more additional polypeptide(s), e.g., a marker and/or an effector molecule. In some embodiments, one polynucleotide contains a nucleic acid sequence encoding a polynucleotide encoding the engineered CARD11-PIK3R3 fusion protein, or portion or components thereof, and a nucleic acid sequence encoding one or more additional polypeptide(s). In some embodiments, one vector or construct contains a nucleic acid sequence encoding a polynucleotide encoding the engineered CARD11-PIK3R3 fusion protein, or portion or components thereof, and a separate vector or construct contains a

nucleic acid sequence encoding one or more additional polypeptide(s). In some embodiments, the nucleic acid sequence encoding the polynucleotide encoding the engineered CARD11-PIK3R3 fusion protein and the nucleic acid sequence encoding the one or more additional polypeptide(s) are operably linked to two different promoters. In some embodiments, the nucleic acid encoding the polynucleotide encoding the engineered CARD11-PIK3R3 fusion protein is present upstream of the nucleic acid encoding the one or more additional polypeptide(s). In some embodiments, the nucleic acid encoding the polynucleotide encoding the engineered CARD11-PIK3R3 fusion protein is present downstream of the nucleic acid encoding one or more additional polypeptide(s).

**[0384]** In some embodiments, polynucleotides can be engineered as a bicistronic unit containing an IRES, which allows coexpression of gene products (e.g. encoding the recombinant receptor and the additional polypeptide) by a message from a single promoter. Alternatively, in some cases, a single promoter may direct expression of an RNA that contains, in a single open reading frame (ORF), two or three genes (e.g. encoding the marker and encoding the recombinant receptor) separated from one another by sequences encoding a self-cleavage peptide (e.g., 2A sequences) or a protease recognition site (e.g., furin). The ORF thus encodes a single polypeptide, which, either during (in the case of 2A) or after translation, is processed into the individual proteins. In some cases, the peptide, such as a T2A, can cause the ribosome to skip (ribosome skipping) synthesis of a peptide bond at the C-terminus of a 2A element, leading to separation between the end of the 2A sequence and the next peptide downstream (see, e.g., de Felipe, Genetic Vaccines and Ther. 2:13 (2004) and de Felipe et al. Traffic 5:616-626 (2004)). Various 2A elements are known. Examples of 2A sequences that can be used in the methods and system disclosed herein, without limitation, 2A sequences from the foot-and-mouth disease virus (F2A), equine rhinitis A virus (E2A), Thosea asigna virus (T2A), and porcine teschovirus-1 (P2A) as described in U.S. Patent Pub. No. 20070116690.

**[0385]** In some embodiments, the polynucleotide comprises one or more modified nucleotides. Non-limiting examples of modified nucleotides include pseudouridine, N1-methylpseudouridine, 4'-thiouridine, 5-methylcytosine, 2-thio-l-methyl-1-deaza-pseudouridine, 2-thio-l-methyl-pseudouridine, 2-thio-5-aza-uridine, 2-thio-dihydropseudouridine, 2-thio-dihydrouridine, 2-thio-pseudouridine, 4-methoxy-2-thio-pseudouridine, 4-methoxy-pseudouridine, 4-thio-l-methyl- pseudouridine, 4-thio-pseudouridine, 5-aza-uridine, dihydropseudouridine, 5-methyluridine, 5-methyluridine, 5-methoxyuridine, 2'-O-methyl uridine. In some embodiments, the one or more modified

nucleotides are selected from the group consisting of pseudouridine, N1-methylpseudouridine, 5-methylcytosine, 5- methoxyuridine, and a combination thereof. In some embodiments, the one or more modified nucleotides are N1- methylpseudouridines.

**[0386]** In some aspects, nucleic acid sequences of interest, including coding and/or noncoding sequences and/or partial coding sequences, that are inserted or integrated at the target location in the genome can also be referred to as "transgene," "transgene sequences," "exogenous nucleic acids sequences," "heterologous sequences" or "donor sequences." In some aspects, the transgene is a nucleic acid sequence that is exogenous or heterologous to an endogenous genomic sequences, such as the endogenous genomic sequences at a specific target locus or target location in the genome, of a T cell, e.g., a human T cell. In some aspects, the transgene is a sequence that is modified or different compared to an endogenous genomic sequence at a target locus or target location of a T cell, e.g., a human T cell. In some aspects, the transgene is a nucleic acid sequence that originates from or is modified compared to nucleic acid sequences from different genes, species and/or origins. In some aspects, the transgene is a sequence that is derived from a sequence from a different locus, e.g., a different genomic region or a different gene, of the same species.

**[0387]** Polynucleotides for insertion can also be referred to as "transgene" or "exogenous sequences" or "donor" polynucleotides or molecules. The template polynucleotide can be DNA, single-stranded and/or double-stranded and can be introduced into a cell in linear or circular form. The template polynucleotide can be RNA single-stranded and/or double-stranded and can be introduced as a RNA molecule (e.g., part of an RNA virus). See also, U.S. Patent Publication Nos. 20100047805 and 20110207221. The template polynucleotide can also be introduced in DNA form, which may be introduced into the cell in circular or linear form. If introduced in linear form, the ends of the template polynucleotide can be protected (e.g., from exonucleolytic degradation) by known methods. For example, one or more dideoxynucleotide residues are added to the 3' terminus of a linear molecule and/or self-complementary oligonucleotides are ligated to one or both ends. See, for example, Chang et al. (1987) Proc. Natl. Acad. Sci. USA 84:4959-4963; Nehls et al. (1996) Science 272:886-889. Additional methods for protecting exogenous polynucleotides from degradation include, but are not limited to, addition of terminal amino group(s) and the use of modified internucleotide linkages such as, for example, phosphorothioates, phosphoramidates, and O-methyl ribose or deoxyribose residues. If introduced in double-stranded form, the template polynucleotide may include one or more nuclease target site(s),

for example, nuclease target sites flanking the transgene to be integrated into the cell's genome. See, e.g., U.S. Patent Publication No. 20130326645.

## V. VECTORS

**[0388]** In another aspect, the present disclosure provides a vector comprising the polynucleotide that encodes the engineered polypeptides disclosed herein. In some embodiments, the vector can be used to transform or transfect a host cell, e.g., a cell for engineering. Exemplary vectors include vectors designed for introduction, propagation and expansion or for expression or both, such as DNA vectors (e.g., a plasmid or closed-end vector) and viral vectors. In some aspects, the vector is an expression vector, e.g., a recombinant expression vector. In some embodiments, the recombinant expression vectors can be prepared using standard recombinant DNA techniques.

**[0389]** The nucleic acid molecules can be contained within a vector that is capable of directing their expression in, for example, a cell that has been transformed/transduced with the vector. Suitable vectors for use in eukaryotic and prokaryotic cells are known in the art and are commercially available, or readily prepared by a skilled artisan. See for example, Sambrook, J., & Russell, D. W. (2012). Molecular Cloning: A Laboratory Manual (4th ed.). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory and Sambrook, J., & Russel, D. W. (2001). Molecular Cloning: A Laboratory Manual (3rd ed.). Cold Spring Harbor, NY: Cold Spring Harbor Laboratory (jointly referred to herein as "Sambrook"); Ausubel, F. M. (1987). Current Protocols in Molecular Biology. New York, NY: Wiley (including supplements through 2014); Bollag, D. M. et al. (1996). Protein Methods. New York, NY: Wiley-Liss; Huang, L. et al. (2005). Nonviral Vectors for Gene Therapy. San Diego: Academic Press; Kaplitt, M. G. et al. (1995). Viral Vectors: Gene Therapy and Neuroscience Applications. San Diego, CA: Academic Press; Lefkovits, I. (1997). The Immunology Methods Manual: The Comprehensive Sourcebook of Techniques. San Diego, CA: Academic Press; Doyle, A. et al. (1998). Cell and Tissue Culture: Laboratory Procedures in Biotechnology. New York, NY: Wiley; Mullis, K. B., Ferré, F. & Gibbs, R. (1994). PCR: The Polymerase Chain Reaction. Boston: Birkhauser Publisher; Greenfield, E. A. (2014). Antibodies: A Laboratory Manual (2nd ed.). New York, NY: Cold Spring Harbor Laboratory Press; Beaucage, S. L. et al. (2000). Current Protocols in Nucleic Acid Chemistry. New York, NY: Wiley, (including supplements through 2014); and Makrides, S. C. (2003). Gene Transfer and Expression in Mammalian Cells. Amsterdam, NL: Elsevier Sciences B.V., the disclosures of which are incorporated herein by reference).

**[0390]** Any methods known to those skilled in the art for the insertion of nucleic acid fragments into a vector can be used to construct expression vectors comprising a polynucleotide disclosed herein. These methods can include in vitro recombinant DNA and synthetic techniques and in vivo (genetic) recombination. The polynucleotide disclosed herein can be operably linked to control sequences in the expression vector(s) to ensure the expression of the polypeptide of the disclosure. Such control sequences may include, but are not limited to, leader or signal sequences, promoters (e.g., naturally associated or heterologous promoters), ribosomal binding sites, enhancer or activator elements, translational start and termination sequences, and transcription start and termination sequences, and are chosen to be compatible with the host cell chosen to express the polypeptide of the disclosure. Constitutive or inducible promoters as known in the art are also contemplated. The promoters may be either naturally occurring promoters, hybrid promoters that combine elements of more than one promoter, or synthetic promoters. An expression construct may be present in a cell on an episome, such as a plasmid, or the expression construct may be inserted in a chromosome such as in a gene locus. In some embodiment, the expression vector includes a selectable marker gene to allow the selection of transformed host cells. Some embodiments, include an expression vector comprising a nucleotide sequence encoding a variant polypeptide operably linked to at least one regulatory control sequence. Regulatory control sequence for use herein include promoters, enhancers, and other expression control elements. In some embodiments, an expression vector is designed for the choice of the host cell to be transformed, the particular variant polypeptide desired to be expressed, the vector's copy number, the ability to control that copy number, and/or the expression of any other protein encoded by the vector, such as antibiotic markers.

**[0391]** Examples of suitable mammalian promoters include, for example, MND promoter, EF1a promoter, sEF1 promoter, gamma retroviral LTR promoter, CD4 promoter, CD8a promoter, CD8b promoter, TCRa promoter, TCRb promoter, CD3d promoter, CD3g promoter, CD3e promoter, CD3z promoter, MND promoter or a short EF1 promoter. In some embodiments, the promoter is a minimal TATA promoter, a pGK, actin promoter, CD25 promoter, IL2 promoter, IL7 promoter, IL15 promoter, KLRG-1 promoter, HLA-DR promoter, CD38 promoter, CD69 promoter, Ki-67 promoter, CD11a promoter, CD58 promoter, CD99 promoter, CD62L promoter, CD103 promoter, CCR4 promoter, CCR5 promoter, CCR6 promoter, CCR9 promoter, CCR10 promoter, CXCR3 promoter, CXCR4 promoter, CLA promoter, Granzyme A promoter, Granzyme B promoter, Perforin promoter,

CD57 promoter, CD161 promoter, IL-18Ra promoter,CD69 promoter, GzmB promoter, T-bet promoter, IFNgamma promoter, TIM3 promoter, IL4 promoter, GATA3 promoter, IL1 promoter, IL5 promoter, IL6 promoter, IL13 promoter, IL10 promoter, IL17A promoter, IL6 promoter, IL21 promoter, IL23R promoter, FoxP3 promoter, CTLA4 promoter, CD25 promoter, PD1 promoter, CD45RO promoter, CCR7 promoter, CD28 promoter, CD95 promoter, CD28 promoter, CD27 promoter, CD127 promoter, PD-1 promoter, CD122 promoter, CD132 promoter, c-Kit promoter, nuclear factor of activated T cells (NFAT) promoter, programmed death 1 (PD-1) promoter, T cell immunoglobulin mucin-3 (TIM-3) promoter, cytotoxic T lymphocyte antigen-4 (CTLA4) promoter, lymphocyte-activation protein 3 (LAG-3) promoter, tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) promoter, B- and T-lymphocyte attenuator (BTLA) promoter, CD25 promoter, CD69 promoter, Fas ligand (FasL) promoter, TIGIT promoter, TGF-beta promoter, T-bet promoter, Eomes promoter, GATA3 promoter, CD45RA promoter, 2B4 promoter, Type I interferon (IFN) alpha, Type I IFN beta promoter, IFN gamma promoter, IRF3 promoter, IRF7 promoter, NF-κB promoter, AP-1 promoter, TNF-alpha promoter, and CD130 promoter, NR4A1 promoter, NR4A2, or NR4A3 promoter. Exemplary constitutive promoters include, e.g., simian virus 40 early promoter (SV40), cytomegalovirus immediate-early promoter (CMV), human Ubiquitin C promoter (UBC), human elongation factor la promoter (EFla), mouse phosphoglycerate kinase 1 promoter (PGK), and chicken P-Actin promoter coupled with CMV early enhancer (CAG). In some embodiments, the constitutive promoter is a synthetic or modified promoter. In some embodiments, the promoter is or comprises an MND promoter, a synthetic promoter that contains the U3 region of a modified MoMuLV LTR with myeloproliferative sarcoma virus enhancer (see Challita et al. (1995) J. Virol. 69(2):748-755). In some embodiments, the promoter is a tissue-specific promoter. In another embodiment, the promoter is a viral promoter. In another embodiment, the promoter is a non-viral promoter. In some embodiments, exemplary promoters can include, but are not limited to, human elongation factor 1 alpha (EFla) promoter or a modified form thereof or the MND promoter.

**[0392]** Examples of suitable mammalian promoters include, for example: TRAC promoter, TRBC1 promoter, TRBC2 promoter, CD3alpha promoter, CD3beta promoter, CD3epsilon promoter, CD3delta promoter, CD3zeta promoter, CARD11 promoter, PIK3R3 promoter, B2M promoter, CIITA promoter, PD-1 promoter, CXCR4 promoter, an albumin promoter, an F3 promoter (also known as CD142), a MICA promoter, a MICB promoter, a LRP1 promoter (also known as CD91), a HMGB1 promoter, an ABO promoter, a RHD promoter,

a FUT1 promoter, a KDM5D promoter (also known as HY), a CCR5 promoter, a PDGFRa promoter, a OLIG2 promoter, and/or a GFAP promoter, elongation factor 1 alpha (EFla) promoter, CAG promoter, ubiquitin/S27a promoter of the hamster (WO 97/15664), Simian vacuolating virus 40 (SV40) early promoter, adenovirus major late promoter, mouse metallothionein-I promoter, the long terminal repeat region of Rous Sarcoma Virus (RSV), mouse mammary tumor virus promoter (MMTV), Moloney murine leukemia virus Long Terminal repeat region, and the early promoter of human Cytomegalovirus (CMV). Examples of other heterologous mammalian promoters are the actin, immunoglobulin or heat shock promoter(s). In additional embodiments, promoters for use in mammalian host cells can be obtained from the genomes of viruses such as polyoma virus, fowlpox virus (UK 2,211,504 published 5 Jul. 1989), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus, hepatitis-B virus and Simian Virus 40 (SV40). In further embodiments, heterologous mammalian promoters are used. Examples include the actin promoter, an immunoglobulin promoter, and heat-shock promoters. The early and late promoters of SV40 are conveniently obtained as an SV40 restriction fragment which also contains the SV40 viral origin of replication (Fiers et al., Nature 273: 113-120 (1978)). The immediate early promoter of the human cytomegalovirus is conveniently obtained as a Hindlll restriction enzyme fragment (Greenaway et al., Gene 18: 355-360 (1982)). The foregoing references are incorporated by reference in their entirety.

**[0393]** In some embodiments, the vector is a pseudotyped, self-inactivating lentiviral vector that carries the polypeptide of the disclosure. In some embodiments, the vector is a selfinactivating lentiviral vector pseudotyped with a vesicular stomatitis VSV-G envelope, and which carries the polynucleotide encoding the polypeptide of the disclosure.

**[0394]** In some of any embodiments, the viral vector is an AAV vector that carries the polypeptide of the disclosure. In some of any embodiments, the AAV vector is an AAV6 vector that carries the encoding the polypeptide of the disclosure.

**[0395]** The vector can include, but is not limited to, viral vectors, plasmid DNA, and closed-end DNA. Viral vectors can include, but are not limited to, adenoviral vectors, lentiviral vectors, retroviral vectors, adeno-associated viral vectors, vaccinia viruses, poxviruses, and herpes simplex viruses, or any of the viruses described elsewhere herein. Commonly, expression vectors contain selection markers such as ampicillin-resistance, hygromycin-resi stance, tetracycline resistance, kanamycin resistance, or neomycin resistance to permit detection of those cells transformed with the desired DNA sequences. Suitable vectors, promoter, and enhancer elements are known in the art; many are commercially available for

generating subject recombinant constructs. In some embodiments, the vector is a polycistronic vector. In some embodiments, the vector is a bicistronic vector or a tricistronic vector. Bicistronic or multi ci str onic expression vectors may include (1) multiple promoters fused to each of the open reading frames; (2) insertion of splicing signals between genes; (3) fusion of genes whose expressions are driven by a single promoter; and (4) insertion of proteolytic cleavage sites between genes (self-cleavage peptide) or insertion of internal ribosomal entry sites (IRESs) between genes.

**[0396]** A polycistronic vector is used to co-express multiple genes in the same cell. Two strategies are most commonly used to construct a multi cistronic vector. First, an Internal Ribosome Entry Site (IRES) element is typically used for bi-cistronic vectors. The IRES element, acting as another ribosome recruitment site, allows initiation of translation from an internal region of the mRNA. Thus, two proteins are translated from one mRNA. IRES elements are quite large (usually 500-600 bp) (Pelletier et al., 1988; Jang et al., 1988). In some embodiments, the polypeptide of the disclosure disclosed herein have a smaller size compared to the endogenous or patient-derived counterpart polypeptides, and thus could be expressed using IRES element in a multi cistronic vectors having limited packaging capacity.

**[0397]** In some embodiments, the polycistronic construct comprises two expression cassettes, i.e., is bicistronic. In some embodiments, the polycistronic construct comprises three expression cassettes, i.e., is tricistronic. In some embodiments, the polycistronic construct comprises four expression cassettes, i.e., is quadcistronic. In some embodiments, the polycistronic construct comprises more than four expression cassettes. In any of these embodiments, each of the expression cassettes comprises a nucleotide sequence encoding a protein of interest (e.g., a polypeptide of the disclosure and/or a CAR). In certain embodiments, the two or more genes being expressed are under the control of a single promoter and are separated from one another by one or more cleavage sites to achieve co-expression of the proteins of interest from one transcript. In other embodiments, the two or more genes may be under the control of separate promoters. As the name suggests, a polycistronic construct allows simultaneous expression of two or more separate proteins from one mRNA transcript in a host cell. Cleavage sites can be used in the design of a polycistronic construct to achieve such co-expression of multiple genes.

**[0398]** In some embodiments, the one or more cleavage sites comprise one or more selfcleaving sites. The second strategy relies on “self-cleaving” 2A peptides. These peptides, first discovered in picomaviruses, are short (about 20 amino acids) and produce equimolar

levels of multiple genes from the same mRNA. The term "self-cleaving" is not entirely accurate, as these peptides are thought to function by making the ribosome skip the synthesis of a peptide bond at the C-terminus of a 2A element, leading to separation between the end of the 2A sequence and the next peptide downstream (Kim et al., 2011). The "cleavage" occurs between the glycine and proline residues found on the C-terminus. Thus, the upstream cistron will have a few additional residues added to the end, while the downstream cistron will start with the proline. There are four 2A peptides commonly employed in molecular biology, T2A, P2A, E2A, and F2A, the sequences of which are summarized in **Table 4**. A glycine-serine-glycine (GSG) linker is optionally added to the N-terminal of a 2A peptide to increase cleavage efficiency. The use of "()" around a sequence in the present disclosure means that the enclosed sequence is optional.

**Table 4. Sequences of 2A Peptides**

| Component | Sequence | SEQ ID NO: |
|---|---|---|
| T2A | (GSG)EGRGSLLTCGDVEENPGP | 376 |
| P2A | (GSG) ATNFSLLKQAGDVEENPGP | 377 |
| E2A | (GSG) QCTNYALLKLAGDVESNPGP | 378 |
| F2A | (GSG)VKQTLNFDLLKLAGDVESNPGP | 379 |

**[0399]** In some embodiments, the one or more cleavage sites additionally comprise one or more protease sites. The one or more protease sites can either precede or follow the self-cleavage sites (e.g., 2 A sites) in the 5' to 3' order. The protease site may be cleaved by a protease after translation of the full transcript or after translation of each expression cassette such that the first expression product is released prior to translation of the next expression cassette. In these embodiments, having a protease site in addition to the 2A site, especially preceding the 2A site in the 5' to 3' order, may reduce the number of extra amino acid residues attached to the expressed proteins of interest. In some embodiments, the protease site comprises a furin site, also known as a Paired basic Amino acid Cleaving Enzyme (PACE) site. There are at least three furin cleavage sequences, FC1, FC2, and FC3, the amino acid sequences of which are summarized in **Table 5**. Similar to the 2A sites, one or more optional glycine-serine-glycine (GSG) sequences can be included for cleavage efficiency.

**Table 5. Sequences of Furin Sites**

| Component | Sequence | SEQ ID NO: |
|---|---|---|
| FC1 | RRRR (GSG) | 380 |
| FC2 | RKRR (GSG) | 381 |
| FC3 | RKRR (GSG) TPDPW (GSG) | 382 |

**[0400]** In some embodiments, the one or more cleavage sites comprise one or more self-cleaving sites, one or more protease sites, and/or any combination thereof. For example, the cleavage site can include a 2A site alone. For another example, the cleavage site can include a FC2 or FC3 site, followed by a 2A site. In these embodiments, the one or more self-cleaving sites may be the same or different. Similarly, the one or more protease sites may be the same or different.

**[0401]** In some embodiments, the polycistronic construct may be in the form of a vector. Any type of vector suitable for introduction of nucleotide sequences into a host cell can be used, including, for example, plasmids, adenoviral vectors, adenoviral-associated vectors, retroviral vectors, lentiviral vectors, phages, and homology-directed repair (HDR)-based donor vectors.

**[0402]** In some embodiments, the vector herein is a nucleic acid molecule capable of transferring or transporting another nucleic acid molecule, including into the cell or into the genome of a cell. The transferred nucleic acid is generally linked to, e.g., inserted into, the vector nucleic acid molecule. A vector may include sequences that direct autonomous replication in a cell or may include sequences sufficient to allow integration into host cell DNA. In some embodiments, the vector herein is a plasmid. In some embodiments, the vector herein is a DNA vector. In some embodiments, the vector herein is a closed linear DNA vector. In some embodiments, the vector is a synthetic RNA vector. In some embodiments, the vector herein is a phagemid vector. In some embodiments, the vector herein is a synthetic DNA vector. Useful vectors include, for example, plasmids (e.g., DNA plasmids or RNA plasmids), transposons, cosmids, bacterial artificial chromosomes, and viral vectors. Useful viral vectors include, e.g., replication defective retroviruses and lentiviruses. Non-viral vectors may require a delivery vehicle to facilitate entry of the nucleic acid molecule into a cell.

**[0403]** A viral vector can comprise a nucleic acid molecule that includes virus-derived nucleic acid elements that typically facilitate transfer of the nucleic acid molecule or

integration into the genome of a cell or to a viral particle that mediates nucleic acid transfer. Viral particles will typically include various viral components and sometimes also host cell components in addition to nucleic acid(s). A viral vector can comprise, e.g., a virus or viral particle capable of transferring a nucleic acid into a cell, or to the transferred nucleic acid (e.g., as naked DNA). Viral vectors and transfer plasmids can comprise structural and/or functional genetic elements that are primarily derived from a virus. A retroviral vector can comprise a viral vector or plasmid containing structural and functional genetic elements, or portions thereof, that are primarily derived from a retrovirus.

**[0404]** In some embodiments, the vector is a viral vector, such as a retroviral vector. In some embodiments, the polynucleotide encoding the polypeptide of the disclosure are introduced into the cell via retroviral or lentiviral vectors, or via transposons (see, e.g., Baum et al. (2006) Molecular Therapy: The Journal of the American Society of Gene Therapy. 13:1050-1063; Frecha et al. (2010) Molecular Therapy 18:1748-1757; and Hackett et al. (2010) Molecular Therapy 18:674-683).

**[0405]** In some vectors described herein, at least part of one or more protein coding regions that contribute to or are essential for replication may be absent compared to the corresponding wild-type virus. This makes the viral vector replication-defective. In some embodiments, the vector is capable of transducing a target non-dividing host cell and/or integrating its genome into a host genome.

**[0406]** In some embodiments, the vector is a retroviral vector. In some embodiments, the vector is a lentiviral vector. In some embodiments, the vector is a VSV-G pseudotyped lentivirus. In some embodiments, the vector is an AAV vector. In some embodiments, the vector is an AAV6 vector. In some embodiments, the vector is an AAV9 vector. In some embodiments, the vector is a split-intein dual AAV vector. In some embodiments, the vector is a redirected lentiviral vector. In some embodiments, the vector is a fusosome. In some embodiments, the vector is a lentiviral particle engineered with the anti-CD3 Cocal glycoprotein. In some embodiments, the vector is an enveloped delivery vehicle. In some embodiments, the vector is a self-replicating RNA virus. In some embodiments, the vector is an mRNA-packaging virus-like particle. In some embodiments, the vector is RNP-packaging virus-like particle. In some embodiments, the vectors used in the context of this disclosure are the vectors described in WO2023193015A1; WO2022164935A1; Raguram, Aditya, Samagya Banskota, and David R. Liu. "Therapeutic in vivo delivery of gene editing agents." *Cell* (2022); Hamilton, Jennifer R., et al. "In vivo human T cell engineering with

enveloped delivery vehicles." *Nature Biotechnology* (2024): 1-9; Lundstrom, Kenneth. "Self-replicating RNA viruses for RNA therapeutics." *Molecules* 23.12 (2018): 3310.

**[0407]** In some embodiments, the retroviral nucleic acid comprises one or more of (e.g., all of): a 5' promoter (e.g., to control expression of the entire packaged RNA), a 5' LTR (e.g., that includes R (polyadenylation tail signal) and/or U5 which includes a primer activation signal), a primer binding site, a psi packaging signal, a RRE element for nuclear export, a promoter directly upstream of the transgene to control transgene expression, a transgene (or other exogenous agent element), a polypurine tract, and a 3' LTR (e.g., that includes a mutated U3, a R, and U5). In some embodiments, the retroviral nucleic acid further comprises one or more of a cPPT, a WPRE, and/or an insulator element (e.g., as described in Browning et al., "Insulators to Improve the Safety of Retroviral Vectors for HIV Gene Therapy," Biomedicines, 4(1):4 (2016)).

**[0408]** A retrovirus typically replicates by reverse transcription of its genomic RNA into a linear double-stranded DNA copy and subsequently covalently integrates its genomic DNA into a host genome. The structure of a wild-type retrovirus genome often comprises a 5' long terminal repeat (LTR) and a 3' LTR, between or within which are located a packaging signal to enable the genome to be packaged, a primer binding site, integration sites to enable integration into a host cell genome and gag, pol and env genes encoding the packaging components which promote the assembly of viral particles. More complex retroviruses have additional features, such as rev and RRE sequences in HIV, which enable the efficient export of RNA transcripts of the integrated provirus from the nucleus to the cytoplasm of an infected target cell. In the provirus, the viral genes are flanked at both ends by regions called long terminal repeats (LTRs). The LTRs are involved in proviral integration and transcription. LTRs also serve as enhancerpromoter sequences and can control the expression of the viral genes. Encapsidation of the retroviral RNAs occurs by virtue of a psi sequence located at the 5' end of the viral genome.

**[0409]** The LTRs themselves are typically similar (e.g., identical) sequences that can be divided into three elements, which are called U3, R and U5. U3 is derived from the sequence unique to the 3' end of the RNA. R is derived from a sequence repeated at both ends of the RNA and U5 is derived from the sequence unique to the 5' end of the RNA. The sizes of the three elements can vary considerably among different retroviruses.

**[0410]** For the viral genome, the site of transcription initiation is typically at the boundary between U3 and R in one LTR and the site of poly (A) addition (termination) is at the boundary between R and U5 in the other LTR. U3 contains most of the transcriptional

control elements of the provirus, which include the promoter and multiple enhancer sequences responsive to cellular and in some cases, viral transcriptional activator proteins. Some retroviruses comprise any one or more of the following genes that code for proteins that are involved in the regulation of gene expression: tot, rev, tax and rex.

**[0411]** With regard to the structural genes gag, pol and env themselves, gag encodes the internal structural protein of the virus. Gag protein is proteolytically processed into the mature proteins MA (matrix), CA (capsid) and NC (nucleocapsid). The pol gene encodes the reverse transcriptase (RT), which contains DNA polymerase, associated RNase H and integrase (IN), which mediate replication of the genome. The env gene encodes the surface (SU) glycoprotein and the transmembrane (TM) protein of the virion, which form a complex that interacts specifically with cellular receptor proteins. This interaction promotes infection, e.g., by fusion of the viral membrane with the cell membrane.

**[0412]** In a replication-defective retroviral vector genome gag, pol and env may be absent or not functional. The R regions at both ends of the RNA are typically repeated sequences. U5 and U3 represent unique sequences at the 5' and 3' ends of the RNA genome respectively. Retroviruses may also contain additional genes which code for proteins other than gag, pol and env. Examples of additional genes include (in HIV), one or more of vif, vpr, vpx, vpu, tat, rev and nef. EIAV has (amongst others) the additional gene S2.

**[0413]** Illustrative retroviruses suitable for use in particular embodiments, include, but are not limited to: Moloney murine leukemia virus (M-MuLV), Moloney murine sarcoma virus (MoMSV), Harvey murine sarcoma virus (HaMuSV), murine mammary tumor virus (MuMTV), gibbon ape leukemia virus (GaLV), feline leukemia virus (FLV), spumavirus, Friend murine leukemia virus, Murine Stem Cell Virus (MSCV) and Rous Sarcoma Virus (RSV) and human immunodeficiency virus (HIV).

**[0414]** In some embodiments the retrovirus is a Gammretrovirus. In some embodiments the retrovirus is an Epsilonretrovirus. In some embodiments the retrovirus is an Alpharetrovirus. In some embodiments the retrovirus is a Betaretrovirus. In some embodiments the retrovirus is a Deltaretrovirus. In some embodiments the retrovirus is a Spumaretrovirus. In some embodiments the retrovirus is an endogenous retrovirus. In some embodiments the retrovirus is a lentivirus.

**[0415]** In some embodiments, a retroviral or lentivirus vector further comprises one or more insulator elements, e.g., an insulator element described in Browning et al., “Insulators to Improve the Safety of Retroviral Vectors for HIV Gene Therapy,” Biomedicines, 4(1):4 (2016). In various embodiments, the vectors comprise a promoter operably linked to a

polynucleotide encoding an exogenous agent. The vectors may have one or more LTRs, wherein either LTR comprises one or more modifications, such as one or more nucleotide substitutions, additions, or deletions. The vectors may further comprise one of more accessory elements to increase transduction efficiency (e.g., a cPPT/FLAP), viral packaging (e.g., a Psi (Y) packaging signal, RRE), and/or other elements that increase exogenous gene expression (e.g., poly (A) sequences), and may optionally comprise a WPRE or HPRE. In some embodiments, a lentiviral nucleic acid comprises one or more of, e.g., all of, e.g., from 5' to 3', a promoter (e.g., CMV), an R sequence (e.g., comprising TAR), a U5 sequence (e.g., for integration), a PBS sequence (e.g., for reverse transcription), a DIS sequence (e.g., for genome dimerization), a psi packaging signal, a partial gag sequence, an RRE sequence (e.g., for nuclear export), a cPPT sequence (e.g., for nuclear import), a promoter to drive expression of the exogenous agent, a gene encoding the exogenous agent, a WPRE sequence (e.g., for efficient transgene expression), a PPT sequence (e.g., for reverse transcription), an R sequence (e.g., for polyadenylation and termination), and a U5 signal (e.g., for integration).

**[0416]** Illustrative lentiviruses include, but are not limited to: HIV (human immunodeficiency virus; including HIV type 1, and HIV type 2); visna-maedi virus (VMV) virus; the caprine arthritis-encephalitis virus (CAEV); equine infectious anemia virus (EIAV); feline immunodeficiency virus (FIV); bovine immune deficiency virus (BIV); and simian immunodeficiency virus (SIV). In some embodiments, HIV based vector backbones (i.e., HIV cis-acting sequence elements) are used. A lentivirus vector can comprise a viral vector or plasmid containing structural and functional genetic elements, or portions thereof, including LTRs that are primarily derived from a lentivirus.

**[0417]** In embodiments, a lentivirus vector (e.g., lentiviral expression vector) may comprise a lentiviral transfer plasmid (e.g., as naked DNA) or an infectious lentiviral particle. With respect to elements such as cloning sites, promoters, regulatory elements, heterologous nucleic acids, etc., it is to be understood that the sequences of these elements can be present in RNA form in lentiviral particles and can be present in DNA form in DNA plasmids.

**[0418]** In embodiments, a lentivirus vector is a vector with sufficient retroviral genetic information to allow packaging of an RNA genome, in the presence of packaging components, into a viral particle capable of infecting a target cell. Infection of the target cell can comprise reverse transcription and integration into the target cell genome. The recombinant lentivirus vector (RLV) typically carries non-viral coding sequences which are to be delivered by the vector to the target cell. In embodiments, an RLV is incapable of

independent replication to produce infectious retroviral particles within the target cell. Usually, the RLV lacks a functional gag-pol and/or env gene and/or other genes involved in replication. The vector may be configured as a split-intron vector, e.g., as described in PCT patent application WO 99/15683, which is herein incorporated by reference in its entirety.

**[0419]** In some embodiments, the lentivirus vector comprises a minimal viral genome, e.g., the viral vector has been manipulated so as to remove the non-essential elements and to retain the essential elements in order to provide the required functionality to infect, transduce and deliver a nucleotide sequence of interest to a target host cell, e.g., as described in WO 98/17815, which is herein incorporated by reference in its entirety.

**[0420]** A minimal lentiviral genome may comprise, e.g., (5')R-U5-one or more first nucleotide sequences-U3-R(3'). However, the plasmid vector used to produce the lentiviral genome within a source cell can also include transcriptional regulatory control sequences operably linked to the lentiviral genome to direct transcription of the genome in a source cell. These regulatory sequences may comprise the natural sequences associated with the transcribed retroviral sequence, e.g., the 5' U3 region, or they may comprise a heterologous promoter such as another viral promoter, for example the CMV promoter. Some lentiviral genomes comprise additional sequences to promote efficient virus production. For example, in the case of HIV, rev and RRE sequences may be included.

**[0421]** In some embodiments, the vector can be a vector of the pUC series (Fermentas Life Sciences), the pBluescript series (Stratagene, LaJolla, Calif.), the pET series (Novagen, Madison, Wis.), the pGEX series (Pharmacia Biotech, Uppsala, Sweden), or the pEX series (Clontech, Palo Alto, Calif.). In some cases, bacteriophage vectors, such as ZG I O. XGT11, XZapII (Stratagene), XEMBL4, and ZNM I 149, also can be used. In some embodiments, plant expression vectors can be used and include pBIOl, pBI101.2, pBI101.3, pBI121 and pBIN19 (Clontech). In some embodiments, animal expression vectors include pEUK-Cl, pMAM and pMAMneo (Clontech).

**[0422]** In some embodiments, the vector (e.g., a viral vector) is a double stranded DNA vector. In some embodiments, the DNA vector is a closed-end vector. In some embodiments, the closed-end vector is Doggybone™ DNA (dbDNA). Closed-end DNA vectors, including dbDNA, are known in the art and have been described in e.g., Karda et al., Gene Ther (2019) 26:86-92.

**[0423]** In some cases, a vector may be used that does not require that the cells, e.g., T cells, are activated. In some such instances, the cells may be selected and/or transduced prior to

activation. Thus, the cells may be engineered prior to, or subsequent to culturing of the cells, and in some cases at the same time as or during at least a portion of the culturing.

**[0424]** In some embodiments, the cells are activated prior to introduction of the polynucleotide or vector. In some embodiments, the T cells are incubated with e.g., anti-CD3/anti-CD28 antibodies. In some embodiments, the T cells are incubated with apololipoprotein E (ApoE) prior to, during, and/or subsequent to introduction of the polynucleotide or vector. For example, in some instances, the T cells are incubated with ApoE (e.g. ApoE4) prior to incubation with LNPs containing the polynucleotide or a composition thereof. In some embodiments, the T cells are incubated with about 1 pg/mL ApoE4 prior to incubation with LNPs containing the polynucleotide or a composition thereof.

## VI. DELIVERY OF NUCLEIC ACIDS

**[0425]** Introduction of the nucleic acid molecules or amino acid molecules of the disclosure into cells can be achieved by methods known to those skilled in the art such as, for example, viral infection, transfection, conjugation, protoplast fusion, lipofection, electroporation, nucleofection, calcium phosphate precipitation, polyethyleneimine (PEI)-mediated transfection, DEAE-dextran mediated transfection, liposome-mediated transfection, particle gun technology, calcium phosphate precipitation, direct micro-injection, nanoparticle-mediated nucleic acid delivery, naked nucleic acid delivery, as nucleic acid complexed with materials such as a liposome, nanoparticle or poloxamer, or can be delivered by viruses (e.g., adenovirus, AAV, herpesvirus, retrovirus, lentivirus and integrase defective lentivirus (IDLV)), and the like. Introduction of the nucleic acid molecules or amino acid molecules of the disclosure into cells can be ex vivo or in vivo.

**[0426]** In some embodiments, introducing the polynucleotides encoding the engineered polypeptides described herein into cells can be achieved by any suitable technique. Suitable techniques include calcium phosphate, lipid-mediated transfection, electroporation, nucleofection, fusogens, transposons (e.g., a Sleeping Beauty system), and transduction or infection using a viral vector, as discussed herein. In some embodiments, the polynucleotides are introduced into a cell via viral transduction (e.g., AAV transduction, lentiviral transduction) or otherwise delivered on a viral vector (e.g., fusogen-mediated delivery). In some embodiments, the polynucleotides are introduced into a cell via a fusogen-mediated delivery or a transposase system selected from the group consisting of conditional or inducible transposases, conditional or inducible PiggyBac transposons,

conditional or inducible Sleeping Beauty (SB11) transposons, conditional or inducible Mosl transposons, and conditional or inducible Tol2 transposons.

**[0427]** The process of introducing the nucleic acid molecule into the cell can be achieved by any suitable technique. Suitable techniques include calcium phosphate or lipid-mediated transfection, electroporation, and transduction or infection using a viral vector. In some embodiments, the nucleic acid molecule comprises DNA. In some embodiments, the nucleic acid molecule comprises a modified DNA. In some embodiments, the nucleic acid molecule comprises RNA. In some embodiments, the nucleic acid molecule comprises mRNA. In some embodiments, the nucleic acid molecule comprises a modified mRNA.

**[0428]** In some embodiments, the nucleic acid molecules can be delivered by conventional transformation or transfection techniques. Suitable methods for transforming or transfecting host cells can be found in Sambrook et al. (2012, supra) and other standard molecular biology laboratory manuals, such as, calcium phosphate transfection, DEAE-dextran mediated transfection, transfection, microinjection, cationic lipid-mediated transfection, electroporation, transduction, scrape loading, ballistic introduction, nucleoporation, hydrodynamic shock, and infection.

**[0429]** In some embodiments, one or more polynucleotide(s) are introduced into a cell using electroporation (see, e.g., Chicaybam et al, (2013) PLoS ONE 8(3): e60298 and Van Tedeloo et al. (2000) Gene Therapy 7(16): 1431-1437). For examples, in some embodiments, one or more RNA molecules is introduced into a T cell using electroporation. Methods and systems for electroporation are known in the art, including e.g. Nucleofection® Technology (Lonza). In some embodiments, recombinant nucleic acids are transferred into T cells via transposition (see, e.g., Manuri et al. (2010) Hum Gene Ther 21(4): 427-437; Sharma et al. (2013) Molec Ther Nucl Acids 2, e74; and Huang et al. (2009) Methods Mol Biol 506: 115-126). Other methods of introducing and expressing genetic material, e.g., polynucleotides and/or vectors, into immune cells include calcium phosphate transfection (e.g., as described in Current Protocols in Molecular Biology, John Wiley & Sons, New York. N.Y.), protoplast fusion, cationic liposome-mediated transfection; tungsten particle-facilitated microparticle bombardment (Johnston, Nature, 346: 776-777 (1990)); and strontium phosphate DNA co-precipitation (Brash et al., Mol. Cell Biol., 7: 2031-2034 (1987) and other approaches described in, e.g., International Pat. App. Pub. No. WO 2014055668, and U.S. Patent No. 7,446,190.

**[0430]** In some embodiments, the nucleic acid molecules can be delivered by viral or non-viral delivery vehicles known in the art. For example, the nucleic acid molecule can be

stably integrated in the host genome, or can be episomally replicating, or present in the recombinant host cell as a mini-circle expression vector for transient expression. Accordingly, in some embodiments, the nucleic acid molecule is maintained and replicated in the recombinant host cell as an episomal unit. In some embodiments, the nucleic acid molecule is stably integrated into the genome of the recombinant cell. Stable integration can be achieved using classical random genomic recombination techniques or with more precise techniques such as guide RNA-directed CRISPR/Cas9 genome editing, or DNA-guided endonuclease genome editing with NgAgo (Natronobacterium gregoryi Argonaute), or TALENs genome editing (transcription activator-like effector nucleases). In some embodiments, the nucleic acid molecule is present in the recombinant host cell as a mini-circle expression vector for transient expression.

**[0431]** In some embodiments, the nucleic acid molecules can be encapsulated in a viral capsid or a lipid nanoparticle, or can be delivered by viral or non-viral delivery means and methods known in the art, such as electroporation. For example, introduction of nucleic acids into cells may be achieved by viral transduction. In a non-limiting example, adeno-associated virus (AAV) is engineered to deliver nucleic acids to target cells via viral transduction. Several AAV serotypes have been described, and all of the known serotypes can infect cells from multiple diverse tissue types. AAV is capable of transducing a wide range of species and tissues *in vivo* with no evidence of toxicity, and it generates relatively mild innate and adaptive immune responses.

**[0432]** In some embodiments, the polypeptide of the disclosure is delivered using viral transduction, for example, with a vector. In some embodiments, the vector is a pseudotyped, self- inactivating lentiviral vector that carries the exogenous polynucleotide. In some embodiments, the vector is a self-inactivating lentiviral vector pseudotyped with a vesicular stomatitis VSV-G envelope, and which carries the exogenous polynucleotide.

**[0433]** In some embodiments, a lentiviral vector can be used for delivery. Lentiviral-derived vector systems are useful for nucleic acid delivery and gene therapy via viral transduction. Lentiviral vectors offer several attractive properties as gene-delivery vehicles, including: (i) sustained gene delivery through stable vector integration into host genome; (ii) the capability of infecting both dividing and non-dividing cells; (iii) broad tissue tropisms, including important gene- and cell-therapy-target cell types; (iv) no expression of viral proteins after vector transduction; (v) the ability to deliver complex genetic elements, such as polycistronic or intron-containing sequences; (vi) a potentially safer integration site profile; and (vii) a relatively easy system for vector manipulation and production.

**[0434]** Methods of lentiviral transduction are known. Exemplary methods are described in, e.g., Wang et al. (2012) J. Immunother. 35(9): 689-701; Cooper et al. (2003) Blood. 101:1637-1644; Verhoeyen et al. (2009) Methods Mol Biol. 506: 97-114; and Cavalieri et al. (2003) Blood. 102(2): 497-505. In some embodiments, the polynucleotide encoding the recombinant receptor and/or one or more additional polypeptide(s), is introduced into a population containing cultured cells, such as by retroviral transduction, transfection, or transformation.

**[0435]** In some embodiments, a recombinant adeno-associated virus (AAV) vector can be used for delivery. Techniques to produce rAAV particles, in which an AAV genome to be packaged that includes the polynucleotide to be delivered, rep and cap genes, and helper virus functions are provided to a cell are standard in the art. Production of rAAV requires that the following components are present within a single cell (denoted herein as a packaging cell): a rAAV genome, AAV rep and cap genes separate from (e.g., not in) the rAAV genome, and helper virus functions. The AAV rep and cap genes can be from any AAV serotype for which recombinant virus can be derived, and can be from a different AAV serotype than the rAAV genome ITRs, including, but not limited to, AAV serotypes AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAV-6, AAV-7, AAV-8, AAV-9, AAV-10, AAV-11, AAV-12, AAV-13 and AAV rh.74. Production of pseudotyped rAAV is disclosed in, for example, international patent application publication number WO 01/83692.

**[0436]** In some embodiments, the nucleic acid encoding the engineered protein of the present disclosure is delivered with a retrovirus. In some embodiments, the nucleic acid encoding the engineered protein of the present disclosure is delivered with a lentivirus. In some embodiments, the nucleic acid encoding the engineered protein of the present disclosure is delivered with a VSV-G pseudotyped lentivirus. In some embodiments, the nucleic acid encoding the engineered protein of the present disclosure is delivered with an AAV. In some embodiments, the nucleic acid encoding the engineered protein of the present disclosure is delivered with an AAV6. In some embodiments, the nucleic acid encoding the engineered protein of the present disclosure is delivered with an AAV9. I In some embodiments, the nucleic acid encoding the engineered protein of the present disclosure is delivered with a split-intein dual AAV. In some embodiments, the nucleic acid encoding the engineered protein of the present disclosure is delivered with a redirected lentivirus. In some embodiments, the nucleic acid encoding the engineered protein of the present disclosure is delivered with a fusosome. In some embodiments, the nucleic acid encoding the engineered protein of the present disclosure is delivered with a lentiviral particle engineered with the

anti-CD3 Cocal glycoprotein. In some embodiments, the nucleic acid encoding the engineered protein of the present disclosure is delivered with a enveloped delivery vehicle. In some embodiments, the nucleic acid encoding the engineered protein of the present disclosure is delivered with a self-replicating RNA virus. In some embodiments, the nucleic acid encoding the engineered protein of the present disclosure is delivered with an mRNA-packaging virus-like particle. In some embodiments, the nucleic acid encoding the engineered protein of the present disclosure is delivered with an RNP-packaging virus-like particle. In some embodiments, the delivery used in the context of this disclosure are described in WO2023193015A1; WO2022164935A1; Raguram, Aditya, Samagya Banskota, and David R. Liu. "Therapeutic in vivo delivery of gene editing agents." *Cell* (2022); Hamilton, Jennifer R., et al. "In vivo human T cell engineering with enveloped delivery vehicles." *Nature Biotechnology* (2024): 1-9; Lundstrom, Kenneth. "Self-replicating RNA viruses for RNA therapeutics." *Molecules* 23.12 (2018): 3310.

**[0437]** In some embodiments, the nucleic acid is or comprises mRNA encoding the engineered protein of the disclosure. The mRNA may be delivered to the cell using any appropriate technique, such as electroporation or using a lipid nanoparticle (LNP). In some embodiments, the nucleic acid is delivered with a lipid nanoparticle (LNP). In some embodiments, the nucleic acid is delivered with a selective organ targeting (SORT) LNP). In some embodiments, the nucleic acid is delivered with an antibody targeted LNP. In some embodiments, the LNP comprises: (i) an ionizable lipid (e.g., an amino lipid), (ii) a sterol or other structural lipid, (iii) a non-cationic helper lipid or phospholipid, and (iv) a PEG-lipid (e.g., a PEG-modified lipid). In some embodiments, the nucleic acid is delivered with a polymer nanoparticle. In some embodiments, the nucleic acid is delivered with a protein nanoparticle. In some embodiments, the delivery used in the context of this disclosure are described in WO2024006960A1; WO2019152557A1; Raguram, Aditya, Samagya Banskota, and David R. Liu. "Therapeutic in vivo delivery of gene editing agents." *Cell* (2022); Rurik, Joel G., et al. "CAR T cells produced in vivo to treat cardiac injury." *Science* 375.6576 (2022): 91-96.

**[0438]** In some embodiments, the polynucleotides disclosed herein can be delivered or introduced using one or more agent(s), capable of inducing a genetic disruption (e.g., CRISPR/Cas and/or gRNA components), to a cell, using any of a number of known delivery methods or vehicles for introduction or transfer to cells, for example, using viral delivery vectors, or any of the known methods or vehicles for delivering CRISPR/Cas molecules and gRNAs. Exemplary methods are described in, e.g., Wang et al. (2012) J. Immunother. 35(9):

689-701; Cooper et al. (2003) Blood. 101:1637-1644; Verhoeyen et al. (2009) Methods Mol Biol. 506: 97-114; and Cavalieri et al. (2003) Blood. 102(2): 497- 505. In some embodiments, nucleic acid sequences encoding one or more components of one or more agent(s) capable of inducing a genetic disruption is introduced into the cells, e.g., by any methods for introducing nucleic acids into a cell described herein or known. In some embodiments, a vector encoding components of one or more agent(s) capable of inducing a genetic disruption such as a CRISPR guide RNA and/or a Cas enzyme can be delivered into the cell.

**[0439]** In some embodiments, the one or more agent(s) capable of inducing a genetic disruption (e.g., one or more agent(s) that is a CRISPR/Cas/gRNA), is introduced into the cell as a ribonucleoprotein (RNP) complex. RNP complexes include a sequence of ribonucleotides, such as an RNA or a gRNA molecule, and a protein, such as a Cas9 protein or variant thereof. For example, the Cas9 protein is delivered as RNP complex that comprises a Cas9 protein and a gRNA molecule targeting the target sequence, e.g., using electroporation or other physical delivery method. In some embodiments, the RNP is delivered into the cell via electroporation or other physical means, e.g., particle gun, Calcium Phosphate transfection, cell compression or squeezing. In some embodiments, the RNP can cross the plasma membrane of a cell without the need for additional delivery agents (e.g., small molecule agents, lipids, etc.). In some embodiments, delivery of the one or more agent(s) capable of inducing genetic disruption, e.g., CRISPR/Cas9, as an RNP offers an advantage that the targeted disruption occurs transiently, e.g., in cells to which the RNP is introduced, without propagation of the agent to cell progenies. For example, delivery by RNP minimizes the agent from being inherited to its progenies, thereby reducing the chance of off-target genetic disruption in the progenies. In such cases, the genetic disruption and the integration of transgene can be inherited by the progeny cells, but without the agent itself, which may further introduce off-target genetic disruptions, being passed on to the progeny cells.

**[0440]** Agent(s) and components capable of inducing a genetic disruption (e.g., a CRISPR/Cas molecule and gRNA molecule), can be introduced into target cells in a variety of forms using a variety of delivery methods and formulations, as set forth in **Tables 6 and 7**, or methods described in, e.g., WO 2015/161276; US 2015/0056705, US 2016/0272999, US 2017/0211075; or US 2017/0016027. As described further herein, the delivery methods and formulations can be used to deliver template polynucleotides and/or other agents to the cell (such as those required for engineering the cells) in prior or subsequent steps of the

methods described herein. When a CRISPR/Cas nuclease or gRNA component is encoded as DNA for delivery, the DNA may typically but not necessarily include a control region, e.g., comprising a promoter, to effect expression. Exemplary promoters for CRISPR/Cas nuclease sequences include, e.g., CMV, EFla, EFS, MSCV, PGK, or CAG promoters. Useful promoters for gRNAs include, e.g., Hl, EF-la, tRNA or U6 promoters. Promoters with similar or dissimilar strengths can be selected to tune the expression of components. Sequences encoding a CRISPR/Cas nuclease molecule may comprise a nuclear localization signal (NLS), e.g., an SV40 NLS. In some embodiments a promoter for a CRISPR/Cas nuclease molecule or a gRNA molecule may be, independently, inducible, tissue specific, or cell specific. In some embodiments, an agent capable of inducing a genetic disruption is introduced RNP complexes.

**Table 6. Exemplary Delivery Methods**

| **Elements** | | **Comments** |
|---|---|---|
| **Cas9 Molecule(s)** | **gNRA Molecule(s)** | |
| DNA | DNA | In this embodiment, a Cas9 molecule and a gRNA are transcribed from DNA. In this embodiment, they are encoded on separate molecules. |
| DNA | | In this embodiment, a Cas9 molecule and a gRNA are transcribed from DNA, here from a single molecule |
| DNA | RNA | In this embodiment, a Cas9 molecule is transcribed from DNA, and a gRNA is provided an in vitro transcribed or synthesized RNA. |
| mRNA | RNA | In this embodiment, a Cas9 molecule is translated from in vitro transcribed mRNA, and a gRNA is provided an in vitro transcribed or synthesized RNA. |
| mRNA | DNA | In this embodiment, a Cas9 molecule is translated from in vitro transcribed mRNA, and a gRNA is transcribed from DNA |
| Protein | DNA | In this embodiment, a Cas9 molecule is provided as a protein, and a gRNA is transcribed from DNA. |
| Protein | RNA | In this embodiment, a Cas9 molecule is provided as a protein, and a gRNA is provided as transcribed or synthesized RNA. |

**Table 7. Comparison of Exemplary Delivery Methods**

| Delivery Vector/Mode | | Delivery into Non-Dividing Cells | Duration of Expression | Genome Integration | Type of Molecule Delivered |
|---|---|---|---|---|---|
| **Physical (e.g., electroporation, particle gun, Calcium Phosphate transfection, cell compression or squeezing)** | | YES | Transient | NO | Nucleic Acids and Proteins |
| **Viral** | **Retrovirus** | YES | Stable | YES/NO with modifications | RNA |
| | **Lentivirus** | YES | Stable | NO | RNA |
| | **Adenovirus** | YES | Transient | NO | DNA |
| | **AAV** | YES | Stable | NO | DNA |
| | **Vaccinia Virus** | YES | Very Transient | NO | DNA |
| | **Herpes Simplex Virus** | YES | Stable | NO | DNA |
| **Non-Viral** | **Cationic Liposomes** | YES | Transient | Depends on what is delivered | Nucleic Acids and Proteins |
| | **Polymeric Nanoparticles** | YES | Transient | Depends on what is delivered | Nucleic Acids and Proteins |
| **Biological Non-Viral Delivery Vehicles** | **Attenuated Bacteria** | YES | Transient | NO | Nucleic Acids |
| | **Engineered Bacteriophages** | YES | Transient | NO | Nucleic Acids |
| | **Mammalian Virus-Like Particles** | YES | Transient | NO | Nucleic Acids |
| | **Biological liposomes: Erythrocyte Ghosts and Exosomes** | YES | Transient | NO | Nucleic Acids |

**[0441]** In some embodiments, the polynucleotide encoding the engineered proteins of the disclosure can be delivered into cells by known methods or as described herein. For example, by vectors (e.g., viral or non-viral vectors), non-vector based methods (e.g., using naked DNA or DNA complexes), or a combination thereof. In some embodiments, the

polynucleotide containing the agent(s) and/or components thereof is delivered by a vector (e.g., viral vector/virus or plasmid). The vector may be any described herein.

**[0442]** In some embodiments, the polynucleotide encoding the engineered proteins of the disclosure is delivered by a non-vector based method (e.g., using naked DNA or DNA complexes). For example, the DNA or RNA or proteins or combination thereof, e.g., ribonucleoprotein (RNP) complexes, can be delivered, e.g., by organically modified silica or silicate (Ormosil), electroporation, transient cell compression or squeezing (such as described in Lee, et al. (2012) Nano Lett 12: 6322-27, Kollmannsperger et al (2016) Nat Comm 7, 10372), gene gun, sonoporation, magnetofection, lipid-mediated transfection, dendrimers, inorganic nanoparticles, calcium phosphates, or a combination thereof.

**[0443]** In some embodiments, delivery via electroporation comprises mixing the cells with the polynucleotide encoding the engineered proteins of the disclosure or RNP complex in a cartridge, chamber or cuvette and applying one or more electrical impulses of defined duration and amplitude. In some embodiments, delivery via electroporation is performed using a system in which cells are mixed with the DNA in a vessel connected to a device (e.g., a pump) which feeds the mixture into a cartridge, chamber or cuvette wherein one or more electrical impulses of defined duration and amplitude are applied, after which the cells are delivered to a second vessel.

**[0444]** In some embodiments, the delivery vehicle is a non-viral vector. In some embodiments, the non-viral vector is an inorganic nanoparticle. Exemplary inorganic nanoparticles include, e.g., magnetic nanoparticles (e.g., FesMnCh) and silica. The outer surface of the nanoparticle can be conjugated with a positively charged polymer (e.g., polyethylenimine, polylysine, polyserine) which allows for attachment (e.g., conjugation or entrapment) of payload. In some embodiments, the non-viral vector is an organic nanoparticle. Exemplary organic nanoparticles include, e.g., SNALP liposomes that contain cationic lipids together with neutral helper lipids which are coated with polyethylene glycol (PEG), and protamine-nucleic acid complexes coated with lipid. Exemplary lipids and polymers for gene transfer include those described in, for example, WO 2019/195492 and WO 2020/223535.

**[0445]** In some embodiments, the vehicle has targeting modifications to increase target cell update of nanoparticles and liposomes, e.g., cell specific antigens, monoclonal antibodies, single chain antibodies, aptamers, polymers, sugars, and cell penetrating peptides. In some embodiments, the vehicle uses fusogenic and endosome-destabilizing peptides/polymers. In some embodiments, the vehicle undergoes acid-triggered conformational changes (e.g., to

accelerate endosomal escape of the cargo). In some embodiments, a stimulus-cleavable polymer is used, e.g., for release in a cellular compartment. For example, disulfide-based cationic polymers that are cleaved in the reducing cellular environment can be used.

**[0446]** In some embodiments, the delivery vehicle is a biological non-viral delivery vehicle. In some embodiments, the vehicle is an attenuated bacterium (e.g., naturally or artificially engineered to be invasive but attenuated to prevent pathogenesis and expressing the transgene (e.g., Listeria monocytogenes, certain Salmonella strains, Bifidobacterium longum, and modified Escherichia coll), bacteria having nutritional and tissue- specific tropism to target specific cells, bacteria having modified surface proteins to alter target cell specificity). In some embodiments, the vehicle is a genetically modified bacteriophage (e.g., engineered phages having large packaging capacity, less immunogenicity, containing mammalian plasmid maintenance sequences and having incorporated targeting ligands). In some embodiments, the vehicle is a mammalian virus-like particle. For example, modified viral particles can be generated (e.g., by purification of the "empty" particles followed by ex vivo assembly of the virus with the desired cargo). The vehicle can also be engineered to incorporate targeting ligands to alter target tissuespecificity. In some embodiments, the vehicle is a biological liposome. For example, the biological liposome is a phospholipid-based particle derived from human cells (e.g., erythrocyte ghosts, which are red blood cells broken down into spherical structures derived from the subject (e.g., tissue targeting can be achieved by attachment of various tissue or cell-specific ligands), or secretory exosomes - subject-derived membrane-bound nanovescicles (30 -100 nm) of endocytic origin (e.g., can be produced from various cell types and can therefore be taken up by cells without the need for targeting ligands).

**[0447]** In some embodiments, an RNA polynucleotide encoding the engineered proteins of the disclosure can be delivered into cells, e.g., target cells described herein, by known methods or as described herein. For example, RNA can be delivered, e.g., by microinjection, electroporation, transient cell compression or squeezing (such as described in Lee, et al. (2012) Nano Let 12: 6322-27), lipid-mediated transfection, peptide-mediated delivery, e.g., cell-penetrating peptides, or a combination thereof.

**[0448]** In some embodiments, delivery via electroporation comprises mixing the cells with the RNA in a cartridge, chamber or cuvette and applying one or more electrical impulses of defined duration and amplitude. In some embodiments, delivery via electroporation is performed using a system in which cells are mixed with the RNA encoding CRISPR/Cas nuclease molecules and/or gRNA molecules in a vessel connected to a device (e.g., a pump)

which feeds the mixture into a cartridge, chamber or cuvette wherein one or more electrical impulses of defined duration and amplitude are applied, after which the cells are delivered to a second vessel.

**[0449]** In some embodiments, engineered polypeptide molecules can be delivered into cells by known methods or as described herein. For example, protein molecules can be delivered, e.g., by microinjection, electroporation, transient cell compression or squeezing (such as described in Lee, et al. (2012) Nano Let 12: 6322-27), lipid-mediated transfection, peptide-mediated delivery, or a combination thereof. Delivery can be accompanied by DNA encoding a polypeptide (e.g., an engineered polypeptide, gRNA, CAR, TCR, etc.).

**[0450]** In some embodiments, the exogenous Cas protein can be introduced into the cell in polypeptide form. In certain embodiments, the Cas proteins can be conjugated to or fused to a cell-penetrating polypeptide or cell-penetrating peptide. As used herein, "cell-penetrating polypeptide" and "cell-penetrating peptide" refers to a polypeptide or peptide, respectively, which facilitates the uptake of molecule into a cell. The cell-penetrating polypeptides can contain a detectable label.

**[0451]** In many embodiments, Cas proteins can be conjugated to or fused to a charged protein (e.g., that carries a positive, negative or overall neutral electric charge). Such linkage may be covalent. In some embodiments, the Cas protein can be fused to a superpositively charged GFP to significantly increase the ability of the Cas protein to penetrate a cell (Cronican et al. ACS Chem Biol. 2010; 5(8):747-52). In certain embodiments, the Cas protein can be fused to a protein transduction domain (PTD) to facilitate its entry into a cell. Exemplary PTDs include Tat, oligoarginine, and penetratin. In some embodiments, the Cas9 protein comprises a Cas9 polypeptide fused to a cell-penetrating peptide. In some embodiments, the Cas9 protein comprises a Cas9 polypeptide fused to a PTD. In some embodiments, the Cas9 protein comprises a Cas9 polypeptide fused to a tat domain. In some embodiments, the Cas9 protein comprises a Cas9 polypeptide fused to an oligoarginine domain. In some embodiments, the Cas9 protein comprises a Cas9 polypeptide fused to a penetratin domain. In some embodiments, the Cas9 protein comprises a Cas9 polypeptide fused to a superpositively charged GFP. In some embodiments, the Casl2a protein comprises a Casl2a polypeptide fused to a cell-penetrating peptide. In some embodiments, the Casl2a protein comprises a Casl2a polypeptide fused to a PTD. In some embodiments, the Casl2a protein comprises a Casl2a polypeptide fused to a tat domain. In some embodiments, the Casl2a protein comprises a Casl2a polypeptide fused to an oligoarginine domain. In some embodiments, the Casl2a protein comprises a Casl2a polypeptide fused to a penetratin

domain. In some embodiments, the Casl2a protein comprises a Casl2a polypeptide fused to a superpositively charged GFP.

**[0452]** In some embodiments, the Cas protein can be introduced into a cell containing the target polynucleotide sequence in the form of a nucleic acid encoding the Cas protein. The process of introducing the nucleic acids into cells can be achieved by any suitable technique. Suitable techniques include those described herein.

## VII. CELLS

**[0453]** In another aspect, the present disclosure provides an engineered cell comprising a polynucleotide encoding the engineered CARD11-PIK3R3 fusion protein disclosed herein, and/or a vector comprising the polynucleotide that encodes the engineered CARD11-PIK3R3 fusion protein disclosed herein. In some embodiments, the engineered cell is transfected or transduced by a vector of the disclosure. In some embodiments, the engineered cell is contacted by a particle of the disclosure.

**[0454]** In some embodiments, activating the engineered cell results in the secretion of IFNg by the cell at a level that is at least 10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold higher than the IFNg secretion level of a control cell lacking the recombinant polypeptide.

**[0455]** In some embodiments, activating the engineered cell results in the secretion of IL-2 by the cell at a level that is at least 10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold higher than the IL-2 secretion level of a control cell lacking the recombinant polypeptide.

**[0456]** In some embodiments, activation of the engineered cell and subsequent removal of the target antigen results in CARD11-PIK3R3 signaling by the cell at a level that is at least 10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold lower than the CARD11-PIK3R3 signaling of a control cell comprising a CARD11-PIK3R3 fusion protein lacking the inhibitory domain.

**[0457]** In some embodiments, activation of the engineered cell and subsequent removal of the target antigen results in CARD11-PIK3R3 signaling by the cell at a level that is at least 10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold lower than the CARD11-PIK3R3 signaling of a control cell comprising a CARD11-PIK3R3 fusion protein lacking one of

more of S466, S512, S535, S559, S644, and S652 according to SEQ ID NO: 518 in the inhibitory domain.

**[0458]** In some embodiments, CARD11-PIK3R3 signaling in the cell in the absence of a target antigen is at a level that is at least 10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold lower than the CARD11-PIK3R3 signaling of a control cell comprising a control CARD11-PIK3R3 fusion protein lacking the inhibitory domain. In some embodiments, CARD11-PIK3R3 signaling in the cell in the absence of a target antigen is at a level that is at least 10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold lower than the CARD11-PIK3R3 signaling of a control cell comprising a control CARD11-PIK3R3 fusion protein lacking one of more of S466, S512, S535, S559, S644, and S652 according to SEQ ID NO: 518 in the inhibitory domain. In some embodiments, CARD11-PIK3R3 signaling is measured by: (a) BCL10 binding, (b) BCL10 and MALT1 complex formation, (c) NFAT, NF-κB and/or AP-1 signaling, (d) cytokine production, (e) JAK/STAT signaling, (f) co-stimulatory molecule signaling, (g) phospholipase gamma signaling, and/or (h) transcription factor activity.

**[0459]** In some embodiments, expression of the recombinant polypeptide by the cell is under the control of an endogenous promoter. Non-limiting examples of endogenous promoters include a TCRa promoter, a TCRb promoter, a CD3d promoter, a CD3g promoter, a CD3e promoter, a CD3z promoter, a CARD9 promoter, a CARD10 promoter, a CARD11 promoter, a CARD14 promoter, or a PIK3R3 promoter. In some embodiments, expression of the recombinant polypeptide by the cell is under the control of an exogenous promoter. Non-limiting examples of exogenous promoters include a minimal TATA promoter, pGK promoter, actin promoter, CD4 promoter, CD8a promoter, CD8b promoter, TCRa promoter, TCRb promoter, CD25 promoter, IL2 promoter, IL7 promoter, IL15 promoter, KLRG-1 promoter, HLA-DR promoter, CD38 promoter, CD69 promoter, Ki-67 promoter, CD11a promoter, CD58 promoter, CD99 promoter, CD62L promoter, CD103 promoter, CCR4 promoter, CCR5 promoter, CCR6 promoter, CCR9 promoter, CCR10 promoter, CXCR3 promoter, CXCR4 promoter, CLA promoter, Granzyme A promoter, Granzyme B promoter, Perforin promoter, CD57 promoter, CD161 promoter, IL-18Ra promoter, CD69 promoter, GzmB promoter, T-bet promoter, IFNgamma promoter, TIM3 promoter, IL4 promoter, GATA3 promoter, IL1 promoter, IL5 promoter, IL6 promoter, IL13 promoter, IL10 promoter, IL17A promoter, IL6 promoter, IL21 promoter, IL23R promoter, FoxP3 promoter, CTLA4 promoter, CD25 promoter, PD1 promoter, CD45RO promoter, CCR7

promoter, CD28 promoter, CD95 promoter, CD28 promoter, CD27 promoter, CD127 promoter, PD-1 promoter, CD122 promoter, CD132 promoter, c-Kit promoter, nuclear factor of activated T cells (NFAT) promoter, programmed death 1 (PD-1) promoter, T cell immunoglobulin mucin-3 (TIM-3) promoter, cytotoxic T lymphocyte antigen-4 (CTLA4) promoter, lymphocyte-activation protein 3 (LAG-3) promoter, tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) promoter, B- and T-lymphocyte attenuator (BTLA) promoter, CD25 promoter, CD69 promoter, Fas ligand (FasL) promoter, TIGIT promoter, TGF-beta promoter, T-bet promoter, Eomes promoter, GATA3 promoter, CD45RA promoter, 2B4 promoter, Type I interferon (IFN) alpha, Type I IFN beta promoter, IFN gamma promoter, IRF3 promoter, IRF7 promoter, NFkB promoter, AP-1 promoter, TNF-alpha promoter, CD130 promoter, NR4A1 promoter, NR4A2, NR4A3 promoter, MND promoter, EF-1alpha promoter, short EF-1alpha promoter, CAG promoter, ubiquitin/S27a promoter, SV40 promoter, SV40 early promoter, adenovirus major late promoter, mouse metallothionein- I promoter, Moloney murine leukemia virus (MMLV) long terminal repeat (LTR) region, CMV promoter, immunoglobulin promoter, heat shock promoter, polyoma virus promoter, fowlpox virus promoter, bovine papilloma virus promoter, avian sarcoma virus promoter, retrovirus promoter, hepatitis-B virus promoter, PGK promoter, vaccinia virus 7.5K promoter, TK promoter of HSV, mouse mammary tumor virus (MMTV) promoter, LTR promoter of HIV, promoter of oloney virus, Epstein Barr virus (EBV) promoter, Rous sarcoma virus (RSV) promoter, U6 promoter, or UBC promoter.

**[0460]** The present disclosure additionally provides engineered cells wherein the nucleic acid construct is inserted in a T-cell receptor (TCR) locus, a CD3 locus, a B2 microglobulin (B2M) locus, a class II transactivator (CIITA) locus, a CARD9 locus, a CARD10 locus, a CARD11 locus, a CARD14 locus, a PIK3R3 locus, or a safe harbor locus. In some embodiments, the TCR locus is or comprises a TRAC locus, a TRBC1 locus, a TRBC2 locus, or a TRAJ splice-acceptor site. In some embodiments, the TRAJ locus is or comprises a TRAJ intron splice acceptor locus. In some embodiments, the cell does not express a gene product of an endogenous TRAC locus. In some embodiments, the cell does not express a gene product of an endogenous TRBC1 locus. In some embodiments, the cell does not express a gene product of an endogenous TRBC2 locus. In some embodiments, the CD3 locus is or comprises a CD3d locus, a CD3g locus, a CD3e locus, or a CD3z locus. In some embodiments, the cell does not express a gene product of an endogenous CD3d locus. In some embodiments, the cell does not express a gene product of an endogenous CD3g locus. In some embodiments, the cell does not express a gene product of an endogenous CD3e

locus. In some embodiments, the cell does not express a gene product of an endogenous CD3z locus. In some embodiments, the safe harbor locus is or comprises an AAVS1, ABO, CCR5, CLYBL, CXCR4, F3, FUT1, HMGB1, KDM5D, LRP1, MICA, MICB, RHD, ROSA26, or SHS231 locus. In some embodiments, the nucleic acid construct is inserted in an exon, an intron, between an intron and an exon, or in a regulatory region.

**[0461]** In some embodiments, the disclosure provides a population of cells comprising one or more engineered cells. In some embodiments, the disclosure provides a population of cells comprising at least 10^2, at least 10^3, at least 10^4, at least 10^5, at least 10^6, at least 10^7, at least 10^8, at least 10^9, at least 10^10, at least 10^11, or at least 10^12, engineered cells of the disclosure.

**[0462]** In some embodiments, the cell further comprises **(i)** a chimeric antigen receptor (CAR) having specificity for a target antigen; and/or **(ii)** a recombinant T cell receptor (TCR) having specificity for a target antigen. In some embodiments, the engineered cell comprises a nucleic acid construct expressing the CAR or recombinant TCR. In some embodiments, the nucleic acid construct is an RNA construct. In some embodiments, the target antigen is CD1, CD1a, CD1b, CD1c, CD1d, CD1e, CD2, CD3d, CD3e, CD3g, CD3ε, CD4, CD5, CD7, CD8a, CD8b, CD19, CD20, CD21, CD22, CD23, CD24, CD25, CD27, CD28, CD30, CD33, CD34, CD38, CD40, CD44v6, CD45, CD46, CD47 CD48, CD52, CD59, CD66, CD70, CD71, CD72, CD73, CD79A, CD79B, CD80 (B7.1), CD86 (B7.2), CD94, CD95, CD97, CD123, CD134, CD140 (PDGFR4), CD152, CD154, CD158, CD171, CD178, CD179, CD179a, CD181 (CXCR1), CD182 (CXCR2), CD183 (CXCR3), CD210, CD213A2, CD246, CD252, CD253, CD261, CD262, CD272, CD273 (PD-L2), CD274 (PD-L1), CD276 (B7H3), CD279, CD295, CD339 (JAG1), CD340 (HER2), CDH17, CEA, CLECL1, CLL-1, CLDN6, CLDN18.2, CS1, DLL3, LY6G6D, GCC, p53R175H, PRAME, EGFR, EGFRvIII, FGFR2, AFP, CA125, MUC-1, MAGE, ALPI, alkaline phosphatase placental-like 2 (ALPPL2), B-cell maturation antigen (BCMA), green fluorescent protein (GFP), enhanced green fluorescent protein (eGFP), KLK2, KLK3, Mesothelin, IL13Ra2, ggcc, signal regulatory protein α (SIRPα), TCRalpha, TCRbeta, TSHR, GD2, GD3, Tn Ag, cMET, Axl, ROR1, ROR2, GPC1, GPC2, GPC3, FLT3, TAG72, CEA, EPCAM, KIT (CD117), IL-13Ra2, IL-11Ra, PSCA, PRSS21, VEGFR2, LewisY, PDGFRβ, SSEA-4, folate receptor alpha, ERBB2 (Her2/neu), MUC1, MUC16, NCAM, prostase, PAP, ELF2M, Ephrin B2, IGF-I receptor, CAIX, LMP2, gp100, bcr-abl, tyrosinase, EphA2, STEAP1, STEAP2, fucosyl GM1, sLe, GM3, TGS5, HMWMAA, o-acetyl-GD2, folate receptor beta, TEM1/CD248, TEM7R, ROPN1, GPRC5D, GPA33, CXORF61, ALK, Polysialic acid,

PLAC1, GloboH, NY-BR-1, UPK2, HAVCR1, ADRB3, PANX3, GPR20, LY6K, OR51E2, TARP, WT1, NY-ESO-1, LAGE-la, MAGE-A4, legumain, HPV E6,E7, MAGE Al, ETV6-AML, sperm protein 17, XAGE1, Tie 2, MAD-CT-1, MAD-CT-2, Fos-related antigen 1, p53, p53 mutant, p53R175H, KRAS, mutant KRAS, KRAS G12D, prostein, surviving, telomerase, PCTA-1/Galectin 8, MelanA/MART1, Ras mutant, hTERT, sarcoma translocation breakpoints, ML-IAP, ERG (TMPRSS2 ETS fusion gene), NA17, PAX3, androgen receptor, cyclin Bl, MYCN, RhoC, TRP-2, CYP1B1, BORIS, SART3, PAX5, OY-TES1, LCK, AKAP-4, SSX2, RAGE-1, human telomerase reverse transcriptase, RU1, RU2, intestinal carboxyl esterase, mut hsp70-2,LAIR1, FCAR, LILRA2, CD300LF, CLEC12A, BST2, EMR2, LY75, FCRL5, IGLL1, PSMA, TROP2, citrullinated vimentin, the extracellular portion of the APRIL protein, or any combinations thereof. In some embodiments, the target antigen is B7H3, BCMA, CD19, CD20, CD22, CD70, CD79a, CD79b, CDH17, CLDN6, CLDN18.2, DLL3, GCC, GD2, GD3, GPC3, GPRC5D, GPA33, KLK3, LY6G6D, p53R175H, PRAME, ROPN1, STEAP1, or STEAP2. In some embodiments, the engineered cell has cell surface expression of the CAR or TCR.

**[0463]** In some embodiments, the cell further comprises a chimeric auto antigen receptor (CAAR) having specificity for a target antigen. In some embodiments, the antigen is pancreatic P-cell antigen, synovial joint antigen, myelin basic protein, proteolipid protein, myelin oligodendritic glycoprotein, MuSK, keratinocyte adhesion protein desmoglein 3 (Dsg3), Ro-RNP complex, La antigen, myeloperoxidase, proteinase 3, cardiolipin, citrullinated proteins, carbamylated proteins, or a3 chain of basement membrane collagen. In some embodiments, the engineered cell has cell surface expression of the CAAR.

**[0464]** In some embodiments, the cell further comprises a B-cell autoantibody receptor (BAR) having specificity for a target antigen. In some embodiments, the engineered cell has cell surface expression of the BAR.

**[0465]** In some embodiments, the engineered cell further comprises a non-coding RNA. In some embodiments, the non-coding RNA comprises a shRNA or a microRNA.

**[0466]** In some embodiments, the cell further comprises an additional potency enhancement polypeptide, a cytokine, a chemokine, a growth factor, a tolerogenic factor, a safety switch, or any combination thereof. In some embodiments, the additional potency enhancement polypeptide is or comprises a dominant negative form of an inhibitor of a cell-mediated immune response of the immune cell (e.g., TGFβR2 DNR), c-Jun, CCL19, CCL21, IL2R, IL7, IL7Ralpha, IL15, IL15RA, IL18, decoy-resistant IL18 (DR-18), MyD88/CD40, PD1-

CD28 switch receptor, PD1-41BB switch receptor, CD40L-CD28 switch receptor, CTBR12 switch receptor, or CD8alpha/beta.

**[0467]** In some embodiments, the safety switch is or comprises a herpesvirus thymidine kinase (HSV-tk) gene, an Escherichia coli cytosine deaminase (EC-CD) gene, or an inducible Caspase9 (iCasp9) protein.

**[0468]** In some embodiments, the engineered cell is a prokaryotic cell or a eukaryotic cell. In some embodiments, the cell is *in vivo*. In some embodiments, the cell is *ex vivo*. In some embodiments, the cell is *in vitro*. In some embodiments, the engineered cell is a eukaryotic cell. In some embodiments, the engineered cell is an animal cell. In some embodiments, the animal cell is a mammalian cell. In some embodiments, the animal cell is a human cell. In some embodiments, the cell is a non-human primate cell. In some embodiments, the cell is a canine cell. In some embodiments, the cell is a non-natural cell or has been genetically engineered (i.e., an engineered cell). In some embodiments, the polynucleotide is an exogenous nucleic acid. In some embodiments, the mammalian cell is an immune cell, a neuron, an epithelial cell, and endothelial cell, or a stem cell.

**[0469]** In some embodiments, the cell is an immune cell selected from the group of consisting of a T cell, a CD4+ T cell, a CD8+ T cell, a regulatory T cell (Treg), a gamma delta T cell (γδT), an invariant natural killer T (iNKT) cell, a mucosal associated invariant T (MAIT) cell, a macrophage, a monocyte, a natural killer (NK) cell, a tumor infiltrating lymphocyte (TIL), a cytotoxic T cell, a T helper cell, a memory T cell, a central memory T (TCM) cell, a stem memory T (TSCM) cell, a stem-cell-like memory T cell (or stem-like memory T cells), an effector memory T (TEM) cell, a TEMRA (CD45RA+) cell, an effector T cell, a Th1 cell, a Th2 cell, a Th9 cell, a Th17 cell, a Th22 cell, a Tfh (follicular helper) cell, a natural killer T (NKT) cell, a transitional memory T (TTM) cell, a terminal effector T (TTE) cell, a naïve T (TN) cell, a hematopoietic stem cell, and a progenitor cell of the lymphoid lineage. In some embodiments, the cell is a T cell, a macrophage, a monocyte, or a natural killer (NK) cell. In some embodiments, the cell is a T cell. In some embodiments, the T cell is a regulatory T cell (Treg), a gamma delta T cell, a CD8+ T cell, an invariant iNKT cell, a MAIT cell, a CAR T cell, a tumor-infiltrating lymphocyte, or an engineered T cell comprising a transcriptional receptor.

**[0470]** Sources for the T cells include, but are not limited to, peripheral blood, bone marrow, or other sources of hematopoietic cells. T cells can be isolated by methods well known in the art, including commercially available isolation methods (see, for example, Rowland-Jones et al., Lymphocytes: A Practical Approach, Oxford University Press, New York (1999), Su et

al., Methods Mol. Biol. 806:287-299 (2012); Bluestone et al., Sci. Transl. Med. 7(315) (doi: 10.1126/scitranslmed.aad4134)(2015); Miyara et al., Nat. Rev. Rheumatol. 10:543-551 (2014); Liu et al., J. Exp. Med. 203:1701-1711 (2006); Seddiki et al., J. Exp. Med. 203:1693-1700 (2006); Ukena et al., Exp. Hematol. 39:1152-1160 (2011); Chen et al., J. Immunol. 183:4094-4102 (2009); Putnam et al., Diabetes 58:652-662 (2009); Putnam et al., Am. Tranplant. 13:3010-3020 (2013); Lee et al., Cancer Res. 71:2871-2881 (2011); MacDonald et al., J Clin. Invest. 126:1413-1424 (2016)).

**[0471]** In some embodiments, the engineered cell is an autologous cell or an allogeneic cell. In some embodiments, the engineered cell is a primary cell. In some embodiments, the engineered cell is derived from a stem cell. In some embodiments, the engineered cell is genetically modified. In some embodiments, the engineered cell has reduced or eliminated expression of an endogenous T cell receptor. In some embodiments, the engineered cell is more prone to display central memory phenotype than a control cell lacking the recombinant polypeptide. In some embodiments, the engineered cell further comprises one or more modifications that inactivate or disrupt one or more alleles of TGFbeta, Regnase-1, FAS, PTPN2, NR4A3, CD52, PD-1, B2M, CIITA, SOCS1, CBLB, DGKalpha, and/or DGKζ.

**[0472]** In some embodiments, the cell further comprises a sequence specific nuclease, a nucleic acid programmable DNA binding protein, an RNA guided nuclease, an RNA-guided nuclease comprising a Cas nuclease and a guide RNA (CRISPR-Cas combination), a ribonucleoprotein (RNP) complex comprising a gRNA and a Cas nuclease, a homing endonuclease, a zinc finger nuclease (ZF) nucleic acid binding entity, a transcription activator-like effector (TALE) nucleic acid binding entity, a meganuclease, a Cas nuclease, a core Cas protein, a homing endonuclease, an endonuclease-deficient-Cas protein, an enzymatically inactive Cas protein, a CRISPR- associated transposase (CAST), a Type II or Type V Cas protein, or a functional portion thereof.

**[0473]** In some embodiments, the cell further comprises Casl, Cas2, Cas3, Cas4, Cas5, Cas6, Cas7, Cas8a, Cas8b, Cas8c, Cas9, CaslO, Casl2, Casl2a (Cpfl), Casl2b (C2cl), Casl2c (C2c3), Casl 2d (CasY), Casl2e (CasX), Casl2f (C2cl0), Cas 12g, Casl2h, Casl2i, Cas 12k (C2c5), Casl3, Casl3a (C2c2), Casl3b, Casl3c, Casl3d, C2c4, C2c8, C2c9, Cmrl, Cmr2, Cmr3, Cmr4, Cmr5, Cmr6, Csdl, Csd2, Cas5d, Csel, Cse2, Cse3, Cse4, Cas5e, Csfl, Csml, Csm2, Csm3, Csm4, Csm5, Csnl, Csn2, Cstl, Cst2, Cas5t, Cshl, Csh2, Cas5h, Csal, Csa2, Csa3, Csa4, Csa5, Cas5a, CsxlO, Csxl l, Csyl, Csy2, Csy3, Csy4, Mad7, SpCas9, eSpCas9, SpCas9-HFl, HypaSpCas9, HeFSpCas9, evoSpCas9 high-fidelity variants of SpCas9, SaCas9, NmeCas9, CjCas9, StCas9, TdCas9, LbCasl2a, AsCasl2a, AacCasl2b, BhCasl2b

v4, TnpB, dCas (D10A), dCas (H840A), dCasl3a, dCasl3b, or a functional fragment thereof. In some embodiments, the RNA-guided nuclease is a Life Edit nuclease (LEG), optionally a LEG14 nuclease.

**[0474]** Various known techniques can be employed to isolate or enrich for desired immune cells such as T cells. If a particular type of T cell is to be isolated, various cell surface markers or combinations of markers, including but not limited to, CD3, CD4, CD8, CD34 (for hematopoietic stem and progenitor cells) and the like, can be used to separate the cells, as is well known in the art (see Kearse, T Cell Protocols: Development and Activation, Humana Press, Totowa N.J. (2000); De Libero, T Cell Protocols, Vol. 514 of Methods in Molecular Biology, Humana Press, Totowa N.J. (2009)) Negative selection methods can be used to remove cells that are not the desired immune cells. Additionally, positive selection methods can be used to isolate or enrich for desired T cells. In some instances, a combination of both positive and negative selection methods can be used.

**[0475]** In some embodiments, the engineered cells provided by the disclosure are modified such that they are able to survive and proliferate when administered to a patient. In some embodiments, the engineered cells provided by the disclosure are modified such that they have increased signaling through certain pathways. These effects on survival, proliferation, and/or signaling may be related to the recombinant polypeptide or functional fragment thereof that is comprised by the engineered cell. The engineered cell may demonstrate one or more of the following characteristics: reduced exhaustion, increased proliferative capacity, enhanced replicative lifespan, decreased replicative senescence, enhanced anti-tumor effect, reduced dysfunction, enhanced persistence, and/or increase intratumoral presence in vivo. In some embodiments, the engineered cell may demonstrate increased signaling through has increased or decreased signaling through the CARD11-BCL10-MALT1 complex, NF-κB, AP-1, NFAT, JAK/STAT, and/or MEK/ERK pathways.

**[0476]** In some embodiments, the provided modified cells are modified such that they are able to evade immune recognition and responses when administered to a patient (e.g., recipient subject). The cells can evade killing by immune cells in vitro and in vivo. In some embodiments, the cells evade killing by macrophages and NK cells. In some embodiments, the cells are ignored by immune cells or a subject's immune system. In other words, the cells administered in accordance with the methods described herein are not detectable by immune cells of the immune system. In some embodiments, the cells are cloaked and therefore avoid immune rejection.

**[0477]** Methods of determining whether a modified cell provided herein evades immune recognition include, but are not limited to, IFN-γ Elispot assays, microglia killing assays, cell engraftment animal models, cytokine release assays, ELISAs, killing assays using bioluminescence imaging or chromium release assay or Xcelligence analysis, mixed-lymphocyte reactions, immunofluorescence analysis, etc. In some embodiments, the immunogenicity of the cells is evaluated in a complement-dependent cytotoxicity (CDC) assay. CDC can be assayed in vitro by incubating cells with IgG or IgM antibodies targeting an HLA-independent antigen expressed on the cell surface in the presence of serum containing complement and analyzing cell killing. In some embodiments, CDC can be assayed by incubating cells with ABO blood type incompatible serum, wherein the cells comprise A antigens or B antigens, and the serum comprises antibodies against the A antigens and/or B antigens of the cells.

**[0478]** The cell may be a vertebrate cell, for example, a mammalian cell, such as a human cell, a canine cell, or a mouse cell. The cell may also be a vertebrate stem cell, for example, a mammalian stem cell, such as a human stem cell (or a cell derived from such a stem cell), a canine stem cell, or a mouse stem cell (or a cell derived from such a stem cell). In embodiments, the cell or stem cell or a cell derived from such a stem cell is amenable to modification.

**[0479]** In some embodiments, the cells that are engineered or modified as provided herein are cells from a subject that is known or suspected of having a particular disease or condition to be treated.

**[0480]** In some embodiments, the cells that are engineered or modified as provided herein are cells from a healthy subject, such as a subject that is not known or suspected of having a particular disease or condition to be treated. For instance, if T cells are isolated or obtained from a donor subject, such as for treating a cancer, the donor subject is a healthy subject not known or suspected of suffering from cancer or another disease or condition.

**[0481]** The polynucleotide encoding the engineered CARD11-PIK3R3 fusion protein of the present disclosure can be introduced into a target cell, such as, for example, a human T lymphocyte, to produce a recombinant or engineered cell containing the nucleic acid molecule.

**[0482]** Among the cells expressing the engineered CARD11-PIK3R3 fusion protein disclosed herein are engineered cells. The genetic engineering generally involves introduction of a nucleic acid encoding the recombinant or engineered component into a

composition containing the cells, such as by retroviral transduction, transfection, or transformation.

**[0483]** In some embodiments, the nucleic acids are heterologous, i.e., normally not present in a cell or sample obtained from the cell, such as one obtained from another organism or cell, which for example, is not ordinarily found in the cell being engineered and/or an organism from which such cell is derived. In some embodiments, the nucleic acids are not naturally occurring, such as a nucleic acid not found in nature, including one comprising chimeric combinations of nucleic acids encoding various domains from multiple different cell types.

**[0484]** The cells generally are eukaryotic cells, such as mammalian cells, and typically are human cells. In some embodiments, the cells are derived from the blood, bone marrow, lymph, or lymphoid organs, are cells of the immune system, such as cells of the innate or adaptive immunity, e.g., myeloid or lymphoid cells, including lymphocytes, typically T cells and/or NK cells. Other exemplary cells include stem cells, such as multipotent and pluripotent stem cells, including induced pluripotent stem cells (iPSCs). The cells typically are primary cells, such as those isolated directly from a subject and/or isolated from a subject and frozen. In some embodiments, the cells include one or more subsets of T cells or other cell types, such as whole T cell populations, CD4+ cells, CD8+ cells, and subpopulations thereof, such as those defined by function, activation state, maturity, potential for differentiation, expansion, recirculation, localization, and/or persistence capacities, antigen-specificity, type of antigen receptor, presence in a particular organ or compartment, marker or cytokine secretion profile, and/or degree of differentiation. With reference to the subject to be treated, the cells may be allogeneic and/or autologous. Among the methods include off-the-shelf methods. In some aspects, such as for off- the-shelf technologies, the cells are pluripotent and/or multipotent, such as stem cells, such as induced pluripotent stem cells (iPSCs). In some embodiments, the methods include isolating cells from the subject, preparing, processing, culturing, and/or engineering them, and re introducing them into the same subject, before or after cryopreservation.

**[0485]** In other embodiments, the methods include isolating cells from a donor, preparing, processing, culturing, and/or engineering them, and introducing them into a subject who is not the donor, before or after cryopreservation.

**[0486]** Among the sub-types and subpopulations of T cells and/or of CD4+ and/or of CD8+ T cells are naive T (TN) cells, effector T cells (TEFF), memory T cells and sub-types thereof, such as stem cell memory T (TSCM), central memory T (TCM), effector memory T

(TEM), or terminally differentiated effector memory T cells, tumor-infiltrating lymphocytes (TIL), immature T cells, mature T cells, helper T cells, cytotoxic T cells, mucosa-associated invariant T (MAIT) cells, naturally occurring and adaptive regulatory T (Treg) cells, helper T cells, such as TH1 cells, TH2 cells, TH3 cells, TH17 cells, TH9 cells, TH22 cells, follicular helper T cells, alpha/beta T cells, and delta/gamma T cells.

**[0487]** In some embodiments, the cells are natural killer (NK) cells. In some embodiments, the cells are monocytes or granulocytes, e.g., myeloid cells, macrophages, neutrophils, dendritic cells, mast cells, eosinophils, and/or basophils.

**[0488]** Among the sub-types and subpopulations of NK cells are CD56+ bright and CD56+ dim NK cells. CD56+ bright NK cells, known for their high expression of CD56, play a pivotal role in immune regulation and cytokine production, particularly in response to interleukin-12 (IL-12), IL-15, and IL-18. These cells are potent producers of cytokines like interferon-gamma (IFN-γ) but have limited cytotoxic capabilities. In contrast, CD56+ dim NK cells, which express CD56 at lower levels, are highly cytotoxic and specialize in directly killing virus-infected cells and tumor cells. Without wishing to be bound by theory, CD56+ dim NK cells achieve this through the release of granules containing perforin and granzymes. Additionally, there are other NK subsets based on the expression of receptors like NKG2A, NKG2C, and KIRs (Killer-cell Immunoglobulin-like Receptors), which modulate NK cell activity and specificity. These NK subsets demonstrate variations in response to viral infections, tumor surveillance, and interaction with other immune cells.

**[0489]** In some embodiments, the cells include one or more nucleic acids introduced via genetic engineering, and thereby express recombinant or genetically engineered products of such nucleic acids. In some embodiments, the nucleic acids are heterologous, i.e., normally not present in a cell or sample obtained from the cell, such as one obtained from another organism or cell, which for example, is not ordinarily found in the cell being engineered and/or an organism from which such cell is derived. In some embodiments, the nucleic acids are not naturally occurring, such as a nucleic acid not found in nature, including one comprising chimeric combinations of nucleic acids encoding various domains from multiple different cell types.

**[0490]** In some embodiments, the cells are isolated from a biological sample from a patient or donor. In some embodiments, the sample include tissue, fluid, and other samples taken directly from the subject or donor, as well as samples resulting from one or more processing steps, such as separation, centrifugation, genetic engineering (e.g., transduction with viral vector), washing, and/or incubation. The biological sample can be a sample obtained directly

from a biological source or a sample that is processed. Biological samples include, but are not limited to, body fluids, such as blood, plasma, serum, cerebrospinal fluid, synovial fluid, urine and sweat, tissue and organ samples, including processed samples derived therefrom.

**[0491]** In some aspects, the sample from which the cells are derived or isolated is blood or a blood-derived sample, or is or is derived from an apheresis or leukapheresis product. Exemplary samples include whole blood, peripheral blood mononuclear cells (PBMCs), leukocytes, bone marrow, thymus, tissue biopsy, tumor, leukemia, lymphoma, lymph node, gut associated lymphoid tissue, mucosa associated lymphoid tissue, spleen, other lymphoid tissues, liver, lung, stomach, intestine, colon, kidney, pancreas, breast, bone, prostate, cervix, testes, ovaries, tonsil, or other organ, and/or cells derived therefrom. Samples include, in the context of cell therapy, e.g., adoptive cell therapy, samples from autologous and allogeneic sources.

**[0492]** In some embodiments, the cells are derived from cell lines, e.g., T cell lines. The cells in some embodiments are obtained from a xenogeneic source, for example, from mouse, rat, non-human primate, dog, and pig.

***Primary Cells***

**[0493]** In some embodiments, the cell is a primary cell. Primary cells are isolated directly from human or animal tissue using enzymatic or mechanical methods. Once isolated, they are placed in an artificial environment in plastic or glass containers supported with specialized medium containing essential nutrients and growth factors to support proliferation. Primary cells could be of two types: adherent or suspension. Adherent cells require attachment for growth and are said to be anchorage-dependent cells. Adherent cells are usually derived from tissues of organs. Suspension cells do not require attachment for growth and are said to be anchorage independent cells. Most suspension cells are isolated from the blood system, but some tissue- derived cells can also be used in suspension, such as hepatocytes or intestinal cells. Although primary cells usually have a limited lifespan, they offer a number of advantages compared to cell lines. Primary cell culture enables researchers to study donors and not just cells. Several factors such as age, medical history, race, and sex can be considered when building an experimental model. With a growing trend towards personalized medicine, such donor variability and tissue complexity can be achieved with use of primary cells, but are difficult to replicate with cell lines that are more systematic and uniform in nature and do not capture the true diversity of a living tissue.

**[0494]** In some embodiments, the starting material is a primary cell collected from a subject (e.g., a patient). In some embodiments, the starting material is a primary blood cell collected from a subject (e.g., a patient), e.g., via a leukopak. For example, in some embodiments, the starting material are unmodified T cells obtained from a subject (e.g., a patient). In some embodiments, the starting material is a primary cell collected from a donor. In some embodiments, the starting material is a primary blood cell collected from a donor, e.g., via a leukopak. For example, in some embodiments, the starting material are unmodified T cells obtained from a donor. In some embodiments, the starting material is otherwise modified or engineered to have altered expression of one or more genes.

**[0495]** In some embodiment the cells that are engineered as provided herein comprise cells derived from primary cells obtained or isolated from one or more individual subjects or donors. In some embodiments, the cells are derived from a pool of isolated primary cells obtained from a subject (e.g., the patient administered the cells). In some embodiments, the cells are derived from a pool of isolated primary cells obtained from one or more (e.g., two or more, three or more, four or more, five or more, ten or more, twenty or more, fifty or more, or one hundred or more) different donor subjects. In some embodiments, the primary cells isolated or obtained from the plurality of different donor subjects (e.g., two or more, three or more, four or more, five or more, ten or more, twenty or more, fifty or more, or one hundred or more) are pooled together in a batch and are engineered in accord with the provided methods.

***Pluripotent Stem Cells and Stem Cell Derived Cells***

**[0496]** In some embodiments, the cell is a stem cell. In some embodiments, the cell is a pluripotent stem cell. In some embodiments, the cell is derived from a pluripotent stem cell. Pluripotent stem cells are cells that have the capacity to self-renew by dividing and to develop into the three primary germ cell layers of the early embryo and therefore into all cells of the adult body, but not extra-embryonic tissues such as the placenta. Embryonic stem cells (ESCs) and induced pluripotent stem cells (iPSCs) are pluripotent stem cells. In some embodiments, the cell is an ESC. ESCs are pluripotent stem cells derived from the inner cell mass of a blastocyst, an early-stage pre-implantation embryo. In some embodiments, the cell is an iPSC. iPSCs are derived from adult somatic cells that have been genetically reprogrammed back into a pluripotent state that enables the development of an unlimited source of any type of cell needed for therapeutic purposes.

**[0497]** "Pluripotent stem cells" as used herein have the potential to differentiate into any of the three germ layers: endoderm (e.g., the stomach lining, gastrointestinal tract, lungs, etc.), mesoderm (e.g., muscle, bone, blood, urogenital tissue, etc.) or ectoderm (e.g., epidermal tissues and nervous system tissues). The term "pluripotent stem cells," as used herein, also encompasses ESCs and iPSCs (or iPS cells). In some embodiments, a pluripotent stem cell is produced or generated from a cell that is not a pluripotent cell. In other words, pluripotent stem cells can be direct or indirect progeny of a non-pluripotent cell. Examples of parent cells include somatic cells that have been reprogrammed to induce a pluripotent, undifferentiated phenotype by various means. Such "iPS" or "iPSC" cells can be created by inducing the expression of certain regulatory genes or by the exogenous application of certain proteins. Methods for the induction of iPS cells are known in the art and are further described below. (See, e.g., Zhou et al., Stem Cells 27 (11): 2667-74 (2009); Huangfu et al., Nature Biotechnol. 26 (7): 795 (2008); Woltjen et al., Nature 458 (7239): 766-770 (2009); and Zhou et al., Cell Stem Cell 8:381-384 (2009); each of which is incorporated by reference herein in their entirety.) As used herein, "hiPSCs" are human induced pluripotent stem cells. In some embodiments, the cell is a mesenchymal stem cell (MSCs).

**[0498]** In some embodiments, the cell is a differentiated cell. In some embodiments, the cell is differentiated from a pluripotent stem cell. Differentiated cells are cells that have undergone differentiation. They are mature cells that perform a specialized function. Some examples of differentiated cells are T cells, NK cells, etc. Generally, these cells have a unique morphology, metabolic activity, membrane potential, and responsiveness to signals facilitating their function in a body tissue or organ.

**[0499]** In some embodiments, the engineered cells described are derived from an ESC or iPSC or a progeny thereof. As used herein, the term “derived from an ESC or iPSC or a progeny thereof” encompasses the initial ESC or iPSC that is generated and any subsequent progeny thereof. As used herein, the term “progeny” encompasses, e.g., a first-generation progeny, i.e., the progeny is directly derived from, obtained from, obtainable from or derivable from the initial ESC or iPSC by, e.g., traditional propagation methods. The term “progeny” also encompasses further generations such as second, third, fourth, fifth, sixth, seventh, or more generations, i.e., generations of cells which are derived from, obtained from, obtainable from or derivable from the former generation by, e.g., traditional propagation methods. The term “progeny” also encompasses modified cells that result from the modification or alteration of the initial ESC or iPSC or a progeny thereof.

**[0500]** In embodiments, the cell or stem cell, or a cell derived from such a stem cell, has or is believed to have therapeutic value, such that the cell or stem cell or a cell derived or differentiated from such stem cell may be used to treat a disease, disorder, defect or injury in a subject in need of treatment for same.

**[0501]** In some embodiments, the cell is a stem cell or progenitor cell (e.g., iPSC, ESC, hematopoietic stem cell, mesenchymal stem cell, endothelial stem cell, epithelial stem cell, adipose stem or progenitor cells, germline stem cells, lung stem or progenitor cells, mammary stem cells, olfactory adult stem cells, hair follicle stem cells, multipotent stem cells, amniotic stem cells, cord blood stem cells, or neural stem or progenitor cells). In some embodiments, the stem cells are adult stem cells (e.g., somatic stem cells or tissue specific stem cells). In some embodiments, the stem or progenitor cell is capable of being differentiated (e.g., the stem cell is totipotent, pluripotent, or multipotent). In some embodiments, the cell is isolated from embryonic or neonatal tissue. In some embodiments, the cell is a fibroblast, monocytic precursor, B cell, exocrine cell, pancreatic progenitor, endocrine progenitor, hepatoblast, myoblast, preadipocyte, progenitor cell, hepatocyte, chondrocyte, smooth muscle cell, K562 human erythroid leukemia cell line, bone cell, synovial cell, tendon cell, ligament cell, meniscus cell, adipose cell, dendritic cells, or natural killer cell. In some embodiments, the cell is manipulated (e.g., converted or differentiated) into a muscle cell, erythroid-megakaryocytic cell, eosinophil, iPS cell, macrophage, T cell, islet beta-cell, neuron, cardiomyocyte, blood cell, endocrine progenitor, exocrine progenitor, ductal cell, acinar cell, alpha cell, beta cell, delta cell, PP cell, hepatocyte, cholangiocyte, or brown adipocyte. In some embodiments, the cell is a muscle cell (e.g., skeletal, smooth, or cardiac muscle cell), erythroid-megakaryocytic cell, eosinophil, iPS cell, macrophage, T cell, islet beta-cell, neuron, cardiomyocyte, blood cell (e.g., red blood cell, white blood cell, or platelet), endocrine progenitor, exocrine progenitor, ductal cell, acinar cell, alpha cell, beta cell, delta cell, PP cell, hepatocyte, cholangiocyte, or white or brown adipocyte. In some embodiments, the cell is a hormone-secreting cell (e.g., a cell that secretes insulin, oxytocin, endorphin, vasopressin, serotonin, somatostatin, gastrin, secretin, glucagon, thyroid hormone, bombesin, cholecystokinin, testosterone, estrogen, or progesterone, renin, ghrelin, amylin, or pancreatic polypeptide), an epidermal keratinocyte, an epithelial cell (e.g., an exocrine secretory epithelial cell, a thyroid epithelial cell, a keratinizing epithelial cell, a gall bladder epithelial cell, or a surface epithelial cell of the cornea, tongue, oral cavity, esophagus, anal canal, distal urethra, or vagina), a kidney cell, a germ cell, a skeletal joint synovium cell, a periosteum cell, a bone cell (e.g., osteoclast or

osteoblast), a perichondrium cell (e.g., a chondroblast or chondrocyte ), a cartilage cell (e.g., chondrocyte), a fibroblast, an endothelial cell, a pericardium cell, a meningeal cell, a keratinocyte precursor cell, a keratinocyte stem cell, a pericyte, a glial cell, an ependymal cell, a cell isolated from an amniotic or placental membrane, or a serosal cell (e.g., a serosal cell lining body cavities).

**[0502]** In some embodiments, the cell is a T cell or NK cell that has been differentiated from an engineered ESC or iPSC. In some embodiments, the cell is an engineered cell that has been modified from a primary cell. In some embodiments, the cell comprises increased expression of polypeptide of the disclosure.

**[0503]** As will be appreciated by those in the art, there are a variety of different methods for the generation of ESCs and iPCSs. The original induction of iPSCs was done from mouse embryonic or adult fibroblasts using the viral introduction of four transcription factors, Oct3/4, Sox2, c-Myc and Klf4; see Takahashi and Yamanaka Cell 126:663-676 (2006), hereby incorporated by reference in its entirety and specifically for the techniques outlined therein. Since then, a number of methods have been developed; see Seki et al, World J. Stem Cells 7(1): 116-125 (2015) for a review, and Lakshmipathy and Vermuri, editors, Methods in Molecular Biology: Pluripotent Stem Cells, Methods and Protocols, Springer 2013, both of which are hereby expressly incorporated by reference in their entirety, and in particular for the methods for generating hiPSCs (see for example Chapter 3 of the latter reference).

**[0504]** Generally, iPSCs are generated by the transient expression of one or more reprogramming factors" in the host cell, usually introduced using episomal vectors. Under these conditions, small amounts of the cells are induced to become iPSCs (in general, the efficiency of this step is low, as no selection markers are used). Once the cells are "reprogrammed", and become pluripotent, they lose the episomal vector(s) and produce the factors using the endogeneous genes.

**[0505]** Any of the pluripotent stem cells described herein can be differentiated into any cells of an organism and tissue. In an aspect, provided herein are engineered cells that are differentiated into different cell types from ESCs or iPSCs for subsequent transplantation into recipient subjects. Differentiation can be assayed as is known in the art, generally by evaluating the presence of cell-specific markers. As will be appreciated by those in the art, the differentiated engineered pluripotent cell derivatives can be transplanted using techniques known in the art that depends on both the cell type and the ultimate use of these cells. In some embodiments, the iPSCs are differentiated into cell types selected from T cells, NK cells.

*T Cells*

**[0506]** In some embodiments, the cell is a T cell. In some embodiments, the T cell comprises a polynucleotide encoding the engineered polypeptide of the disclosure. T cells of the disclosure or precursors thereof can be immune cells of the lymphoid lineage. Relevant information concerning T cells as referred to in the context of the present disclosure is known in the art, including certain information regarding desired features of T cells when used for cell therapy. It will be understood that embodiments concerning T cells described herein may be readily and appropriately combined. It will be understood that embodiments concerning T cells described herein may be readily and appropriately combined with other embodiments described herein (for example, but not limited to, the polypeptide of the disclosure).

**[0507]** The T cells described herein may be used to treat or prevent a disease in a subject.

**[0508]** A T cell is a type of lymphocyte. T cells are one of the white blood cells of the immune system and play a central role in the adaptive immune response. T cells can be derived from T cells in a patient's own blood (autologous) or derived from the T cells of another healthy donor (allogeneic). T cells can be obtained from a number of sources including, but not limited to, peripheral blood mononuclear cells, bone marrow, lymph nodes tissue, cord blood, thymus issue, tissue from a site of infection, ascites, pleural effusion, spleen tissue, and tumors. In certain embodiments, T cells can be obtained from a unit of blood collected from a subject using any number of techniques known to the skilled person, such as sedimentation, e.g., FICOLL™ separation, antibody-conjugated bead-based methods such as MACS™ separation (Miltenyi).

**[0509]** In some embodiments, the T cells can be CD4+ or CD8+ and can include, but are not limited to, regulatory T cells, cytotoxic T cells, T helper cells (CD4+), and memory T cells, including central memory T cells (TCM), stem memory T cells (TSCM), stem-cell-like memory T cells (or stem-like memory T cells), and effector memory T cells such as, for example, TEM cells and TEMRA (CD45RA+) cells, effector T cells, Th1 cells, Th2 cells, Th9 cells, Th17 cells, Th22 cells, Tfh (follicular helper) cells, T regulatory cells, natural killer T cells, mucosal associated invariant T cells (MAIT), and γδ T cells. Major T cell subtypes include TSCM (stem cell memory), TCM (central memory), TTM (Transitional Memory), TEM (Effector memory), TTE (Terminal Effector) and TN (naive). In some embodiments, the T cells express CCR7, CD27, CD28, and CD45RA. In some embodiments, the central T cells express CCR7, CD27, CD28, and CD45RO. In other

embodiments, the effector memory T cells express PD-1, CD27, CD28, and CD45RO. In other embodiments, the effector memory RA T cells express PD-1, CD57, and CD45RA.

**[0510]** In some embodiments, the T cells of the disclosure can be hematopoietic stem and/or progenitor cells of the lymphoid lineage that can differentiate into T cells. Hematopoietic stem and/or progenitor cells can be derived from bone marrow, umbilical cord blood, and adult peripheral blood.

**[0511]** In some embodiments, the T cells as provided herein are primary T cells. In some embodiments, the primary T cells are isolated or obtained from and individual subject or one or more donors, such as one or more healthy donors (e.g., a subject that is not known or suspected of, e.g., not exhibiting clinical signs of, a disease or infection). In some instances, the T cells are populations of primary T cells. In some instances, the T cells are subpopulations or sub-types or subsets of primary T cells. Subpopulations and sub-types and subsets of primary T cells are herein described in further detail below. As will be appreciated by those in the art, methods of isolating or obtaining T lymphocytes from an individual can be achieved using known techniques. Provided herein are engineered primary T lymphocytes that contain modifications (e.g., genetic modifications) described herein for subsequent transplantation or engraftment into subjects (e.g., recipients).

**[0512]** In some embodiments, primary T cells are obtained (e.g., harvested, extracted, removed, or taken) from a subject or an individual. In some embodiments, primary T cells are produced from a pool of T cells such that the T cells are from one or more subjects (e.g., one or more human including one or more healthy humans). In some embodiments, the pool of primary T cells is from 1-100, 1-50, 1-20, 1-10, 1 or more, 2 or more, 3 or more, 4 or more, 5 or more, 10 or more, 20 or more, 30 or more, 40 or more, 50 or more, or 100 or more subjects. In some embodiments, the donor subject is different from the patient (e.g., the recipient that is administered the therapeutic cells). In some embodiments, the pool of T cells does not include cells from the patient. In some embodiments, one or more of the donor subjects from which the pool of T cells is obtained are different from the patient.

**[0513]** In some embodiments, primary T cells are primary T cells of a T cell subset or a sub-type or subpopulation. Such T cell subsets may be found in US2018/0319862A1 and/or WO20 16/090190A1, the entire contents of which are hereby incorporated by reference. Accordingly, in some embodiments, the T cell subset, such as a CD62L+ T cell subset, that is increased in subjects upon administration of the genetically engineered cells are or include or share phenotypic characteristics with memory T cells or particular subsets thereof, such as long-lived memory T cells. In some embodiments, such memory T cells are central memory

T cells (Tcm) or T memory stem cells (Tsc) cells. In some embodiments, the memory T cells are Tsc cells. Tsc cells may be described as having one or more phenotypic differences or functional features compared to other memory T cell subsets or compared to naive T cells, such as being less differentiated or more naive (see e.g., Ahlers and Belyakov (2010) Blood, 115: 1678); Cieri et al (2015) Blood, 125:2865; Flynn et al. (2014) Clinical & Translational Immunology, 3, e20; Gattinoni et al. (2012) Nat. Med., 17: 1290-1297; Gattinoni et al. (2012) Nat .Reviews, 12:671; Li et al. (2013) PLOS ONE, 8:e67401; and published PCT Appl. No. W02014/039044). In some cases, Tsc cells are thought to be the only memory T cells able to generate effector T cells and all three subsets of memory T cells (Tsc, Tcm, and Tern). In some aspects, Tsc cells have the highest survival and proliferation response to antigenic or homeostatic stimuli of all the memory T cell subsets, and the least attrition absent cognate antigen. In some embodiments, the less - differentiated Tsc cells may exhibit greater expansion, long-term viability, and target cell destruction following adoptive transfer than other memory T cells, and thus may be able to mediate more effective treatment with fewer transferred cells than would be possible for either Tcm or Tern cells.

**[0514]** In some embodiments, the cells as provided herein are T lymphocytes differentiated from engineered pluripotent cells that contain modifications (e.g., genetic modifications) described herein and that are differentiated into T lymphocyte. As will be appreciated by those in the art, the methods for differentiation depend on the desired cell type using known techniques. In some embodiments, the cells differentiated into a T lymphocyte may be used for subsequent transplantation or engraftment into subjects (e.g., recipients).

**[0515]** Methods for generating T cells from pluripotent stem cells (e.g., iPSC) are described, for example, in Iriguchi et al., Nature Communications 12, 430 (2021); Themeli et al. 16(4):357-366 (2015); Themeli et al., Nature Biotechnology 31 :928-933 (2013). In some embodiments, the cell is an iPSC-derived T cell that is engineered to contain modifications (e.g., genetic modifications) described herein. In some embodiments, the cell is a primary T cell that is engineered to contain modifications (e.g., genetic modifications) described herein. In some embodiments, the cell comprises overexpression or increased expression of one or more complement inhibitor. In some embodiments, the T cell can be engineered with a chimeric antigen receptor (CAR), including any as described herein. In some embodiments, the engineered T cell can be used to treat a variety of indications with allogenic cell therapy, including any as described herein. In some embodiments, the engineered T cell can be used to treat cancer.

**[0516]** Cells disclosed herein may be T cells, for example a population of T cells. It will be understood that any reference to "a cell" e.g. "a T cell" below also applies to "a population of cells" e.g. "a population of T cells" as described in the present application.

***NK Cells***

**[0517]** In some embodiments, the cell is a natural killer (NK) cell. In some embodiments, the NK cell comprises a polynucleotide encoding the polypeptide of the disclosure. Relevant information concerning NK cells as referred to in the context of the present disclosure is known in the art, including certain information regarding desired features of NK cells when used for cell therapy and for example may be found from WO2017214569A1, WO 2011/068896A1, the contents of which are herein incorporated by reference. It will be understood that embodiments concerning NK cells described herein may be readily and appropriately combined with other embodiments described herein (for example, but not limited to, the engineered polypeptide of the disclosure).

**[0518]** The NK cells described herein may be used to treat or prevent a disease in a subject.

**[0519]** NK cells, which arise through the lymphoid lineage and are part of the innate immune system, may be used in anti-cancer therapy as they have been found to detect and kill certain types of tumor cells. Natural killer (NK) cells are cytotoxic lymphocytes capable of human immune surveillance. WO2017214569A1 (the contents of which are incorporated herein by reference in their entirety) describes genome-edited primary NK cells, methods of making those cells, and methods of administering those cells.

**[0520]** In some embodiments, the cells that are engineered or modified as provided herein are Natural Killer (NK) cells. In some embodiments, the NK cells are isolated or obtained from an individual subject or one or more donors, such as one or more healthy donor (e.g., a donor that is not known or suspected of, e.g., not exhibiting clinical signs of, a disease or infection). As will be appreciated by those in the art, methods of isolating or obtaining NK cells from an individual can be achieved using known techniques. Provided herein are engineered primary NK cells that contain modifications (e.g., genetic modifications) described herein for subsequent transplantation or engraftment into subjects (e.g., recipients. For instance, the engineered NK cells are administered to a subject (e.g., recipient, such as a patient), by infusion of the engineered NK cells into the subject.

**[0521]** In some embodiments, the NK cells are primary NK cells. In some embodiments, NK cells, including primary NK cells isolated from an individual subject or one or more donors (e.g., healthy donors) or NK cells differentiated from iPSCs derived from one or

more donors (e.g., healthy donors) express CD56 (e.g., CDSb^111 or CD56bright) and lack CD3 (e.g., CD3neg). In some embodiments, NK cells as described herein may also express the low-affinity Fey receptor CD16, which, without wishing to be bound by theory, mediate ADCC. In some embodiments, the NK cells also express one or more natural killer cell receptors NKG2A and NKG2D or one or more natural cytotoxicity receptors NKp46, NKp44, NKp30. For example, for the case of primary NK cells, in specific cases, the primary cells may be isolated from a starting source of NK cells, such as a sample containing peripheral blood mononuclear cells (PBMCs), by depletion of cells positive for CD3, CD14, and/or CD19. For instance, the cells may be subject to depletion using immunomagnetic beads having attached thereto antibodies to CD3, CD 14, and/or CD19, respectively), thereby producing an enriched population of NK cells. In other cases, primary NK cells may be isolated from a starting source that is a mixed population (e.g., PBMCs) by selecting cells for the presence of one or more markers on the NK cells, such as CD56, CD16, NKp46, and/or NKG2D.

**[0522]** In some embodiments, the NK cells can be immature NK cells and can be CD56+ and CD16-. In some embodiments, the NK cells can be mature NK cells and can be CD56- and CD16+, or CD561o and CD16+ (as described in WO 2011/068896A1 (the contents of which are incorporated herein by reference in their entirety)).

**[0523]** A primary NK cell may express CD16 and/or CD56. In some embodiments, an NK cell does not express CD3. In some embodiments, an "NK cell" is preferably defined as a cell that is CD56+ and CD3-. In some embodiments, an "NK cell" is defined as a cell that is CD16+ and CD3-. NK cells are lymphocytes of the innate immune system that kill virally infected or transformed cells. Like T cells, NK cells are cytotoxic lymphocytes. Unlike T cells, NK cells do not require antigen recognition, and require integration of signals from many activating and inhibitory receptors to perform their function. Despite their similarities to T cells, NK cells behave differently under stimulation conditions and do not tolerate electroporation in the same way as T cells (as described in WO2017214569A1 (the contents of which are incorporated herein by reference in their entirety)).

**[0524]** A primary NK cell may be isolated from, for example, peripheral blood, umbilical cord cells, ascites, and/or a solid tumor (as described in WO2017214569A1, the contents of which are incorporated herein by reference in their entirety). In some embodiments, a "primary NK cell" is an NK cell that is freshly isolated. In some embodiments, a "primary NK cell" is an NK cell that has undergone up to 5 replications or divisions after being isolated, up to 10 replications or divisions after being isolated, up to 15 replications or

divisions after being isolated, up to 20 replications or divisions after being isolated, up to 25 replications or divisions after being isolated, up to 30 replications or divisions after being isolated, up to 35 replications or divisions after being isolated, or up to 40 replications or divisions after being isolated. In some embodiments, the primary NK cell is a non-clonal cell. In some embodiments, primary NK cell is a proliferating cell. In some embodiments, primary NK cell is an expanded cell.

**[0525]** Some desired features of NK cells when used for cell therapy are described herein. NK cells may be distinguished using a number of assays, for example as set out in WO 2011/068896A1 (the contents of which is hereby incorporated by reference in its entirety). For example, IL12-p70 secretion may be measured: IL12p70 is secreted by mature DCs in order to elicit a Thl-directed response from CD4+ T cells. Human BM-derived DCs can produce >500pg/ml of IL12p70 yet blast-derived DCs did not produce any detectable IL12p70 upon maturation. Mixed lymphocyte reaction (MLR) assay: The MLR assay determines the ability of DCs to stimulate proliferation of allogenic T cells. Cord blood mononuclear cells (CBMCs, which include T cells) were used as responders, fluorescently labeled, and their proliferation was measured after 4-5 days coculture with immature or mature blast- derived DCs. Preliminary results show that the responder cells proliferate in response to mature (m)DCs. Hemangioblast-derived natural killer cells: Cell surface markers CD45, CD7, CD94, CD56, CD16, and NKG2D were evaluated in blast-derived and human bone marrow-derived NK cells.

**[0526]** Methods of generating natural killer (NK) cells are known in the art, for example methods described in WO 2011/068896A1 (the contents of which are incorporated by reference herein in their entirety) comprising: providing hemangioblasts; culturing the hemangioblasts on methylcellulose and a first cytokine mixture comprising IL2, IL3, IL6, IL7, IL15, SCF and FL; harvesting the cultured cells; and culturing the harvested cells in liquid media comprising human serum, and a second cytokine mixture comprising IL7, IL15, SCF and FL to generate NK cells.

**[0527]** In some embodiments, the cells as provided herein are NK cells differentiated from engineered pluripotent cells that contain modifications (e.g., genetic modifications) described herein and that are differentiated into NK cells. As will be appreciated by those in the art, the methods for differentiation depend on the desired cell type using known techniques. In some embodiments, the cells differentiated into NK cells may be used for subsequent administration to a subject (e.g., recipient, such as a patient), such as by infusion of the differentiated NK cells into the subject. Methods for generating NK cells from

pluripotent stem cells (e.g., iPSC) are described, for example, in U.S. Patent No. 10626373; Shankar et al. Stem Cell Res Ther. 2020; 11 : 234; Euchner et al. Frontiers in Immunology, 2021; 12, Article 640672. doi=10.3389/fimmu.2021.640672. Methods of differentiating NK cells are known in the art, for example as described in WO 2011/068896A1 (the contents of which are hereby incorporated by reference in their entirety). An exemplary method as described in WO 2011/068896A1. In one embodiment, the initial differentiation procedure for both cell types may be the same and may involve a 4 day culture of hESCs in Stemline II (Sigma) plus cytokines in order to generate embryoid bodies (EBs). The cytokines, VEGF and BMP4 may be used throughout the EB culture while bFGF may be added after the first 2 days. After 4 days total, the resulting EBs may be disaggregated with 0.05% trypsin and then the trypsin may be inactivated with serum- containing media. Individual cells may be subsequently filtered through a 40 pM cell strainer, counted, and seeded into H4436 or H4536 methylcellulose (Stem Cell Technologies) containing additional cytokines, such as TPO (50pg/ml), VEGF(50pg/ml), FL (50pg/ml) and bFGF (20-50pg/ml). For NK differentiation, the cytokines IL2 (l-10pg/ml), IL7 (l-20pg/ml), and/or IL15 (l-10pg/ml) may also be added at this stage. Methylcellulose cultures may be plated at a concentration of 50,000 to 150,000 cells per ml for the production and expansion of a hemangioblastic population. Blast-like cells may be harvested from methylcellulose between day 6 and 10 and further differentiated by one of the following procedures.

**[0528]** Cells disclosed herein may be natural killer (NK) cells, for example a population of NK cells. It will be understood that any reference to "a cell" e.g. "a NK cell" below also applies to "a population of cells" e.g. "a population of NK cells" as described in the present application.

## VIII. METHODS OF GENOME MODIFICATION

**[0529]** In some embodiments, a polynucleotide encoding the engineered CARD11-PIK3R3 fusion protein of the present disclosure may be inserted into the genome of a cell. The insertion may be non-targeted (i.e., insertion into a random genomic locus of the cell) or targeted (i.e., insertion into a specific genomic locus of the cell), using any of the non-targeted or site-directed (targeted) insertion methods described herein.

### *Non-Targeted Insertion*

**[0530]** In some embodiments, t a polynucleotide encoding the engineered CARD11-PIK3R3 fusion protein of the present disclosure can be inserted into a random genomic locus of a

host cell. As known to a person skilled in the art, viral vectors, including, for example, retroviral vectors, lentiviral vectors, adenoviral vectors, and adeno-associated viral vectors, are commonly used to deliver genetic material into host cells and randomly insert the foreign or exogenous nucleic acid into the genome of the cell to facilitate stable expression and replication of the foreign or exogenous nucleic acid.

***Homology-Directed Repair (HDR)***

**[0531]** In some embodiments, homology-directed repair (HDR) can be utilized for targeted integration or insertion of one or more nucleic acid sequences, e.g., transgene sequences, at one or more target site(s) in the genome. In some embodiments, the nuclease-induced HDR can be used to alter a target sequence, integrate a transgene at a particular target location, and/or to edit or repair a mutation in a particular target gene.

**[0532]** Alteration of nucleic acid sequences at the target site can occur by HDR with an exogenously provided polynucleotide (also referred to as donor polynucleotide or template sequence). For example, the template polynucleotide provides for alteration of the target sequence, such as insertion of the transgene contained within the template polynucleotide. In some embodiments, a plasmid or a vector can be used as a template for homologous recombination. In some embodiments, a linear DNA fragment can be used as a template for homologous recombination. In some embodiments, a single stranded template polynucleotide can be used as a template for alteration of the target sequence by alternate methods of homology directed repair (e.g., single strand annealing) between the target sequence and the template polynucleotide. Template polynucleotide-effected alteration of a target sequence depends on cleavage by a nuclease, e.g., a targeted nuclease such as CRISPR/Cas9. Cleavage by the nuclease can comprise a double strand break or two single strand breaks.

**[0533]** In some embodiments, a template polynucleotide comprises the following components: [5’ homology arm] -[transgene] -[3’ homology arm]. The homology arms provide for recombination into the chromosome, thus insertion of the transgene into the DNA at or near the cleavage site, e.g., target site(s). In some embodiments, the homology arms flank the most distal target site(s).

**[0534]** In some embodiments, “recombination” refers to a process of exchange of genetic information between two polynucleotides. In some embodiments, “homologous recombination (HR)” refers to the specialized form of such exchange that takes place, for example, during repair of double-strand breaks in cells via homology-directed repair

mechanisms. This process requires nucleotide sequence homology, uses a template polynucleotide to template repair of a target DNA (i.e., the one that experienced the double-strand break, e.g., target site in the endogenous gene), and is variously known as "non-crossover gene conversion" or "short tract gene conversion," because it leads to the transfer of genetic information from the template polynucleotide to the target. In some embodiments, such transfer can involve mismatch correction of heteroduplex DNA that forms between the broken target and the template polynucleotide, and/or "synthesis-dependent strand annealing," in which the template polynucleotide is used to resynthesize genetic information that will become part of the target, and/or related processes. Such specialized HR often results in an alteration of the sequence of the target molecule such that part or all of the sequence of the template polynucleotide is incorporated into the target polynucleotide. As described herein, the genetic disruption of the target site or target position can be created by any mechanisms, such as ZFNs, TALENs, CRISPR/Cas9 system, or TtAgo nucleases.

**[0535]** In some embodiments, double strand cleavage is affected by a nuclease, e.g., a Cas9 molecule having cleavage activity associated with an HNH-like domain and cleavage activity associated with a RuvC-like domain, e.g., an N-terminal RuvC-like domain, e.g., a wild type Cas9.

**[0536]** In some embodiments, DNA repair mechanisms can be induced by a nuclease after (1) a single double-strand break, (2) two single strand breaks, (3) two double stranded breaks with a break occurring on each side of the target site, (4) one double stranded break and two single strand breaks with the double strand break and two single strand breaks occurring on each side of the target site (5) four single stranded breaks with a pair of single stranded breaks occurring on each side of the target site, or (6) one single stranded break. In some embodiments, a single-stranded template polynucleotide is used and the target site can be altered by alternative HDR.

**[0537]** Template polynucleotide-effected alteration of a target site depends on cleavage by a nuclease molecule. Cleavage by the nuclease can comprise a nick, a double strand break, or two single strand breaks, e.g., one on each strand of the DNA at the target site. After introduction of the breaks on the target site, resection occurs at the break ends resulting in single stranded overhanging DNA regions.

**[0538]** In canonical HDR, a double-stranded template polynucleotide is introduced, comprising homologous sequence to the target site that will either be directly incorporated into the target site or used as a template to insert the transgene or correct the sequence of the target site. After resection at the break, repair can progress by different pathways, e.g., by

the double Holliday junction model (or double strand break repair, DSBR, pathway) or the synthesisdependent strand annealing (SDSA) pathway.

**[0539]** In some embodiments, other DNA repair pathways such as single strand annealing (SSA), single- stranded break repair (SSBR), mismatch repair (MMR), base excision repair (BER), nucleotide excision repair (NER), intrastrand cross-link (ICL), translesion synthesis (TLS), error-free postreplication repair (PRR) can be employed by the cell to repair a doublestranded or single-stranded break created by the nucleases.

***Site-Directed Insertion***

**[0540]** In some embodiments, the methods for genetically modifying cells to knock out, knock down, or otherwise modify one or more genes comprise using a site-directed nuclease, including, for example, zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), meganucleases, transposases, and clustered regularly interspaced short palindromic repeat (CRISPR)/Cas systems, as well as nickase systems, base editing systems, prime editing systems, and any other gene editing systems known in the art.

ZFN

**[0541]** ZFNs are fusion proteins comprising an array of site-specific DNA binding domains adapted from zinc finger-containing transcription factors attached to the endonuclease domain of the bacterial FokI restriction enzyme. A ZFN may have one or more (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, or more) DNA binding domains or zinc finger domains. See, e.g., Carroll et al., Genetics Society of America (2011) 188:773-782; Kim et al., Proc. Natl. Acad. Sci. USA (1996) 93: 1156-1160. The individual DNA binding domains are typically referred to as "fingers" or "zinc fingers." Each zinc finger domain is a small protein structural motif stabilized by one or more zinc ions and usually recognizes a 3- to 4-bp DNA sequence. Tandem domains can thus potentially bind to an extended nucleotide sequence that is unique within a cell's genome. Each finger binds from two to four base pairs of DNA, typically three or four base pairs of DNA. A DNA binding domain binds to a nucleic acid sequence called a target site or target segment. Each finger typically comprises an approximately 30 amino acid, zinc-chelating, DNA-binding subdomain. Studies have demonstrated that a single zinc finger of this class consists of an alpha helix containing the two invariant histidine residues coordinated with zinc along with the two cysteine residues of a single beta turn (see, e.g., Berg & Shi, Science 271 : 1081-1085 (1996)).

**[0542]** Various zinc fingers of known specificity can be combined to produce multifinger polypeptides which recognize about 6, 9, 12, 15, or 18-bp sequences. Various selection and modular assembly techniques are available to generate zinc fingers (and combinations thereof) recognizing specific sequences, including phage display, yeast one-hybrid systems, bacterial one-hybrid and two-hybrid systems, and mammalian cells. Zinc fingers can be engineered to bind a predetermined nucleic acid sequence. Criteria to engineer a zinc finger to bind to a predetermined nucleic acid sequence are known in the art. See, e.g., Sera et al., Biochemistry (2002) 41 :7074-7081; Liu et al., Bioinformatics (2008) 24: 1850-1857.

**[0543]** ZFNs containing FokI nuclease domains or other dimeric nuclease domains function as a dimer. Thus, a pair of ZFNs are required to target non-palindromic DNA sites. The two individual ZFNs must bind opposite strands of the DNA with their nucleases properly spaced apart. See Bitinaite < / al., Proc. Natl. Acad. Sci. USA (1998) 95: 10570-10575. To cleave a specific site in the genome, a pair of ZFNs are designed to recognize two sequences flanking the site, one on the forward strand and the other on the reverse strand. Upon binding of the ZFNs on either side of the site, the nuclease domains dimerize and cleave the DNA at the site, generating a DSB with 5' overhangs. HDR can then be utilized to introduce a specific mutation, with the help of a repair template containing the desired mutation flanked by homology arms. The repair template is usually an exogenous double-stranded DNA vector introduced to the cell. See Miller et al., Nat. Biotechnol. (2011) 29: 143-148; Hockemeyer et al., Nat. Biotechnol. (2011) 29:731-734.

TALENS

**[0544]** TALENs are another example of an artificial nuclease which can be used to edit a target gene. A "TALE-nuclease" (TALEN) is a fusion protein consisting of a nucleic acidbinding domain typically derived from a Transcription Activator Like Effector (TALE) and one nuclease catalytic domain to cleave a nucleic acid target sequence. The catalytic domain is preferably a nuclease domain and more preferably a domain having endonuclease activity, for instance I-TevI, ColE7, NucA and Fok-I. In numerous embodiments, the TALE domain can be fused to a meganuclease, for instance LCrel and I-Onul or functional variant thereof. In a more preferred embodiment, said nuclease is a monomeric TALE-Nuclease. A monomeric TALE- Nuclease is a TALE-Nuclease that does not require dimerization for specific recognition and cleavage, such as the fusions of engineered TAL repeats with the catalytic domain of I-TevI described in WO2012138927.

**[0545]** Transcription Activator like Effector (TALE) are proteins from the bacterial species Xanthomonas and comprise a plurality of repeated sequences. Each repeat is 33 to 35 amino acids in length, with two adjacent amino acids (termed the repeat-variable di-residue, or RVD) in position 12 and 13 that are specific to each nucleotide base of the nucleic acid targeted sequence. Binding domains with similar modular base-per-base nucleic acid binding properties (MBBBD) can also be derived from new modular proteins recently discovered in a different bacterial species. The new modular proteins have the advantage of displaying more sequence variability than TAL repeats. Preferably, RVDs associated with recognition of the different nucleotides are HD for recognizing C, NG for recognizing T, NI for recognizing A, NN for recognizing G or A, NS for recognizing A, C, G or T, HG for recognizing T, IG for recognizing T, NK for recognizing G, HA for recognizing C, ND for recognizing C, HI for recognizing C, HN for recognizing G, NA for recognizing G, SN for recognizing G or A and YG for recognizing T, TL for recognizing A, VT for recognizing A or G and SW for recognizing A. In another embodiment, critical amino acids 12 and 13 can be mutated towards other amino acid residues in order to modulate their specificity towards nucleotides A, T, C and G and in particular to enhance this specificity. TALEN kits are sold commercially.

**[0546]** TALENs are produced artificially by fusing one or more TALE DNA binding domains (e.g., 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more) to a nuclease domain, for example, a FokI endonuclease domain. See Zhang, Nature Biotech. (2011) 29: 149-153. Several mutations to FokI have been made for its use in TALENs; these, for example, improve cleavage specificity or activity. See Cermak et al., Nucl. Acids Res. (2011) 39:e82; Miller et al., Nature Biotech. (2011) 29: 143-148; Hockemeyer et al., Nature Biotech. (2011) 29:731-734; Wood et al., Science (2011) 333 :307; Doyon et al., Nature Methods (2010) 8:74-79; Szczepek et al., Nature Biotech (2007) 25:786-793; Guo et al., J. Mol. Biol. (2010) 200:96. The FokI domain functions as a dimer, requiring two constructs with unique DNA binding domains for sites in the target genome with proper orientation and spacing. Both the number of amino acid residues between the TALE DNA binding domain and the FokI nuclease domain and the number of bases between the two individual TALEN binding sites appear to be important parameters for achieving high levels of activity. Miller et al., Nature Biotech. (2011) 29: 143-148.

**[0547]** By combining engineered TALE repeats with a nuclease domain, a site-specific nuclease can be produced specific to any desired DNA sequence. Similar to ZFNs, TALENs can be introduced into a cell to generate DSBs at a desired target site in the genome, and so

can be used to knock out genes or knock in mutations in similar, HDR-mediated pathways. See Boch, Nature Biotech. (2011) 29: 135-136; Boch et al., Science (2009) 326: 1509-1512; Moscou et al., Science (2009) 326:3501.

Meganucleases

**[0548]** Meganucleases are sequence-specific enzymes in the endonuclease family which are characterized by their capacity to recognize and cut large DNA sequences (from 14 to 40 base pairs). (Chevalier, B. S. and B. L. Stoddard, Nucleic Acids Res., 2001, 29, 3757-3774). They can cleave unique sites in living cells, thereby enhancing gene targeting by 1000-fold or more in the vicinity of the cleavage site (Puchta et al., Nucleic Acids Res., 1993, 21, 5034-5040; Rouet et al., Mol. Cell. Biol., 1994, 14, 8096-8106; Choulika et al., Mol. Cell. Biol., 1995, 15, 1968-1973; Puchta et al., Proc. Natl. Acad. Sci. USA, 1996, 93, 5055-5060; Sargent et al., Mol. Cell. Biol., 1997, 17, 267-77; Donoho et al., Mol. Cell. Biol, 1998, 18, 4070-4078; Elliott et al., Mol. Cell. Biol., 1998, 18, 93-101; Cohen-Tannoudji et al., Mol. Cell. Biol., 1998, 18, 1444- 1448).

**[0549]** Meganucleases are grouped into families based on their structural motifs which affect nuclease activity and/or DNA recognition. The most widespread and best known meganucleases are the proteins in the LAGLID ADG family, which owe their name to a conserved amino acid sequence. See Chevalier et aL, Nucleic Acids Res. (2001) 29(18): 3757- 3774. On the other hand, the GIY-YIG family members have a GIY-YIG module, which is 70- 100 residues long and includes four or five conserved sequence motifs with four invariant residues, two of which are required for activity. See Van Roey et al., Nature Struct. Biol. (2002) 9:806-811. The His-Cys family meganucleases are characterized by a highly conserved series of histidines and cysteines over a region encompassing several hundred amino acid residues. See Chevalier et al., Nucleic Acids Res. (2001) 29(18):3757-3774. Members of the NHN family are defined by motifs containing two pairs of conserved histidines surrounded by asparagine residues. See Chevalier et al, Nucleic Acids Res. (2001) 29(18):3757-3774.

**[0550]** Because the chance of identifying a natural meganuclease for a particular target DNA sequence is low due to the high specificity requirement, various methods including mutagenesis and high throughput screening methods have been used to create meganuclease variants that recognize unique sequences. Strategies for engineering a meganuclease with altered DNA-binding specificity, e.g., to bind to a predetermined nucleic acid sequence are known in the art. See, e.g., Chevalier et al., Mol. Cell. (2002) 10:895-905; Epinat et aL,

Nucleic Acids Res (2003) 31 :2952-2962; Silva et al., J Mol. Biol. (2006) 361 :744-754; Seligman et al., Nucleic Acids Res (2002) 30:3870-3879; Sussman et aL, J Mol Biol (2004) 342:31-41; Doyon et aL, J Am Chem Soc (2006) 128:2477-2484; Chen et al, Protein Eng Des Sei (2009) 22:249-256; ArnovAd et aL, JMol Biol. (2006) 355:443-458; Smith et aL, Nucleic Acids Res. (2006) 363(2):283-294.

**[0551]** Like ZFNs and TALENs, Meganucleases can create DSBs in the genomic DNA, which can create a frame-shift mutation if improperly repaired, e.g., via NHEJ, leading to a decrease in the expression of a target gene in a cell. Alternatively, foreign DNA can be introduced into the cell along with the meganuclease. Depending on the sequences of the foreign DNA and chromosomal sequence, this process can be used to modify the target gene. See Silva et al., Current Gene Therapy (2011) 11 : 11-27.

Transposases

**[0552]** Transposases are enzymes that bind to the end of a transposon and catalyze its movement to another part of the genome by a cut and paste mechanism or a replicative transposition mechanism. By linking transposases to other systems such as the CRISPER/Cas system, new gene editing tools can be developed to enable site specific insertions or manipulations of the genomic DNA. There are two known DNA integration methods using transposons which use a catalytically inactive Cas effector protein and Tn7-like transposons. The transposase-dependent DNA integration does not provoke DSBs in the genome, which may guarantee safer and more specific DNA integration.

CRISPR/Cas

**[0553]** The CRISPR system was originally discovered in prokaryotic organisms (e.g., bacteria and archaea) as a system involved in defense against invading phages and plasmids that provides a form of acquired immunity. Now it has been adapted and used as a popular gene editing tool in research and clinical applications.

**[0554]** CRISPR/Cas systems generally comprise at least two components: one or more guide RNAs (gRNAs) and a Cas protein. The Cas protein is a nuclease that introduces a DSB into the target site. CRISPR-Cas systems fall into two major classes: class 1 systems use a complex of multiple Cas proteins to degrade nucleic acids; class 2 systems use a single large Cas protein for the same purpose. Class 1 is divided into types I, III, and IV; class 2 is divided into types II, V, and VI. Different Cas proteins adapted for gene editing applications include, but are not limited to, Casl, Cas2, Cas3, Cas4, Cas5, Cas6, Cas7, Cas8a, Cas8b, Cas8c, Cas9, CaslO, Casl2, Casl2a (Cpfl), Casl2b (C2cl), Casl2c (C2c3), Cas12d (CasY),

Casl2e (CasX), Casl2f (C2cl0), Cas 12g, Casl2h, Casl2i, Cas 12k (C2c5), Casl3, Casl3a (C2c2), Casl3b, Casl3c, Casl3d, C2c4, C2c8, C2c9, Cmrl, Cmr2, Cmr3, Cmr4, Cmr5, Cmr6, Csdl, Csd2, Cas5d, Csel, Cse2, Cse3, Cse4, Cas5e, Csfl, Csml, Csm2, Csm3, Csm4, Csm5, Csnl, Csn2, Cstl, Cst2, Cas5t, Cshl, Csh2, Cas5h, Csal, Csa2, Csa3, Csa4, Csa5, Cas5a, CsxlO, Csxl l, Csyl, Csy2, Csy3, Cys4, Mad7, SpCas9, eSpCas9, SpCas9-HFl, HypaSpCas9, HeFSpCas9, and evoSpCas9 high-fidelity variants of SpCas9, SaCas9, NmeCas9, CjCas9, StCas9, TdCas9, LbCasl2a, AsCasl2a, AacCasl2b, BhCasl2b v4, TnpB, dCas (D10A), dCas (H840A), dCasl3a, dCasl3b, and LEG14. The most widely used Cas9 is described herein as illustrative. These Cas proteins may be originated from different source species. For example, Cas9 can be derived from *S. pyogenes* or *S. aureus*.

**[0555]** In the original microbial genome, the type II CRISPR system incorporates sequences from invading DNA between CRISPR repeat sequences encoded as arrays within the host genome. Transcripts from the CRISPR repeat arrays are processed into CRISPR RNAs (crRNAs) each harboring a variable sequence transcribed from the invading DNA, known as the "protospacer" sequence, as well as part of the CRISPR repeat. Each crRNA hybridizes with a second transactivating CRISPR RNA (tracrRNA), and these two RNAs form a complex with the Cas9 nuclease. The protospacer-encoded portion of the crRNA directs the Cas9 complex to cleave complementary target DNA sequences, provided that they are adjacent to short sequences known as "protospacer adjacent motifs" (PAMs).

**[0556]** Since its discovery, the CRISPR system has been adapted for inducing sequence specific DSBs and targeted genome editing in a wide range of cells and organisms spanning from bacteria to eukaryotic cells including human cells. In its use in gene editing applications, artificially designed, synthetic gRNAs have replaced the original crRNA:tracrRNA complex. For example, the gRNAs can be single guide RNAs (sgRNAs) composed of a crRNA, a tetraloop, and a tracrRNA. The crRNA usually comprises a complementary region (also called a spacer, usually about 20 nucleotides in length) that is user-designed to recognize a target DNA of interest. The tracrRNA sequence comprises a scaffold region for Cas nuclease binding. The crRNA sequence and the tracrRNA sequence are linked by the tetraloop and each have a short repeat sequence for hybridization with each other, thus generating a chimeric sgRNA. One can change the genomic target of the Cas nuclease by simply changing the spacer or complementary region sequence present in the gRNA. The complementary region will direct the Cas nuclease to the target DNA site through standard RNA-DNA complementary base pairing rules.

**[0557]** In order for the Cas nuclease to function, there must be a PAM immediately downstream of the target sequence in the genomic DNA. Recognition of the PAM by the Cas protein is thought to destabilize the adjacent genomic sequence, allowing interrogation of the sequence by the gRNA and resulting in gRNA-DNA pairing when a matching sequence is present. The specific sequence of PAM varies depending on the species of the Cas gene. For example, the most commonly used Cas9 nuclease derived from S. pyogenes recognizes a PAM sequence of 5'-NGG-3' or, at less efficient rates, 5'-NAG-3', where "N" can be any nucleotide. Other Cas nuclease variants with alternative PAMs have also been characterized and successfully used for genome editing, which are summarized in **Table 8** below.

**Table 8. Exemplary Cas nuclease variants and their PAM sequences**

| CRISPR Nuclease | Source Organism | PAM Sequence (5'to 3') | SEQ ID NO |
|---|---|---|---|
| SpCas9 | Streptococcus pyogenes | NGG OR NAG | N/A |
| SaCas9 | Staphylococcus aureus | NGRRT OR NGRRN | N/A |
| NmeCas9 | Neisseria meningitidis | NNNNGATT | N/A |
| CjCas9 | Campylobacter jejuni | NNNNRYAC | N/A |
| StCas9 | Streptococcus thermophilus | NNAGAAW | N/A |
| TdCas9 | Treponema denticola | NAAAC | N/A |
| LbCas12a (Cpf1) | Lachnospiraceae bacterium | TTTV | N/A |
| AsCas12a (Cpf1) | Acidaminococcus sp. | TTTV | N/A |
| AacCas12b | Alicyclobacillus acidiphilus | TTN | N/A |
| BhCas12b v4 | Bacillus Hisashi | AAATN, TTTN, OR GTTN | N/A |
| NmeCas9 | Neisseria meningitidis | NNNNGATT | N/A |

R = A or G; Y = C or T; W = A or T; V = A or C or G; N = any base

**[0558]** In some embodiments, Cas nucleases may comprise one or more mutations to alter their activity, specificity, recognition, and/or other characteristics. For example, the Cas

nuclease may have one or more mutations that alter its fidelity to mitigate off- target effects (e.g., eSpCas9, SpCas9-HFl, HypaSpCas9, HeFSpCas9, and evoSpCas9 high-fidelity variants of SpCas9). For another example, the Cas nuclease may have one or more mutations that alter its PAM specificity.

**[0559]** Nuclease domains of the Cas, in particular the Cas9, nuclease can be mutated independently to generate enzymes referred to as DNA "nickases". Nickases are capable of introducing a single-strand cut with the same specificity as a regular CRISPR/Cas nuclease system, including for example CRISPR/Cas9. Nickases can be employed to generate doublestrand breaks which can find use in gene editing systems (Mali et al., Nat Biotech, 31(9):833-838 (2013); Mali et al. Nature Methods, 10:957-963 (2013); Mali et al, Science, 339(6121):823-826 (2013)). In some instances, when two Cas nickases are used, long overhangs are produced on each of the cleaved ends instead of blunt ends which allows for additional control over precise gene integration and insertion (Mali et al., Nat Biotech, 31(9):833-838 (2013); Mali et al. Nature Methods, 10:957-963 (2013); Mali et al., Science, 339(6121):823-826 (2013)). As both nicking Cas enzymes must effectively nick their target DNA, paired nickases can have lower off-target effects compared to the double-strand-cleaving Cas-based systems (Ran et al., Cell, 155(2):479- 480(2013); Mali et al, Nat Biotech, 31(9):833-838 (2013); Mali et al. Nature Methods, 10:957- 963 (2013); Mali et al., Science, 339(6121):823-826 (2013)).

**[0560]** The molecular machinery of such Cas proteins that allows the CRISPR/Cas system to alter target polynucleotide sequences in cells include RNA binding proteins, endo- and exo-nucleases, helicases, and polymerases. In some embodiments, the CRISPR/Cas system includes a Cas protein and at least one to two ribonucleic acids that are capable of directing the Cas protein to and hybridizing to a target motif of a target polynucleotide sequence. As used herein, "protein" and "polypeptide" are used interchangeably to refer to a series of amino acid residues joined by peptide bonds (i.e., a polymer of amino acids) and include modified amino acids (e.g., phosphorylated, glycated, glycosylated, etc.) and amino acid analogs. Exemplary polypeptides or proteins include gene products, naturally occurring proteins, homologs, paralogs, fragments and other equivalents, variants, and analogs of the above.

**[0561]** In some embodiments, a Cas protein comprises one or more amino acid substitutions or modifications. In some embodiments, the one or more amino acid substitutions comprises a conservative amino acid substitution. In some instances, substitutions and/or modifications can prevent or reduce proteolytic degradation and/or extend the half-life of the polypeptide

in a cell. In some embodiments, the Cas protein can comprise a peptide bond replacement (e.g., urea, thiourea, carbamate, sulfonyl urea, etc.). In some embodiments, the Cas protein can comprise a naturally occurring amino acid. In some embodiments, the Cas protein can comprise an alternative amino acid (e.g., D-amino acids, beta-amino acids, homocysteine, phosphoserine, etc.). In some embodiments, a Cas protein can comprise a modification to include a moiety (e.g., PEGylation, glycosylation, lipidation, acetylation, end-capping, etc.).

**[0562]** In some embodiments, a Cas protein comprises a core Cas protein, isoform thereof, or any Cas-like protein with similar function or activity of any Cas protein or isoform thereof. In some embodiments, a Cas protein comprises a core Cas protein. Exemplary Cas core proteins include, but are not limited to Casl, Cas2, Cas3, Cas4, Cas5, Cas6, Cas7, Cas8 and Cas9. In some embodiments, a Cas protein comprises type V Cas protein. In some embodiments, a Cas protein comprises a Cas protein of an E. coli subtype (also known as CASS2). Exemplary Cas proteins of the E. Coli subtype include, but are not limited to Csel, Cse2, Cse3, Cse4, and Cas5e. In some embodiments, a Cas protein comprises a Cas protein of the Ypest subtype (also known as CASS3). Exemplary Cas proteins of the Ypest subtype include, but are not limited to Csyl, Csy2, Csy3, and Csy4. In some embodiments, a Cas protein comprises a Cas protein of the Nmeni subtype (also known as CASS4). Exemplary Cas proteins of the Nmeni subtype include, but are not limited to Csnl and Csn2. In some embodiments, a Cas protein comprises a Cas protein of the Dvulg subtype (also known as CASS1). Exemplary Cas proteins of the Dvulg subtype include Csdl, Csd2, and Cas5d. In some embodiments, a Cas protein comprises a Cas protein of the Tneap subtype (also known as CASS7). Exemplary Cas proteins of the Tneap subtype include, but are not limited to, Cstl, Cst2, Cas5t. In some embodiments, a Cas protein comprises a Cas protein of the Hmari subtype. Exemplary Cas proteins of the Hmari subtype include, but are not limited to Cshl, Csh2, and Cas5h. In some embodiments, a Cas protein comprises a Cas protein of the Apem subtype (also known as CASS5). Exemplary Cas proteins of the Apem subtype include, but are not limited to Csal, Csa2, Csa3, Csa4, Csa5, and Cas5a. In some embodiments, a Cas protein comprises a Cas protein of the Mtube subtype (also known as CASS6). Exemplary Cas proteins of the Mtube subtype include, but are not limited to Csml, Csm2, Csm3, Csm4, and Csm5. In some embodiments, a Cas protein comprises a RAMP module Cas protein. Exemplary RAMP module Cas proteins include, but are not limited to, Cmrl, Cmr2, Cmr3, Cmr4, Cmr5, and Cmr6. See, e.g., Klompe et al., Nature 571, 219-225 (2019); Strecker et al., Science 365, 48-53 (2019). Examples of Cas proteins include, but are not limited to: Cas3, Cas8a, Cas5, Cas8b, Cas8c, CaslOd, Csel, Cse2, Csyl, Csy2, Csy3, and/or GSU0054.

In some embodiments, a Cas protein comprises Cas3, Cas8a, Cas5, Cas8b, Cas8c, CaslOd, Csel, Cse2, Csyl, Csy2, Csy3, and/or GSU0054. Examples of Cas proteins include, but are not limited to: Cas9, Csn2, and/or Cas4. In some embodiments, a Cas protein comprises Cas9, Csn2, and/or Cas4. In some embodiments, Examples of Cas proteins include, but are not limited to: CaslO, Csm2, Cmr5, CaslO, Csxl 1, and/or CsxlO. In some embodiments, a Cas protein comprises a CaslO, Csm2, Cmr5, CaslO, Csxl 1, and/or CsxlO. In some embodiments, examples of Cas proteins include, but are not limited to: Csfl. In some embodiments, a Cas protein comprises Csfl.In some embodiments, examples of Cas proteins include, but are not limited to: Casl2a, Casl2b, Casl2c, C2c4, C2c8, C2c5, C2cl0, and C2c9; as well as CasX (Casl2e) and CasY (Casl2d). Also see, e.g., Koonin et al., Curr Opin Microbiol. 2017; 37:67-78: "Diversity, classification and evolution of CRISPR- Cas systems." In some embodiments, a Cas protein comprises Casl2a, Casl2b, Casl2c, Casl2d, Casl2e, Casl2d, and/or Casl2e. In some embodiments, a Cas protein comprises Casl3, Casl3a, C2c2, Casl3b, Casl3c, and/or Casl3d. In some embodiments, the CRISPR/Cas system comprises a Cas effector protein selected from the group consisting of: a) Cas3, Cas8a, Cas5, Cas8b, Cas8c, CaslOd, Csel, Cse2, Csyl, Csy2, Csy3, and GSU0054; b) Cas9, Csn2, and Cas4; c) CaslO, Csm2, Cmr5, CaslO, Csxl l, and CsxlO; d) Csfl; e) Casl2a, Casl2b, Casl2c, C2c4, C2c8, C2c5, C2cl0, C2c9, CasX (Casl2e), and CasY (Casl2d); and f) Casl3, Casl3a, C2c2, Casl3b, Casl3c, and Casl3d.

**[0563]** In some embodiments, a Cas protein comprises any one of the Cas proteins described herein or a functional portion thereof. As used herein, "functional portion" when used in reference to a Cas protein refers to a portion of a peptide which retains its ability to complex with at least one ribonucleic acid (e.g., guide RNA (gRNA)) and cleave a target polynucleotide sequence. In some embodiments, the functional portion comprises a combination of operably linked Cas9 protein functional domains selected from the group consisting of a DNA binding domain, at least one RNA binding domain, a helicase domain, and an endonuclease domain. In some embodiments, the functional portion comprises a combination of operably linked Casl2a (also known as Cpfl) protein functional domains selected from the group consisting of a DNA binding domain, at least one RNA binding domain, a helicase domain, and an endonuclease domain. In some embodiments, the functional domains form a complex. In some embodiments, a functional portion of the Cas9 protein comprises a functional portion of a RuvC-like domain. In some embodiments, a functional portion of the Cas9 protein comprises a functional portion of the HNH nuclease

domain. In some embodiments, a functional portion of the Casl2a protein comprises a functional portion of a RuvC-like domain.

**[0564]** In some embodiments, the Cas protein is complexed with one to two ribonucleic acids. In some embodiments, the Cas protein is complexed with two ribonucleic acids. In some embodiments, the Cas protein is complexed with one ribonucleic acid. In some embodiments, the Cas protein is encoded by a modified nucleic acid, as described herein (e.g., a synthetic, modified mRNA).

**[0565]** The methods of the present disclosure contemplate the use of any ribonucleic acid that is capable of directing a Cas protein to and hybridizing to a target motif of a target polynucleotide sequence. In some embodiments, at least one of the ribonucleic acids comprises tracrRNA. In some embodiments, at least one of the ribonucleic acids comprises CRISPR RNA (crRNA). In some embodiments, a single ribonucleic acid comprises a guide RNA that directs the Cas protein to and hybridizes to a target motif of the target polynucleotide sequence in a cell. In some embodiments, at least one of the ribonucleic acids comprises a guide RNA that directs the Cas protein to and hybridizes to a target motif of the target polynucleotide sequence in a cell. In some embodiments, both of the one to two ribonucleic acids comprise a guide RNA that directs the Cas protein to and hybridizes to a target motif of the target polynucleotide sequence in a cell. The ribonucleic acids of the present disclosure can be selected to hybridize to a variety of different target motifs, depending on the particular CRISPR/Cas system employed, and the sequence of the target polynucleotide, as will be appreciated by those skilled in the art. The one to two ribonucleic acids can also be selected to minimize hybridization with nucleic acid sequences other than the target polynucleotide sequence. In some embodiments, the one to two ribonucleic acids hybridize to a target motif that contains at least two mismatches when compared with all other genomic nucleotide sequences in the cell. In some embodiments, the one to two ribonucleic acids hybridize to a target motif that contains at least one mismatch when compared with all other genomic nucleotide sequences in the cell. In some embodiments, the one to two ribonucleic acids are designed to hybridize to a target motif immediately adjacent to a deoxyribonucleic acid motif recognized by the Cas protein. In some embodiments, each of the one to two ribonucleic acids are designed to hybridize to target motifs immediately adjacent to deoxyribonucleic acid motifs recognized by the Cas protein which flank a mutant allele located between the target motifs.

**[0566]** In some embodiments, each of the one to two ribonucleic acids comprises guide RNAs that directs the Cas protein to and hybridizes to a target motif of the target polynucleotide sequence in a cell.

**[0567]** In some embodiments, one or two ribonucleic acids (e.g., guide RNAs) are complementary to and/or hybridize to sequences on the same strand of a target polynucleotide sequence. In some embodiments, one or two ribonucleic acids (e.g., guide RNAs) are complementary to and/or hybridize to sequences on the opposite strands of a target polynucleotide sequence. In some embodiments, the one or two ribonucleic acids (e.g., guide RNAs) are not complementary to and/or do not hybridize to sequences on the opposite strands of a target polynucleotide sequence. In some embodiments, the one or two ribonucleic acids (e.g., guide RNAs) are complementary to and/or hybridize to overlapping target motifs of a target polynucleotide sequence. In some embodiments, the one or two ribonucleic acids (e.g, guide RNAs) are complementary to and/or hybridize to offset target motifs of a target polynucleotide sequence.

**[0568]** In some embodiments, CRISPR systems of the present disclosure comprise TnpB polypeptides. In some embodiments, TnpB polypeptides may comprise a Ruv-C-like domain. The RuvC domain may be a split RuvC domain comprising RuvC-I, RuvC-II, and RuvC-III subdomains. In some embodiments, a TnpB may further comprise one or more of a HTH domain, a bridge helix domain and a zinc finger domain. TnpB polypeptides do not comprise an HNH domain. In some embodiments, a TnpB protein comprises, starting at the N-terminus: a HTH domain, a RuvC-I subdomain, a bridge helix domain, a RuvC-II sub-domain, a zinger finger domain, and a RuvC-III sub-domain. In some embodiments, a RuvC-III sub-domain forms the C- terminus of a TnpB polypeptide. In some embodiments, a TnpB polypeptide is from Epsilonproteobacteria bacterium, Actinoplanes lobatus strain DSM 43150, Actinomadura celluolosilytica strain DSM 45823, Actinomadura namibiensis strain DSM 44197, Alicyclobacillus macrosprangiidus strain DSM 17980, Lipingzhangella hal ophila strain DSM 102030, or Ktedonobacter recemifer. In some embodiments, a TnpB polypeptide is from Ktedonobacter racemifer, or comprises a conserved RNA region with similarity to the 5' ITR of K. racemifer TnpB loci. In some embodiments, a TnpB may comprise a Fanzor protein, a TnpB homolog found in eukaryotic genomes. In some embodiments, a CRISPR system comprising a TnpB polypeptide binds a target adjacent motif (TAM) sequence 5' of a target polynucleotide. In some embodiments, a TAM is a transposon-associated motif. In some embodiments, a TAM sequence comprises TCA. In

some embodiments, a TAM sequence comprises TTCAN. In some embodiments, a TAM sequence comprises TTGAT. In some embodiments, a TAM sequence comprises ATAAA.

**[0569]** In certain embodiments, the first and/or the second transgene may function as a DNA repair template to be integrated into the target site through HDR in associated with a gene editing system (e.g., the CRISPR/Cas system) as described. Generally, the transgene to be inserted would comprise at least the expression cassette encoding the protein of interest (e.g., the tolerogenic factor or CAR) and would optionally also include one or more regulatory elements (e.g., promoters, insulators, enhancers). In certain of these embodiments, the transgene to be inserted would be flanked by homologous sequence immediately upstream and downstream of the target, i.e., left homology arm (LHA) and right homology arm (RHA), specifically designed for the target genomic locus to serve as template for HDR. The length of each homology arm is generally dependent on the size of the insert being introduced, with larger insertions requiring longer homology arms.

**[0570]** In some embodiments, target-primed reverse transcription (TPRT) or prime editing may be used to engineer exogenous genes into specific loci. In some embodiments, prime editing mediates targeted insertions, deletions, all 12 possible base-to-base conversions, and combinations thereof in human cells without requiring DSBs or donor DNA templates.

**[0571]** Prime editing is a genome editing method that directly writes new genetic information into a specified DNA site using a nucleic acid programmable DNA binding protein ("napDNAbp") working in association with a polymerase (i.e., in the form of a fusion protein or otherwise provided in trans with the napDNAbp), wherein the prime editing system is programmed with a prime editing (PE) guide RNA ("PEgRNA") that both specifies the target site and templates the synthesis of the desired edit in the form of a replacement DNA strand by way of an extension (either DNA or RNA) engineered onto a guide RNA (e.g., at the 5' or 3' end, or at an internal portion of a guide RNA). The replacement strand containing the desired edit (e.g., a single nucleobase substitution) shares the same sequence as the endogenous strand of the target site to be edited (with the exception that it includes the desired edit). Through DNA repair and/or replication machinery, the endogenous strand of the target site is replaced by the newly synthesized replacement strand containing the desired edit. In some cases, prime editing may be thought of as a "search-and- replace" genome editing technology since the prime editors search and locate the desired target site to be edited, and encode a replacement strand containing a desired edit which is installed in place of the corresponding target site endogenous DNA strand at the same time. For example, prime editing can be adapted for conducting precision

CRISPR/Cas-based genome editing in order to bypass double stranded breaks. In some embodiments, a homologous protein is or encodes for a Cas protein-reverse transcriptase fusions or related systems to target a specific DNA sequence with a guide RNA, generate a single strand nick at the target site, and use the nicked DNA as a primer for reverse transcription of an engineered reverse transcriptase template that is integrated with the guide RNA. In some embodiments, a prime editor protein is paired with two prime editing guide RNAs (pegRNAs) that template the synthesis of complementary DNA flaps on opposing strands of genomic DNA, resulting in the replacement of endogenous DNA sequence between the PE-induced nick sites with pegRNA-encoded sequences.

**[0572]** In some embodiments, a gene editing technology is associated with a prime editor that is a reverse transcriptase, or any DNA polymerase known in the art. Thus, in one aspect, a prime editor may comprise Cas9 (or an equivalent napDNAbp) which is programmed to target a DNA sequence by associating it with a specialized guide RNA (i.e., PEgRNA) containing a spacer sequence that anneals to a complementary protospacer in the target DNA. Such methods include any disclosed in Anzalone et al., (doi.org/10.1038/s41586-019-1711-4), or in PCT publication Nos. WO2020191248, WO2021226558, or W02022067130, which are hereby incorporated in their entirety.

**[0573]** In some embodiments, the base editing technology may be used to introduce singlenucleotide variants (SNVs) into DNA or RNA in living cells. Base editing is a CRISPR-Cas9- based genome editing technology that allows the introduction of point mutations in RNAs or DNAs without generating DSBs. Base editors (BEs) are typically fusions of a Cas ("CRISPR- associated") domain and a nucleobase modification domain (e.g., a natural or evolved deaminase, such as a cytidine deaminase that include AP0BEC1 ("apolipoprotein B mRNA editing enzyme, catalytic polypeptide 1"), CD A ("cytidine deaminase"), and AID ("activation-induced cytidine deaminase")) domains. In some embodiments, base editors may also include proteins or domains that alter cellular DNA repair processes to increase the efficiency and/or stability of the resulting single-nucleotide change. Two major classes of base editors have been developed: cytidine base editors (CBEs) (e.g., BE4) that allow C:G to T:A conversions and adenine base editors (ABEs) (e.g., ABE7.10) that allow A:T to G:C conversions. Base editors are composed by a catalytically dead Cas9 (dCas9) or a nickase Cas9 (nCas9) fused to a deaminase and guided by a sgRNA to the locus of interest. The d/nCas9 recognizes a specific PAM sequence and the DNA unwinds thanks to the complementarity between the sgRNA and the DNA sequence usually located upstream of the PAM (also called protospacer). Then, the opposite

DNA strand is accessible to the deaminase that converts the bases located in a specific DNA stretch of the protospacer. Compared to HDR- based strategies, base editing is a promising tool to precisely correct genetic mutations as it avoids gene disruption by NHEJ associated with failed HDR-mediated gene correction. Rat deaminase AP0BEC1 (rAPOBECl) fused to deactivated Cas9 (dCas9) has been used to successfully convert cytidines to thymidines upstream of the PAM of the sgRNA. In some embodiments, this first BE system was optimized by changing the dCas9 to a "nickase" Cas9 D10A, which nicks the strand opposite the deaminated cytidine. Without being bound by theory, this is expected to initiate long- patch base excision repair (BER), where the deaminated strand is preferentially used to template the repair to produce a U:A base pair, which is then converted to T:A during DNA replication.

**[0574]** In some embodiments, a base editor is a nucleobase editor containing a first DNA binding protein domain that is catalytically inactive, a domain having base editing activity, and a second DNA binding protein domain having nickase activity, where the DNA binding protein domains are expressed on a single fusion protein or are expressed separately (e.g., on separate expression vectors). In some embodiments, a base editor is a fusion protein comprising a domain having base editing activity (e.g., cytidine deaminase or adenosine deaminase), and two nucleic acid programmable DNA binding protein domains (napDNAbp), a first comprising nickase activity and a second napDNAbp that is catalytically inactive, wherein at least the two napDNAbp are joined by a linker. In some embodiments, a base editor is a fusion protein that comprises a DNA domain of a CRISPR-Cas (e.g., Cas9) having nickase activity (nCas; nCas9), a catalytically inactive domain of a CRISPR-Cas protein (e.g., Cas9) having nucleic acid programmable DNA binding activity (dCas; e.g., dCas9), and a deaminase domain, wherein the dCas is joined to the nCas by a linker, and the dCas is immediately adjacent to the deaminase domain. In some embodiments, a base editor is an adenine-to-thymine or "ATBE" (or thymine-to-adenine or "TABE") transversion base editor. Exemplary base editor and base editor systems include any as described in patent publication Nos. US20220127622, US20210079366, US20200248169, US20210093667, US20210071163, W02020181202, WO2021158921, WO2019126709, W02020181178, W02020181195, WO2020214842, W02020181193, which are hereby incorporated in their entirety.

**[0575]** In some embodiments, a gene editing technology is Programmable Addition via Site-specific Targeting Elements (PASTE). In some aspects, PASTE is platform in which genomic insertion is directed via a CRISPR-Cas9 nickase fused to both a reverse

transcriptase and serine integrase. As described in Ioannidi et al. (doi.org/10.1101/2021.11.01.466786), PASTE does not generate double stranded breaks, but allows for integration of sequences as large as ~36 kb. In some embodiments, a serine integrase can be any known in the art. In some embodiments, a serine integrase has sufficient orthogonality such that PASTE can be used for multiplexed gene integration, simultaneously integrating at least two different genes at at least two genomic loci. In some embodiments, PASTE has editing efficiencies comparable to or better than those of homology directed repair or non-homologous end joining based integration, with activity in non-dividing cells and fewer detectable off-target events.

***Genomic Loci for Insertion***

**[0576]** The specific site for insertion within a gene locus may be located within any suitable region of the gene, including but not limited to a gene coding region (also known as a coding sequence or "CDS"), an exon, an intron, a sequence spanning a portion of an exon and a portion of an adjacent intron, or a regulatory region (e.g., promoter, enhancer). In some embodiments, the insertion occurs in one allele of the specific genomic locus. In some embodiments, the insertion occurs in both alleles of the specific genomic locus. In either of these embodiments, the orientation of the transgene inserted into the target genomic locus can be either the same or the reverse of the direction of the endogenous gene in that locus.

**[0577]** In some embodiments, polynucleotides of the present disclosure are inserted into a T cell receptor alpha chain (TRAC) locus. The TRAC gene encodes the TCR alpha chain constant region. The human TRAC gene resides on chromosome 14 at 22,547,506-22,552,156, forward strand. The TRAC genomic sequence is set forth in Ensembl ID ENSG00000277734.

**[0578]** In some embodiments, polynucleotides of the present disclosure are inserted into a T cell receptor beta chain (TRBC) locus. The TRBC genes encode the TCR beta chain constant region. TRBC1 and TRBC2 are analogs of the same gene, and T cells mutually exclusively express either TRBC1 or TRBC2. The human TRBC1 gene resides on chromosome 7 at 142,791,694-142,793,368, forward strand, and its genomic sequence is set forth in Ensembl ID ENSG00000211751. The human TRBC2 gene resides on chromosome 7 at 142,801,041-142,802,748, forward strand, and its genomic sequence is set forth in Ensembl ID ENSG00000211772.

**[0579]** In some embodiments, polynucleotides of the present disclosure are inserted into a TRAJ splice acceptor site locus. The human TRAC gene resides on chromosome 14 at

22,545,037-22,545,098, forward strand. The TRAJ genomic sequence is set forth in Ensembl ID ENSG00000211888.

**[0580]** In some embodiments, polynucleotides of the present disclosure are inserted into a CD247 (also known as the CD3 zeta chain) locus. The CD247 gene encodes the zeta chain of the T-cell receptor. The CD247 gene resides on chromosome 1: 167,425,027-167,518,640, reverse strand, and its genomic sequence is set forth in Ensembl ID ENSG00000198821.

**[0581]** In some embodiments, polynucleotides of the present disclosure are inserted into a beta-2-microglobulin (B2M) locus. The B2M gene encodes the B2M component of class I major histocompatibility complex (MHC I). The B2M gene resides on chromosome 15: 45,003,675-45,011,075, forward strand, and its genomic sequence is set forth in Ensembl ID ENSG00000166710.

**[0582]** In some embodiments, polynucleotides of the present disclosure are inserted into a class II, major histocompatibility complex, transactivator (CIITA) locus. The CIITA gene encodes the CIITA protein that is a positive regulator of class II major histocompatibility complex (MHC II) gene transcription. The CIITA gene resides on chromosome 16: 10,866,222-10,943,021, forward strand, and its genomic sequence is set forth in Ensembl ID ENSG00000179583.

**[0583]** In some embodiments, polynucleotides of the present disclosure are inserted into a caspase recruitment domain family member 11 (CARD11) locus. In some embodiments, polynucleotides encoding the PIK3R3 protein, or a portion thereof, of the present disclosure are inserted into a CARD11 locus. The CARD11 gene encodes the CARD11 protein which plays a role in various immune responses including, amoung others, lymphocyte activation, proliferation, and differentiation, and plays a role as a positive regulator of NF-kB activation. The CARD11 gene resides on chromosome 7: 2,906,142-3,044,228, reverse strand, and its genomic sequence is set forth in Ensembl ID ENSG00000198286. In some embodiments, a polynucleotide of the present disclosure is integrated at exon 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 or intron 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 of an endogenous CARD11 locus, In some embodiments, the polynucleotide is integrated at exon 13 of an endogenous CARD11 locus. In some embodiments, the polynucleotide is integrated at intron 12 of an endogenous CARD11 locus. In some embodiments, the polynucleotide is integrated at exon 14 of an endogenous CARD11 locus. In some embodiments, the polynucleotide is integrated at intron 13 of an endogenous CARD11 locus.In some embodiments,

polynucleotides of the present disclosure are inserted into a phosphoinositide-3-kinase regulatory subunit 3 (PIK3R3) locus. In some embodiments, polynucleotides encoding the CARD11 protein, or a portion thereof, of the present disclosure are inserted into a PIK3R3 locus. The PIK3R3 gene encodes the PIK3R3 protein which plays a role in inhibiting cell senescence and enhancing cell proliferation. The PIK3R3 gene resides on chromosome 1: 46,040,140-46,133,036, reverse strand, and its genomic sequence is set forth in Ensembl ID ENSG00000117461. In some embodiments, a polynucleotide of the present disclosure is integrated at exon 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or intron 1, 2, 3, 4, 5, 6, 7, 8, or 9 of an endogenous PIK3R3 locus. In some embodiments, the polynucleotide is integrated at exon 8 of an endogenous PIK3R3 locus. In some embodiments, the polynucleotide is integrated at intron 7 of an endogenous PIK3R3 locus.

**[0584]** In some embodiments, polynucleotides of the present disclosure are inserted into a safe harbor locus. Non-limiting examples of safe harbor loci include, but are not limited to, an AAVS1 (also known as PPP1R12C; Ensembl ID: ENSG00000125503), ABO (Ensembl ID: ENSG00000175164), CCR5 (Ensembl ID: ENSG00000160791), CLYBL (Ensembl ID: ENSG00000125246), CXCR4 (Ensembl ID: ENSG00000121966), F3 (also known as CD142; Ensembl ID: ENSG00000117525), FUT1 (Ensembl ID: ENSG00000174951), HMGB1 (Ensembl ID: ENSG00000189403), KDM5D (Ensembl ID: ENSG00000012817), LRP1 (also known as CD91; Ensembl ID: ENSG00000123384), MICA (Ensembl ID: ENSG00000204520 ), MICB (Ensembl ID: ENSG00000206449), RHD (Ensembl ID: ENSG00000187010), human ROSA26 (GRCh37/hg19, chr3: 9,415,082–9,414,043), and SHS 231 (GRCh37/hg19, chr4: 58,976,613–58,976,632).

***gRNAs for Site-Directed Insertion***

**[0585]** In some embodiments, provided are gRNAs for use in site-directed insertion of a polynucleotides according to various embodiments provided herein, especially in association with the CRISPR/Cas system. The gRNAs comprise a crRNA sequence, which in turn comprises a complementary region (also called a spacer) that recognizes and binds a complementary target DNA of interest. The length of the spacer or complementary region is generally between 15 and 30 nucleotides, usually about 20 nucleotides in length, although will vary based on the requirements of the specific CRISPR/Cas system. In certain embodiments, the spacer or complementary region is fully complementary to the target DNA sequence. In other embodiments, the spacer is partially complementary to the target DNA sequence, for example at least 80%, 85%, 90%, 95%, 98%, or 99% complementary.

**[0586]** In certain embodiments, the gRNAs provided herein further comprise a tracrRNA sequence, which comprises a scaffold region for binding to a nuclease. The length and/or sequence of the tracrRNA may vary depending on the specific nuclease being used for editing. In certain embodiments, nuclease binding by the gRNA does not require a tracrRNA sequence. In those embodiments where the gRNA comprises a tracrRNA, the crRNA sequence may further comprise a repeat region for hybridization with complementary sequences of the tracrRNA.

**[0587]** In some embodiments, the gRNAs provided herein comprise two or more gRNA molecules, for example, a crRNA and a tracrRNA, as two separate molecules. In other embodiments, the gRNAs are single guide RNAs (sgRNAs), including sgRNAs comprising a crRNA and a tracrRNA on a single RNA molecule. In certain of these embodiments, the crRNA and tracrRNA are linked by an intervening tetraloop.

**[0588]** In some embodiments, one gRNA can be used in association with a site-directed nuclease for targeted editing of a gene locus of interest. In other embodiments, two or more gRNAs targeting the same gene locus of interest can be used in association with a site-directed nuclease.

**[0589]** In some embodiments, exemplary gRNAs (e.g., sgRNAs) for use with various common Cas nucleases that require both a crRNA and tracrRNA, including Cas9 are provided in **Table 9** below. See, e.g., Jinek et al., Science (2012) 337 (6096):816-821; Dang et al., Genome Biology (2015) 16:280; Ran et al., Nature (2015) 520: 186-191; Strecker et al., Nature Comm. (2019) 10:212. For each exemplary gRNA, sequences for different portions of the gRNA, including the complementary region or spacer, crRNA repeat region, tetraloop, and tracrRNA, are shown.

**Table 9. Exemplary gRNA Structure and Sequences for CRISPR/Cas**

| SEQ ID NO: | Sequence (5' to 3') | Description |
|---|---|---|
| N/A | nnnnnnnnnnnnnnnnnnnn | Exemplary spCas9 1 complementary region (spacer) |
| 365 | guuuuagagcua | Exemplary spCas9 1 crRNA repeat region |
| N/A | gaaa | Exemplary spCas9 1 crRNA tetraloop |
| 367 | uagcaaguuaaaauaaggcuaguccguuaucaac uugaaaaaguggcaccgagucggugcuuuuuu | Exemplary spCas9 1 crRNA tracrRNA |
| N/A | nnnnnnnnnnnnnnnnnnnn | Exemplary spCas9 2 complementary region (spacer) |

| | | |
|---|---|---|
| 369 | guuusagagcuaugcug | Exemplary spCas9 2 crRNA repeat region |
| N/A | gaaa | Exemplary spCas9 2 crRNA tetraloop |
| 371 | cagcauagcaaguusaaauaaggcuaguccguua ucaacuugaaaaaguggcaccgagucggugcuu uuuu | Exemplary spCas9 2 crRNA tracrRNA |
| N/A | nnnnnnnnnnnnnnnnnnnn | Exemplary saCas9 complementary region (spacer) |
| 373 | guuuuaguacucug | Exemplary saCas9 crRNA repeat region |
| N/A | gaaa | Exemplary saCas9 tetraloop |
| 375 | cagaaucuacuaaaacaaggcaaaaugccguguu uaucucgucaacuuguuggcgagauuuuuu | Exemplary saCas9 tracrRNA |

s = c or g; n = any base

**[0590]** In some embodiments, the gRNA comprises a complementary region specific to a target gene locus of interest, for example, a TRAC locus, a TRBC1 locus, a TRBC2 locus, a TRAJ locus, a CD3zeta locus, a B2M locus, a CIITA locus, a CARD11 locus, a PIK3R3 locus, or a safe harbor locus selected from the group consisting of an AAVS1 locus, an ABO locus, a CCR5 locus, a CLYBL locus, a CXCR4 locus, a F3 locus, a FUT1 locus, a HMGB1 locus, a KDM5D locus, a LRP1 locus, a MICA locus, a MICB locus, a RHD locus, a ROSA26, locus and a SHS231 locus. The complementary region may bind a sequence in any region of the target gene locus, including for example, a CDS, an exon, an intron, a sequence spanning a portion of an exon and a portion of an adjacent intron, or a regulatory region (e.g., promoter, enhancer). Where the target sequence is a CDS, exon, intron, or sequence spanning portions of an exon and intron, the CDS, exon, intron, or exon/intron boundary may be defined according to any splice variant of the target gene. In some embodiments, the genomic locus targeted by the gRNA is located within 4000 bp, within 3500 bp, within 3000 bp, within 2500 bp, within 2000 bp, within 1500 bp, within 1000 bp, or within 500 bp of any of the loci or regions thereof as described. Further provided herein are compositions comprising one or more gRNAs provided herein and a Cas protein or a nucleotide sequence encoding a Cas protein. In certain of these embodiments, the one or more gRNAs and a nucleotide sequence encoding a Cas protein are comprised within a vector, for example, a viral vector.

**[0591]** In some embodiments, provided are methods of identifying new loci and/or gRNA sequences for use in the site-directed genomic insertion approaches as described. For example, for CRISPR/Cas systems, when an existing gRNA for a particular locus (e.g., within an endogenous TCR gene locus) is known, an "inch worming" approach can be used to identify additional loci for targeted insertion of transgenes by scanning the flanking regions on either side of the locus for PAM sequences, which usually occurs about every 100 base pairs (bp) across the genome. The PAM sequence will depend on the particular Cas nuclease used because different nucleases usually have different corresponding PAM sequences. The flanking regions on either side of the locus can be between about 500 to 4000 bp long, for example, about 500 bp, about 1000 bp, about 1500 bp, about 2000 bp, about 2500 bp, about 3000 bp, about 3500 bp, or about 4000 bp long. When a PAM sequence is identified within the search range, a new guide can be designed according to the sequence of that locus for use in site-directed insertion of transgenes. Although the CRISPR/Cas system is described as illustrative, any gene editing approaches as described can be used in this method of identifying new loci, including those using ZFNs, TALENs, meganucleases, and transposases.

**[0592]** In some embodiments, the activity, stability, and/or other characteristics of gRNAs can be altered through the incorporation of chemical and/or sequential modifications. As one example, transiently expressed or delivered nucleic acids can be prone to degradation by, e.g., cellular nucleases. Accordingly, the gRNAs described herein can contain one or more modified nucleosides or nucleotides which introduce stability toward nucleases. While not being bound by a particular theory, it is believed that certain modified gRNAs described herein can exhibit a reduced innate immune response when introduced into a population of cells, particularly the cells of the present technology. As used herein, the term "innate immune response" includes a cellular response to exogenous nucleic acids, including single stranded nucleic acids, generally of viral or bacterial origin, which involves the induction of cytokine expression and release, e.g., the interferons, and cell death. Other common chemical modifications of gRNAs to improve stabilities, increase nuclease resistance, and/or reduce immune response include 2'-O-methyl modification, 2'-fluoro modification, 2'-O-methyl phosphorothioate linkage modification, and 2'- O-methyl 3' thioPACE modification.

**[0593]** One common 3' end modification is the addition of a poly(A) tract comprising one or more (and typically 5-200) adenine (A) residues. The poly(A) tract can be contained in the nucleic acid sequence encoding the gRNA or can be added to the gRNA during chemical synthesis or following in vitro transcription using a polyadenosine polymerase (e.g., E. coll

poly(A) polymerase). In vivo, poly(A) tracts can be added to sequences transcribed from DNA vectors through the use of polyadenylation signals. Examples of such signals are provided in Maeder. Other suitable gRNA modifications include, without limitations, those described in U.S. Patent Application No. US 2017/0073674 Al and International Publication No. WO 2017/165862 Al, the entire contents of each of which are incorporated by reference herein.

**[0594]** In some embodiments, a tool for designing a gRNA as disclosed herein comprises: Benchling, Broad Institute GPP, CasOFFinder, CHOPCHOP, CRISPick, CRISPOR, Deskgen, E- CRISP, Geneious, Guides, Horizon Discovery, IDT, Off-Spotter, Synthego, or TrueDesign (ThermoFisher). One of ordinary skill in the art would understand that a tool that predicts both activity and specificity (e.g., to limit off-target modification) would be useful for designing a gRNA in certain instances as disclosed herein.

## IX. OTHER FEATURES

### *Expression of Polynucleotides*

**[0595]** In some embodiments, the polynucleotides of the present disclosure are expressed under the control of an endogenous promoter. In some embodiments, the endogenous promoter, is a TRAC promoter, TRBC1 promoter, TRBC2 promoter, CD3alpha promoter, CD3beta promoter, CD3epsilon promoter, CD3delta promoter, CD3zeta promoter, CARD11 promoter, PIK3R3 promoter, B2M promoter, CIITA promoter, PD-1 promoter, CXCR4 promoter, an albumin promoter, an F3 promoter (also known as CD142), a MICA promoter, a MICB promoter, a LRP1 promoter (also known as CD91), a HMGB1 promoter, an ABO promoter, a RHD promoter, a FUT1 promoter, a KDM5D promoter (also known as HY), a CCR5 promoter, a PDGFRa promoter, a OLIG2 promoter, and/or a GFAP promoter.

**[0596]** In some embodiments, the polynucleotides of the present disclosure are expressed under the control of a contitutive promoter. Examples of suitable promoters include, for example, MND promoter, EF1a promoter, sEF1 promoter, gamma retroviral LTR promoter, CD4 promoter, CD8a promoter, CD8b promoter, TCRa promoter, TCRb promoter, CD3d promoter, CD3g promoter, CD3e promoter, or a CD3z promoter. In some embodiments, the promoter is a minimal TATA promoter, a pGK, actin promoter, CD25 promoter, IL2 promoter, IL7 promoter, IL15 promoter, KLRG-1 promoter, HLA-DR promoter, CD38 promoter, CD69 promoter, Ki-67 promoter, CD11a promoter, CD58 promoter, CD99 promoter, CD62L promoter, CD103 promoter, CCR4 promoter, CCR5 promoter, CCR6 promoter, CCR9 promoter, CCR10 promoter, CXCR3 promoter, CXCR4 promoter, CLA

promoter, Granzyme A promoter, Granzyme B promoter, Perforin promoter, CD57 promoter, CD161 promoter, IL-18Ra promoter,CD69 promoter, GzmB promoter, T-bet promoter, IFNgamma promoter, TIM3 promoter, IL4 promoter, GATA3 promoter, IL1 promoter, IL5 promoter, IL6 promoter, IL13 promoter, IL10 promoter, IL17A promoter, IL6 promoter, IL21 promoter, IL23R promoter, FoxP3 promoter, CTLA4 promoter, CD25 promoter, PD1 promoter, CD45RO promoter, CCR7 promoter, CD28 promoter, CD95 promoter, CD28 promoter, CD27 promoter, CD127 promoter, PD-1 promoter, CD122 promoter, CD132 promoter, c-Kit promoter, nuclear factor of activated T cells (NFAT) promoter, programmed death 1 (PD-1) promoter, T cell immunoglobulin mucin-3 (TIM-3) promoter, cytotoxic T lymphocyte antigen-4 (CTLA4) promoter, lymphocyte-activation protein 3 (LAG-3) promoter, tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) promoter, B- and T-lymphocyte attenuator (BTLA) promoter, CD25 promoter, CD69 promoter, Fas ligand (FasL) promoter, TIGIT promoter, TGF-beta promoter, T-bet promoter, Eomes promoter, GATA3 promoter, CD45RA promoter, 2B4 promoter, Type I interferon (IFN) alpha, Type I IFN beta promoter, IFN gamma promoter, IRF3 promoter, IRF7 promoter, NF-κB promoter, AP-1 promoter, TNF-alpha promoter, and CD130 promoter, NR4A1 promoter, NR4A2, or NR4A3 promoter. Exemplary constitutive promoters include, e.g., simian virus 40 early promoter (SV40), cytomegalovirus immediate-early promoter (CMV), human Ubiquitin C promoter (UBC), human elongation factor la promoter (EFla), mouse phosphoglycerate kinase 1 promoter (PGK), and chicken P-Actin promoter coupled with CMV early enhancer (CAG). In some embodiments, the constitutive promoter is a synthetic or modified promoter. In some embodiments, the promoter is or comprises an MND promoter, a synthetic promoter that contains the U3 region of a modified MoMuLV LTR with myeloproliferative sarcoma virus enhancer (see Challita et al. (1995) J. Virol. 69(2):748-755). In some embodiments, the promoter is a tissue-specific promoter. In another embodiment, the promoter is a viral promoter. In another embodiment, the promoter is a non-viral promoter. In some embodiments, exemplary promoters can include, but are not limited to, human elongation factor 1 alpha (EFla) promoter or a modified form thereof or the MND promoter.

***Cell Therapy Potency Enhancements***

**[0597]** In some embodiments, the disclosure provides polynucleotides, polypeptides, and methods for improving adoptive immune cell (*e.g.,* T cell) therapies. In some embodiments, the polynucleotides and polypeptides of the disclosure improves the therapeutic efficacy of

the immune cells (*e.g.,* T cells) by altering T cell signaling, decreasing T cell exhaustion, and/or by enhancing *in vivo* persistence and fitness of engineered T cells.

**[0598]** Adoptive cell therapies comprising a chimeric antigen receptor (CAR) or recombinant T cell receptor (TCR)) have shown therapeutic success in hematological malignancies. However, effectively targeting solid tumors has been limited, in part due to challenges related to the immune suppressive tumor microenvironment, T cell exhaustion, poor T cell activity, and/or poor *in vivo* persistence and fitness of engineered T cells. T cell-based therapies, such as adoptive T cell therapies (including those involving the administration of engineered cells expressing recombinant, engineered or chimeric receptors specific for a disease or disorder of interest, such as a recombinant T cell receptor (TCR) or other recombinant, engineered or chimeric receptors, such as a chimeric antigen receptor (CAR)) can be effective in the treatment of cancer and other diseases and disorders. In certain contexts, other approaches for generating engineered cells for adoptive cell therapy may not always be entirely satisfactory. In some aspects, efficacy or potency of the engineered cells can depend on various factors, including T cell exhaustion, immunosuppressive tumor microenvironment (TME), poor cell infiltration into the target, e.g., tumor, and a lack of endogenous anti-tumor immune response.

**[0599]** In some contexts, optimal activity or outcome can depend on the ability of the administered cells to express the recombinant receptor on the surface, recognize and bind to a target, to traffic, localize to and successfully enter appropriate sites within the subject, tumors, and environments thereof. In some contexts, optimal activity or outcome can depend on the ability of the administered cells to uniformly and/or continuously express the recombinant receptor, become activated, expand, to exert various effector functions, including cytotoxic killing and secretion of various factors such as cytokines, to persist, including long-term, to differentiate, transition or engage in reprogramming into certain phenotypic states (such as long-lived memory, less-differentiated, and effector states), to avoid or reduce immunosuppressive conditions in the local microenvironment of a disease, to provide effective and robust recall responses following clearance and re-exposure to target ligand or antigen, and avoid or reduce exhaustion, anergy, peripheral tolerance, terminal differentiation, and/or differentiation into a suppressive state.

In some embodiments, the polypeptides of the disclosure, or the polynucleotides of the disclosure can alter the therapeutic efficacy of engineered T cells. Alteration of therapeutic efficacy can include, without limitation, a decrease in T cell exhaustion, increased proliferative capacity, enhanced anti-tumor effect, enhanced replicative lifespan, decreased

replicative senescence, enhanced ability to kill, enhanced fitness of engineered T cells and/or other functions or activities of T cells.

***Potency Enhancements***

**[0600]** In some embodiments, the polypeptides or recombinant nucleic acid constructs of the disclosure can alter the *in vivo* persistence of T cells in tumors or the other functions or activities of T cells described herein.

**[0601]** In some embodiments of the recombinant nucleic constructs and/or polypeptides of the disclosure, the polypeptides can include more than one mutation. For example, one or two or three or four or five or more mutations. In some embodiments, the mutations are a combination of different types of mutations. The different types of mutations can be point mutations, gene fusions, substitutions, gain-of-function mutations, stop-gain mutations, insertion mutations, deletion mutations, duplication mutations or translocations. The mutation can be a T cell lymphoma mutation or a mutation in a clonally expanded population of T cells. In some embodiments, the mutation is a point mutation or substitution.

**[0602]** In some embodiments, the mutation is a T cell lymphoma mutation. T cell lymphoma mutation can be a mutation occurring in, or identified in, a T cell lymphoma. T cell lymphomas are a heterogeneous group of lymphoid malignancies that occur in nodal and extranodal sites. There are two main types of T cell lymphomas namely T-lymphoblastic lymphoma and Peripheral T-cell lymphomas classified based on clinical manifestations and cytogenetic mutations. Peripheral T cell lymphoma can be further divided into cutaneous T cell lymphoma, adult T cell lymphoma, angioimmunoblastic T cell lymphoma, natural killer T cell lymphoma, enteropathy associated T cell lymphoma, and anaplastic large cell lymphoma. In some embodiments, the T cell lymphoma mutation occurs in a T lymphoblastic lymphoma. In some embodiments, the T cell lymphoma mutation occurs in a Peripheral T cell lymphoma.

**[0603]** In some embodiments, the mutation is a mutation in a clonally expanded population of T cells. As used herein, a clonally expanded population of T cells can be a population of T cells that are descended from a single progenitor cell. T cells within the clonally expanded population can express the same T cell receptor.

**[0604]** In some embodiments, the mutation is a gene fusion. In some embodiments, the gene fusion comprises a polypeptide comprising a caspase-associated recruitment domain (CARD). In some embodiments, the gene fusion comprises a polypeptide comprising a CARD containing protein or a functional fragment thereof. A functional fragment of a

CARD containing protein can be, for example, a fragment that provides NF-κB transcriptional activity, AP-1 transcriptional activity, and/or MALT1 proteolytic activity activity at a level at least 70%, 75%, 80%, 85%, 90%, or 95% of that of a full-length protein, as determined by the in vitro CAR Jurkat assay. In some embodiments, the gene fusion comprises a domain capable of binding to (i) a substrate localized to the intracellular side of the plasma membrane of a cell and/or (ii) a target polypeptide comprising a phosphorylated tyrosine (pTyr). In some embodiments, the gene fusion comprises a CARD containing protein and a domain capable of binding to (i) a substrate localized to the intracellular side of the plasma membrane of a cell and/or (ii) a target polypeptide comprising a pTyr.

**[0605]** In some embodiments, the domain is capable of binding to a substrate indirectly localized to the intracellular side of the plasma membrane. Indirect localization can refer to localization of the substrate to the intracellular side of the plasma membrane through, for example, binding of the substrate to another polypeptide or lipid that is directly localized to the intracellular side of the plasma membrane. Indirect localization can refer to localization of the substrate to the intracellular side of the plasma membrane through, for example, interaction of the substrate with another polypeptide or lipid that is directly localized to the intracellular side of the plasma membrane. In some embodiments, the domain is capable of binding to a substrate directly localized to the intracellular side of the plasma membrane. Direct localization can refer to localization of the substrate to the intracellular side of the plasma membrane through, for example, binding of the substrate to the intracellular side of the plasma membrane itself. Direct localization can refer to localization of the substrate to the intracellular side of the plasma membrane through, for example, interaction of the substrate with the intracellular side of the plasma membrane itself.

**[0606]** The polypeptides of the disclsoure, or the recombinant nucleic acid constructs of the disclosure, can alter T cell signaling in various ways. In some embodiments, the altering of the T cell signaling can be by enhancing, promoting, improving, reducing, regulating, or modulating the signaling pathway within a T cell. In some embodiments, the altering of the T cell signaling can be by activating, increasing, suppressing, inhibiting, or other means of changing the signaling.

**[0607]** The polypeptides of the disclsoure, or the recombinant nucleic acid constructs of the disclosure, can alter T cell signaling through one or more T cell signaling pathways. In some embodiments, the polypeptides or recombinant nucleic acid constructs of the disclosure can alter T cell signaling by increasing one or more pathways and/or reducing one or more pathways, or by a combination of enhancing and/or reducing various pathways, for example

as shown in FIG 2F. In some embodiments, the mutation can alter cytokine production. The cytokine can be, without limitation, IL-2, IL-4, IL-5, TNF alpha, IFN- gamma, IL-13 and/or other cytokines. In some embodiments, the cytokine is IL-2. In some embodiments, the cytokine is IL-2, and the production of IL-2 is increased.

**[0608]** In some embodiments, the polypeptides or recombinant nucleic acid constructs of the disclosure can alter T cell signaling through one or more T cell signaling pathways. Signaling pathways can be, without limitation, the NFAT pathway, NF-κB pathway, AP-1 pathway, JAK/STAT pathway, RAS/MEK/ERK, and/or phospholipase gamma signaling, or described elsewhere herein.

**[0609]** In some embodiments, the polypeptides or recombinant nucleic acid constructs of the disclosure can alter CARD11-BCL10-MALT1 complex signaling, co-stimulatory molecule signaling, cytokine production and/or transcription factor activity in T cells.

**[0610]** The disclosure also relates to polypeptides having one or more mutations that can reduce T cell exhaustion, increase proliferation, alter effector function, resist T cell dysfunction, increase T cell fitness, enhance *in vivo* persistence, and/or increase intratumoral presence of therapeutic T cells. The disclosure further relates to recombinant nucleic acids encoding polypeptides having one or more mutations, that can reduce T cell exhaustion, increase proliferation, alter effector function, resist T cell dysfunction, increase T cell fitness, enhance *in vivo* persistence, and/or increse intratumoral presence of therapeutic T cells.

**[0611]** T cell fitness can refer to the ability of a T cell to generate an immune response. The ability of a T cell to perform T cell functions such as signaling, cytokine production, survival, and persistence in a tumor, may contribute to the fitness of a T cell. T cell exhaustion may contribute to reducing its fitness.

**[0612]** In some embodiments, polypeptides of the disclosure, or the recombinant nucleic acid constructs of the disclosure can alter *in vivo* persistence in tumors of therapeutic T cells including the mutation. The *in vivo* persistence of therapeutic T cells can refer to the length of time that the therapeutic T cells exist within the tumor of the host after infusion. In some embodiments, *in vivo* persistence of therapeutic T cells is enhanced. Enhanced *in vivo* persistence of therapeutic T cells can include at least a positive log2 fold change in therapeutic T cells as compared to the input total.

**[0613]** In some embodiments, the polypeptides or recombinant nucleic acid constructs of the disclosure can alter the *in vivo* persistence of T cells in tumors or the other functions or activities of T cells described herein, through one or more of the following genes: BCL6,

BCOR, BRAF, CARD11, CARMIL2, CCND3, CD28, CD3E, CSNK1A1, CSNK2B, ECSIT, EIFS1, FYN, GATA, GNAQ, IRF4, ITGB2, JAK1, JAK3, JUNB, KCNQ1, LATS1, MSC, MYCN, NFKB1, NFKB2, NRAS, PDCD1, PLCG1, PRKCB1 , RARA, RASGRP1, RHOA, SMARCB1, STAT3, STAT5, TBL1XR1, TNFRSF1B, TP53 and VAV1.

**[0614]** In some embodiments, the genes include caspase recruitment domain family member 11 (CARD11). In some embodiments, the genes include capping protein regulator and myosin 1 linker 2 (CARMIL2), mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1), B-cell lymphoma 6 (BCL6), B-cell lymphoma 10 (BCL10), and MYCN. In some embodiments, the gene can be a STAT3, STAT5B, JAK1, JAK2, or JAK3 gene. In some embodiments, the gene can be BRAF, or RASGRP1. In some embodiments, the gene can be phospholipase C gamma 1 (PLCG1) gene. In some embodiments, the gene can be NFKB1, NFKB2, or JUNB gene. In some embodiments, the gene can be TNFRSF1B.

**[0615]** In some embodiments, the disclosure relates to polypeptides, or to recombinant nucleic acids encoding polypeptides, comprising a caspase-associated recruitment domain (CARD) containing protein, or a functional fragment thereof. CARD is a conserved homology domain comprising a 6-helix bundle or 5-helix bundle. CARDs can mediate protein-protein interactions between key apoptotic signaling molecules. Non-limiting examples of CARDs include CARD6, CARD8, CARD9, CARD10, CARD11, CARD14, CARD16, CARD18, and CARD19. Non-limiting examples of proteins comprising CARDs include those described in Boyle and Monie *bioRxiv* 087908; doi: https://doi.org/10.1101/087908; and/or Park. *In J Mol Med.* 2019 Mar; 43(3):1119-1127, such as human caspase-1, -2, -4, and -5, mouse caspase-1, -2, -11, and -12, ASC, NOD1, NOD2, Apaf-1, BCL-10, and RIG-I. In some embodiments, the CARD containing protein, or functional fragment thereof, is derived from a CARD protein selected from CARD9 (UniProt # Q9H257), CARD10 (UniProt # Q9BWT7) CARD11 (UniProt # Q9BXL7), or CARD14 (UniProt # Q9H257) (Wang et al. J Biol Chem. 2001 Jun 15;276(24):21405-9; Bertin et al. J Biol Chem. 2001 Apr 13;276(15):11877-82.). In some embodiments, the CARD containing protein, or functional fragment thereof, is derived from a CARD11 protein (UniProt #Q9BXL7), or a functional fragment thereof.

**[0616]** In some embodiments, the disclosure relates to polypeptides, or to recombinant nucleic acids encoding polypeptides, comprising a domain capable of binding to (i) a substrate localized to the intracellular side of the plasma membrane of a cell and/or (ii) a target polypeptide comprising a phosphorylated tyrosine. Non-limiting examples of domains capable of binding to (i) a substrate localized to the intracellular side of the plasma

membrane of a cell and/or (ii) a target polypeptide comprising a phosphorylated tyrosine include Src Homology region 2 (SH2) domains, Src Homology region 3 (SH3) domains, pleckstrin homology (PH) domains, and phosphytyrosine-binding (PTB) domains. SH2 and PTB domains mediate protein-protein interactions involved in many signal transduction pathways. SH2, SH3, and PTB domains that can be used in the disclosure include, for example, those disclosed in Schlessinger et al. *Sci STKE.* 2003 Jul 15;2003(191):RE12. SH2 containing proteins can bind to plasma membrane lipids through a different binding pocket than the pTyr binding pocket; most SH2 domains bind plasma membrane lipids and many have high phosphoinositide specificity (Park et al., Cell. 2016 Apr 7;62(1):7-20). In some embodiments, the domain is, or comprises, an SH3 domain. In some embodiments, the domain is, or comprises, a PTB domain. In some embodiments, the domain is, or comprises, a PH domain. In some embodiments, the domain is, or comprises, an SH2 domain. In some embodiments, the SH2 domain is from a PIK3R3 protein (UniProt # Q92569). In some embodiments, the SH2 domain comprises the motif of a conserved arginine residue in the FLVR motif (Arg βB5 or Arg175 in the v-Src SH2 domain). Most conserved resides are clustered on the βB strand, and the conserved arginine residue in the FLVR motif plays the central role in forming a double hydrogen bond with the phosphate group of pTyr. Additional residues that are key for phosphopeptide binding are His βD4, Lys βD6, and Arg αA2, which coordinate and anchor the aromatic ring of the phospho-tyrosine (Diop et al. Int J Mol Sci. 2022 Dec 15;23(24):15944). In some embodiments, the SH2 domain is an engineered SH2 domain with an enhanced affinity for phosphotyorsine (e.g., Veggiani et al. Protein Sci. 2019 Feb;28(2):403-413).

**[0617]** Furthermore, the disclosure relates to polypeptides comprising a CARD containing protein or a functional fragment thereof, and a domain capable of binding to (i) a substrate localized to the intracellular side of the plasma membrane of a cell and/or (ii) a target polypeptide comprising a phosphorylated tyrosine (also called a CARD fusion polypeptide), as disclosed herein. In some embodiments, the polypeptide comprises a CARD containing protein, or a functional fragment thereof, and an SH2 domain (also called a CARD-SH2 fusion polypeptide). In some embodiments, the disclosure relates to a polypeptide comprising a functional fragment of a CARD containing protein derived from a CARD11 protein and an SH2 domain from a PIK3R3 protein (also called a CARD11-PIK3R3 fusion polypeptide).

**[0618]** Furthermore, the disclosure relates to recombinant nucleic acids encoding polypeptides comprising a CARD containing protein or a functional fragment thereof and a

domain capable of binding to (i) a substrate localized to the intracellular side of the plasma membrane of a cell and/or (ii) a target polypeptide comprising a phosphorylated tyrosine, as disclosed herein. In some embodiments, the recombinant nucleic acid encodes a polypeptide comprising a CARD containing protein or a functional fragment thereof and an SH2 domain. In some embodiments, the recombinant nucleic acid encodes a functional fragment of a CARD containing protein derived from a CARD11 protein and an SH2 domain from a PIK3R3 protein. In some embodiments, the recombinant nucleic acid encodes a functional fragment of a CARD containing protein derived from a CARD9 protein and an SH2 domain from a PIK3R3 protein.

**[0619]** In some embodiments, the polypeptide is a CARD11-PIK3R3 fusion polypeptide. In some embodiments, the recombinant nucleic acid constructs encode a CARD11-PIK3R3 fusion polypeptide or any truncations thereof.

**[0620]** The disclosure further relates to a second polypeptide portion derived from PIK3R3. Non-limiting examples of polypeptides derived from PIK3R3 include either of two SH2 domains, a catalytic subunit, and a regulatory subunit. In some embodiments, the polypeptide does not comprise a Coiled-coil domain or a portion thereof. In some embodiments, the polypeptide comprises a Coiled-coil domain or a portion thereof.

**[0621]** The present disclosure further relates to polypeptides wherein the domain capable of binding to the target polypeptide comprising the phosphorylated tyrosine, or the SH2 domain, or the second polypeptide portion, is located at the N-terminus of the CARD containing protein or functional fragment thereof, between the CARD containing protein and the Coiled-coil domain, or at the C-terminus of the CARD containing protein and/or the Coiled-coil domain. In some embodiments, the polypeptide comprises the CARD domain derived from a CARD11 protein followed by the Coiled-coil domain derived from the CARD11 protein. The present disclosure further relates to polypeptides wherein the domain capable of binding to the target polypeptide comprising the phosphorylated tyrosine, or the SH2 domain, or the second polypeptide portion, is located close to the C-terminus of the polypeptide, wherein the polypeptide has no more than 50, 40, 30, 20, 15, 10, or 5 amino acids at the C-terminus of the domain in (b), or the SH2 domain, or the second polypeptide portion.

**[0622]** In some embodiments, the polypeptide does not comprise an inhibitory domain (ID) or a portion thereof. In other embodiments, the polypeptide comprises an ID or a portion thereof.

**[0623]** In some embodiments, the CARD11-PIK3R3 fusion polypeptide includes a CARD domain (i.e., containing protein or functional fragment thereof), a CARD inhibitory domain (ID), a coiled-coil domain, and an SH2 domain from PIK3R3. In some embodiments, the CARD11-PIK3R3 fusion polypeptide includes a functional fragment of a CARD containing protein derived from CARD11domain, a coiled-coil domain, and an SH2 domain from PIK3R3.

**[0624]** In some embodiments, the polypeptide of the disclosure has a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to SEQ ID NO: 3-150 or a functional variant thereof.

**[0625]** In some embodiments, the polypeptide of the disclosure has, or the recombinant nucleic acid constructs of the disclosure encodes, a polypeptide comprising a CARD11 polypeptide having a substitution at amino acid 357, 361, 615, 634, and/or 655. In some embodiments, the substitution at amino acid 357 is D357N. In some embodiments, the substitution at amino acid 361 is Y361C. In some embodiments, the substitution at amino acid 615 is S615F. In some embodiments, the substitution at amino acid 634 is E634K. In some embodiments, the substitution at amino acid 655 is S655C.

**[0626]** In some embodiments, the polypeptide of the disclosure has, or the recombinant nucleic acid constructs of the disclosure encodes, a polypeptide comprising a BCL3 polypeptide having a substitution at amino acid 647. In some embodiments, the substitution at amino acid 647 is S647R.

**[0627]** In some embodiments, the polypeptide of the disclosure has, or the recombinant nucleic acid constructs of the disclosure encodes, a polypeptide comprising a CARMIL2 polypeptide having a substitution at amino acid 575. In some embodiments, the substitution at amino acid 575 is Q575E.

**[0628]** In some embodiments, the polypeptide of the disclosure has, or the recombinant nucleic acid constructs of the disclosure encodes a polypeptide comprising a MYCN polypeptide having a substitution at amino acid 44. In some embodiments, the substitution at amino acid 44 is P44L.

**[0629]** In some embodiments, the polypeptide of the disclosure has, or the recombinant nucleic acid constructs of the disclosure encodes a polypeptide comprising a JAK1 polypeptide having a substitution at amino acid 1097. In some embodiments, the substitution at amino acid 1097 is an G1097A substitution.

**[0630]** In some embodiments, the polypeptide of the disclosure has, or the recombinant nucleic acid constructs of the disclosure encodes a polypeptide comprising a JAK3

polypeptide having a substitution at amino acid 573. In some embodiments, the substitution at amino acid 573 is an A573V substitution.

**[0631]** In some embodiments, the polypeptide of the disclosure has, or the recombinant nucleic acid constructs of the disclosure encodes a polypeptide comprising a STAT3 polypeptide having a substitution at amino acid 618. In some embodiments, the substitution at amino acid 618 is an G618R substitution. In some embodiments, the polypeptide of the disclosure has, or the recombinant nucleic acid constructs of the disclosure encodes a polypeptide comprising a STAT3 polypeptide having a substitution at amino acid 647. In some embodiments, the substitution at amino acid 647 is an N647I substitution. In some embodiments, the polypeptide of the disclosure has, or the recombinant nucleic acid constructs of the disclosure encodes a polypeptide comprising a STAT3 polypeptide having a substitution at amino acid 661. In some embodiments, the substitution at amino acid 661 is an D661I substitution.

**[0632]** In some embodiments, the polypeptide of the disclosure has, or the recombinant nucleic acid constructs of the disclosure encodes a polypeptide comprising a STAT5 polypeptide having a substitution at amino acid 628. In some embodiments, the substitution at amino acid 628 is an T628S substitution. In some embodiments, the polypeptide of the disclosure has, or the recombinant nucleic acid constructs of the disclosure encodes a polypeptide comprising a STAT5 polypeptide having a substitution at amino acid 665. In some embodiments, the substitution at amino acid 665 is an Y665F substitution.

**[0633]** In some embodiments of the recombinant nucleic acid constructs of the disclosure, the nucleic acid encodes a polypeptide comprising a TNFRSF1B polypeptide having a substitution at amino acid 256 and/or 377. In some embodiments of the recombinant nucleic acid constructs of the disclosure, the nucleic acid encodes a polypeptide comprising a TNFRSF1B polypeptide having a substitution at amino acid 256. In some embodiments, the substitution at amino acid 256 is an G256C substitution. In some embodiments of the recombinant nucleic acid constructs of the disclosure, the nucleic acid encodes a polypeptide comprising a TNFRSF1B polypeptide having a substitution at amino acid 337. In some embodiments, the substitution at amino acid 337 is an T337I substitution.

**[0634]** In some embodiments of the recombinant nucleic acid constructs of the disclosure, the nucleic acid encodes a polypeptide comprising a CD28 polypeptide having a substitution at amino acid 51, 77, and/or 195. In some embodiments of the recombinant nucleic acid constructs of the disclosure, the nucleic acid encodes a polypeptide comprising a CD28 polypeptide having a substitution at amino acid 51. In some embodiments, the substitution at

amino acid 51 is an F51V substitution. In some embodiments, the substitution at amino acid 51 is an F51I substitution. In some embodiments of the recombinant nucleic acid constructs of the disclosure, the nucleic acid encodes a polypeptide comprising a CD28 polypeptide having a substitution at amino acid 77. In some embodiments, the substitution at amino acid 77 is an Q77P substitution. In some embodiments of the recombinant nucleic acid constructs of the disclosure, the nucleic acid encodes a polypeptide comprising a CD28 polypeptide having a substitution at amino acid 195. In some embodiments, the substitution at amino acid 195 is an T195P substitution.

**[0635]** In some embodiments of the recombinant nucleic acid constructs of the disclosure, the nucleic acid encodes a polypeptide comprising an ICOS-CD28 polypeptide.

**[0636]** In some embodiments of the recombinant nucleic acid constructs of the disclosure, the nucleic acid encodes a polypeptide comprising a CD28-CTLA4 polypeptide.

**[0637]** In some embodiments of the recombinant nucleic acid constructs of the disclosure, the nucleic acid encodes a polypeptide comprising a BRAF polypeptide having a substitution at amino acid 469 and/or 594. In some embodiments of the recombinant nucleic acid constructs of the disclosure, the nucleic acid encodes a polypeptide comprising a BRAF polypeptide having a substitution at amino acid 469. In some embodiments, the substitution at amino acid 469 is an G469R substitution. In some embodiments, the substitution at amino acid 469 is an G469A substitution. In some embodiments, the substitution at amino acid 594 is an D594N substitution.

**[0638]** In some embodiments of the recombinant nucleic acid constructs of the disclosure, the nucleic acid encodes a polypeptide comprising a RASGRP1 polypeptide having a substitution at amino acid 261. In some embodiments, the substitution at amino acid 261 is an M261I substitution.

**[0639]** In some embodiments of the recombinant nucleic acid constructs of the disclosure, the nucleic acid encodes a polypeptide comprising a PLCG1 polypeptide having a substitution at amino acid 47, 48, 520, 1163, 1165 or any combination thereof. In some embodiments, the substitution at amino acid 47 is an E47K substitution. In some embodiments, the substitution at amino acid 48 is an R48W substitution. In some embodiments, the substitution at amino acid 520 is an S520F substitution. In some embodiments, the substitution at amino acid 1163 is an E1163K substitution. In some embodiments, the substitution at amino acid 1165 is an D1165H substitution.

**[0640]** In some embodiments of the recombinant nucleic acid constructs of the disclosure, the nucleic acid encodes a polypeptide comprising a NFKB1 polypeptide having a

substitution at amino acid 67. In some embodiments, the substitution at amino acid 67 is an H67Y substitution.

**[0641]** In some embodiments of the recombinant nucleic acid constructs of the disclosure, the nucleic acid encodes a polypeptide comprising a NFKB2 polypeptide having a substitution at amino acid 656. In some embodiments, the substitution at amino acid 656 is an K656X substitution.

**[0642]** In some embodiments of the recombinant nucleic acid constructs of the disclosure, the nucleic acid encodes a polypeptide comprising a JUNB polypeptide having a substitution at amino acid 282. In some embodiments, the substitution at amino acid 282 is an A282V substitution.

***Additional Potency Enhancements and/or Cell Armours***

**[0643]** In some embodiments, the cells disclosed herein further comprise an additional potency enhancement or cell armours. In some embodiments, the cells disclosed herein comprise polypeptides comprising additional potency enhancement or cell armour. In some embodiments, the cells disclosed herein comprise polynucleotides encoding the polypeptides comprising additional potency enhancement or cell armours. In some embodiments, the additional potency enhancement or cell armours that can reduce T cell exhaustion, increase proliferation, alter effector function, resist T cell dysfunction, increase T cell fitness, enhance *in vivo* persistence, and/or increase intratumoral presence of therapeutic T cells. The disclosure further relates to recombinant nucleic acids encoding polypeptides having one or more mutations, that can reduce T cell exhaustion, increase proliferation, alter effector function, resist T cell dysfunction, increase T cell fitness, enhance *in vivo* persistence, and/or increase intratumoral presence of therapeutic T cells. Such disclosure is applicable to the methods, uses and cells described herein. In some embodiments, the cells are T cells (e.g., CAR-T cells) or NK cells.

**[0644]** Described here are engineered cells that comprise one or more modifications. In some embodiments, the engineered cells comprise a modification to increase expression of one or more additional potency enhancement or cell armours. In some embodiments, the additional potency enhancement or cell armours is one or more of a dominant-negative transforming growth factor-β receptor type II (dnTGFβRII), c-Jun, CCL19, CCL21, IL2R, IL7, IL7Ralpha,IL15, IL15RA, IL18, decoy-resistant IL18 (DR-18), MyD88/CD40, PD1-CD28 switch receptor, PD1-41BB switch receptor, CD40L-CD28 switch receptor, CTBR12 switch receptor, or CD8alpha/beta.

**[0645]** Described here are engineered cells that comprise one or more modifications. In some embodiments, the engineered cells comprise a modification to inactivate or disrupt one or more alleles of TGFbeta receptor II (TGFBRII), Regnase-1, FAS, PTPN2, NR4A3, CD52, PD-1, B2M, CIITA, SOCS1, CBLB, DGKalpha, and DGKζ.

***Chimeric Antigen Receptors, T Cell Receptors, and Synthetic Receptors***

**[0646]** In some embodiments, a provided engineered cell is further modified to express a chimeric antigen receptor (CAR), a T cell receptor (TCR) such as a recombinant TCR, a chimeric cytokine receptor (CCR), a chimeric chemokine receptor, a synthetic RIP family receptor, a synthetic notch receptor (synNotch), a synthetic intramembrane proteolysis receptor (SNIPR), a synthetic Robo receptor (SynRobo receptor), a synthetic RoboNotch receptor (SynRobo receptor), a Modular Extracellular Sensor Architecture (MESA) receptor, a Tango receptor, a ChaCha receptor, a generalized extracellular molecule sensor (GEMS) receptor, a growth factor receptor, a cytokine receptor, a chemokine receptor, a switch receptor, an adhesion molecule, an integrin, an inhibitory receptor, a stimulatory receptor, an immunoreceptor tyrosine-based activation motif (ITAM)-containing receptor, an immunoreceptor tyrosine-based inhibition motif (ITIM)-containing receptor, a hormone receptor, a receptor tyrosine kinase.

**[0647]** It will be understood that embodiments concerning CAR modified cells may be readily applied to any suitable cell type as described herein, as well as safety switches and other modified/ gene edited cells as described herein.

**[0648]** In some embodiments, a polynucleotide encoding a CAR is introduced into the cell. In some embodiments, the cell is a T cell, such as a primary T cell or a T cell differentiated from a pluripotent cell (e.g., iPSC). In some embodiments, the cell is a Natural Killer (NK) cell, such as a primary NK cell or an NK cell differentiated from a pluripotent cell (e.g., iPSC).

**[0649]** In some embodiments, the CAR is selected from the group consisting of a first generation CAR, a second generation CAR, a third generation CAR, and a fourth generation CAR. In some embodiments, the CAR is or comprises a first generation CAR comprising an antigen binding domain, a transmembrane domain, and at least one signaling domain (e.g., one, two or three signaling domains). In some embodiments, the CAR comprises a second generation CAR comprising an antigen binding domain, a transmembrane domain, and at least two signaling domains. In some embodiments, the CAR comprises a third generation CAR comprising an antigen binding domain, a transmembrane domain, and at least three

signaling domains. In some embodiments, the CAR is or comprises a fourth generation CAR comprising an antigen binding domain, a transmembrane domain, three or four signaling domains, and a domain which upon successful signaling of the CAR induces expression of a cytokine gene.

**[0650]** In some embodiments, the CAR is or comprises a first generation CAR. In some embodiments, a first generation CAR comprises an antigen binding domain, a transmembrane domain, and signaling domain. In some embodiments, a signaling domain mediates downstream signaling during T cell activation.

**[0651]** In some embodiments, the CAR is or comprises a second generation CAR. In some embodiments, a second generation CAR comprises an antigen binding domain, a transmembrane domain, and two signaling domains. In some embodiments, a signaling domain mediates downstream signaling during T cell activation. In some embodiments, a signaling domain is a costimulatory domain. In some embodiments, a costimulatory domain enhances cytokine production, CAR T cell proliferation, and/or CAR T cell persistence during T cell activation.

**[0652]** In some embodiments, the CAR is or comprises a third generation CAR. In some embodiments, a third generation CAR comprises an antigen binding domain, a transmembrane domain, and at least three signaling domains. In some embodiments, a signaling domain mediates downstream signaling during T cell activation. In some embodiments, a signaling domain is a costimulatory domain. In some embodiments, a costimulatory domain enhances cytokine production, CAR T cell proliferation, and or CAR T cell persistence during T cell activation. In some embodiments, a third generation CAR comprises at least two costimulatory domains. In some embodiments, the at least two costimulatory domains are not the same.

**[0653]** In some embodiments, the CAR is or comprises a fourth generation CAR. In some embodiments, a fourth generation CAR comprises an antigen binding domain, a transmembrane domain, and at least two, three, or four signaling domains. In some embodiments, a signaling domain mediates downstream signaling during T cell activation. In some embodiments, a signaling domain is a costimulatory domain. In some embodiments, a costimulatory domain enhances cytokine production, CAR T cell proliferation, and or CAR T cell persistence during T cell activation.

**[0654]** In some embodiments of the provided methods and uses, the engineered cells, such as T cells, express a chimeric receptors, such as a chimeric antigen receptors (CAR), that contains one or more domains that combine a ligand-binding domain (e.g., derived from an

antibody or antibody fragment) that provides specificity for a desired antigen (e.g., tumor antigen) with intracellular signaling domains. In some embodiments, the intracellular signaling domain is an activating intracellular domain portion, such as a T cell activating domain, providing a primary activation signal. In some embodiments, the intracellular signaling domain contains or additionally contains a costimulatory signaling domain to facilitate effector functions. Upon specific binding to the molecule, e.g., antigen, the receptor generally delivers an immunostimulatory signal, such as an ITAM-transduced signal, into the cell, thereby promoting an immune response targeted to the disease or condition. In some embodiments, chimeric receptors when genetically engineered into immune cells can modulate T cell activity, and, in some cases, can modulate T cell differentiation or homeostasis, thereby resulting in genetically engineered cells with improved longevity, survival and/or persistence in vivo, such as for use in adoptive cell therapy methods.

**[0655]** In some embodiments, the CAR is constructed with a specificity for a particular antigen (or marker or ligand), such as an antigen expressed in a particular cell type to be targeted by adoptive therapy, e.g., a cancer marker, and/or an antigen intended to induce a dampening response, such as an antigen expressed on a normal or non-diseased cell type. Thus, the CAR typically includes in its extracellular portion one or more antigen binding molecules, such as one or more antigen-binding fragment, domain, or portion, or one or more antibody-derived variable domains, and/or antibody-derived molecules.

**[0656]** The term "antibody" herein is used in the broadest sense and includes polyclonal and monoclonal antibodies, including intact antibodies and functional (antigen-binding) antibody fragments, including fragment antigen binding (Fab) fragments, F(ab')2 fragments, Fab' fragments, Fv fragments, recombinant IgG (rlgG) fragments, heavy chain variable (VH) regions capable of specifically binding the antigen, single chain antibody fragments, including single chain variable fragments (scFv), and single domain antibodies ( e.g ., sdAb, sdFv, nanobody) fragments. The term encompasses genetically engineered and/or otherwise modified forms of immunoglobulins, such as intrabodies, peptibodies, chimeric antibodies, fully human antibodies, humanized antibodies, and heteroconjugate antibodies, multispecific, e.g., bispecific or trispecific, antibodies, diabodies, triabodies, and tetrabodies, tandem di-scFv, tandem tri-scFv. Unless otherwise stated, the term "antibody" should be understood to encompass functional antibody fragments thereof also referred to herein as "antigen-binding fragments." The term also encompasses intact or full-length antibodies,

including antibodies of any class or sub-class, including IgG and sub-classes thereof, IgM, IgE, IgA, and IgD.

**[0657]** The terms "complementarity determining region," and "CDR," synonymous with "hypervariable region" or "HVR," are known in the art to refer to non-contiguous sequences of amino acids within antibody variable regions, which confer antigen specificity and/or binding affinity. In general, there are three CDRs in each heavy chain variable region (CDR-H1, CDR- H2, CDR-H3) and three CDRs in each light chain variable region (CDR-L1, CDR-L2, CDR- L3). "Framework regions" and "FR" are known in the art to refer to the non-CDR portions of the variable regions of the heavy and light chains. In general, there are four FRs in each full- length heavy chain variable region (FR-H1, FR-H2, FR-H3, and FR-H4), and four FRs in each full-length light chain variable region (FR-L1, FR-L2, FR-L3, and FR-L4).

**[0658]** The precise amino acid sequence boundaries of a given CDR or FR can be readily determined using any of a number of well-known schemes, including those described by Kabat et al. (1991), "Sequences of Proteins of Immunological Interest," 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD ("Kabat" numbering scheme); Al-Lazikani et al, (1997) JMB 273,927-948 ("Chothia" numbering scheme); MacCallum et al, J. Mol. Biol. 262:732-745 (1996), "Antibody-antigen interactions: Contact analysis and binding site topography," J. Mol. Biol. 262, 732-745." ("Contact" numbering scheme); Lefranc MP el ah, "IMGT unique numbering for immunoglobulin and T cell receptor variable domains and Ig superfamily V-like domains," Dev Comp Immunol, 2003 Jan;27(l):55-77 ("IMGT" numbering scheme); Honegger A and Pliickthun A, "Yet another numbering scheme for immunoglobulin variable domains: an automatic modeling and analysis tool," J Mol Biol, 2001 Jun 8;309(3):657- 70, ("Aho" numbering scheme); Martin et al, "Modeling antibody hypervariable loops: a combined algorithm," PNAS, 1989, 86(23):9268-9272, ("AbM" numbering scheme); Abhinandan and Martin, "Analysis and improvements to Kabat and structurally correct numbering of antibody variable domains," Mol Immunol, 2008 Aug 45(14): 3832-9 ("enhanced Chothia" numbering scheme); and identification of the minimal set of amino acids comprising of the putative antigen binding interface using structural modeling applications, for example AlphaFold ("structural modeling" approach).

**[0659]** The boundaries of a given CDR or FR may vary depending on the scheme used for identification. For example, the Kabat scheme is based on structural alignments, while the Chothia scheme is based on structural information. Numbering for both the Kabat and

Chothia schemes is based upon the most common antibody region sequence lengths, with insertions accommodated by insertion letters, for example, "30a," and deletions appearing in some antibodies. The two schemes place certain insertions and deletions ("indels") at different positions, resulting in differential numbering. The Contact scheme is based on analysis of complex crystal structures and is similar in many respects to the Chothia numbering scheme.

**[0660]** The AbM scheme is a compromise between Kabat and Chothia definitions based on that used by Oxford Molecular's AbM antibody modeling software.

**[0661]** **Table 10**, below, lists exemplary position boundaries of CDR-L1, CDR-L2, CDR-L3 and CDR-H1, CDR-H2, CDR-H3 as identified by Kabat, Chothia, AbM, and Contact schemes, respectively. For CDR-H1, residue numbering is listed using both the Kabat and Chothia numbering schemes. FRs are located between CDRs, for example, with FR-L1 located before CDR-L1, FR-L2 located between CDR-L1 and CDR-L2, FR-L3 located between CDR-L2 and CDR-L3 and so forth. It is noted that because the shown Kabat numbering scheme places insertions at H35A and H35B, the end of the Chothia CDR-H1 loop when numbered using the shown Kabat numbering convention varies between H32 and H34, depending on the length of the loop.

**Table 10. Boundaries of CDRs according to various numbering schemes**

| **Table 1.** Boundaries of CDRs according to various numbering schemes. | | | | |
|---|---|---|---|---|
| **CDR** | **Kabat** | **Chothia** | **AbM** | **Contact** |
| CDR-L1 | L24--L34 | L24--L34 | L24--L34 | L30--L36 |
| CDR-L2 | L50--L56 | L50--L56 | L50--L56 | L46--L55 |
| CDR-L3 | L89--L97 | L89--L97 | L89--L97 | L89--L96 |
| CDR-H1 (Kabat Numbering[1]) | H31--H35B | H26--H32.34 | H26--H35B | H30--H35B |
| CDR-H1 | H31--H35 | H26--H32 | H26--H35 | H30--H35 |
| (Chothia Numbering[2]) | | | | |
| CDR-H2 | H50--H65 | H52--H56 | H50--H58 | H47--H58 |
| CDR-H3 | H95--H102 | H95--H102 | H95--H102 | H93--H101 |

1 - Kabat et al. (1991), "Sequences of Proteins of Immunological Interest," 5th Ed. Public Health Service, National Institutes of Health, Bethesda, MD 2 - Al-Lazikani et al, (1997) JMB 273,927-948

**[0662]** Thus, unless otherwise specified, a "CDR" or "complementary determining region," or individual specified CDRs (e.g., CDR-H1, CDR-H2, CDR-H3), of a given antibody or region thereof, such as a variable region thereof, should be understood to encompass a (or the specific) complementary determining region as defined by any of the aforementioned schemes, or other known schemes. For example, where it is stated that a particular CDR

(e.g., a CDR-H3) contains the amino acid sequence of a corresponding CDR in a given VH or VL region amino acid sequence, it is understood that such a CDR has a sequence of the corresponding CDR (e.g., CDR-H3) within the variable region, as defined by any of the aforementioned schemes, or other known schemes. In some embodiments, specific CDR sequences are specified. Exemplary CDR sequences of provided antibodies are described using various numbering schemes, although it is understood that a provided antibody can include CDRs as described according to any of the other aforementioned numbering schemes or other numbering schemes known to a skilled artisan.

**[0663]** Likewise, unless otherwise specified, a FR or individual specified FR(s) (e.g., FR-Hl, FR-H2, FR-H3, FR-H4), of a given antibody or region thereof, such as a variable region thereof, should be understood to encompass a (or the specific) framework region as defined by any of the known schemes. In some instances, the scheme for identification of a particular CDR, FR, or FRs or CDRs is specified, such as the CDR as defined by the Kabat, Chothia, AbM, IMGT, Contact method, enhanced Chothia, or structural modeling approach, or other known schemes. In other cases, the particular amino acid sequence of a CDR or FR is given.

**[0664]** The term "variable region" or "variable domain" refers to the domain of an antibody heavy or light chain that is involved in binding the antibody to antigen. The variable regions of the heavy chain and light chain (VH and VL, respectively) of a native antibody generally have similar structures, with each domain comprising four conserved framework regions (FRs) and three CDRs. (See, e.g., Kindt et al. Kuby Immunology, 6th ed., W.H. Freeman and Co., page 91 (2007). A single VH or VL domain may be sufficient to confer antigen-binding specificity. Furthermore, antibodies that bind a particular antigen may be isolated using a VH or VL domain from an antibody that binds the antigen to screen a library of complementary VL or VH domains, respectively. See, e.g., Portolano et ah, J. Immunol. 150:880-887 (1993); Clarkson et al, Nature 352:624-628 (1991).

**[0665]** Among the antigen binding domains included in the CARs are those derived from antibody fragments. An "antibody fragment" or "antigen-binding fragment" refers to a molecule other than an intact antibody that comprises a portion of an intact antibody that binds the antigen to which the intact antibody binds. Examples of antibody fragments include but are not limited to Fv, Fab, Fab', Fab'-SH, F(ab')2; diabodies; linear antibodies; heavy chain variable (VH) regions, single-chain antibody molecules such as scFvs and single-domain antibodies comprising only the VH region; and multispecific antibodies formed from antibody fragments. In particular embodiments, the antibodies from which the

CARs are derived are single-chain antibody fragments comprising a heavy chain variable (VH) region and/or a light chain variable (VL) region, such as scFvs.

**[0666]** Single-domain antibodies (sdAbs) are antibody fragments comprising all or a portion of the heavy chain variable region or all or a portion of the light chain variable region of an antibody. In certain embodiments, a single-domain antibody is a human single-domain antibody.

**[0667]** Antibody fragments can be made by various techniques, including but not limited to proteolytic digestion of an intact antibody as well as production by recombinant host cells. In some embodiments, the antibodies are recombinantly-produced fragments, such as fragments comprising arrangements that do not occur naturally, such as those with two or more antibody regions or chains joined by synthetic linkers, e.g., peptide linkers, and/or that are may not be produced by enzyme digestion of a naturally-occurring intact antibody. In some aspects, the antibody fragments are scFvs.

**[0668]** In some embodiments, the CAR includes an antigen-binding portion or portions of an antibody molecule, such as a single-chain antibody fragment (scFv) derived from the variable heavy (VH) and variable light (VL) chains of a monoclonal antibody (mAh), or a single domain antibody (sdAb), such as sdFv, nanobody, VHH and VNAR. In some embodiments, an antigen binding fragment comprises antibody-derived variable regions joined by a flexible linker.

**[0669]** In some embodiments, the CAR comprises an antigen binding region and a transmembrane domain, but does not comprise any signaling domain. In some embodiments, the transmembrane domain of such CARs binds to another signaling protein. In some embodiments, such CARs comprise a domain that interacts with another signaling protein. In some embodiments, such CARs are capable of activating the signaling protein it interacts with in the presence of the antigen.

**[0670]** In some embodiments, the antigen binding region of the CAR is or comprises an antigen-binding fragment of an antibody. In some embodiments, the antibody is a human antibody or humanized antibody. In some embodiment, the antibody is a monoclonal antibody.

**[0671]** In some embodiments, the antibody or antigen-binding fragment thereof is a single chain antibody fragment, such as a single chain variable fragment (scFv) or a diabody or a single domain antibody (sdAb). In some embodiments, the antibody or antigen-binding fragment is a single domain antibody comprising only the VH region. In some embodiments,

the antibody or antigen binding fragment is an scFv comprising a heavy chain variable (VH) region and a light chain variable (VL) region.

**[0672]** A "humanized" antibody isan antibody with an amino acid sequence corresponding to that of an antibody produced by a human or a human cell, or non-human source that utilizes human antibody repertoires or other human antibody-encoding sequences, including human antibody libraries. The term excludes humanized forms of non-human antibodies comprising non-human antigen-binding regions, such as those in which all or substantially all CDRs are non-human. The term includes antigen-binding fragments of human antibodies. Human antibodies may be prepared by administering an immunogen to a transgenic animal that has been modified to produce intact human antibodies or intact antibodies with human variable regions in response to antigenic challenge. Such animals typically contain all or a portion of the human immunoglobulin loci, which replace the endogenous immunoglobulin loci, or which are present extrachromosomally or integrated randomly into the animal's chromosomes. In such transgenic animals, the endogenous immunoglobulin loci have generally been inactivated. Human antibodies also may be derived from human antibody libraries, including phage display and cell-free libraries, containing antibody-encoding sequences derived from a human repertoire.

**[0673]** Among the antibodies included from which the provided CARs described herein are derive, are human antibodies. A "human antibody" is an antibody with an amino acid sequence corresponding to that of an antibody produced by a human or a human cell, or non-human source that utilizes human antibody repertoires or other human antibody-encoding sequences, including human antibody libraries. The term excludes humanized forms of non-human antibodies comprising non-human antigen-binding regions, such as those in which all or substantially all CDRs are non-human. The term includes antigen-binding fragments of human antibodies.

**[0674]** Human antibodies may be prepared by administering an immunogen to a transgenic animal that has been modified to produce intact human antibodies or intact antibodies with human variable regions in response to antigenic challenge. Such animals typically contain all or a portion of the human immunoglobulin loci, which replace the endogenous immunoglobulin loci, or which are present extrachromosomally or integrated randomly into the animal's chromosomes. In such transgenic animals, the endogenous immunoglobulin loci have generally been inactivated. Human antibodies also may be derived from human antibody libraries, including phage display and cell-free libraries, containing antibody-encoding sequences derived from a human repertoire.

**[0675]** Among the antibodies from which the provided CARs derive are those that are monoclonal antibodies, including monoclonal antibody fragments. The term "monoclonal antibody" as used herein refers to an antibody obtained from or within a population of substantially homogeneous antibodies, i.e., the individual antibodies comprising the population are identical, except for possible variants containing naturally occurring mutations or arising during production of a monoclonal antibody preparation, such variants generally being present in minor amounts. In contrast to polyclonal antibody preparations, which typically include different antibodies directed against different epitopes, each monoclonal antibody of a monoclonal antibody preparation is directed against a single epitope on an antigen. The term is not to be construed as requiring production of the antibody by any particular method. A monoclonal antibody may be made by a variety of techniques, including but not limited to generation from a hybridoma, recombinant DNA methods, phage-display and other antibody display methods.

**[0676]** In some embodiments, the CAR includes a binding portion or portions of an antibody molecule, such as a heavy chain variable (VH) region and/or light chain variable (VL) region of the antibody, e.g., an scFv antibody fragment. The chimeric receptors, such as CARs, generally include an extracellular antigen binding domain, such as a portion of an antibody molecule, generally a variable heavy (VH) chain region and/or variable light (VL) chain region of the antibody, e.g., an scFv antibody fragment.

**[0677]** In some embodiments, the antibody or antigen binding fragment, contains a heavy and/or light chain variable (VH or VL) region sequence as described, or a sufficient antigen -binding portion thereof. In some embodiments, the antigen-binding fragment, contains a VH region sequence or sufficient antigen -binding portion thereof that contains a CDR-H1, CDR-H2 and/or CDR-H3 as described. In some embodiments, the antigen-binding fragment, contains a VL region sequence or sufficient antigen-binding portion that contains a CDR-L1, CDR-L2 and/or CDR-L3 as described. In some embodiments, the antigen-binding fragment, contains a VH region sequence that contains a CDR-H1, CDR-H2 and/or CDR-H3 as described and contains a VL region sequence that contains a CDR-L1, CDR-L2 and/or CDR-L3 as described. Also, among the antibodies are those having sequences at least at or about 90%, about 91%, about 92%, about 93%, about 94%, about 95%, about 96%, about 97%, about 98%, or about 99% identical to such a sequence.

**[0678]** In some embodiments, the antibody is a single domain antibody (sdAb) comprising only a VH region sequence or a sufficient antigen-binding portion thereof, such as any of the above described VH sequences (e.g., a CDR-H1, a CDR-H2, a CDR-H3 and/or a CDR-H4).

**[0679]** In some embodiments, an antibody provided herein or antigen -binding fragment thereof comprising a VH region further comprises a light chain or a sufficient antigen binding portion thereof. For example, in some embodiments, the antibody or antigen -binding fragment thereof contains a VH region and a VL region, or a sufficient antigen binding portion of a VH and VL region. In such embodiments, a VH region sequence can be any of the above described VH sequence. In some such embodiments, the antibody is an antigen binding fragment, such as a Fab or an scFv. In some such embodiments, the antibody is a full- length antibody that also contains a constant region.

**[0680]** In some embodiments, the nucleotide sequence encoding a CAR may be derived from a mammalian sequence, for example, a mouse sequence, a primate sequence, a human sequence, or combinations thereof. In the cases where the nucleotide sequence encoding a CAR is non-human, the sequence of the CAR may be humanized. The nucleotide sequence encoding a CAR may also be codon-optimized for expression in a mammalian cell, for example, a human cell. In any of these embodiments, the nucleotide sequence encoding a CAR may be at least 80% identical (e.g., at least 80%, at least 85%, at least 90%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99%, or 100% identical) to any of the nucleotide sequences disclosed herein. The sequence variations may be due to codon-optimalization, humanization, restriction enzymebased cloning scars, and/or additional amino acid residues linking the functional domains, etc.

**[0681]** In certain embodiments, the CAR can be re-engineered as a chimeric autoantibody receptor (CAAR) to selectively deplete autoreactive immune cells. In certain embodiments, CAARs are engineered to target autoantibodies present on immune cells. Exemplary target antigens for CAARs include, but are not limited to, DSG3 (associated with pemphigus volgaris); factor VIII (FVIII)(associated with haemophilia). In any of these embodiments, the extracellular binding domain of the CAR can be codon-optimized for expression in a host cell or have variant sequences to increase functions of the extracellular binding domain.

**[0682]** In some embodiments, an engineered cell provided herein (e.g., primary or iPSC-derived T cell or primary or iPSC-derived NK cell) includes a polynucleotide encoding a CAR, wherein the polynucleotide is inserted in a genomic locus. In some embodiments, the polynucleotide is inserted into a safe harbor locus, such as but not limited to, an AAVS1 locus, CCR5 locus, CLYBL locus, ROSA26 locus, SHS231 locus, F3 (also known as CD142) locus, MICA locus, MICB locus, LRP1 (also known as CD91) locus, HMGB1 locus, ABO locus, RHD locus, FUT1 locus, or KDM5D locus. In some embodiments, the polynucleotide is inserted in a B2M locus, CIITA locus, TRAC locus, TRBC1 locus,

TRBC2 locus, TRAJ locus, CARD11 locus, PIK3R3 locus, PD-1 locus, CTLA-4 locus. Any suitable method can be used to insert the CAR into the genomic locus of the cell including the gene editing methods described herein (e.g., a CRISPR/Cas system).

**[0683]** In accordance with the present disclosure, on-off or other types of control switch techniques may be incorporated herein. These techniques may comprise use of dimerization domains and optional activators of such domain dimerization, e.g., as disclosed by Wu et al., Science 2015 Oct; 350(6258):aab4077 utilizing FKBP/Rapalog dimerization systems in certain cells, the contents of which are incorporated by reference herein in their entirety. Additional dimerization technology is described in, e.g., Fegan et al. Chem. Rev. 2010, 110, 3315-3336 as well as U.S. Pat. Nos. 5,830,462; 5,834,266; 5,869,337; and 6,165,787, the contents of each of which is also incorporated by reference herein with respect to dimerization technology. Additional dimerization pairs may comprise cyclosporine-A/cyclophilin, receptor, estrogen/estrogen receptor (optionally using tamoxifen, 4-hydroxytamoxifen, or endoxifen), glucocorticoids/glucocorticoid receptor, tetracycline/tetracycline receptor, and/or vitamin D/vitamin D receptor. Further examples of dimerization technology may be found in e.g., WO 2014/127261, WO 2015/090229, US 2014/0286987, US 2015/0266973, US 2016/0046700, U.S. Pat. No. 8,486,693, US 2014/0171649, and US 2012/0130076, the contents of which are further incorporated by reference herein in their entirety.

**[0684]** In some embodiments, the CAR is a bicistronic CAR. In some embodiments, the immune cell of the disclosure comprises a bicistronic CAR. Bicistronic CARs can comprise two CARs that bind different targets and are encoded by a single vector. A bicistronic CAR may comprise a first CAR sequence and a second CAR sequence expressed as a single polypeptide comprising a cleavable linker between the first and second CARs. Non-limiting examples of first and/or second CAR sequences include CD19,CD20, BCMA, CD22, CD70, DLL3, LY6G6D, Claudin 6, GCC, p53R175H, and PRAME. An exemplary cleavable linker is Furin-GSG-T2A (see, e.g., Chng et al. MAbs. 2015 Mar-Apr; 7(2): 403-412, which is herein incorporated by reference with respect to cleavable linkers; see also Guedan et al. Mol Ther Methods Clin Dev. 2019 Mar 15; 12: 145-156, which is incorporated herein by reference with respsect to bicistronic CAR design).

**[0685]** In some embodiments, the CAR is a bispecific CAR. In some embodiments, a first binding motif and a second binding motif (e.g., distinct anti-CD20 and anti-CD19 binding motifs) are both comprised in single bispecific CAR. In some embodiments, the immune cell of the disclosure comprises a bispecific CAR. In such bispecific CARs, a CAR molecule

itself may be engineered to recognize more than one antigen. In tandem bispecific CARs, the first and second binding motifs are extracellular and may be characterized as a membrane-proximal binding motif and a membrane-distal binding motif.

**[0686]** In some embodiments, the CAR further comprises a domain which upon successful signaling of the CAR induces expression of a cytokine gene. In some embodiments, a cytokine gene is endogenous or exogenous to a target cell comprising a CAR which comprises a domain which upon successful signaling of the CAR induces expression of a cytokine gene. In some embodiments, a cytokine gene encodes a pro-inflammatory cytokine. In some embodiments, a cytokine gene encodes IL-1, IL-2, IL-9, IL-12, IL-18, TNF, or IFN-gamma, or functional fragment thereof. In some embodiments, a domain which upon successful signaling of the CAR induces expression of a cytokine gene is or comprises a transcription factor or functional domain or fragment thereof. In some embodiments, a domain which upon successful signaling of the CAR induces expression of a cytokine gene is or comprises a transcription factor or functional domain or fragment thereof. In some embodiments, a transcription factor or functional domain or fragment thereof is or comprises a nuclear factor of activated T cells (NFAT), an NF-kB, or functional domain or fragment thereof. See, e.g., Zhang. C. et al., Engineering CAR-T cells. Biomarker Research. 5:22 (2017); WO 2016126608; Sha, H. et al. Chimaeric antigen receptor T-cell therapy for tumour immunotherapy. Bioscience Reports Jan 27, 2017, 37 (1).

**[0687]** A skilled artisan is familiar with CARs and different components and configurations of CARs. Any known CAR can be employed in connection with the provided embodiments. In addition to the CARs described herein, various CARs and nucleotide sequences encoding the same are known in the art and would be suitable for engineering cells as described herein. See, e.g., W02013040557; W02012079000; W02016030414; Smith T, et al., Nature Nanotechnology. 2017. DOI: 10.1038/NNAN0.2017.57, the disclosures of which are herein incorporated by reference. Exemplary features and components of a CAR are described in the following subsections.

**[0688]** In some embodiments, the cell expresses an engineered immune receptor that binds to a target on a tumor cell. In some embodiments, the cell can express an exogenous T cell receptor (TCR). A TCR as known by the skilled in the art can be composed of two different, an alpha chain and a beta chain, each consisting of a constant region that anchors the chain inside the T cell surface membrane, and a variable region which can recognize and bind an antigen presented by MHCs. The TCR complex can be associated with six polypeptides forming two heterodimers, CD3γε and CD3δε, and one homodimer, CD3ζ, which together

forms the CD3 complex. TCRs can be engineered to utilize the modification of T cells that retain these complexes to specifically target the antigens expressed by particular tumor cells. As used herein, a TCR can be a naturally-occurring or an engineered TCR.

**[0689]** TCRs are disulfide-linked membrane anchored heterodimeric proteins, typically comprising highly variable alpha (a) and beta (β) chains expressed as a complex with invariant CD3 chain molecules. T cells expressing these types of TCRs are referred to as α:β (or αβ) T cells. A minority of T cells express an alternative TCR comprising variable gamma (γ) and delta (δ) chains and are referred to as γδ T cells. TCR is not able to mediate signal transduction itself due to its short cytoplasmic tail, so TCR still requires CD3 and zeta to carry out the signal transduction in its place. A TCR receptor complex is an octomeric complex of variable TCR receptor α and β chains with three dimeric signaling modules CD3δ/ε, CD3γ/ε and CD247 ζ/ζ or ζ/η.

**[0690]** According to embodiments of the application, suitable TCRs bind specifically to a major histocompatibility complex (MHC) on the surface of cancer cells that displays a peptide fragment of a tumor antigen. An MHC is a set of cell-surface proteins which allow the acquired immune system to recognize 'foreign' molecules. Proteins are intracellularly degraded and presented on the surface of cells by the MHC. MHCs displaying "foreign" peptides, such a viral or cancer associated peptides, are recognized by T cells with the appropriate TCRs, prompting cell destruction pathways. MHCs on the surface of cancer cells can display peptide fragments of tumor antigen i.e. an antigen which is present on a cancer cell but not the corresponding non-cancerous cell. T cells which recognize these peptide fragments can exert a cytotoxic effect on the cancer cell.

**[0691]** According to embodiments of the application, the T cells can be modified to express a heterologous TCR that binds specifically to MHCs displaying peptide fragments of a tumor antigen expressed by the cancer cells in a specific cancer patient. Tumor antigens expressed by cancer cells in the cancer patient may be identified using standard techniques. For example, a polypeptide, such as an FSH fragment, which binds specifically to an FSHR, is fused to CD3 epsilon, CD3 gamma or CD3 delta chain. The fusion protein forms a TCR complex on T cells through the interaction of CD3 with TCR α/β chains. The TCR complex binds specifically to FSHR on tumor cells via the FSH fragment, and the binding initiates TCR signaling against the tumor cells. According to yet another embodiment of the invention, the heterologous TCR is expressed together with a CAR that binds specifically to mesothelin. Heterologous TCRs can include αβTCR heterodimers.

**[0692]** The TCR can be engineered to increase its affinity or avidity for a tumor antigen (i.e. an affinity enhanced TCR). The affinity enhanced TCR can comprise one or more mutations relative to a naturally occurring TCR, for example, one or more mutations in the hypervariable complementarity determining regions (CDRs) of the variable regions of the TCR α and β chains. These mutations increase the affinity of the TCR for MHCs that display a peptide fragment of a tumor antigen expressed by cancer cells. Suitable methods of generated affinity enhanced TCRs include screening libraries of TCR mutants using phage or yeast display and are well known in the art (see for example Robbins et al J Immunol (2008) 180 (9) : 6116; San Miguel et al (2015) Cancer Cell 28 (3) 281-283; Schmitt et al (2013) Blood 122 348-256; Jiang et al (2015) Cancer Discovery 5 901).

**[0693]** In some of embodiments, the cell comprises a polynucleotide encoding a TCRa and/or TCRb chain or a portion thereof that has been codon-optimized. In some embodiments, the transgene encodes a portion of a TCRa and/or TCRb chain with less than 100% amino acid sequence identity to a corresponding portion of a native or endogenous TCRa and/or TCRb chain. In some embodiments, the encoded TCRa and/or TCRb chain contains an amino acid sequence with, with about, or with at least 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99%, or greater than 99% identity but less than 100% identity to a corresponding native or endogenous TCRa and/or TCRb chain. In particular embodiments, the transgene encodes a TCRa and/or TCRb constant domain or portion thereof with less than 100% amino acid sequence identity to a corresponding native or endogenous TCRa and/or TCRb constant domain. In some embodiments, the TCRa and/or TCRb constant domain contains an amino acid sequence with, with about, or with at least 70%, 75%, 80%, 85%, 90%, 95%, 98%, 99%, or greater than 99% identity but less than 100% identity to a corresponding native or endogenous TCRa and/or TCRb chain.

**[0694]** In some embodiments, the polynucleotide contains one or more modifications(s) to introduce one or more cysteine residues that are capable of forming one or more non-native disulfide bridges between the TCRa chain and TCRB chain. In some embodiments, the one or more non-native cysteine residues are capable of forming non-native disulfide bonds, e.g., with a TCRa chain encoded by the transgene.

**[0695]** In some embodiments, the transgene encodes all or a portion of a TCRa constant domain (Ca) and/or a TCRb constant domain (Cb) with one or more modifications to remove or prevent a native disulfide bond, e.g., between the TCRb chain encoded by the transgene and the endogenous TCRa chain. In some embodiments, one or more native cysteines that form and/or are capable of forming a native interchain disulfide bond are

substituted to another residue, e.g., serine or alanine. In some embodiments, the portion of a TCRa constant domain (Ca) and/or a TCRb constant domain (Cb) is modified to replace one or more non-cysteine residues to a cysteine. Exemplary of modified encoded TCRa constant domain (Ca) and/or TCRb constant domain (Cb) include any of those described herein, for example, in Section III.A, and those described in W02006/000830, WO 2006/037960 and Kuball et al. (2007) Blood, 109:2331- 2338.

**[0696]** Among such recombinant TCRs or antigen-binding fragments thereof are TCRs or antigen-binding fragments thereof that contain any of the variable alpha (Va) regions and/or a variable beta (Vb) regions as described, individually, or a sufficient antigen-binding portion of such chain(s). In some embodiment, the provided recombinant TCRs comprise a TCR alpha (TCRa) chain comprising a variable alpha (Va) region, and a TCR beta (TCRb) chain comprising a variable beta (Vb) region.

**[0697]** In some embodiments, the TCR or antigen-binding fragment thereof is isolated or purified or is recombinant. In particular embodiments, any of the provided TCR or antigenbinding fragment thereof is recombinant. In some aspects, the TCR or antigen-binding fragment thereof is human. In some embodiments, the recombinant TCR is monoclonal. In some aspects, the recombinant TCR is a single chain. In other embodiments, the recombinant TCR contains two chains. In some embodiments, the TCR or antigen-binding fragment thereof is expressed on the surface of a cell.

**[0698]** Unless otherwise stated, the term “TCR” should be understood to encompass full TCRs as well as antigen-binding portions or antigen-binding fragments thereof. In some embodiments, the TCR is an intact or full-length TCR, such as a TCR containing the a chain and b chain. In some embodiments, the TCR is an antigen-binding portion that is less than a full- length TCR but that binds to a specific peptide bound in an MHC molecule, such as binds to an MHC-peptide complex. In some cases, an antigen-binding portion or fragment of a TCR can contain only a portion of the structural domains of a full-length or intact TCR, but yet is able to bind the peptide epitope, such as MHC-peptide complex, to which the full TCR binds. In some cases, an antigen-binding portion contains the variable domains of a TCR, such as variable a (Va) chain and variable b (Vb) chain of a TCR, or antigen-binding fragments thereof sufficient to form a binding site for binding to a specific MHC-peptide complex.

**[0699]** In some embodiments, the variable domains of the TCR contain complementarity determining regions (CDRs), which generally are the primary contributors to antigen recognition and binding capabilities and specificity of the peptide, MHC and/or MHC-

peptide complex. In some embodiments, a CDR of a TCR or combination thereof forms all or substantially all of the antigen-binding site of a given TCR molecule. The various CDRs within a variable region of a TCR chain generally are separated by framework regions (FRs), which generally display less variability among TCR molecules as compared to the CDRs (see, e.g., lores et al., Proc. Nat'l Acad. Sci. U.S.A. 57:9138, 1990; Chothia et al., EMBO J. 737^5, 1988; see also Lefranc et al., Dev. Comp. Immunol. 27:55, 2003). In some embodiments, CDR3 is the main CDR responsible for antigen binding or specificity, or is the most important among the three CDRs on a given TCR variable region for antigen recognition, and/or for interaction with the processed peptide portion of the peptide-MHC complex. In some contexts, the CDR1 of the alpha chain can interact with the N-terminal part of certain antigenic peptides. In some contexts, CDR1 of the beta chain can interact with the C-terminal part of the peptide. In some contexts, CDR2 contributes most strongly to or is the primary CDR responsible for the interaction with or recognition of the MHC portion of the MHC-peptide complex. In some embodiments, the variable region of the b-chain can contain a further hypervariable region (CDR4 or HVR4), which generally is involved in superantigen binding and not antigen recognition (Kotb (1995) Clinical Microbiology Reviews, 8:411-426).

**[0700]** In some embodiments, the TCRa chain and/or TCRb chain also can contain a constant domain, a transmembrane domain and/or a short cytoplasmic tail (see, e.g., Janeway et al., Immunobiology: The Immune System in Health and Disease, 3rd Ed., Current Biology Publications, p. 4:33, 1997). In some aspects, each chain (e.g. alpha or beta) of the TCR can possess one N-terminal immunoglobulin variable domain, one immunoglobulin constant domain, a transmembrane region, and a short cytoplasmic tail at the C-terminal end. In some embodiments, a TCR, for example via the cytoplasmic tail, is associated with invariant proteins of the CD3 complex involved in mediating signal transduction. In some cases, the structure allows the TCR to associate with other molecules like CD3 and subunits thereof. For example, a TCR containing constant domains with a transmembrane region may anchor the protein in the cell membrane and associate with invariant subunits of the CD3 signaling apparatus or complex. The intracellular tails of CD3 signaling subunits (e.g. CD3y, CD36, CD3s and CD3(^ chains) contain one or more immunoreceptor tyrosine-based activation motif or IT AM and generally are involved in the signaling capacity of the TCR complex.

**[0701]** The various domains or regions of a TCR can be identified. In some cases, the exact locus of a domain or region can vary depending on the particular structural or homology

modeling or other features used to describe a particular domain. It is understood that reference to amino acids, including to a specific sequence set forth as a SEQ ID NO: used to describe domain organization of a TCR are for illustrative purposes and are not meant to limit the scope of the embodiments provided. In some cases, the specific domain (e.g. variable or constant) can be several amino acids (such as one, two, three or four) longer or shorter. In some aspects, residues of a TCR are known or can be identified according to the International Immunogenetics Information System (IMGT) numbering system (see e.g. www.imgt.org; see also, Lefranc et al. (2003) Developmental and Comparative Immunology, 27(l);55-77; and The T Cell Factsbook 2nd Edition, Lefranc and LeFranc Academic Press 2001).

**[0702]** In some embodiments, the TCRa chain and/or TCRb chain each further contain a constant domain. In some embodiments, the a chain constant domain (Ca) and b chain constant domain (Cb) individually are mammalian, such as is a human or murine constant domain. In some embodiments, the constant domain is adjacent to the cell membrane. For example, in some cases, the extracellular portion of the TCR formed by the two chains contains two membrane-proximal constant domains, and two membrane-distal variable domains, which variable domains each contain CDRs.

**[0703]** In some aspects, provided herein are TCRs that contains a human constant region, such as an alpha chain containing a human Ca region and a beta chain containing a human Cb. In some embodiments, the provided TCRs are fully human. Among the provided TCRs are TCRs containing a human constant region, such as fully human TCRs, whose expression and/or activity, such as when expressed in human cells, e.g. human T cells, such as primary human T cells, are not impacted by or are not substantially impacted by the presence of an endogenous human TCR.

**[0704]** In some embodiments, the TCR may be a heterodimer of two chains TCRa and TCRb that are linked, such as by a disulfide bond or disulfide bonds. In some embodiments, the constant domain of the TCR may contain short connecting sequences in which a cysteine residue forms a disulfide bond, thereby linking the two chains of the TCR. In some embodiments, a TCR may have an additional cysteine residue in each of the TCRa and TCRb chains, such that the TCR contains two disulfide bonds in the constant domains. In some embodiments, each of the constant and variable domains contains disulfide bonds formed by cysteine residues.

**[0705]** In some embodiments, the TCR can contain an introduced disulfide bond or bonds. In some embodiments, the native disulfide bonds are not present. In some embodiments, the

one or more of the native cysteines (e.g. in the constant domain of the a chain and b chain) that form a native interchain disulfide bond are substituted to another residue, such as to a serine or alanine. In some embodiments, an introduced disulfide bond can be formed by mutating noncysteine residues on the alpha and beta chains, such as in the constant domain of the a chain and b chain, to cysteine. Opposing cysteines in the TCRa and TCRb chains provide a disulfide bond that links the constant regions of TCRa and TCRb chains of the substituted TCR to one another and which is not present in a TCR comprising the unsubstituted constant region in which the native disulfide bonds are present, such as unsubstituted native human constant region or the unsubstituted native mouse constant region. In some embodiments, the presence of non-native cysteine residues (e.g. resulting in one or more non-native disulfide bonds) in a recombinant TCR can favor production of the desired recombinant TCR in a cell in which it is introduced over expression of a mismatched TCR pair containing a native TCR chain. In some embodiments, the TCRa and/or TCRb chain and/or a TCRa and/or TCRb chain constant domains are modified to replace one or more non-cysteine residues to a cysteine.Non-limiting examples of TCRs, TCRa, and/or TCRb chains can be found, for example, in WO2019067243A1, WO2019067242A1, WO2022236050A1, WO2021163434A1, WO2020264269A1, WO2022177961A1, WO2022072760A1, WO2022236050A1, WO2020264269A1, and WO2024206274A2, which are incorporated herein by reference in their entirety.

**[0706]** In some embodiments, the invention provides engineered cells comprising a synthetic receptor comprising an extracellular antigen recognition domain that recognises an antigen on a target cell, a transmembrane domain, and an intracellular signalling domain that activates expression of a response element in the cell when the extracellular antigen recognition domain binds the target cell. The synthetic receptors allow the cells to recognise and bind specific target cells and to activate immune responses upon binding.

**[0707]** In some embodiments, the synthetic receptor is a synthetic RIP family receptor, a synthetic notch receptor (synNotch), a synthetic intramembrane proteolysis receptor (SNIPR), a synthetic Robo receptor (SynRobo receptor), a synthetic RoboNotch receptor (SynRobo receptor).

**[0708]** SNIPRs comprise an extracellular antigen binding domain, which may be an scFv with specificity for a particular cell surface antigen, such as a tumour antigen, a transmembrane domain, which controls proteolysis and cleavage, and an intracellular signalling domain, which may be a transcription factor. SNIPRs comprise a transmembrane domain from the regulated intracellular proteolysis (RIP) family of proteins. For example, a

SNIPR may comprise a transmembrane domain selected from the group consisting of Notchl, Notch2 and Robol. In some embodiments, the synthetic receptor is a SynNotch receptor. Binding of the antigen recognition domain releases a transcription factor. In some embodiments, the SNIPR may further comprise a juxtamembrane domain, such as a human NOTCH2 juxtamembrane domain.

**[0709]** In some embodiments, the T cells of the disclosure can be engineered to express a transgene, such as a CAR or a transcriptional regulator. Transcriptional regulators include synthetic receptors such as the synthetic notch receptors described in US 11,202,801 or other synthetic receptors, such as, for example, those described in provisional application nos: 62/905,251, 63/15,428, 62/905,268, 62/905,263, 62/935,024 and 62/905,248, which are herein incorporated by reference in their entirety.

**[0710]** In some embodiments, the synthetic receptor comprises a Notch domain encoded by a Notch gene. A preferred Notch gene is NOTCH1. Alternative Notch genes include NOTCH2, NOTCH3 and NOTCH4. In other embodiments, the synthetic receptor comprises a Robol domain.

**[0711]** In certain embodiments, the synthetic receptor system is multiplexed with multiple transcription factor domains and/or response elements. In certain embodiments, the engineered cell expresses on its surface a synthetic receptor, optionally a SynNotch receptor, comprising an extracellular antigen binding domain that recognises an antigen on a target cell, a transmembrane domain, and two or more intracellular signalling domains that activate expression of a two or more response elements in the cell when the extracellular antigen binding domain binds the target cell, wherein at least one response element encodes a gene that activates an immune response. In certain embodiments, the engineered cell expresses on its surface a synthetic receptor, optionally a SynNotch receptor, comprising an extracellular antigen binding domain that recognises an antigen on a target cell, a transmembrane domain, and two or more intracellular signalling domains that activate expression of a two or more response elements in the cell when the extracellular antigen binding domain binds the target cell, wherein at least one response element encodes a transcription factor.

Antigen Binding Domain

**[0712]** In some embodiments, a CAR antigen binding domain (ABD) is or comprises an antibody or antigen-binding portion thereof. In some embodiments, a CAR antigen binding domain is or comprises an scFv or Fab.

**[0713]** In some embodiments, an antigen binding domain binds to a cell surface antigen of a cell. In some embodiments, a cell surface antigen is characteristic of (e.g., expressed by) a particular or specific cell type. In some embodiments, a cell surface antigen is characteristic of more than one type of cell.

**[0714]** In some embodiments, the antigen may be an antigen that is expressed on tumor cells, or an antigen that is characteristic of an autoimmune or inflammatory disease. In some embodiments, the antigen may be an antigen that is exclusively or preferentially expressed on tumor cells, or an antigen that is characteristic of an autoimmune or inflammatory disease. In some embodiments, the antigen binding domain (ABD) targets an antigen characteristic of a neoplastic cell. For instance, the antigen binding domain targets an antigen expressed by a neoplastic or cancer cell. In some embodiments, the ABD binds a tumor associated antigen. In some embodiments, the antigen characteristic of a neoplastic cell (e.g., antigen associated with a neoplastic or cancer cell) or a tumor associated antigen is selected from a cell surface receptor, an ion channel-linked receptor, an enzyme-linked receptor, a G protein-coupled receptor, receptor tyrosine kinase, tyrosine kinase associated receptor, receptor-like tyrosine phosphatase, receptor serine/ threonine kinase, receptor guanylyl cyclase, histidine kinase associated receptor.

**[0715]** In some embodiments, the target antigen is an antigen that includes, but is not limited to, ALPPL2, Epidermal Growth Factor Receptors (EGFR), EGFRvIII, ErbBl/EGFR, ErbB2/HER2, ErbB3/HER3, ErbB4/HER4, Fibroblast Growth Factor Receptors (FGFR), FGFR1, FGFR2, FGFR3, FGFR4, FGFR5, FGFR6, FGFR7, FGFR18, FGFR21, Vascular Endothelial Growth Factor Receptors (VEGFR), VEGFR1, VEGFR2, VEGR3, VEGF-A, VEGF-B, VEGF-C, VEGF-D, PIGF, RET Receptor, Eph Receptor Family, EphAl, EphA2, Eph A3, EphA4, EphA5, EphA6, EphA7, EphA8, EphA9, EphAlO, EphBl, EphB2. EphB3, EphB4, EphB6, ELF2M, CXCR1, CXCR2, CXCR3, CXCR4, CXCR6, CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR8, CFTR, CIC-1, CIC-2, CIC-4, CIC-5, CIC-7, CIC-Ka, CIC-Kb, Bestrophins, TMEM16A, GAB A receptor, glycin receptor, ABC transporters, NAV1.1, NAVI.2, NAVI.3, NAVI.4, NAVI.5, NAVI.6, NAVI.7, NAVI.8, NAVI.9, sphingosine-1 -phosphate receptor (S1P1R), NMDA channel, transmembrane protein, multispan transmembrane protein, T-cell receptor motifs; T-cell alpha chains; T-cell beta chains; T-cell y chains; T-cell 6 chains, GCC, CCR7, CD1, CD1a, CD1b, CD1c, CD1d, CD1e, CD2, CD3d, CD3e, CD3g, CD3ε, CD3, CD4, CD5, CD7, CD8a, CD8b, CD8, CD1lb, CD11c, CD16, CD19, CD20, CD21, CD22, CD23, CD24, CD25, CD27, CD28, CD30, CD33, CD34, CD35, CD38, CD40, CD45RA, CD45RO, CD46, CD47, CD48, CD52, CD56,

CD59, CD62L, CD66, CD68, CD70, CD71, CD72, CD73, CD79A, CD79B, CD80 (B7.1), CD86 (B7.2), CD94, CD95, CD97, CD117, CD123, CD127, CD133, CD134, CD140 (PDGFR4), CD152, CD154, CD158, CD171, CD178, CD179, CD179a, CD181 (CXCR1), CD182 (CXCR2), CD183 (CXCR3), CD210, CD213A2, CD134, CD137 (4-1 BB), CD163, CD246, CD252, CD253, CD261, CD262, CD272, CD273 (PD-L2), CD274 (PD-L1), CD276 (B7H3), CD279, CD295, CD339 (JAG1), CD340 (HER2), F4/80, IL-4Ra, Sca-1 , CTLA-4, GITR, GARP, LAP, granzyme B, LFA-1, transferrin receptor, NKp46, perforin, CD4+, Thl, Th2, Thl7, Th40, Th22, Th9, Tfh, Canonical Treg, FoxP3+, Tri, Th3, Tregl7, TREG, CDCP, NT5E, EpCAM, CEA, gpA33, Mucins, Carbonic anhydrase IX, Folate binding protein, Gangliosides (e.g., CD2, CD3, GM2), Lewis-y2, aVp3, a5pl, ErbBl/HER2, ErB3, c-MET, IGF1R, TRAIL-R1, TRAIL-R2, RANKL, FAP, Tenascin, PDL-1, BAFF, HDAC, ABL, FLT3, KIT, MET, RET, IL- Ip, ALK, RANKL, mTOR, CTLA-4, IL-6, IL-6R, JAK3, BRAF, PTCH, Smoothened, PIGF, ANPEP, TIMP1, PLAUR, PTPRJ, LTBR, or ANTXR1, Folate receptor alpha (Fra), ERBB2 (Her2/neu), IL-13Ra2, Mesothelin, TSHR, CD171, CD179a, CLECL1, CS-1, CLL-1, GD2, GD3, BCMA (CD269), MUC16 (CA125), L1CAM, LeY, MSLN, IL13Ral, Ll-CAM, Tn Ag, prostate specific membrane antigen (PSMA), ROR1, GM3, CD44v6, B7H3 (CD276), KIT, interleukin- 11 receptor a (IL-l lRa), PSCA, PRSS21, LewisY, platelet-derived growth factor receptor-beta (PDGFR-beta), SSEA-4, DLL3, MUC1, NCAM, Prostase, PAP, Ephrin B2, IGF-1 receptor, CAIX, LMP2, gplOO, bcr-abl, tyrosinase, Fucosyl GM1, sLe, TGS5, HMWMAA, o-acetyl-GD2, Folate receptor beta, TEM1/CD248, TEM7R, CLDN6, CLDN18.2, CX0RF61, CD97, CD 179a, ALK, Polysialic acid, PLAC1, GloboH, NY-BR-1, UPK2, HAVCR1, ADRB3, PANX3, GPR20, LY6K, OR51E2, TARP, WT1, NY-ESO- 1, LAGE-la, MAGE-A1, legumain, HPV E6, E7, ETV6-AML, sperm protein 17, XAGE1, Tie 2, MAD-CT-1, MAD-CT-2, Major histocompatibility complex class I-related gene protein (MR1), urokinase-type plasminogen activator receptor (uPAR), Fos-related antigen 1, p53, p53 mutant, prostein, survivin, telomerase, PCTA-l/Galectin 8, MelanA/MARTl, Ras mutant, hTERT, sarcoma translocation breakpoints, ML-IAP, ERG (TMPRSS2 ETS fusion gene), NA17, PAX3, Androgen receptor, Cyclin Bl, MYCN, RhoC, TRP-2, CYPIB I, BORIS, SART3, PAX5, OY- TES1, LCK, AKAP-4, SSX2, RAGE-1, human telomerase reverse transcriptase, RU1, RU2, intestinal carboxyl esterase, mut hsp70-2, CD79a, CD79b, CD72, LAIR1, FCAR, LILRA2, CD300LF, CLEC12A, BST2, EMR2, LY75, GPC3, FCRL5, a neoantigen, CD15, CD184, CD56, CD26, CD29, CD44, HLA-A, HLA-B, HLA-C, (HLA-A,B,C) CD49f, CD151 CD340, CD200, tkrA, trkB, trkC, GPRC5D, IGLL1, IL-11Rα, KLK2, KLK3,

LY6G6D, p53, p53R175H, KRAS, KRAS G12D, PRAME, SIRPα, TAG72, ALPI, citrullinated vimentin, Axl, or an antigenic fragment or antigenic portion thereof.

**[0716]** In some embodiments, exemplary target antigens include, but are not limited to, CD1, CD1a, CD1b, CD1c, CD1d, CD1e, CD2, CD3d, CD3e, CD3g, CD3ε, CD4, CD5, CD7, CD8a, CD8b, CD19, CD20, CD21, CD22, CD23, CD24, CD25, CD27, CD28, CD30, CD33, CD34, CD35, CD38, CD40, CD44v6, CD45, CD45RA, CD45RO, CD46, CD47, CD48, CD52, CD56, CD59, CD62L, CD66, CD68, CD70, CD71, CD72, CD73, CD79A, CD79B, CD80 (B7.1), CD86 (B7.2), CD94, CD95, CD97, CD117, CD123, CD213A2, CD127, CD133, CD134, CD140 (PDGFR4), CD152, CD154, CD158, CD171, CD178, CD179, CD179a, CD181 (CXCR1), CD182 (CXCR2), CD183 (CXCR3), CD210, CD213A2, CD246, CD252, CD253, CD261, CD262, CD272, CD273 (PD-L2), CD274 (PD-L1), CD276 (B7H3), CD279, CD295, CD339 (JAG1), CD340 (HER2), CDH17, CEA, CLL-1, CS1, EGFR, FGFR2, AFP, CA125, MUC-1, MAGE, alkaline phosphatase, placental-like 2 (ALPPL2), B-cell maturation antigen (BCMA), green fluorescent protein (GFP), enhanced green fluorescent protein (eGFP), Claudin18.2, PSMA, Mesothelin, IL13Ra2, FAP, signal regulatory protein α (SIRPα), TSHR, EGFRvIII, GD2, GD3, Tn Ag, ROR1, ROR2, GPC1, GPC2, FLT3, FAP, TAG72, CEA, EPCAM, B7H3, KIT, IL-13Ra2, IL-11Ra, PSCA, PRSS21, VEGFR2, LewisY, PDGFR-beta, SSEA-4, , folate receptor alpha, ERBB2 (Her2/neu), MUC1, MUC16, EGFR, NCAM, prostase, PAP, ELF2M, Ephrin B2, IGF-1 receptor, CAIX, LMP2, gp100, bcr-abl, tyrosinase, EphA2, fucosyl GM1, sLe, GM3, TGS5, HMWMAA, o-acetyl-GD2, folate receptor beta, TEM1/CD248, TEM7R, CLDN6, GPRC5D, CXORF61, ALK, Polysialic acid, PLAC1, GloboH, NY-BR-1, UPK2, HAVCR1, ADRB3, PANX3, GPR20, LY6K, OR51E2, TARP, WT1, NY-ESO-1, LAGE-la, MAGE-Al, legumain, HPV E6,E7, MAGE Al, ETV6-AML, sperm protein 17, XAGE1, Tie 2, MAD-CT-1, MAD-CT-2, Fos-related antigen 1, p53, p53 mutant, KRAS, mutant KRAS, KRAS G12D, prostein, surviving, telomerase, PCTA-1/Galectin 8, MelanA/MART1, Ras mutant, hTERT, sarcoma translocation breakpoints, ML-IAP, ERG (TMPRSS2 ETS fusion gene), NA17, PAX3, androgen receptor, cyclin Bl, MYCN, RhoC, TRP-2, CYP1B1, BORIS, SART3, PAX5, OY-TES1, LCK, AKAP-4, SSX2, RAGE-1, human telomerase reverse transcriptase, RU1, RU2, intestinal carboxyl esterase, mut hsp70-2,LAIR1, FCAR, LILRA2, CD300LF, CLEC12A, BST2, EMR2, LY75, GPC3, FCRL5, IGLL1, AFP, folate receptor alpha, ERBB2 (Her2/neu), MUC1, MUC16, EGFR, NCAM, prostase, PAP, ELF2M, Ephrin B2, IGF-I receptor, CAIX, LMP2, gp100, bcr-abl, tyrosinase, EphA2, fucosyl GM1, sLe, GM3, TGS5, HMWMAA, o-acetyl-GD2, folate receptor beta,

TEM1/CD248, TEM7R, CLDN6, GPRC5D, GPA33, CXORF61, , ALK, Polysialic acid, PLAC1, GloboH, NY-BR-1, UPK2, HAVCR1, ADRB3, PANX3, GPR20, LY6K, OR51E2, TARP, WT1, NY-ESO-1, LAGE-la, MAGE-Al, legumain, HPV E6,E7, MAGE-A4, ETV6-AML, sperm protein 17, XAGE1, Tie 2, MAD-CT-1, MAD-CT-2, Fos-related antigen 1, p53, p53 mutant, prostein, surviving, telomerase, PCTA-1/Galectin 8, MelanA/MART1, Ras mutant, hTERT, sarcoma translocation breakpoints, ML-IAP, ERG (TMPRSS2 ETS fusion gene), NA17, PAX3, androgen receptor, cyclin Bl, MYCN, RhoC, TRP-2, CYP1B1, BORIS, SART3, PAX5, OY-TES1, LCK, AKAP-4, SSX2, RAGE-1, human telomerase reverse transcriptase, RU1, RU2, intestinal carboxyl esterase, mut hsp70-2, LAIR1, FCAR, LILRA2, CD300LF, CLEC12A, BST2, EMR2, LY75, GPC3, FCRL5, IGLL1, the extracellular portion of the APRIL protein, or any combinations thereof.

**[0717]** In some embodiments, the target antigen is an antigen that includes, but is not limited to,B7H3, BCMA, CD19, CD20, CD22, CD70, CD79a, CD79b, CDH17, CLDN6, CLDN18.2, DLL3, GCC, GD2, GD3, GPC3, GPRC5D, GPA33, KLK3, LY6G6D, p53R175H, PRAME, ROPN1, STEAP1, and STEAP2.

**[0718]** In some aspects, the target antigen is AFP, CD19, TRAC, TCRβ, BCMA, CLL-1, CS1, CD38, CD19, TSHR, CD123, CD22, CD30, CD171, CD33, EGFRvIII, GD2, GD3, Tn Ag, PSMA, ROR1, ROR2, GPC1, GPC2, FLT3, FAP, TAG72, CD44v6, CEA, EPCAM, B7H3, KIT, IL-13Ra2, IL-11Ra, PSCA, PRSS21, VEGFR2, LewisY, CD24, PDGFR-beta, SSEA-4, CD20, folate receptor alpha, ERBB2 (Her2/neu), MUC1, MUC16, EGFR, NCAM, prostase, PAP, ELF2M, Ephrin B2, IGF-I receptor, CAIX, LMP2, gplOO, bcr-abl, tyrosinase, EphA2, fucosyl GM1, sLe, GM3, TGS5, HMWMAA, o-acetyl-GD2, folate receptor beta, TEM1/CD248, TEM7R, CLDN6, GPRC5D, CXORF61, CD97, CD179a, ALK, Polysialic acid, PLAC1, GloboH, NY-BR-1, UPK2, HAVCR1, ADRB3, PANX3, GPR20, LY6K, OR51E2, TARP, WT1, NY-ESO-1, LAGE-la, MAGE-Al, legumain, HPV E6,E7, MAGE Al, ETV6-AML, sperm protein 17, XAGE1, Tie 2, MAD-CT-1, MAD-CT-2, Fos-related antigen 1, p53, p53 mutant, KRAS, mutant KRAS, KRAS G12D, prostein, surviving, telomerase, PCTA-1/Galectin 8, MelanA/MART1, Ras mutant, hTERT, sarcoma translocation breakpoints, ML-IAP, ERG (TMPRSS2 ETS fusion gene), NA17, PAX3, androgen receptor, cyclin Bl, MYCN, RhoC, TRP-2, CYP1B1, BORIS, SART3, PAX5, OY-TES1, LCK, AKAP-4, SSX2, RAGE-1, human telomerase reverse transcriptase, RU1, RU2, intestinal carboxyl esterase, mut hsp70-2, CD79a, CD79b, CD72, LAIR1, FCAR, LILRA2, CD300LF, CLEC12A, BST2, EMR2, LY75, GPC3, FCRL5, IGLL1, the extracellular portion of the APRIL protein, or any combinations thereof.

**[0719]** In some aspects, the CAR or TCR targets AFP, CD19, TRAC, TCRβ, BCMA, CLL-1, CS1, CD38, CD19, TSHR, CD123, CD22, CD30, CD171, CD33, EGFRvIII, GD2, GD3, Tn Ag, PSMA, ROR1, ROR2, GPC1, GPC2, FLT3, FAP, TAG72, CD44v6, CEA, EPCAM, B7H3, KIT, IL-13Ra2, IL-11Ra, PSCA, PRSS21, VEGFR2, LewisY, CD24, PDGFR-beta, SSEA-4, CD20, folate receptor alpha, ERBB2 (Her2/neu), MUC1, MUC16, EGFR, NCAM, prostase, PAP, ELF2M, Ephrin B2, IGF-I receptor, CAIX, LMP2, gplOO, bcr-abl, tyrosinase, EphA2, fucosyl GM1, sLe, GM3, TGS5, HMWMAA, o-acetyl-GD2, folate receptor beta, TEM1/CD248, TEM7R, CLDN6, GPRC5D, CXORF61, CD97, CD179a, ALK, Polysialic acid, PLAC1, GloboH, NY-BR-1, UPK2, HAVCR1, ADRB3, PANX3, GPR20, LY6K, OR51E2, TARP, WT1, NY-ESO-1, LAGE-la, MAGE-Al, legumain, HPV E6,E7, MAGE Al, ETV6-AML, sperm protein 17, XAGE1, Tie 2, MAD-CT-1, MAD-CT-2, Fos-related antigen 1, p53, p53 mutant, KRAS, mutant KRAS, KRAS G12D, prostein, surviving, telomerase, PCTA-1/Galectin 8, MelanA/MART1, Ras mutant, hTERT, sarcoma translocation breakpoints, ML-IAP, ERG (TMPRSS2 ETS fusion gene), NA17, PAX3, androgen receptor, cyclin Bl, MYCN, RhoC, TRP-2, CYP1B1, BORIS, SART3, PAX5, OY-TES1, LCK, AKAP-4, SSX2, RAGE-1, human telomerase reverse transcriptase, RU1, RU2, intestinal carboxyl esterase, mut hsp70-2, CD79a, CD79b, CD72, LAIR1, FCAR, LILRA2, CD300LF, CLEC12A, BST2, EMR2, LY75, GPC3, FCRL5, IGLL1, the extracellular portion of the APRIL protein, or any combinations thereof.

**[0720]** In some embodiments, the CAR is a DLL3 CAR. In some embodiments, the extracellular binding domain of the DLL3 CAR comprises an antibody that specifically binds to DLL3, for example, human DLL3. DLL3 antibodies, scFvs, and CARs have been described in PCT Application Publication Nos. WO201193097, WO2013126746, WO2014125273, WO2019195408, WO2019200007, WO2019217145, WO202076977, WO2020180591, WO2020210067, WO2020247873, WO2021226204, WO202284915, WO202298979, WO202108610, WO202107371, and WO202411177, the entire content of each of which is incorporated by reference herein.

**[0721]** In some embodiments, the CAR is a LY6G6D CAR. In some embodiments, the extracellular binding domain of the LY6G6D CAR comprises an antibody that specifically binds to LY6G6D, for example, human LY6G6D. LY6 antibodies, scFvs, and CARs have been described in PCT Application Publication Nos. WO2021119505 and WO2023150510, the entire content of each of which is incorporated by reference herein.

**[0722]** In some embodiments, the CAR is a CLDN6 CAR. In some embodiments, the extracellular binding domain of the CLDN6 CAR comprises an antibody that specifically

binds to CLDN6, for example, human CLDN6. CLDN6 antibodies, scFvs, and CARs have been described in PCT Application Publication Nos. WO2012156018A1, WO2014075788A1, WO2015014376A1, WO2015014870A1, WO2015150327A1, WO2018054484A1, WO2019056023A2, WO2019135404A1, WO2020161186A1, WO2020191344A1, WO2020191342A1, WO2021006328A1, and WO2021200939A1, the entire content of each of which is incorporated by reference herein.

**[0723]** In some embodiments, the CAR is a CLDN18.2 CAR. In some embodiments, the extracellular binding domain of the CLDN18.2 CAR comprises an antibody that specifically binds to CLDN18.2, for example, human CLDN18.2.

**[0724]** In some embodiments, the CAR is a GCC CAR. In some embodiments, the extracellular binding domain of the GCC CAR comprises an antibody that specifically binds to GCC, for example, human GCC. GCC antibodies, scFvs, and CARs have been described in PCT Application Publication Nos. WO2011050242, WO2013163633, WO2014134311, WO2017167217, WO2018064921, WO2019178580, WO2020146743, WO2019224716, WO2021205325, WO2022123307, WO2022123316, WO2022127871, WO2022172085, WO2022216079, WO2024044202, WO2023116802, WO2023125349, and WO2023150510, the entire content of each of which is incorporated by reference herein, as well as as well as US Patent Nos. US9662405 and US9156915, the entire content of each of which is incorporated by reference herein.

**[0725]** In some embodiments, the CAR is a PRAME CAR. In some embodiments, the extracellular binding domain of the PRAME CAR comprises an antibody that specifically binds to PRAME, for example, human PRAME.

**[0726]** In some embodiments, the CAR is a KLK2 CAR. In some embodiments, the extracellular binding domain of the KLK2 CAR comprises an antibody that specifically binds to KLK2, for example, human KLK2. KLK2 antibodies, scFvs, and CARs have been described in PCT Application Publication Nos. WO2023144723, WO2022162549, WO2021019389, and WO2021019386, the entire content of each of which is incorporated by reference herein.

**[0727]** In some embodiments, the CAR is a KLK3 CAR. In some embodiments, the extracellular binding domain of the KLK3 CAR comprises an antibody that specifically binds to KLK3, for example, human KLK3.

**[0728]** In some embodiments, the CAR is a CD70 CAR. In some embodiments, the extracellular binding domain of the CD70 CAR comprises an antibody that specifically binds to CD70, for example, human CD70.

**[0729]** In some embodiments, the CAR is a STEAP1 CAR. In some embodiments, the extracellular binding domain of the STEAP1 CAR comprises an antibody that specifically binds to STEAP1, for example, human STEAP1.

**[0730]** In some embodiments, the CAR is a STEAP2 CAR. In some embodiments, the extracellular binding domain of the STEAP2 CAR comprises an antibody that specifically binds to STEAP2, for example, human STEAP2. CLDN6 antibodies, scFvs, and CARs have been described in PCT Application Publication No. WO2018058001, the entire content of each of which is incorporated by reference herein.

**[0731]** In some embodiments, the CAR is a GPC3 CAR. In some embodiments, the extracellular binding domain of the GPC3 CAR comprises an antibody that specifically binds to GPC3, for example, human GPC3.

**[0732]** In some embodiments, the CAR is a TROP2 CAR. In some embodiments, the extracellular binding domain of the TROP2 CAR comprises an antibody that specifically binds to TROP2, for example, human TROP2.

**[0733]** In some embodiments, the CAR is a ROPN1 CAR. In some embodiments, the extracellular binding domain of the ROPN1 CAR comprises an antibody that specifically binds to ROPN1, for example, human ROPN1.

**[0734]** In some embodiments, the CAR is a ROR1 CAR. In some embodiments, the extracellular binding domain of the ROR1 CAR comprises an antibody that specifically binds to ROR1, for example, human ROR1.

**[0735]** In some embodiments, the CAR is a IGF2BP3 CAR. In some embodiments, the extracellular binding domain of the IGF2BP3 CAR comprises an antibody that specifically binds to IGF2BP3, for example, human IGF2BP3.

**[0736]** In some embodiments, the CAR is a CD79a CAR. In some embodiments, the extracellular binding domain of the CD79a CAR comprises an antibody that specifically binds to CD79a, for example, human CD79a.

**[0737]** In some embodiments, the CAR is a CD79b CAR. In some embodiments, the extracellular binding domain of the CD79b CAR comprises an antibody that specifically binds to CD79b, for example, human CD79b.

**[0738]** In some embodiments, the CAR is a GD2 CAR. In some embodiments, the extracellular binding domain of the GD2 CAR comprises an antibody that specifically binds to GD2 for example, human GD2. I.

**[0739]** In some embodiments, the CAR is a GD3 CAR. In some embodiments, the extracellular binding domain of the GD3 CAR comprises an antibody that specifically binds to GD3 for example, human GD3.

**[0740]** In some embodiments, the CAR is a CDH17 CAR. In some embodiments, the extracellular binding domain of the CDH17 CAR comprises an antibody that specifically binds to CSH17 for example, human CDH17. CDH17 antibodies, scFvs, and CARs have been described in PCT Application Publication Nos. WO2017120557, WO2019210155, WO2023177821, and WO2024100294, the entire content of each of which is incorporated by reference herein.

**[0741]** In some embodiments, the CAR is a B7H3 CAR. In some embodiments, the extracellular binding domain of the B7H3 CAR comprises an antibody that specifically binds to B7H3 for example, human B7H3.

**[0742]** In some embodiments, the CAR is a CD19 CAR. In some embodiments, the extracellular binding domain of the CD19 CAR comprises an antibody that specifically binds to CD19, for example, human CD19. In some embodiments, the extracellular binding domain of the CD19 CAR comprises an scFv antibody fragment derived from the FMC63 monoclonal antibody (FMC63), which comprises the heavy chain variable region (VH) and the light chain variable region (VL) of FMC63 connected by a linker peptide. In some embodiments, the linker peptide is a "Whitlow" linker peptide. FMC63 and the derived scFv have been described in Nicholson et al., Mal. lmmun. 34(16-17): 1157-1165 (1997) and PCT Application Publication No. WO2018/213337 A 1, the entire content of each of which is incorporated by reference herein. In some embodiments, the extracellular binding domain of the CD19 CAR comprises an antibody derived from one of the CD19-specific antibodies including, for example, SJ25C1 (Bejcek et al., Cancer Res. 55:2346-2351 (1995)), HD37 (Pezutto et al., J. Immunol. 138(9):2793-2799 (1987)), 4G7 (Meeker et al., Hybridoma 3:305-320 (1984)), B43 (Bejcek (1995)), BLY3 (Bejcek (1995)), B4 (Freedman et al., 70:418-427 (1987)), B4 HB12b (Kansas & Tedder, J. Immunol. 147:4094-4102 (1991); Yazawa et al., Proc. Natl. Acad. Sci. USA 102: 15178- 15183 (2005); Herbst et al., J. Pharmacol. Exp. Ther. 335:213-222 (2010)), BU12 (Gallard et al., J. Immunology, 148(10): 2983-2987 (1992)), and CLB-CD19 (De Rie Cell. Immunol. 118:368- 381(1989)).

**[0743]** In some embodiments, the CAR is CD22 CAR. CD22, which is a transmembrane protein found mostly on the surface of mature B cells that functions as an inhibitory receptor for B cell receptor (BCR) signaling. CD22 is expressed in 60-70% of B cell lymphomas and leukemias (e.g., B-chronic lymphocytic leukemia, hairy cell leukemia, acute lymphocytic

leukemia (ALL), and Burkitt's lymphoma) and is not present on the cell surface in early stages of B cell development or on stem cells. In some embodiments, the CD22 CAR comprises an extracellular binding domain that specifically binds CD22, a transmembrane domain, an intracellular signaling domain, and/or an intracellular costimulatory domain. In some embodiments, the extracellular binding domain of the CD22 CAR comprises an scFv antibody fragment derived from the m971 monoclonal antibody (m971), which comprises the heavy chain variable region (VH) and the light chain variable region (VL) of m971 connected by a linker. In some embodiments, the extracellular binding domain of the CD22 CAR comprises an scFv antibody fragment derived from m971-L7, which an affinity matured variant of m971 with significantly improved CD22 binding affinity compared to the parental antibody m971 (improved from about 2 nM to less than 50 pM). In some embodiments, the scFv antibody fragment derived from m971-L7 comprises the VH and the VL of m971-L7 connected by a 3xG4S linker. In some embodiments, the extracellular binding domain of the CD22 CAR comprises immunotoxins HA22 or BL22. Immunotoxins BL22 and HA22 are therapeutic agents that comprise an scFv specific for CD22 fused to a bacterial toxin, and thus can bind to the surface of the cancer cells that express CD22 and kill the cancer cells. BL22 comprises a dsFv of an anti-CD22 antibody, RFB4, fused to a 38-kDa truncated form of Pseudomonas exotoxin A (Bang et al., Clin. Cancer Res., 11 : 1545-50 (2005)). HA22 (CAT8015, moxetumomab pasudotox) is a mutated, higher affinity version of BL22 (Ho et al., J. Biol. Chem., 280(1): 607-17 (2005)). Suitable sequences of antigen binding domains of HA22 and BL22 specific to CD22 are disclosed in, for example, U.S. Patent Nos. 7,541,034; 7,355,012; and 7,982,011, which are hereby incorporated by reference in their entirety.

**[0744]** In some embodiments, the CAR is BCMA CAR. BCMA is a tumor necrosis family receptor (TNFR) member expressed on cells of the B cell lineage, with the highest expression on terminally differentiated B cells or mature B lymphocytes. BCMA is involved in mediating the survival of plasma cells for maintaining long-term humoral immunity. The expression of BCMA has been recently linked to a number of cancers, such as multiple myeloma, Hodgkin's and non-Hodgkin's lymphoma, various leukemias, and glioblastoma. In some embodiments, the BCMA CAR comprises an extracellular binding domain that specifically binds BCMA, a transmembrane domain, an intracellular signaling domain, and/or an intracellular costimulatory domain. In some embodiments, the extracellular binding domain of the BCMA CAR comprises an antibody that specifically binds to BCMA, for example, human BCMA. CARs directed to BCMA have been described in PCT

Application Publication Nos. WO2016/014789, WO2016/014565, WO2013/154760, and WO 2015/128653. BCMA-binding antibodies are also disclosed in PCT Application Publication Nos. WO2015/166073 and W02014/068079. In some embodiments, the extracellular binding domain of the BCMA CAR comprises an scFv antibody fragment derived from a murine monoclonal antibody as described in Carpenter et al., Clin. Cancer Res. 19(8):2048-2060 (2013). In some embodiments, the scFv antibody fragment is a humanized version of the murine monoclonal antibody (Sommermeyer et al., Leukemia 31 :2191-2199 (2017)). In some embodiments, the extracellular binding domain of the BCMA CAR comprises single variable fragments of two heavy chains (VHH) that can bind to two epitopes of BCMA as described in Zhao et al., J. Hematol. Oneal. 11(1): 141 (2018). In some embodiments, the extracellular binding domain of the BCMA CAR comprises a fully human heavy-chain variable domain (FHVH) as described in Lam et al., Nat. Commun. 11 (1) :283 (2020).

**[0745]** In some embodiments, the CAR is a GPRC5D CAR. In some embodiments, the extracellular binding domain of the GPRC5D CAR comprises an antibody that specifically binds to GPRC5D, for example, human GPRC5D.

**[0746]** In some embodiments, the CAR is a CD20 CAR. In some embodiments, the extracellular binding domain of the CD20 CAR comprises an antibody that specifically binds to CD20, for example, human CD20.

**[0747]** In some embodiments, the antigen binding domain targets an antigen characteristic of an autoimmune or inflammatory disorder. In some embodiments, the ABD binds an antigen associated with an autoimmune or inflammatory disorder. In some instances, the antigen is expressed by a cell associated with an autoimmune or inflammatory disorder. In some embodiments, the autoimmune or inflammatory disorder is selected from chronic graft-vs-host disease (GVHD), lupus, arthritis, immune complex glomerulonephritis, goodpasture, uveitis, hepatitis, systemic sclerosis or scleroderma, type I diabetes, multiple sclerosis, cold agglutinin disease, Pemphigus vulgaris, Grave's disease, autoimmune hemolytic anemia, Hemophilia A, Primary Sjogren's Syndrome, thrombotic thrombocytopenia purrpura, neuromyelits optica, Evan's syndrome, IgM mediated neuropathy, cyroglobulinemia, dermatomyositis, idiopathic thrombocytopenia, ankylosing spondylitis, bullous pemphigoid, acquired angioedema, chronic urticarial, antiphospholipid demyelinating polyneuropathy, and autoimmune thrombocytopenia or neutropenia or pure red cell aplasias, while exemplary non-limiting examples of alloimmune diseases include allosensitization (see, for example, Blazar et al., 2015, Am. J. Transplant, 15(4):931-41) or

xenosensitization from hematopoietic or solid organ transplantation, blood transfusions, pregnancy with fetal allosensitization, neonatal alloimmune thrombocytopenia, hemolytic disease of the newborn, sensitization to foreign antigens such as can occur with replacement of inherited or acquired deficiency disorders treated with enzyme or protein replacement therapy, blood products, and gene therapy. Allosensitization, in some instances, refers to the development of an immune response (such as circulating antibodies) against human leukocyte antigens that the immune system of the recipient subject or pregnant subject considers to be non-self-antigens. In some embodiments, the antigen characteristic of an autoimmune or inflammatory disorder is selected from a cell surface receptor, an ion channel-linked receptor, an enzyme-linked receptor, a G protein-coupled receptor, receptor tyrosine kinase, tyrosine kinase associated receptor, receptor-like tyrosine phosphatase, receptor serine/ threonine kinase, receptor guanylyl cyclase, or histidine kinase associated receptor.

**[0748]** In some embodiments, the antigen binding domain targets an antigen characteristic of an infectious disease. In some embodiments, the ABD binds an antigen associated with an infectious disease. In some instances, the antigen is expressed by a cell affected by an infectious disease. In some embodiments, wherein the infectious disease is selected from HIV, hepatitis B virus, hepatitis C virus, Human herpes virus, Human herpes virus 8 (HHV-8, Kaposi sarcoma- associated herpes virus (KSHV)), Human T-lymphotrophic virus-1 (HTLV-1), Merkel cell polyomavirus (MCV), Simian virus 40 (SV40), Epstein-Barr virus, CMV, human papillomavirus. In some embodiments, the antigen characteristic of an infectious disease is selected from a cell surface receptor, an ion channel-linked receptor, an enzyme-linked receptor, a G protein-coupled receptor, receptor tyrosine kinase, tyrosine kinase associated receptor, receptor-like tyrosine phosphatase, receptor serine/ threonine kinase, receptor guanylyl cyclase, histidine kinase associated receptor, HIV Env, gpl20, or CD4-induced epitope on HIV-1 Env.

**[0749]** In any of these embodiments, the extracellular binding domain of the CAR can be codon-optimized for expression in a host cell or have variant sequences to increase functions of the extracellular binding domain.

**[0750]** In some embodiments, the CAR is bispecific to two target antigens (e.g., comprising binding region(s) for two target antigens). In some embodiments, the target antigens are different target antigens. In some of any such embodiments, the two different target antigens are any two different antigens described above. In some embodiments, the extracellular binding domains are different and bind two different antigens from (i) CD19 and CD20, (ii)

CD20 and LI -CAM, (iii) LI -CAM and GD2, (iv) EGFR and LI -CAM, (v) CD19 and CD22, (vi) EGFR and C-MET, (vii) EGFR and HER2, (viii) C-MET and HER2, or (ix) EGFR and ROR1. In some embodiments, each of the two different antigen binding domains is an scFv. In some embodiments, the C-terminus of one variable domain (VH or VL) of a first scFv is tethered to the N-terminus of the second scFv (VL or VH, respectively) via a polypeptide linker. In some embodiments, the linker connects the N-terminus of the VH with the C-terminus of VL or the C-terminus of VH with the N-terminus of VL. These scFvs lack the constant regions (Fc) present in the heavy and light chains of the native antibody. The scFvs, specific for at least two different antigens, are arranged in tandem and linked to the co-stimulatory domain and the intracellular signaling domain via a transmembrane domain. In some embodiments, an extracelluar spacer domain may be linked between the antigen-specific binding region and the transmembrane domain.

**[0751]** In some embodiments, each antigen-specific targeting region of the CAR comprises a divalent (or bivalent) single-chain variable fragment (di-scFvs, bi-scFvs). In CARs comprising di-scFVs, two scFvs specific for each antigen are linked together by producing a single peptide chain with two VH and two VL regions, yielding tandem scFvs. (Xiong, Cheng- Yi; Natarajan, A; Shi, X B; Denardo, G L; Denardo, S J (2006). "Development of tumor targeting anti-MUC-1 multimer: effects of di-scFv unpaired cysteine location on PEGylation and tumor binding". Protein Engineering Design and Selection 19 (8): 359-367; Kufer, Peter; Lutterbiise, Ralf; Baeuerle, Patrick A. (2004). "A revival of bispecific antibodies". Trends in Biotechnology 22 (5): 238-244). CARs comprising at least two antigen-specific targeting regions would express two scFvs specific for each of the two antigens. The resulting antigen-specific targeting region, specific for at least two different antigens, is joined to the co-stimulatory domain and the intracellular signaling domain via a transmembrane domain. In some embodiments, an extracelluar spacer domain may be linked between the antigen-specific binding domain and the transmembrane domain.

**[0752]** In some embodiments, each antigen-specific targeting region of the CAR comprises a diabody. In a diabody, the scFvs are created with linker peptides that are too short for the two variable regions to fold together, driving the scFvs to dimerize. Still shorter linkers (one or two amino acids) lead to the formation of trimers, the so-called triabodies or tribodies. Tetrabodies may also be used.

**[0753]** In some embodiments, the cell is engineered to express more than one CAR, such as two different CARs, in which each CAR has an antigen-binding domain directed to a different target antigen. In some of any such embodiments, the two different target antigens

are any two different antigens described above. In some embodiments, the extracellular binding domains are different and bind two different antigens from (i) CD19 and CD20, (ii) CD20 and LI -CAM, (iii) LI -CAM and GD2, (iv) EGFR and LI -CAM, (v) CD19 and CD22, (vi) EGFR and C-MET, (vii) EGFR and HER2, (viii) C-MET and HER2, or (ix) EGFR and ROR1.

**[0754]** In some embodiments, two different engineered cells are prepared that contain the provided modifications with each engineered with a different CAR. In some embodiments, each of the two different CARs has an antigen-binding domain directed to a different target antigen. In some of any such embodiments, the two different target antigens are any two different antigens described above. In some embodiments, the extracellular binding domains are different and bind two different antigens from (i) CD19 and CD20, (ii) CD20 and LI -CAM, (iii) LI -CAM and GD2, (iv) EGFR and LI -CAM, (v) CD19 and CD22, (vi) EGFR and C-MET, (vii) EGFR and HER2, (viii) C-MET and HER2, or (ix) EGFR and R0R1. In some embodiments, a population of engineered cells expressing a first CAR directed against a first target antigen and a population of engineered cells expressing a second CAR directed against a second target antigen are separately administered to the subject. In some embodiments, the first and second population of cells are administered sequentially in any order. For instance, the population of cells expressing the second CAR is administered a after administration of the population of cells expressing the first CAR.

Linker Sequences

**[0755]** In some embodiments, the antigen binding domain of the CAR may comprise one or more linker sequences. In some embodiments, the antigen binding domain of the CAR may comprise one or more linker sequences connecting a heavy chain variable region (VH) and a light chain variable region (VL). The VH and the VL may be connected in either order, i.e., VH-linker-VL or VL-linker-VH. Nonlimiting examples of linkers include Whitlow linker (SEQ ID NO: 401; GSTSGSGKPGSGEGSTKG), (G4S)n (n can be a positive integer, e.g., 1, 2, 3, 4, 5, 6, etc.) linker, and variants thereof.

**[0756]** Examples of exemplary linker sequences are described in **Table 11**. In provided aspects, each linker in **Table 11** can be combined with any of the other CAR component sequences described here.

**Table 11. Exemplary Linker Sequences**

| Component | Sequence | SEQ ID NO: |
|---|---|---|
| G4Sx3 Linker – amino acid | GGGGSGGGGS GGGGS | 416 |

| | | |
|---|---|---|
| G4S Linker – amino acid | GGTGGAGGCG GTTCT | 417 |
| SG4S Linker – amino acid | SGGGGS | 418 |
| Whitlow Linker – amino acid | GSTSGSGKPGSGEGSTKG | 401 |
| G4Sx3 Linker – nucleotide | ggcggtggag gatctggagg tggcggaagt ggaggaggtg gttct | 504 |
| G4S Linker – nucleotide | GGTGGAGGCG GTTCT | 505 |
| SG4S Linker – nucleotide | AGCGGAGGCG GAGGCTCT | 506 |

Spacer (Hinge) Sequences

**[0757]** In some embodiments, the CAR further comprises one or more spacers, e.g., wherein the spacer is a first spacer between the antigen binding domain and the transmembrane domain. In some embodiments, the first spacer includes at least a portion of an immunoglobulin constant region or variant or modified version thereof. In some embodiments, the spacer is a second spacer between the transmembrane domain and a signaling domain. In some embodiments, the second spacer is an oligopeptide, e.g., wherein the oligopeptide comprises glycine and serine residues such as but not limited to glycine-serine doublets. In some embodiments, the CAR comprises two or more spacers, e.g., a spacer between the antigen binding domain and the transmembrane domain and a spacer between the transmembrane domain and a signaling domain. The terms "hinge" and "spacer" may be used interchangeably in the present disclosure. Non-limiting examples of hinge domains include CD8a hinge domain, CD28 hinge domain, IgG4 hinge domain, IgG4 hinge-CH2-CH3 domain, and variants thereof.

**[0758]** Examples of exemplary spacer (hinge) sequences are described in **Table 12**. In provided aspects, the sequences of each spacer (hinge) in **Table 12** can be combined with any of the other CAR component sequences described here.

**Table 12. Exemplary Spacer (Hinge) Sequences**

| **Component** | **Sequence** | SEQ ID NO: |
|---|---|---|
| IgG4 Hinge | ESKYGPPCPPCP | 355 |
| IgG4 Hinge 2 | ESKYGPPCPSCP | 356 |

| | | |
|---|---|---|
| CD8 Hinge – amino acid | TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD | 420 |
| CD8alpha Hinge | TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD | 358 |
| CD28 Hinge | IEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPSKP | 359 |
| CD28 Hinge | AAAIEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPSKP | 360 |
| IgG4 hing-CH2-CH3 domain | ESKYGPPCPPCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVD VSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVL HQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPS QEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLD SDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLS LGK | 357 |

Transmembrane Domain

**[0759]** In some embodiments, the CAR transmembrane domain comprises at least a transmembrane region of the TCR alpha chain, TCR beta chain, TCR zeta chain, CD28, CD3 epsilon, CD3 zeta, 1.xxCD3 zeta, CD45, CD4, CD5, CD8, CD9, CD16, CD22, CD28, CD33, CD37, CD64, CD80, CD86, CD134, CD 137, CD 154, or functional variant thereof. In some embodiments, the transmembrane domain comprises at least a transmembrane region(s) of CD8a, CD8P, 4-1BB/CD137, CD28, CD34, CD4, FcsRIy, CD16, OX40/CD134, CD3< CD3s, CD3y, CD38, TCRa, TCRb, TCR^, CD32, CD64, CD64, CD45, CD5, CD9, CD22, CD37, CD80, CD86, CD40, CD40L/CD154, VEGFR2, FAS, and FGFR2B, or functional variant thereof.

**[0760]** Examples of exemplary transmembrane domain sequences are described in **Table 13**. In provided aspects, each transmembrane domain in T**able 13** can be combined with any of the other CAR component sequences described here.

**Table 13. Exemplary Transmembrane Domain Sequences**

| Component | Sequence | SEQ ID NO: |
|---|---|---|
| CD8 TM – amino acid | IYIWAPLAGTCGVLLLSLVI TLYC | 421 |
| CD28 TM – amino acid | FWVLVVVGGVLACYSLLVTVAFIIFWV | 413 |

Signaling Domain(s)

**[0761]** In some embodiments, a CAR described herein comprises one or more signaling domains. A CAR may comprise a costimulatory signaling domain, e.g., to increase signaling potency. See U.S. Pat. Nos. 7,741,465, and 6,319,494, as well as Krause et al. and Finney et al. (supra), Song et al., Blood 119:696-706 (2012); Kalos et al, Sci Transl. Med. 3:95 (2011); Porter et al, N. Engl. J. Med. 365:725-33 (2011), and Gross et al, Annu. Rev. Pharmacol. Toxicol. 56:59-83 (2016). Signals generated through a TCR alone may be insufficient for full activation of a T cell and a secondary or co-stimulatory signal may increase activation. Thus, in some embodiments, a signaling domain further comprises one or more additional signaling domains (e.g., costimulatory signaling domains) that activate one or more immune cell effector functions (e.g. , a native immune cell effector function described herein). In some embodiments, a portion of such costimulatory signaling domains may be used, as long as the portion transduces the effector function signal. In some embodiments, a cytoplasmic domain described herein comprises one or more cytoplasmic sequences of a T cell co-receptor (or fragment thereof). Non-limiting examples of such T cell co-receptors comprise CD27, CD28, 4-1BB (CD137), 0X40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA- 1), MYD88, CD2, CD7, LIGHT, NKG2C, B7-H3, and a ligand that binds with CD83. An exemplary costimulatory protein has the amino acid sequence of a costimulatory protein found naturally on T cells, the complete native amino acid sequence of which costimulatory protein is described in NCBI Reference Sequence: NP_006130.1.

**[0762]** In some embodiments, a CAR described herein comprises one or more signaling domain selected from one or more of B7-1/CD80; B7-2/CD86; B7-H1/PD-L1; B7-H2; B7-H3; B7-H4; B7-H6; B7-H7; BTLA/CD272; CD28; CTLA-4; Gi24/VISTA/B7-H5; ICOS/CD278; PD-1; PD-L2/B7-DC; PDCD6); 4-1BB/TNFSF9/CD137; 4-1BB Ligand/TNFSF9; BAFF/BLyS/TNFSF13B; BAFF R/TNFRSF13C; CD27/TNFRSF7; CD27 Ligand/TNFSF7; CD30/TNFRSF8; CD30 Ligand/TNFSF8; CD40/TNFRSF5; CD40/TNFSF5; CD40 Ligand/TNFSF5; DR3/TNFRSF25; GITR/TNFRSF18; GITR Ligand/TNFSF18; HVEM/TNFRSF14; LIGHT/TNFSF14; Lymphotoxin-alpha/TNF-beta; OX40/TNFRSF4; 0X40 Ligand/TNFSF4; RELT/TNFRSF19L; TACI/TNFRSF13B; TL1A/TNFSF15; TNF-alpha; TNF RII/TNFRSF1B); 2B4/CD244/SLAMF4; BLAME/SLAMF8; CD2; CD2F-10/SLAMF9; CD48/SLAMF2; CD58/LFA-3; CD84/SLAMF5; CD229/SLAMF3; CRACC/SLAMF7; NTB- A/SLAMF6; SLAM/CD150); CD2; CD7; CD53; CD82/Kai-1; CD90/Thyl; CD96; CD160; CD200; CD300a/LMIRl; HLA

Class I; HLA-DR; Ikaros; Integrin alpha 4/CD49d; Integrin alpha 4 beta 1; Integrin alpha 4 beta 7/LPAM-l; LAG-3; TCL1A; TCL1B; CRTAM; DAP12; Dectin- 1/CLEC7A; DPPIV/CD26; EphB6; TIM- 1 /KIM- 1 /HA VCR; TIM-4; TSLP; TSLP R; lymphocyte function associated antigen-1 (LFA-1); NKG2C, a CD3 zeta domain, a 1.xxCD3 zeta domain,an immunoreceptor tyrosinebased activation motif (ITAM), CD27, CD28, 4-1BB, CD134/OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD2, CD7, LIGHT, NKG2C, B7-H3, a ligand that specifically binds with CD83, or functional fragment thereof.

**[0763]** In some embodiments, a CAR comprises a signaling domain which is a costimulatory domain. In some embodiments, a CAR comprises a second costimulatory domain. In some embodiments, a CAR comprises at least two costimulatory domains. In some embodiments, a CAR comprises at least three costimulatory domains. In some embodiments, a CAR comprises a costimulatory domain derived from a protein selected from the group comprising CD3zeta, CD28, 4-1BB, OX40, IL2Rb turbodomains, MYD88/CD40, MYD88/CD40 inducible constimulatory domain, CD2, B7-1/CD80; B7-2/CD86; B7-H1/PD-L1; B7-H2; B7-H3; B7-H4; B7-H6; B7-H7; BTLA/CD272; CD28; CTLA-4; Gi24/VISTA/B7-H5; ICOS/CD278; PD-1; PD-L2/B7-DC; PDCD6); 4-1BB/TNFSF9/CD137; 4-1BB Ligand/TNFSF9; BAFF/BLyS/TNFSF13B; BAFF R/TNFRSF13C; CD27/TNFRSF7; CD27 Ligand/TNFSF7; CD30/TNFRSF8; CD30 Ligand/TNFSF8; CD40/TNFRSF5; CD40/TNFSF5; CD40 Ligand/TNFSF5; DR3/TNFRSF25; GITR/TNFRSF18; GITR Ligand/TNFSF18; HVEM/TNFRSF14; LIGHT/TNFSF14; Lymphotoxin-alpha/TNF-beta; OX40/TNFRSF4; 0X40 Ligand/TNFSF4; RELT/TNFRSF19L; TACI/TNFRSF13B; TL1A/TNFSF15; TNF-alpha; TNF RII/TNFRSF1B); 2B4/CD244/SLAMF4; BLAME/SLAMF8; CD2; CD2F-10/SLAMF9; CD48/SLAMF2; CD58/LFA-3; CD84/SLAMF5; CD229/SLAMF3; CRACC/SLAMF7; NTB- A/SLAMF6; SLAM/CD150); CD2; CD7; CD53; CD82/Kai-1; CD90/Thyl; CD96; CD160; CD200; CD300a/LMIRl; HLA Class I; HLA-DR; Ikaros; Integrin alpha 4/CD49d; Integrin alpha 4 beta 1; Integrin alpha 4 beta 7/LPAM-l; LAG-3; TCL1A; TCL1B; CRTAM; DAP12; Dectin- 1/CLEC7A; DPPIV/CD26; EphB6; TIM-1/KIM-1/HAVCR; TIM-4; TSLP; TSLP R; lymphocyte function associated antigen-1 (LFA-1); NKG2C, an immunoreceptor tyrosinebased activation motif (ITAM), CD27, CD134/OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD7, LIGHT, NKG2C, and any combinations thereof. In some embodiments, if a CAR comprises two or more costimulatory

domains, two costimulatory domains are different. In some embodiments, if a CAR comprises two or more costimulatory domains, two costimulatory domains are the same.

**[0764]** In other embodiments, the at least one signaling domain comprises (i) a 4-1BB domain, or functional variant thereof; and (ii) a CD3 zeta domain, or functional variant thereof. In other embodiments, the at least one signaling domain comprises (i) a CD28 domain, or functional variant thereof; and (ii) a CD3 zeta domain, or functional variant thereof. In other embodiments, the at least one signaling domain comprises (i) a CD28 domain, or functional variant thereof; and (ii) a 1.xxCD3 zeta domain, or functional variant thereof.

**[0765]** [0535] In some embodiments, the CAR comprises an extracellular antigen binding domain (e.g., antibody or antibody fragment, such as an scFv) that binds to an antigen (e.g., tumor antigen), a spacer (e.g., containing a hinge domain, such as any as described herein), a transmembrane domain (e.g., any as described herein), and one or more intracellular signaling domains (e.g., any intracellular signaling domain, such as a primary signaling domain or costimulatory signaling domain as described herein). In some embodiments, the one or more intracellular signaling domains is or comprises a primary cytoplasmic signaling domain. In some embodiments, the one or more intracellular signaling domains additionally comprises an intracellular signaling domain of a costimulatory molecule (e.g., a costimulatory domain). Any of such components can be any as described above.

**[0766]** Examples of exemplary signaling domain sequences are described in **Table 14**. In provided aspects, the sequences of each signaling domain in **Table 14** can be combined with any of the other CAR component sequences described here.

**Table 14. Exemplary Signaling Domain Sequences**

| Component | Sequence | SEQ ID NO: |
|---|---|---|
| 4-1BB costimulatory domain | KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRF PEEEEGGCEL | 422 |
| CD28 costimulatory domain | RSKRSRLLHSDYMNMTPRRPGPTRKHYQPY APPRDFAAYRS | 361 |
| CD3zeta signaling domain | RVKFSRSADAPAYQQGQNQLYNELNLGRREE YDVLDKRRGRDPEMGGKPRRKNPQEGLYNEL QKDKMAEAYSEIGMKGERRRGKGHDGLYQG LSTATKDTYDALHMQALPPR | 423 |
| CD3zeta (with Q to K mutation at position 14) | RVKFSRSADAPAYKQGQNQLYNELNLGRREE YDVLDKRRGRDPEMGGKPRRKNPQEGLYNEL QKDKMAEAYSEIGMKGERRRGKGHDGLYQG LSTATKDTYDALHMQALPPR | 488 |

| | | |
|---|---|---|
| 1.xxCD3zeta signaling domain | RVKFSRSADAPAYQQGQNQLYNELNLGRREE YDVLDKRRGRDPEMGGKPRRKNPQEGLFNEL QKDKMAEAFSEIGMKGERRRGKGHDGLFQGL STATKDTFDALHMQALPPR | 424 |

Signal Peptide

**[0767]** In certain embodiments, the CAR may further comprise one or more signal peptides. In certain embodiments, the CAR may comprise a signal peptide at the N-terminus. Non-limiting examples of signal peptides include CD8a signal peptide, IgK signal peptide, and granulocyte-macrophage colony-stimulating factor receptor subunit alpha (GMCSFR-a, also known as colony stimulating factor 2 receptor subunit alpha (CSF2RA)) signal peptide, and variants thereof, the amino acid sequences of which are provided in **Table 15** below.

**[0768]** Examples of exemplary signal peptide sequences are described in **Table 15**. In provided aspects, the sequences of each signal peptide in **Table 15** can be combined with any of the other CAR component sequences described here.

**Table 15. Exemplary Signal Peptide Sequences**

| Component | Sequence | SEQ ID NO: |
|---|---|---|
| CD8alpha signal peptide | MALPVTALLLPLALLLHAARP | 414 |
| IgK signal peptide | METDTLLLWVLLLWVPGSTG | 362 |
| GMCSFR-alpha (CSF2RA) signal peptide | MLLLVTSLLLCELPHPAFLLIP | 363 |

***Tolerogenic Factors***

**[0769]** In some embodiments, the cells are further modified to enable immune evasion. When transplanted in vivo without immunosuppression, immune evasive cells have reduced expression of one or more MHC class I molecules and/or MHC class II molecules and thus there may be no evidence of a systemic immune response against the transplanted cells from the recipient's immune system, such as no T cell activation, antibody production, or NK cell activity. Disclosure relating to edited cells is provided herein. Such disclosure is applicable to the methods, uses and cells described herein. In some embodiments, the cells are T cells (e.g., CAR-T cells).

**[0770]** One modification that may be desirable for the cell therapy product is that the engineered cells and populations thereof exhibit reduced expression of one or more MHC

class I molecules and/or MHC class II molecules. A further modification considered desirable for the cell therapy product is that the engineered cells and populations thereof exhibit increased expression of one or more tolerogenic factor.

**[0771]** Provided herein are methods and compositions for alleviating and/or evading the effects of immune system reactions to allogenic transplants. To overcome the problem of immune rejection of cell-derived and/or tissue transplants, disclosed herein is an engineered immune- evasive cell, or population or pharmaceutical composition thereof, that represents a viable source for any transplantable cell type. The engineered cells disclosed herein provide for reduced recognition the recipient subject's immune system, regardless of the subject's genetic make-up, or any existing response within the subject to one or more previous allogeneic transplants, previous autologous chimeric antigen receptor (CAR) T rejection, and/or other autologous or allogenic therapies wherein a transgene is expressed. The engineered cells may include, but are not limited to, T cells, NK cells, and blood cells.

**[0772]** Described here are engineered cells that comprise one or more modifications. In some embodiments, the provided engineered cells also contain a modification of one or more target polynucleotide sequences that regulates the expression of one or more MHC class I molecules, one or more MHC class II molecules, or one or more MHC class I molecules and one or more MHC class II molecules.

**[0773]** In some embodiments, the provided engineered cells also include a modification to increase expression of one or more tolerogenic factor. In some embodiments, the tolerogenic factor is one or more of A20/TNFAIP3, B2M-HLA-E, CD16, CD16 Fc receptor, CD24, CD27, CD35, CD39, CD46, CD47, CD52, CD55, CD59, CD64, CD200, CCL21, CCL22, CTLA4-Ig, Cl inhibitor, CR1, DUX4, FASL, HLA-C, HLA-E, HLA-E heavy chain, HLA-F, HLA-G, H2-M3, IDO1, IL-10, IL15-RF, IL-35, IL-39, MANF, Mfge8, PD-L1, Serpinb9, or any combination thereof.

***Safety Features***

Safety Switch

**[0774]** In some embodiments, the cells comprise a “suicide gene” or “suicide switch”. A suicide gene or suicide switch can be incorporated to function as a “safety switch” that can cause the death of the engineered cell (e.g., primary engineered cell or cell differentiated from an engineered pluripotent stem cell), such as after the engineered cell is administered to a subject and if they cells should grow and divide in an undesired manner. The “suicide gene” ablation approach includes a suicide gene in a gene transfer vector encoding a protein

that results in cell killing only when activated by a specific compound. A suicide gene may encode an enzyme that selectively converts a nontoxic compound into highly toxic metabolites. The result is specifically eliminating cells expressing the enzyme. In some embodiments, the suicide gene is the herpesvirus thymidine kinase (HSV-tk) gene, and the trigger is ganciclovir. In other embodiments, the suicide gene is the Escherichia coli cytosine deaminase (EC-CD) gene, and the trigger is 5 -fluorocytosine (5-FC) (Barese et al, Mol. Therap. 20(10): 1932-1943 (2012), Xu et al, Cell Res. 8:73-8 (1998), both incorporated herein by reference in their entirety).

**[0775]** In other embodiments, the suicide gene is an inducible Caspase protein. An inducible Caspase protein comprises at least a portion of a Caspase protein capable of inducing apoptosis. In some embodiments, the inducible Caspase protein is iCasp9. It comprises the sequence of the human FK506-binding protein, FKBP12, with an F36V mutation, connected through a series of amino acids to the gene encoding human caspase 9. FKBP12-F36V binds with high affinity to a small-molecule dimerizing agent, API 903. Thus, the suicide function of iCasp9 is triggered by the administration of a chemical inducer of dimerization (CID). I n some embodiments, the CID is the small molecule drug API 903. Dimerization causes the rapid induction of apoptosis. (See WO2011146862; Stasi et al, N. Engl. J. Med 365; 18 (2011); Tey et al, Biol. Blood Marrow Transplant. 13:913-924 (2007), each of which are incorporated by reference herein in their entirety.)

**[0776]** Inclusion of a safety switch or suicide gene allows for controlled killing of the cells in the event of cytotoxicity or other negative consequences to the recipient, thus increasing the safety of cell-based therapies, including those using the engineered polypeptides disclosed herein.

**[0777]** In some embodiments, a safety switch can be incorporated into, such as introduced into, the engineered cells provided herein to provide the ability to induce death or apoptosis of engineered cells containing the safety switch, for example if the cells grow and divide in an undesired manner or cause excessive toxicity to the host. Thus, the use of safety switches enables one to conditionally eliminate aberrant cells in vivo and may improve the use of certain cell therapies in the clinic. Safety switches and their uses thereof are described in, for example, Duzgune§, Origins of Suicide Gene Therapy (2019); Duzgune§ (eds), Suicide Gene Therapy. Methods in Molecular Biology, vol. 1895 (Humana Press, New York, NY) (for HSV-tk, cytosine deaminase, nitroreductase, purine nucleoside phosphorylase, and horseradish peroxidase); Zhou and Brenner, Exp Hematol 44(11): 1013-1019 (2016) (for iCaspase9); Wang et al., Blood 18(5): 1255-1263 (2001) (for huEGFR); U.S. Patent

Application Publication No. 20180002397 (for HERl); and Philip et al., Bloodl24(8): 1277-1287 (2014) (for RQR8).

**[0778]** In some embodiments, the safety switch can cause cell death in a controlled manner, for example, in the presence of a drug or prodrug or upon activation by a selective exogenous compound. In some embodiments, the safety switch is selected from the group consisting of herpes simplex virus thymidine kinase (HSV-tk), cytosine deaminase (CyD), nitroreductase (NTR), purine nucleoside phosphorylase (PNP), horseradish peroxidase, inducible caspase 9 (iCasp9), and rapamycin-activated caspase 9 (rapaCasp9).

**[0779]** In some embodiments, the safety switch may be a transgene encoding a product with cell killing capabilities when activated by a drug or prodrug, for example, by turning a nontoxic prodrug to a toxic metabolite inside the cell. In these embodiments, cell killing is activated by contacting an engineered cell with the drug or prodrug. In some cases, the safety switch is HSV- tk, which converts ganciclovir (GCV) to GCV-triphosphate, thereby interfering with DNA synthesis and killing dividing cells. In some cases, the safety switch is CyD or a variant thereof, which converts the antifungal drug 5 -fluorocytosine (5-FC) to cytotoxic 5 -fluorouracil (5-FU) by catalyzing the hydrolytic deamination of cytosine into uracil. 5-FU is further converted to potent anti-metabolites (5- FdUMP, 5-FdUTP, 5-FUTP) by cellular enzymes. These compounds inhibit thymidylate synthase and the production of RNA and DNA, resulting in cell death. In some cases, the safety switch is NTR or a variant thereof, which can act on the prodrug CB 1954 via reduction of the nitro groups to reactive N-hydroxylamine intermediates that are toxic in proliferating and nonproliferating cells. In some cases, the safety switch is PNP or a variant thereof, which can turn prodrug 6-methylpurine deoxyriboside or fludarabine into toxic metabolites to both proliferating and nonproliferating cells. In some cases, the safety switch is horseradish peroxidase or a variant thereof, which can catalyze indole-3 -acetic acid (IAA) to a potent cytotoxin and thus achieve cell killing.

**[0780]** In some embodiments, the safety switch may be an iCasp9. Caspase 9 is a component of the intrinsic mitochondrial apoptotic pathway which, under physiological conditions, is activated by the release of cytochrome C from damaged mitochondria. Activated caspase 9 then activates caspase 3, which triggers terminal effector molecules leading to apoptosis. The iCasp9 may be generated by fusing a truncated caspase 9 (without its physiological dimerization domain or caspase activation domain) to a FK506 binding protein (FKBP), FKBP12- F36V, via a peptide linker. The iCasp9 has low dimer-independent basal activity and can be stably expressed in host cells (e.g., human T cells)

without impairing their phenotype, function, or antigen specificity. However, in the presence of chemical inducer of dimerization (CID), such as rimiducid (API 903), AP20187, and rapamycin, iCasp9 can undergo inducible dimerization and activate the downstream caspase molecules, resulting in apoptosis of cells expressing the iCasp9. See, e.g., PCT Application Publication No. WO2011/146862; Stasi et al., N. Engl. J. Med. 365; 18 (2011); Tey et al., Biol. Blood Marrow Transplant 13:913-924 (2007). For example, the rapamycininducible caspase 9 variant is called rapaCasp9. See Stavrou et al., Mai. Ther. 26(5): 1266- 1276 (2018). Thus, iCasp9 can be used as a safety switch to achieve controlled killing of the host cells.

Graft Versus Host Disease (GVHD)

**[0781]** In some embodiments, the cells expressing the engineered polypeptide of the disclosure disclosed herein do not comprise a functional endogenous TCR. T cell therapies have shown clinical efficacy in bone marrow and organ transplantation, cancer immunotherapy, viral infections, and autoimmune diseases. Unfortunately, T cells may also engage in deleterious side effects. "On-target but off-tumor" adverse events have been reported in cancer immunotherapy clinical trials using both engineered T cell receptor (TCR) T cells and engineered chimeric antigen receptor (CAR) T cells. These include B cell aplasia in chronic lymphocytic leukemia patients treated with T cells expressing a CD19 CAR, fatal acute respiratory distress syndrome after anti-ERBB2 CAR T cell infusion thought to result from cross- reactivity on lung epithelium, and TCR-induced fatalities from cardiac myonecrosis or neurological toxicity incurred in patients treated with TCRs recognizing cancer-testis antigens. Similarly, the curative gains of donor lymphocyte infusion (DLI) in allogeneic bone marrow transplantation are hampered by the induction of both acute and chronic graft-versus-host disease (GVHD) and bone marrow aplasia.

**[0782]** In some embodiments, cells of the present disclosure comprise shRNA targeting TRAC, TRBC1, TRBC2, and/or CD3, thereby generating a cell that does not comprise a functional endogenous TCR. In some embodiments, cells of the present disclosure have been modified, using methods described herein, to eliminate expression of TRAC, TRBC1, TRBC2, and/or CD3, thereby generating a cell that does not comprise a functional endogenous TCR.

Depletion Tag

**[0783]** In some embodiments, the safety switch may be a membrane-expressed protein which allows for cell depletion after administration of a specific antibody to that protein.

Safety switches of this category may include, for example, one or more transgene encoding CCR4, CD16, CD19, CD20, CD30, EGFR, GD2, HER1, HER2, MUC1, PSMA, or RQR8 for surface expression thereof. These proteins may have surface epitopes that can be targeted by specific antibodies. In some embodiments, the safety switch comprises CCR4, which can be recognized by an anti-CCR4 antibody. Non-limiting examples of suitable anti-CCR4 antibodies include mogamulizumab and biosimilars thereof. In some embodiments, the safety switch comprises CD16 or CD30, which can be recognized by an anti-CD16 or anti-CD30 antibody. Non-limiting examples of such antiCD16 or anti-CD30 antibody include AFM13 and biosimilars thereof. In some embodiments, the safety switch comprises CD19, which can be recognized by an anti-CD19 antibody. Non-limiting examples of such anti-CD19 antibody include MOR208 and biosimilars thereof. In some embodiments, the safety switch comprises CD20, which can be recognized by an anti-CD20 antibody. Non-limiting examples of such anti-CD20 antibody include binutuzumab, ublituximab, ocaratuzumab, rituximab, rituximab-Rllb, and biosimilars thereof. Cells that express the safety switch are thus CD20-positive and can be targeted for killing through administration of an anti- CD20 antibody as described. In some embodiments, the safety switch comprises EGFR, which can be recognized by an anti-EGFR antibody. Non-limiting examples of such anti-EGFR antibody include tomuzotuximab, RO5083945 (GA201), cetuximab, and biosimilars thereof. In some embodiments, the safety switch comprises GD2, which can be recognized by an anti-GD2 antibody. Non-limiting examples of such anti-GD2 antibody include Hul4.18K322A, Hul4.18- IL2, Hu3F8, dinituximab, c.60C3-Rllc, and biosimilars thereof. In some embodiments, the safety switch may be an exogenously administered agent that recognizes one or more tolerogenic factor on the surface of the engineered cell. In some embodiments, the exogenously administered agent is an antibody directed against or specific to a tolerogenic agent (e.g., an anti-CD47 antibody). By recognizing and blocking a tolerogenic factor on the engineered cell, an exogenously administered antibody may block the immune inhibitory functions of the tolerogenic factor thereby re-sensitizing the immune system to the engineered cells. For instance, for an engineered cell that overexpresses CD47 an exogenously administered anti-CD47 antibody may be administered to the subject, resulting in masking of CD47 on the engineered cell and triggering of an immune response to the engineered cell.

Reduced Expression of Target genes

**[0784]** In some embodiments, knocking down (e.g., decreasing, eliminating, or inhibiting) gene expression can be achieved by RNA silencing or RNA interference (RNAi). Useful RNAi methods include those that utilize synthetic RNAi molecules, short interfering RNAs (siRNAs), PlWI-interacting NRAs (piRNAs), short hairpin RNAs (shRNAs), microRNAs (miRNAs), and other transient knock down methods recognized by those skilled in the art. Reagents for RNAi including sequence specific shRNAs, siRNA, miRNAs and the like are commercially available.

## X. COMPOSITIONS

**[0785]** In another aspect, the present disclosure provides a composition comprising the recombinant polypeptide of the disclosure. In another aspect, the present disclosure provides a composition comprising the engineered cells comprising the recombinant nucleic acid encoding the recombinant polypeptide of the disclosure, and/or a vector comprising the recombinant nucleic acid.

**[0786]** The disclosure additionally provides compositions comprising or consisting of a population of engineered cells, within which some portion of the engineered cells express a recombinant polypeptide of the disclosure. In some embodiments, at least at or about 70%, 75%, 80%, 85%, 90%, or 95% of the engineered cells, or of the total cells, in the composition express the recombinant polypeptide. In some embodiments, at least at or about 70%, 75%, 80%, 85%, 90%, or 95% of the engineered cells, or of the total cells, in the composition express the recombinant polypeptide. In some embodiments, at least at or about 70%, 75%, 80%, 85%, 90%, or 95% of the engineered cells, or of the total cells, in the composition do not express a gene product of an endogenous TRAC locus. In some embodiments, at least at or about 70%, 75%, 80%, 85%, 90%, or 95% of the engineered cells, or of the total cells, in the composition express a recombinant polypeptide disclosed herein; at least at or about 70%, 75%, 80%, 85%, 90%, or 95% of the engineered cells, or of the total cells, in the composition express the CAR or recombinant TCR; and/or at least at or about 70%, 75%, 80%, 85%, 90%, or 95% of the engineered cells, or of the total cells, in the composition do not express a gene product of an endogenous TRAC locus.

**[0787]** In some embodiments, the composition comprises CD4+ T cells and CD8+ T cells. In some embodiments of a composition comprising CD4+ T cells and CD8+ T cells, the percentage of CD4+ T cells in the composition is between at or about 20% and at or about

80%, or at or about 20%, 30%, 40%, 50%, 60%, 70%, or 80% of the total cells in the composition; and/or the percentage of CD8+ T cells in the composition is between at or about 20% and at or about 80%, or at or about 20%, 30%, 40%, 50%, 60%, 70%, or 80% of the total cells in the composition. In some embodiments, the ratio of CD4+ T cells to CD8+ T cells is from at or about 1:3 to at or about 3:1, optionally at or about 1:1

**[0788]** As used herein, the term "composition" includes, but is not limited to, a pharmaceutical composition. A "pharmaceutical composition" refers to an active pharmaceutical agent formulated in pharmaceutically acceptable or physiologically acceptable solutions for administration to a cell or an animal, either alone, or in combination with one or more other modalities of therapy. It will also be understood that, if desired, the compositions of the invention may be administered in combination with other agents, such as, e.g., cytokines, growth factors, hormones, small molecules, chemotherapeutics, pro-drugs, drugs, antibodies, or other various pharmaceutically active agents. There is virtually no limit to other components that may also be included in the compositions, provided that the additional agents do not adversely affect the ability of the composition to deliver the intended therapy. The phrase "pharmaceutically acceptable" is used herein to refer to those compounds, materials, compositions, and/or dosage forms which are, within the scope of sound medical judgment, suitable for use in contact with the tissues of human beings and animals without excessive toxicity, irritation, allergic response, or other problem or complication, commensurate with a reasonable benefit/risk ratio.

**[0789]** The compositions may also comprise a pharmaceutically acceptable carrier, diluent, or excipient. As used herein "pharmaceutically acceptable carrier, diluent, or excipient" includes, without limitation, any adjuvant, carrier, excipient, glidant, sweetening agent, diluent, preservative, dye/colorant, flavor enhancer, surfactant, wetting agent, dispersing agent, suspending agent, stabilizer, isotonic agent, solvent, surfactant, or emulsifier which has been approved by the United States Food and Drug Administration as being acceptable for use in humans or domestic animals. Exemplary pharmaceutically acceptable carriers include, but are not limited to, to sugars, such as lactose, glucose, and sucrose; starches, such as com starch and potato starch; cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose, and cellulose acetate; tragacanth; malt; gelatin; talc; cocoa butter; waxes; animal and vegetable fats; paraffins; silicones; bentonites; silicic acid; zinc oxide; oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil, and soybean oil; glycols, such as propylene glycol; polyols, such as glycerin, sorbitol, mannitol, and polyethylene glycol; esters, such as ethyl oleate, and ethyl laurate; agar; buffering

agents, such as magnesium hydroxide and aluminum hydroxide; alginic acid; pyrogen-free water; isotonic saline; Ringer's solution; ethyl alcohol; phosphate buffer solutions; and any other compatible substances employed in pharmaceutical formulations.

**[0790]** The liquid pharmaceutical compositions, whether they be solutions, suspensions or other like form, may include one or more of the following: sterile diluents such as water for injection, saline solution, preferably physiological saline; Ringers solution; isotonic sodium chloride; fixed oils such as synthetic mono or diglycerides which may serve as the solvent or suspending medium; polyethylene glycols; glycerin; propylene glycol or other solvents; antibacterial agents, such as benzyl alcohol or methyl paraben; antioxidants such as ascorbic acid or sodium bisulfite; chelating agents, such as ethylenediaminetetraacetic acid; buffers such as acetates, citrates, or phosphates; and agents for the adjustment of tonicity, such as sodium chloride or dextrose. The parenteral preparation can be enclosed in ampoules, disposable syringes, or multiple dose vials made of glass or plastic. An injectable pharmaceutical composition is preferably sterile.

**[0791]** The composition may be suitably developed for intravenous, intratumoral, oral, rectal, vaginal, parenteral, topical, pulmonary, intranasal, buccal, ophthalmic, or another route of administration.

**[0792]** In some embodiments, the recombinant nucleic acid constructs of the disclosure and recombinant cells of the disclosure can be incorporated into compositions, including pharmaceutical compositions. Such compositions generally include the recombinant nucleic acids, recombinant polypeptides, and/or recombinant cells of the disclosure, and a pharmaceutically acceptable excipient, e.g., carrier.

**[0793]** Pharmaceutical compositions suitable for injectable use include sterile aqueous solutions (where water soluble) or dispersions and sterile powders for the extemporaneous preparation of sterile injectable solutions or dispersion. For intravenous administration, suitable carriers include physiological saline, bacteriostatic water, Cremophor EL™. (BASF, Parsippany, N.J.), or phosphate buffered saline (PBS). In all cases, the composition should be sterile and should be fluid to the extent that easy syringability exists. It should be stable under the conditions of manufacture and storage and must be preserved against the contaminating action of microorganisms such as bacteria and fungi. The carrier can be a solvent or dispersion medium containing, for example, water, ethanol, polyol (for example, glycerol, propylene glycol, and liquid polyethylene glycol, and the like), and suitable mixtures thereof. The proper fluidity can be maintained, for example, by the use of a coating such as lecithin, by the maintenance of the required particle size in the case of dispersion and

by the use of surfactants, e.g., sodium dodecyl sulfate. Prevention of the action of microorganisms can be achieved by various antibacterial and antifungal agents, for example, parabens, chlorobutanol, phenol, ascorbic acid, thimerosal, and the like. In many cases, it will be generally to include isotonic agents, for example, sugars, polyalcohols such as mannitol, sorbitol, and sodium chloride in the composition. Prolonged absorption of the injectable compositions can be brought about by including in the composition an agent which delays absorption, for example, aluminum monostearate and gelatin.

**[0794]** Sterile injectable solutions can be prepared by incorporating the active compound in the required amount in an appropriate solvent with one or a combination of ingredients enumerated above, as required, followed by filtered sterilization. Generally, dispersions are prepared by incorporating the active compound into a sterile vehicle, which contains a basic dispersion medium and the required other ingredients from those enumerated above. In the case of sterile powders for the preparation of sterile injectable solutions, the preferred methods of preparation are vacuum drying and freeze-drying which yields a powder of the active ingredient plus any additional desired ingredient from a previously sterile-filtered solution thereof.

**[0795]** Systemic administration of the subject recombinant nucleic acid constructs and recombinant cells of the disclosure can also be by transmucosal or transdermal means. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration, detergents, bile salts, and fusidic acid derivatives. Transmucosal administration can be accomplished through the use of nasal sprays or suppositories. For transdermal administration, the active compounds are formulated into ointments, salves, gels, or creams as generally known in the art.

**[0796]** In some embodiments, the recombinant nucleic acid constructs and recombinant cells of the disclosure can also be administered by transfection or infection using methods known in the art, including but not limited to the methods described in McCaffrey et al. (Nature 418:6893, 2002), Xia et al. (Nature Biotechnol. 20: 1006-1010, 2002), or Putnam (Am. J. Health Syst. Pharm. 53: 151-160, 1996, erratum at Am. J. Health Syst. Pharm. 53:325, 1996).

**[0797]** In some embodiments, the recombinant nucleic acid constructs and recombinant cells of the disclosure are prepared with carriers that will protect the recombinant polypeptides against rapid elimination from the body, such as a controlled release formulation, including implants and microencapsulated delivery systems. Biodegradable, biocompatible polymers

can be used, such as ethylene vinyl acetate, polyanhydrides, polyglycolic acid, collagen, polyorthoesters, and polylactic acid. Such formulations can be prepared using standard techniques. The materials can also be obtained commercially from Alza Corporation and Nova Pharmaceuticals, Inc. Liposomal suspensions (including liposomes targeted to infected cells with monoclonal antibodies to viral antigens) can also be used as pharmaceutically acceptable carriers. These can be prepared according to methods known to those skilled in the art, for example, as described in U.S. Pat. No. 4,522,811. As described in greater detail below, the polypeptides of the present disclosure may also be modified to achieve extended duration of action such as by PEGylation, acylation, Fc fusions, linkage to molecules such as albumin, etc. In some embodiments, the recombinant polypeptides can be further modified to prolong their half-life in vivo and/or ex vivo. Non-limiting examples of known strategies and methodologies suitable for modifying the recombinant polypeptides of the disclosure include (1) chemical modification of a recombinant polypeptide described herein with highly soluble macromolecules such as polyethylene glycol ("PEG") which prevents the recombinant polypeptides from contacting with proteases; and (2) covalently linking or conjugating a recombinant polypeptide described herein with a stable protein such as, for example, albumin. Accordingly, in some embodiments, the polypeptides of the disclosure can be fused to a stable protein, such as, albumin. For example, human albumin is known as one of the most effective proteins for enhancing the stability of polypeptides fused thereto and there are many such fusion proteins reported.

**[0798]** In some embodiments, the pharmaceutical compositions of the disclosure include one or more PEGylation reagents. In some embodiments, the PEGylation reagent is selected from methoxy polyethylene glycol-succinimidyl propionate (mPEG-SPA), mPEG-succinimidyl butyrate (mPEG-SBA), mPEG-succinimidyl succinate (mPEG-SS), mPEG-succinimidyl carbonate (mPEG-SC), mPEG-Succinimidyl Glutarate (mPEG-SG), mPEG-N-hydroxyl-succinimide (mPEG-NHS), mPEG-tresylate and mPEG-aldehyde. In some embodiments, the PEGylation reagent is polyethylene glycol. In some embodiments, the PEGylation reagent is polyethylene glycol with an average molecular weight of 20 kD covalently bound to the N-terminal methionine residue of the recombinant polypeptides of the disclosure., or about 80 kD covalently bound to the N-terminal methionine residue of the polypeptides of the disclosure. In some embodiments, the PEGylation reagent is polyethylene glycol with an average molecular weight of about 40 kD covalently bound to the N-terminal methionine residue of the polypeptides of the disclosure.

**[0799]** Accordingly, in some embodiments, recombinant nucleic acid constructs and recombinant cells of the disclosure are chemically modified with one or more polyethylene glycol moieties, e.g., PEGylated; or with similar modifications, e.g. PASylated. In some embodiments, the PEG molecule or PAS molecule is conjugated to one or more amino acid side chains of the disclosed recombinant polypeptide. In some embodiments, the PEGylated or PASylated polypeptide contains a PEG or PAS moiety on only one amino acid. In other embodiments, the PEGylated or PASylated polypeptide contains a PEG or PAS moiety on two or more amino acids, e.g., attached to two or more, five or more, ten or more, fifteen or more, or twenty or more different amino acid residues. In some embodiments, the PEG or PAS chain is 2000, greater than 2000, 5000, greater than 5,000, 10,000, greater than 10,000, greater than 10,000, 20,000, greater than 20,000, and 30,000 Da. The PASylated polypeptide may be coupled directly to PEG or PAS (e.g., without a linking group) through an amino group, a sulfhydryl group, a hydroxyl group, or a carboxyl group. In some embodiments, the recombinant polypeptide of the disclosure is covalently bound to a polyethylene glycol with an average molecular weight of 20,000 Daltons. In some embodiments, the recombinant polypeptide of the disclosure is covalently bound to a polyethylene glycol with an average molecular weight ranging from about 1 kD to about 200 kD such as, e.g., about 10 kD to about 150 kD, about 50 kD to about 100 kD, about 5 kD to about 100 kD, about 20 kD to about 80 kD, about 30 kD to about 70 kD, about 40 kD to about 60 kD, about 50 kD to about 100 kD, about 100 kD to about 200 kD, or about 1 150 kD to about 200 kD. In some embodiments, the recombinant polypeptide of the disclosure is covalently bound to a polyethylene glycol with an average molecular weight of about 5 kD, about 10 kD, about 20 kD, about 30 kD, about 40 kD, about 50 kD, about 60 kD, about 70 kD, or about 80 kD. In some embodiments, the recombinant polypeptide of the disclosure is covalently bound to a polyethylene glycol with an average molecular weight of about 40 kD.

## XI. KITS, COMPONENTS, AND ARTICLES OF MANUFACTURE

**[0800]** Systems or kits of the present disclosure include one or more of any of the recombinant nucleic acids, recombinant polypeptides, engineered cells, or pharmaceutical compositions disclosed herein as well as syringes (including pre-filled syringes) and/or catheters (including pre-filled syringes) used to administer any of the recombinant nucleic acids, recombinant polypeptides, engineered cells, or pharmaceutical compositions to a subject. The kits also include written instructions for using of any of the recombinant nucleic

acids, recombinant polypeptides, engineered cells, or pharmaceutical compositions disclosed herein as well as syringes and/or catheters for use with their administration.

**[0801]** Any of the above-described systems and kits can further include one or more additional reagents, where such additional reagents can be selected from: dilution buffers; reconstitution solutions, wash buffers, control reagents, control expression vectors, negative control polypeptides, positive control polypeptides, reagents for *in vitro* production of the chimeric receptor polypeptides.

**[0802]** In some embodiments, the components of a system or kit can be in separate containers. In some other embodiments, the components of a system or kit can be combined in a single container.

**[0803]** In some embodiments, a system or kit can further include instructions for using the components of the kit to practice the methods. The instructions for practicing the methods are generally recorded on a suitable recording medium. For example, the instructions can be printed on a substrate, such as paper or plastic, etc. The instructions can be present in the kits as a package insert, in the labeling of the container of the kit or components thereof (i.e., associated with the packaging or sub-packaging), etc. The instructions can be present as an electronic storage data file present on a suitable computer readable storage medium, e.g. CD-ROM, diskette, flash drive, etc. In some instances, the actual instructions are not present in the kit, but means for obtaining the instructions from a remote source (e.g., via the internet), can be provided. An example of this embodiment is a kit that includes a web address where the instructions can be viewed and/or from which the instructions can be downloaded. As with the instructions, this means for obtaining the instructions can be recorded on a suitable substrate.

## XII. METHODS OF THE DISCLOSURE

**[0804]** The disclosure also relates to methods including the recombinant polypeptides, recombinant nucleic acids, vectors, particles, or the cells of the disclosure.

### *Methods for Preparing T cells for Use in Cell Therapy*

**[0805]** The disclosure also provides methods for preparing an immune cell (*e.g.*, a T cell) for use in cell therapy. The immune cell (*e.g.,* T cell) can further comprise a CAR, a TCR, and a transcriptional receptor. The T cell can be an NK cell or a tumor infiltrating lymphocyte from a patient with cancer. Preparing the cell can include introducing (e.g., by transducing)

into a cell any one or more of the recombinant nucleic acid constructs of the disclosure. Preparing the cell can include introducing (e.g., by transducing) into a cell any one or more vectors comprising any one or more of the recombinant nucleic acid constructs of the disclosure. Preparing the cell can include contacting the cell with any one or more particles comprising any one or more of the recombinant nucleic acids of the disclosure. Preparing the cell can include introducing to the cell any one or more of the recombinant nucleic acids of the disclosure. Preparing the cell can include expressing in the cell any one or more of the polypeptides of the disclosure. The cell can be genetically modified for expression of the polypeptide.

**[0806]** In some embodiments, the cell is in vivo. In some embodiments, the cell is prepared in vivo. In some embodiments, the cell is prepared in the body of a subject in need of treatment.

**[0807]** In some embodiments, the recombinant polypeptide is expressed in the cell, and/or the nucleic acid construct is introduced into the cell, by contacting the cell with a vector or a particle. In some embodiments, the vector is a plasmid, a synthetic DNA vector, a linear DNA vector, a closed linear DNA vector, a phagemid vector, an RNA vector, an mRNA vector, or a viral vector. In some embodiments, the viral vector is selected from a retrovirus vector, an adenovirus vector, and an adeno-associated virus (AAV) vector. In some embodiments, the retrovirus is a lentivirus, optionally a VSV-G pseudotyped lentivirus. In some embodiments, the AAV vector is an AAV6 vector or an AAV9 vector. In some embodiments, the vector is a fusosome, a lentiviral particle engineered with the anti-CD3 Cocal glycoprotein, or an enveloped delivery vehicle. In some embodiments, the vector is a self-replicating RNA virus, an mRNA-packaging virus-like particle, or a ribonucleoprotein (RNP)-packaging virus-like particle.

**[0808]** In some embodiments, the particle is a lipid nanoparticle (LNP), a selective organ targeting (SORT) LNP, or an antibody targeted LNP. In some embodiments, the LNP comprises: (i) an ionizable lipid (e.g., an amino lipid), (ii) a sterol or other structural lipid, (iii) a non-cationic helper lipid or phospholipid, and (iv) a PEG-lipid (e.g., a PEG-modified lipid). In some embodiments, the particle is a polymer nanoparticle or a protein nanoparticle.

**[0809]** In some embodiments, the cell is *ex vivo*. In some embodiments, the cell is prepared *ex vivo*. In some embodiments, the cell is is prepared outside of the body of a subject in need of treatment. In some embodiments, the nucleic acid construct is introduced into the cell by electroporation or transfection. In some embodiments, the transfection is lipofection. In some embodiments, the nucleic acid construct is a plasmid, a synthetic DNA vector, a linear

DNA vector, a closed linear DNA vector, a phagemid vector, an RNA vector, or an mRNA vector. In some embodiments, the nucleic acid construct is introduced via a viral vector. In some embodiments, the viral vector is selected from a retrovirus vector, an adenovirus vector, and an adeno-associated virus (AAV) vector. In some embodiments, the retrovirus is a lentivirus, optionally a VSV-G pseudotyped lentivirus. In some embodiments, the AAV vector is an AAV6 vector or an AAV9 vector.

**[0810]** In some embodiments, the method comprises contacting the cell with an AAV vector comprising a recombinant nucleic acid encoding a recombinant polypeptide of the disclosure.

**[0811]** In some embodiments, the method comprises contacting the cell with a particle comprising a recombinant nucleic acid encoding a recombinant polypeptide of the disclosure; optionally, wherein the particle is a lipid nanoparticle (LNP).

**[0812]** In some embodiments, the method comprises genetically modifying the cell for expression of the recombinant polypeptide. In some embodiments, the method comprises introducing into the cell one or more nucleic acids encoding a sequence specific nuclease or a nucleic acid programmable DNA binding protein. In some embodiments, the method additionally comprises introducing into the cell one or more guide RNAs.

**[0813] [0870]** In some embodiments, the sequence-specific nuclease is an RNA guided nuclease. In some embodiments, the equence-specific nuclease or nucleic acid programmable DNA binding domain is a homing endonuclease, a zinc finger nuclease (ZF) nucleic acid binding entity, a transcription activator-like effector (TALE) nucleic acid binding entity, a meganuclease, a Cas nuclease, a core Cas protein, a homing endonuclease, an endonuclease-deficient-Cas protein, an enzymatically inactive Cas protein, a CRISPR-associated transposase (CAST), a Type II or Type V Cas protein, or a functional portion thereof.

**[0814]** In some embodiments, the Cas nuclease is Casl, Cas2, Cas3, Cas4, Cas5, Cas6, Cas7, Cas8a, Cas8b, Cas8c, Cas9, CaslO, Casl2, Casl2a (Cpfl), Casl2b (C2cl), Casl2c (C2c3), Casl 2d (CasY), Casl2e (CasX), Casl2f (C2cl0), Cas 12g, Casl2h, Casl2i, Cas 12k (C2c5), Casl3, Casl3a (C2c2), Casl3b, Casl3c, Casl3d, C2c4, C2c8, C2c9, Cmrl, Cmr2, Cmr3, Cmr4, Cmr5, Cmr6, Csdl, Csd2, Cas5d, Csel, Cse2, Cse3, Cse4, Cas5e, Csfl, Csml, Csm2, Csm3, Csm4, Csm5, Csnl, Csn2, Cstl, Cst2, Cas5t, Cshl, Csh2, Cas5h, Csal, Csa2, Csa3, Csa4, Csa5, Cas5a, CsxlO, Csxl l, Csyl, Csy2, Csy3, Csy4, Mad7, SpCas9, eSpCas9, SpCas9-HFl, HypaSpCas9, HeFSpCas9, and evoSpCas9 high-fidelity variants of SpCas9, SaCas9, NmeCas9, CjCas9, StCas9, TdCas9, LbCasl2a, AsCasl2a, AacCasl2b, BhCasl2b v4, TnpB,

dCas (D10A), dCas (H840A), dCasl3a, dCasl3b, or a functional portion thereof . In some embodiments, the RNA-guided nuclease is a Life Edit nuclease (LEG), optionally a LEG14 nuclease.

**[0815]** In some embodiments, the method comprises introducing into the cell a ribonucleoprotein (RNP) complex comprising the sequence-specific nuclease or nucleic acid programmable DNA binding. In some embodiments, introducing the nucleic acid construct comprises contacting the cell with a particle or a vector disclosed herein. In some embodiments, the nucleic acid construct is introduced via electroporation. In some embodiments, the nucleic acid construct is introduced via AAV. In some embodiments, the nucleic acid construct is introduced via lentivirus, optionally a VSV-G pseudotyped lentivirus. In some embodiments, the nucleic acid construct is introduced via LNP.

**[0816]** In some embodiments, the nucleic acid construct is inserted into an endogenous locus of the cell. In some embodiments, the endogenous locus is or comprises a TRAC gene locus, a TRBC1 gene locus, or a TRBC2 gene locus. In some embodiments, the endogenous locus is or comprises a TRAC gene locus. In some embodiments, the endogenous locus is or comprises a safe harbor locus. In some embodiments, the safe harbor locus is an AAVS1, ABO, CCR5, CLYBL, CXCR4, F3, FUT1, HMGB1, KDM5D, LRP1, MICA, MICB, RHD, ROSA26, or SHS231 locus. In some embodiments, the irst insertion site is an exon. In some embodiments, first insertion site is an intron. In some embodiments, the first insertion site is between an intron and an exon. In some embodiments, the first insertion site is in a regulatory region.

**[0817]** In some embodiments, the first insertion site is 25 nucleotides or less from a protospacer adjacent motif (PAM) sequence, wherein the PAM sequence is ngg, nag, ngrrt, ngrm, nnnngatt, nnnnryac, nnagaaw, naaaac, tttv, ttn, attn, tttn, gttn, or yttn and wherein: (i) r = a or g, (ii) y = c or t, (iii) w = a or t, (iv) v = a or c or g, and (v) n = a, c, t, or g.

**[0818]** In some embodiments, the cell produced by the method comprises a modified TRAC gene locus. In some embodiments, the cell does not express an endogenous TCR.

In some embodiments, the method further comprises introducing a gene editing machinery into the cell. In some embodiments, the step of introducing comprises homology-directed repair (HDR)-mediated insertion using the gene editing machinery. In some embodiments, the HDR occurs in 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 95%, at least 98%, or at least 99%, of a population of the cells; optionally, wherein the HDR occurs in between about 20%-30%, about 30%-40%, about 40%-50%, about 50%-60%, about 60%-70%, about 70%-80%, about

80%-90%, or about 90%-100%, of the cells. In some embodiments, the step of introducing comprises HDR-mediated insertion in the absence of an HDR enhancer; optionally, wherein the HDR enhancer is M3814 or AZD7648.

**[0819]** In some embodiments, the nucleic acid construct comprises a first homology arm before the 5' end of the nucleic acid sequence encoding the recombinant polypeptide and a second homology arm after the 3' end of the nucleic acid sequence encoding the recombinant polypeptide, as described herein. In some embodiments, the nucleic acid construct comprises, from 5' to 3', the first homology arm, a promoter, a CAR encoding sequence, a polynucleotide sequence encoding the recombinant polypeptide, and the second homology arm. In some embodiments, the nucleic acid construct comprises, from 5' to 3', the first homology arm, a cleavable linker encoding sequence, a CAR encoding sequence, a polynucleotide sequence encoding the recombinant polypeptide, and the second homology arm. In some embodiments, the cleavable linker comprises a 2A family (e.g., P2A, E2A, F2A, or T2A) self-cleaving peptide. In some embodiments, the nucleic acid construct comprises, from 5' to 3', the first homology arm, a promoter, a polynucleotide sequence encoding the recombinant polypeptide, a CAR encoding sequence, and the second homology arm. In some embodiments, the nucleic acid construct comprises, from 5' to 3', the first homology arm, a cleavable linker encoding sequence, a polynucleotide sequence encoding the recombinant polypeptide, a CAR encoding sequence, and the second homology arm; optionally, wherein the cleavable linker comprises a 2A family (e.g., P2A, E2A, F2A, or T2A) self-cleaving peptide.

**[0820]** In some embodiments, the gene editing machinery is CRISPR/Cas9. In some embodiments, the gene editing machinery is prime-editing assisted site-specific integrase gene editing (PASSIGE) system.

**[0821]** In some embodiments, the gene editing machinery is introduced via electroporation. In some embodiments, the nucleic acid construct is introduced via AAV. In some embodiments, the nucleic acid construct is introduced via LNP. In some embodiments, the method thereby produces an engineered cell comprising: (i) the recombinant polypeptide; (ii) the engineered receptor (e.g., CAR); and (iii) the modified TRAC gene locus. In some embodiments, the cell is for use in a cell therapy. In some embodiments, the method thereby produces an engineered cell comprising: (i) the recombinant polypeptide; (ii) the immune receptor (e.g., CAR); and (iii) the modified TRAJ intron splice acceptor locus.

**[0822]** The method can further comprise expressing an engineered immune receptor that binds to an antign target in a target cell. In some embodiments, expressing the immune receptor comprises administering a nucleic acid construct encoding the immune receptor to the cell. In some embodiments, the nucleic construct is an RNA construct. In some embodiments, the immune receptor and the recombinant polypeptide are encoded by the same nucleic acid construct. In some embodiments, the immune receptor and the recombinant polypeptide are encoded by different nucleic acid constructs. In some embodiments, expressing the immune receptor comprises introducing to the cell a nucleic acid construct encoding the immune receptor. In some embodiments, the nucleic acid construct encoding the immune receptor is introduced via a viral vector. In some embodiments, activating the immune receptor results in the secretion of IFNg by the cell at a level that is at least 10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold higher than the IFNg secretion level of a control cell lacking the recombinant polypeptide. In some embodiments, activating the immune receptor results in the secretion of IL-2 by the cell at a level that is at least 10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold higher than the IL-2 secretion level of a control cell lacking the recombinant polypeptide.

**[0823]** In some embodiments, the cells can be transduced with a recombinant nucleic acid construct that can alter T cell signaling via the NFAT pathway, NF-κB pathway, AP-1 pathway, JAK/STAT pathway, RAS/MEK/ERK, phospholipase gamma signaling or other T cell signaling pathways.

**[0824]** The methods of preparing an immune cell (*e.g.,* T cell) according to the disclosure can include transducing immune cells with recombinant nucleic acid constructs capable of enhancing or promoting or improving or reducing or regulating or modulating the pathway or activating or increasing or suppressing or inhibiting or other means of changing the immune cell signaling. The methods of preparing an immune *(e.g,* T cell) according to the disclosure can include transducing cells with recombinant nucleic acid constructs that can alter CARD11-BCL10-MALT1 complex signaling, co-stimulatory molecule signaling, and cytokine production and/or transcription factor activity in the immune cell (*e.g.,* T cell).

**[0825]** In an embodiment, the method of preparing an immune cell (*e.g.,* T cell) for use in a cell therapy includes transducing the immune cell with a recombinant nucleic acid construct having a mutation capable of altering (i) immune cell signaling through NFAT, NF-κB

and/or AP-1 pathways, (ii) cytokine production, and/or (iii) *in vivo* persistence of immune cells in tumors.

**[0826]** In some embodiments of the methods of preparing an immune cell (*e.g.,* T cell) for use in a cell therapy includes polypeptides and/or recombinant nucleic acid constructs that can alter *in vivo* persistence in tumors of therapeutic immune cells including the mutation. In some embodiments, the recombinant nucleic acid constructs of the disclosure can alter the therapeutic efficacy, decrease immune cell exhaustion, increase proliferative capacity, enhance anti-tumor effect, enhance the replicative lifespan, decrease replicative senescence, and enhance ability to kill, enhance the fitness of engineered immune cells and/or other functions or activities of immune cells.

**[0827]** In some embodiments, the polypeptides or recombinant nucleic acid constructs of the disclosure can alter the *in vivo* persistence in tumors, *in vivo* accumulation in tumors, or the other functions or activities of immune cells described herein, through a mutation in one or more of the genes described herein. In some embodiments, the genes include caspase recruitment domain family member 11 (CARD11). In some embodiments, the genes include capping protein regulator and myosin 1 linker 2 (CARMIL2), mucosa-associated lymphoid tissue lymphoma translocation protein 1 (MALT1), B-cell lymphoma 6 (BCL6), B-cell lymphoma 10 (BCL10) and MYCN. The gene can be a signal transducer and activator of transcription gene (STAT) including STAT3, STAT5B. The gene can be a janus kinase gene such as JAK1, JAK2, or JAK3 gene. The gene can be v-raf murine sarcoma viral oncogene homolog B1 (BRAF). The gene can be RAS Guanyl Releasing Protein 1 (RASGRP1). The gene can be Tumor necrosis factor receptor superfamily member 1B (TNFRSF1B). The gene can be phospholipase C gamma 1 (PLCG1) gene. The gene can be nuclear factor kappa light chain enhancer of activated B cells (NF-κB1), or (NF-κB2) or JunB Proto-Oncogene, AP-1 Transcription Factor Subunit (JUNB) gene.

***Methods of Treating a Subject***

**[0828]** The disclosure also provides methods of treating a subject in need of cell therapy, comprising administering to the subject a cell (e.g., T-cell) as described herein or a cell (e.g., T-cell) prepared by any of the methods described herein. The disclosure also provides methods for enhancing the *in vivo* persistence of a cell (*e.g.*, a T cell, or specifically a therapeutic T cell) in a subject in need thereof, by administering to the subject a therapeutically effective amount of the cells of the disclosure. The cells can include any of the polypeptides and/or recombinant nucleic acid constructs of the disclosure.

**[0829]** In some embodiments, the method comprises, before the administration, generating engineered cells according to a method of preparing an engineered cell described herein. In some embodiments, the engineered cells are derived from immune cells: (i) obtained and/or isolated from the subject, (ii) obtained and/or isolated from one or more donors, and/or (iii) derived from one or more stem cells.

**[0830]** The disclosure also provides methods of treating cancer or an autoimmune disease in a subject in need thereof, comprising administering a therapeutically effective number of the engineered cells disclosed herein.

**[0831]** The cancer may be any kind of cancer known in the art. In some embodiments, the cancer is a hematological cancer. Non-limiting examples of hematological cancer include myeloid neoplasm, myelodysplastic syndromes (MDS), myeloproliferative/myelodysplastic syndromes, acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), chronic myelogenous leukemia (CML), blast crisis chronic myelogenous leukemia (bcCML), B-cell acute lymphoid leukemia (B-ALL), T-cell acute lymphoid leukemia (T-ALL), T-cell lymphoma, or B-cell lymphoma. In some embodiments, the cancer is a solid tumor. Non-limiting examples of a solid tumor include small cell lung cancer, colorectal cancer, testicular cancer, ovarian cancer, melanoma, lymphoma, leukemia, multiple myeloma, prostate cancer, breast cancer, non-small cell lung cancer, gastric cancer, esophageal cancer, liver cancer, kidney cancer, head & neck cancer, glioblastoma, neuroblastoma, soft tissue sarcoma, uterine cancer, brain cancer, skin cancer, renal cancer, bladder cancer, pancreatic cancer, thyroid cancer, eye cancer, gastrointestinal cancer, carcinoma, or sarcoma.

**[0832]** The cancer may express one or more cancer-associated antigen. Non-limiting examples of cancer-associated antigens include CD1, CD1a, CD1b, CD1c, CD1d, CD1e, CD2, CD3d, CD3e, CD3g, CD3ε, CD4, CD5, CD7, CD8a, CD8b, CD19, CD20, CD21, CD22, CD23, CD24, CD25, CD27, CD28, CD30, CD33, CD34, CD38, CD40, CD44v6, CD45, CD46, CD47 CD48, CD52, CD59, CD66, CD70, CD71, CD72, CD73, CD79A, CD79B, CD80 (B7.1), CD86 (B7.2), CD94, CD95, CD97, CD123, CD134, CD140 (PDGFR4), CD152, CD154, CD158, CD171, CD178, CD179, CD179a, CD181 (CXCR1), CD182 (CXCR2), CD183 (CXCR3), CD210, CD213A2, CD246, CD252, CD253, CD261, CD262, CD272, CD273 (PD-L2), CD274 (PD-L1), CD276 (B7H3), CD279, CD295, CD339 (JAG1), CD340 (HER2), CDH17, CEA, CLECL1, CLL-1, CLDN6, CLDN18.2, CS1, DLL3, LY6G6D, GCC, p53R175H, PRAME, EGFR, EGFRvIII, FGFR2, AFP, CA125, MUC-1, MAGE, ALPI, alkaline phosphatase placental-like 2 (ALPPL2), B-cell maturation

antigen (BCMA), green fluorescent protein (GFP), enhanced green fluorescent protein (eGFP), KLK2, KLK3, Mesothelin, IL13Ra2, ggcc, signal regulatory protein α (SIRPα), TCRalpha, TCRbeta, TSHR, GD2, GD3, Tn Ag, cMET, Axl, ROR1, ROR2, GPC1, GPC2, GPC3, FLT3, TAG72, CEA, EPCAM, KIT (CD117), IL-13Ra2, IL-11Ra, PSCA, PRSS21, VEGFR2, LewisY, PDGFRβ, SSEA-4, folate receptor alpha, ERBB2 (Her2/neu), MUC1, MUC16, NCAM, prostase, PAP, ELF2M, Ephrin B2, IGF-I receptor, CAIX, LMP2, gp100, bcr-abl, tyrosinase, EphA2, STEAP1, STEAP2, fucosyl GM1, sLe, GM3, TGS5, HMWMAA, o-acetyl-GD2, folate receptor beta, TEM1/CD248, TEM7R, ROPN1, GPRC5D, GPA33, CXORF61, ALK, Polysialic acid, PLAC1, GloboH, NY-BR-1, UPK2, HAVCR1, ADRB3, PANX3, GPR20, LY6K, OR51E2, TARP, WT1, NY-ESO-1, LAGE-la, MAGE-Al, legumain, HPV E6,E7, MAGE-A4, ETV6-AML, sperm protein 17, XAGE1, Tie 2, MAD-CT-1, MAD-CT-2, Fos-related antigen 1, p53, p53 mutant, p53R175H, KRAS, mutant KRAS, KRAS G12D, prostein, surviving, telomerase, PCTA-1/Galectin 8, MelanA/MART1, Ras mutant, hTERT, sarcoma translocation breakpoints, ML-IAP, ERG (TMPRSS2 ETS fusion gene), NA17, PAX3, androgen receptor, cyclin Bl, MYCN, RhoC, TRP-2, CYP1B1, BORIS, SART3, PAX5, OY-TES1, LCK, AKAP-4, SSX2, RAGE-1, human telomerase reverse transcriptase, RU1, RU2, intestinal carboxyl esterase, mut hsp70-2,LAIR1, FCAR, LILRA2, CD300LF, CLEC12A, BST2, EMR2, LY75, FCRL5, IGLL1, PSMA, TROP2, citrullinated vimentin, the extracellular portion of the APRIL protein, or any combination thereof. In some embodiments, the cancer expresses B7H3, BCMA, CD19, CD20, CD22, CD70, CD79a, CD79b, CDH17, CLDN6, CLDN18.2, DLL3, GCC, GD2, GD3, GPC3, GPRC5D, GPA33, KLK3, LY6G6D, p53R175H, PRAME, ROPN1, STEAP1, or STEAP2.

**[0833]** This administering step can be accomplished using any method of implantation delivery in the art. For example, the engineered cells of the disclosure can be infused directly in the individual's bloodstream or otherwise administered to the individual.

**[0834]** In some embodiments, the methods disclosed herein include administering which term is used interchangeably with the terms "introducing," implanting," and "transplanting," engineered cells into an individual, by a method or route that results in at least partial localization of the introduced cells at a desired site such that a desired effect(s) is/are produced. The engineered cells or their differentiated progeny can be administered by any appropriate route that results in delivery to a desired location in the individual where at least a portion of the administered cells or components of the cells remain viable. The period of viability of the cells after administration to an individual can be as short as a few hours, e.g.,

twenty-four hours, to a few days, to as long as several years, or even the lifetime of the individual, i.e., long-term engraftment.

**[0835]** When provided prophylactically, the engineered cells described herein can be administered to an individual in advance of any symptom of a disease or condition to be treated. Accordingly, in some embodiments the prophylactic administration of an engineered cell population prevents the occurrence of symptoms of the disease or condition.

**[0836]** When provided therapeutically in some embodiments, engineered cells are provided at (or after) the onset of a symptom or indication of a disease or condition, e.g., upon the onset of disease or condition.

**[0837]** For use in the various embodiments described herein, an effective amount of engineered cells as disclosed herein, can be at least $10^2$ cells, at least $5 \times 10^2$ cells, at least $10^3$ cells, at least $5 \times 10^3$ cells, at least $10^4$ cells, at least $5 \times 10^4$ cells, at least $10^5$ cells, at least $2 \times 10^5$ cells, at least $3 \times 10^5$ cells, at least $4 \times 10^5$ cells, at least $5 \times 10^5$ cells, at least $6 \times 10^5$ cells, at least $7 \times 10^5$ cells, at least $8 \times 10^5$ cells, at least $9 \times 10^5$ cells, at least $1 \times 10^6$ cells, at least $2 \times 10^6$ cells, at least $3 \times 10^6$ cells, at least $4 \times 10^6$ cells, at least $5 \times 10^6$ cells, at least $6 \times 10^6$ cells, at least $7 \times 10^6$ cells, at least $8 \times 10^6$ cells, at least $9 \times 10^6$ cells, or multiples thereof. The engineered cells can be derived from one or more donors or can be obtained from an autologous source. In some embodiments, the engineered cells are expanded in culture prior to administration to an individual in need thereof.

**[0838]** In some embodiments, the delivery of an engineered cell composition (e.g., a composition including a plurality of engineered cells according to any of the cells described herein) into an individual by a method or route results in at least partial localization of the cell composition at a desired site. A composition including engineered cells can be administered by any appropriate route that results in effective treatment in the individual, e.g., administration results in delivery to a desired location in the individual where at least a portion of the composition delivered, e.g., at least $1 \times 10^4$ cells, is delivered to the desired site for a period of time. Modes of administration include injection, infusion, and instillation. "Injection" includes, without limitation, intravenous, intramuscular, intra-arterial, intrathecal, intraventricular, intracapsular, intraorbital, intracardiac, intradermal, intraperitoneal, transtracheal, subcutaneous, subcuticular, intraarticular, subcapsular, subarachnoid, intraspinal, intracerebrospinal, and intrasternal injection and infusion. In some embodiments, the route is intravenous. For the delivery of cells, delivery by injection or infusion is a preferred mode of administration.

**[0839]** In some embodiments, the engineered cells are administered systemically, e.g., via infusion or injection. For example, a population of engineered cells are administered other than directly into a target site, tissue, or organ, such that it enters the individual's circulatory system and, thus, is subject to metabolism and other similar biological processes.

**[0840]** The efficacy of a treatment including any of the compositions provided herein for the treatment of a disease or condition can be determined by a skilled clinician. However, one skilled in the art will appreciate that a treatment is considered effective if any one or all of the signs or symptoms or markers of disease are improved or ameliorated. Efficacy can also be measured by failure of an individual to worsen as assessed by decreased hospitalization or need for medical interventions (e.g., progression of the disease is halted or at least slowed). Methods of measuring these indicators are known to those of skill in the art and/or described herein. Treatment includes any treatment of a disease in an individual or an animal (some non-limiting examples include a human, or a mammal) and includes: (1) inhibiting the disease, e.g., arresting, or slowing the progression of symptoms; or (2) relieving the disease, e.g., causing regression of symptoms; and (3) preventing or reducing the likelihood of the development of symptoms.

**[0841]** As discussed above, a therapeutically effective amount includes an amount of a therapeutic composition that is sufficient to promote a particular beneficial effect when administered to an individual, such as one who has, is suspected of having, or is at risk for a disease. In some embodiments, an effective amount includes an amount sufficient to prevent or delay the development of a symptom of the disease, alter the course of a symptom of the disease (for example but not limited to, slow the progression of a symptom of the disease), or reverse a symptom of the disease. It is understood that for any given case, an appropriate effective amount can be determined by one of ordinary skill in the art using routine experimentation.

**[0842]** In some embodiments of the disclosed methods, the individual is a mammal. In some embodiments, the mammal is a human. In some embodiments, the individual has or is suspected of having a disease associated with inhibition of cell signaling mediated by a cell surface ligand or antigen. The diseases suitable for being treated by the compositions and methods of the disclosure include, but are not limited to, cancers, autoimmune diseases, inflammatory diseases, and infectious diseases. In some embodiments, the disease is a cancer or a chronic infection.

**[0843]** Further descriptions of methods for CAR design, delivery and expression in T cells, and the manufacturing of clinical-grade CAR-T cell populations can be found, for example,

in Lee et al., Clin Cancer Res (2012) 18(10):2780-90, hereby incorporated by reference in its entirety. For example, the engineered CARs may be introduced into T cells using retroviruses, which efficiently and stably integrate a nucleic acid sequence encoding the chimeric antigen receptor into the target cell genome.

**[0844]** Other methods known in the art include, but are not limited to, lentiviral transduction, transposon-based systems, direct RNA transfection, and CRISPR/Cas systems (e.g., type I, type II, or type III systems using a suitable Cas protein such Cas3, Cas4, Cas5, Cas5e (or CasD), Cas6, Cas6e, Cas6f, Cas7, Cas8a1, Cas8a2, Cas8b, Cas8c, Cas9, Cas10, Cas10d, Cas12a (Cpf1), Cas13a (C2c2), Cas13b, Cas13d, CasF, CasG, CasH, Csy1, Csy2, Csy3, Cse1 (or CasA), Cse2 (or CasB), Cse3 (or CasE), CasX, CasY, Cse4 (or CasC), Csc1, Csc2, Csa5, Csn2, Csm2, Csm3, Csm4, Csm5, Csm6, Cmr1, Cmr3, Cmr4, Cmr5, Cmr6, Csb1, Csb2, Csb3, Csx17, Csx14, Csx10, Csx16, CsaX, Csx3, Csz1, Csx15, Csf1, Csf2, Csf3, Csf4, and Cu1966, etc.).

**[0845]** In some embodiments, a recombinant adeno-associated virus (AAV) vector can be used for delivery. Techniques to produce rAAV particles, in which an AAV genome to be packaged that includes the polynucleotide to be delivered, rep and cap genes, and helper virus functions are provided to a cell are known in the art. Production of rAAV requires that the following components are present within a single cell (denoted herein as a packaging cell): a rAAV genome, AAV rep and cap genes separate from (e.g., not in) the rAAV genome, and helper virus functions. The AAV rep and cap genes can be from any AAV serotype for which recombinant virus can be derived, and can be from a different AAV serotype than the rAAV genome ITRs, including, but not limited to, AAV serotypes AAV-1, AAV-2, AAV-3, AAV-4, AAV-5, AAV-6, AAV-7, AAV-8, AAV-9, AAV-10, AAV-11, AAV-12, AAV-13 and AAV rh.74. Production of pseudotyped rAAV is disclosed in, for example, international patent application publication number WO 01/83692.

**[0846]** The engineered cells (*e.g.*, CAR-T cells), once they have been expanded ex vivo in response to, for example, an autoimmune disease antigen, can be reinfused into the subject in a therapeutically effective amount. The precise amount of engineered cells (*e.g.,* CAR T cells) to be administered can be determined by a physician with consideration of individual differences in age, weight, extent of disease and condition of the subject.

**[0847]** Administration of cell (*e.g.,* T cell) therapies may be defined by number of total cells per infusion or number of cells per kilogram of body weight, especially for pediatric subjects (e.g., patients). As immune cells (*e.g.,* T cells) replicate and expand after transfer, the administered cell dose may not resemble the final steady-state number of cells. In some

embodiments, a pharmaceutical composition including the immune cells (*e.g.,* CAR T cells) of the present disclosure may be administered at a dosage of $10^4$ to $10^{10}$ total cells. In another embodiment, a pharmaceutical composition including the immune cells (*e.g.,* CAR T cells) of the present disclosure may be administered at a dosage of $10^3$ to $10^8$ cells/kg body weight, including all integer values within those ranges.

**[0848]** Compositions including the CAR T cells of the present disclosure may also be administered multiple times at these dosages. The cells can be administered by using infusion techniques that are known in the art (see, for example, Rosenberg et al., New Engl J Med, (1988) 319:1676). The optimal dosage and treatment regimen for a particular subject can be determined by one skilled in the art by monitoring the subject for signs of disease and adjusting the treatment accordingly.

**[0849]** In some embodiments, administration of any of the compositions embodied herein, for the treatment of, for example, an autoimmune or inflammatory disease, can be combined with other cell-based therapies, for example, stem cells, antigen presenting cells, pancreatic islets etc.

**[0850]** The composition of the present disclosure may be prepared in a manner known in the art and in a manner suitable for parenteral administration to mammals, particularly humans, including a therapeutically effective amount of the composition alone, with one or more pharmaceutically acceptable carriers or diluents.

**[0851]** The term "pharmaceutically acceptable carrier" as used herein means any suitable carriers, diluents or excipients. These include all aqueous and non-aqueous isotonic sterile injection solutions, which may contain anti-oxidants, buffers and solutes, which render the composition isotonic with the blood of the intended recipient; aqueous and non-aqueous sterile suspensions, which may include suspending agents and thickening agents, dispersion media, antifungal and antibacterial agents, isotonic and absorption agents and the like. It will be understood that compositions of the present disclosure may also include other supplementary physiologically active agents.

**[0852]** The carrier must be pharmaceutically "acceptable" in the sense of being compatible with the other ingredients of the composition and not injurious to the subject. Compositions include those suitable for parenteral administration, including subcutaneous, intramuscular, intravenous and intradermal administration. The compositions may conveniently be presented in unit dosage form and may be prepared by any method well known in the art of pharmacy. Such methods include preparing the carrier for association with the CAR-T cells.

In general, the compositions are prepared by uniformly and intimately bringing into association any active ingredients with liquid carriers.

**[0853]** In some embodiments, the composition is suitable for parenteral administration. In another embodiment, the composition is suitable for intravenous administration.

**[0854]** Compositions suitable for parenteral administration include aqueous and nonaqueous isotonic sterile injection solutions which may contain anti-oxidants, buffers, bactericides and solutes, which render the composition isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents.

**[0855]** Enhancing the *in vivo* persistence of immune cells (*e.g.,* T cells) in a subject can be enhancing the fitness or enhancing the function and/or enhancing the efficacy of the therapeutic immune cells. The enhancing can be measured by determining the accumulation of the number of T cell in a tumor. In some embodiments, *in vivo* persistence can be measured by promoting the intra-tumoral increase of effector cytokines. In some embodiments, the *in vivo* persistence can be by increasing the expression of the stemness-associated transcription factor TCF1 in TILs. In some embodiments, the *in vivo* persistence can be by increasing the expression of TNF-α, IFN-γ, and/or IL-2. In some embodiments, enhancing the *in vivo* persistence can be measured by measuring differences in gene expression. In some embodiments, the genes include but are not limited to activation markers (IL2RA, CD69), cytotoxic and effector molecules (IFNG, TNF, IL4, IL5, IL13, GZMA, GZMB), chemokines (CCL4, CCL20, CCL8), and co-stimulatory molecules (ICOS, OX40, 4-1BB, GITR).

**[0856]** In some embodiments, the subject has cancer or an autoimmune disease. In some embodiments, the cancer can be a solid tumor. In some embodiments, the cancer can be a hematological cancer. In some embodiments, the cancer expresses a tumor-associated antigen, as described herein. In some embodiments, the cancer expresses DLL3, LY6G6D, Claudin 6, GCC, p53R175H, and/or PRAME.

**[0857]** In some embodiments, the polypeptides, recombinant nucleic acids, vectors, particles, or engineered cells of the disclosure are for treating cancer or tumor. In some embodiments, the disclosure provides methods for using the engineered cells for treating cancer or tumor. In some embodiments, the engineered cells comprise T cells. In some embodiments, the engineered cells comprise NK cells.

**[0858]** In some embodiments, the cancer or tumor is metastatic or has metastasized.

**[0859]** In some embodiments, the tumor is a solid tumor. Exemplary solid tumors include, without limitation, small cell lung cancer, colorectal cancer, testicular cancer, ovarian cancer, or melanoma, lymphoma, leukemia, multiple myeloma, prostate cancer, breast cancer, non-small cell lung cancer, gastric cancer, esophageal cancer, liver cancer, kidney cancer, head & neck cancer, glioblastoma, neuroblastoma, soft tissue sarcoma, uterine cancer, brain cancer, skin cancer, renal cancer, bladder cancer, pancreatic cancer, thyroid cancer, eye cancer, gastrointestinal cancer, carcinoma, and sarcoma.

**[0860]** In some embodiments, the cancer is selected from the group consisting of B cell acute lymphoblastic leukemia (B-ALL), diffuse large B-cell lymphoma, liver cancer, pancreatic cancer, breast cancer, ovarian cancer, colorectal cancer, lung cancer, non-small cell lung cancer, acute myeloid lymphoid leukemia, multiple myeloma, gastric cancer, gastric adenocarcinoma, pancreatic adenocarcinoma, glioblastoma, neuroblastoma, lung squamous cell carcinoma, hepatocellular carcinoma, and bladder cancer.

**[0861]** In some embodiments, the method of treatment does not comprise administration of lymphodepletive agents within 7 days prior to administration of the cell therapy. Non-limiting examples of lymphodepletive agents include cyclophosphamide, fludarabine, and/or bendamustine within 7 days prior to administration of the cell therapy. In some embodiments, the method of treatment does not comprise administration of at least 600,000 IU/kg of IL-2 every 8 hours. In some embodiments, the method of treatment does not comprise a checkpoint therapy which blocks PD-1 or CTLA-4 signaling.

**[0862]** Procedures for separation of cells include, but are not limited to, flow cytometry, affinity chromatography, density gradient centrifugation, magnetic separation with antibody-coated magnetic beads, conjugation to particles that modify cell density, cytotoxic agents joined to or used in conjunction with a monoclonal antibody (mAb) or any other appropriate technique.

**[0863]** In some embodiments, the isolated immune cells (*e.g.*, T cells are genetically engineered ex vivo for recombinant expression of a transgene. In some embodiments, isolated immune cells (*e.g.*, T cells) are genetically engineered ex vivo for recombinant expression of a CAR or a transcriptional receptor as described in more details supra. In some embodiments, the cells can be genetically engineered for recombinant expression. Methods suitable for genetically engineering cells for recombinant expression are well known in the art.

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**[0864]** All publications and patent applications mentioned in this disclosure are herein incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

**[0865]** No admission is made that any reference cited herein constitutes prior art. The discussion of the references states what their authors assert, and the inventors/disclosers reserve the right to challenge the accuracy and pertinence of the cited documents. It will be clearly understood that, although a number of information sources, including scientific journal articles, patent documents, and textbooks, are referred to herein; this reference does not constitute an admission that any of these documents forms part of the common general knowledge in the art.

**[0866]** The discussion of the general methods given herein is intended for illustrative purposes only. Other alternative methods and alternatives will be apparent to those of skill in the art upon review of this disclosure, and are to be included within the spirit and purview of this application.

## EXEMPLARY EMBODIMENTS

Embodiment 1. A recombinant polypeptide comprising a caspase-associated recruitment domain (CARD) containing protein or a functional fragment thereof,

wherein the recombinant polypeptide comprises no more than 724 amino acids.

Embodiment 2. A recombinant polypeptide comprising a caspase-associated recruitment domain (CARD) containing protein or a functional fragment thereof,

wherein the recombinant polypeptide does not comprise a full length CARD containing protein.

Embodiment 3. A recombinant polypeptide comprising a recruitment domain or a functional fragment thereof, wherein the recruitment domain is capable of binding to (i) a substrate localized to the intracellular side of the plasma membrane of a cell and/or (ii) a target polypeptide comprising a phosphorylated tyrosine,

wherein the recombinant polypeptide comprises no more than 724 amino acids.

Embodiment 4. A recombinant polypeptide comprising a recruitment domain or a functional fragment thereof, wherein the recruitment domain is capable of binding to (i) a substrate localized to the intracellular side of the plasma membrane of a cell and/or (ii) a target

polypeptide comprising a phosphorylated tyrosine,

wherein the recombinant polypeptide does not comprise a full length sequence of an endogenous protein comprising the recruitment domain.

Embodiment 5. A recombinant polypeptide comprising:

(a) a caspase-associated recruitment domain (CARD), and

(b) a recruitment domain or a functional fragment thereof, wherein the recruitment domain is capable of binding to (i) a substrate localized to the intracellular side of the plasma membrane of a cell and/or (ii) a target polypeptide comprising a phosphorylated tyrosine.

Embodiment 6. A recombinant polypeptide comprising:

(a) a caspase-associated recruitment domain (CARD), and

(b) a recruitment domain or a functional fragment thereof, wherein the recruitment domain is capable of binding to (i) a substrate localized to the intracellular side of the plasma membrane of a cell and/or (ii) a target polypeptide comprising a phosphorylated tyrosine,

wherein the recombinant polypeptide comprises no more than 724 amino acids.

Embodiment 7. A recombinant polypeptide comprising:

(a) a caspase-associated recruitment domain (CARD), and

(b) a recruitment domain or a functional fragment thereof, wherein the recruitment domain is capable of binding to (i) a substrate localized to the intracellular side of the plasma membrane of a cell and/or (ii) a target polypeptide comprising a phosphorylated tyrosine,

wherein the recombinant polypeptide does not comprise a full length sequence of an endogenous protein comprising the CARD and/or a full length sequence of an endogenous protein comprising the recruitment domain.

Embodiment 8. A recombinant polypeptide comprising:

(a) a first polypeptide capable of binding to a caspase-associated recruitment domain (CARD) on BCL10, and

(b) a recruitment domain or a functional fragment thereof, wherein the recruitment domain is capable of binding to (i) a substrate localized to the intracellular side of the plasma membrane of a cell and/or (ii) a target polypeptide comprising a phosphorylated tyrosine,

wherein the recombinant polypeptide comprises no more than 724 amino acids.

Embodiment 9. A recombinant polypeptide comprising:

(a) a first polypeptide capable of binding to a caspase-associated recruitment domain

(CARD) on BCL10, and

(b) a recruitment domain or a functional fragment thereof, wherein the recruitment domain is capable of binding to (i) a substrate localized to the intracellular side of the plasma membrane of a cell and/or (ii) a target polypeptide comprising a phosphorylated tyrosine,

wherein the recombinant polypeptide does not comprise a full length sequence of an endogenous protein capable of binding the CARD recruitment domain of BCL10 .

Embodiment 10. The recombinant polypeptide of any one of embodiments 1-9, wherein when the recombinant polypeptide is expressed in an immune cell and the immune cell binds a target antigen, the immune cell exhibits decreased:

(a) BCL10 binding,

(b) BCL10 and MALT1 complex formation,

(c) NFAT, NF-κB and/or AP-1 signaling,

(d) cytokine production,

(e) JAK/STAT signaling,

(f) co-stimulatory molecule signaling,

(g) phospholipase gamma signaling, and/or

(h) transcription factor activity,

compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1.

Embodiment 11. The recombinant polypeptide of any one of embodiments 1-10, wherein when the recombinant polypeptide is expressed in an immune cell and the immune cell binds a target antigen, the immune cell exhibits decreased Interferon gamma (IFNg) and/or Interleukin-2 (IL-2) production compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1.

Embodiment 12. The recombinant polypeptide of any one of embodiments 1-9, wherein when the recombinant polypeptide is expressed in an immune cell and the immune cell binds a target antigen, the immune cell exhibits equivalent or enhanced:

(a) BCL10 binding,

(b) BCL10 and MALT1 complex formation,

(c) NF-κB and/or AP-1 signaling,

(d) MALT1 paracaspase activity,

(e) cytokine production,

(f) peak tumor expansion,

(g) peak peripheral blood expansion,

(h) contraction following peak tumor expansion, and/or

(i) contraction following peak peripheral blood expansion,

compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1.

Embodiment 13. The recombinant polypeptide of any one of embodiments 1-9 and 12, wherein when the recombinant polypeptide is expressed in an immune cell and the immune cell binds a target antigen, the immune cell exhibits equivalent or higher IFNg and/or IL-2 production compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1.

Embodiment 14. The recombinant polypeptide of any one of embodiments 1-13, wherein the recombinant polypeptide comprises no more than 700, no more than 650, no more than 600, no more than 550, no more than 500, no more than 450, no more than 400, no more than 350, no more than 300, no more than 250, no more than 200, or no more than 150 amino acids.

Embodiment 15. The recombinant polypeptide of any one of embodiments 1-14, wherein the CARD containing protein or the functional fragment thereof, the CARD, or the recombinant polypeptide, is capable of binding to a CARD domain on BCL10.

Embodiment 16. The recombinant polypeptide of any one of embodiments 1-15, wherein the CARD containing protein or the functional fragment thereof, the CARD, or the recombinant polypeptide, is capable of forming a complex with BCL10 and MALT1.

Embodiment 17. The recombinant polypeptide of any one of embodiments 1-16, wherein the CARD containing protein or the functional fragment thereof, or the CARD, comprises no more than 40, no more than 50, no more than 60, no more than 70, no more than 80, no more than 90, no more than 100, no more than 150, no more than 200, no more than 300, no more than 400, or no more than 500 amino acids.

Embodiment 18. The recombinant polypeptide of any one of embodiments 1-17, wherein the CARD containing protein or the functional fragment thereof, or the CARD, comprises or

consists of a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to any one of SEQ ID NO: 293-323.

Embodiment 19. The recombinant polypeptide of any one of embodiments 1-18, wherein the CARD containing protein is selected from, or wherein the CARD is derived from a protein selected from,CARD9, CARD10, CARD11, and CARD14.

Embodiment 20. The recombinant polypeptide of embodiment 19, wherein the CARD containing protein is, or wherein the CARD is derived from, CARD11.

Embodiment 21. The recombinant polypeptide of any one of embodiments 1-20, wherein the recombinant polypeptide comprises a full CARD domain or a truncated portion thereof, wherein the start of the full CARD domain corresponds to amino acid position 11 of SEQ ID NO: 1, and wherein the end of the full CARD domain corresponds to amino acid position 103 of SEQ ID NO: 1.

Embodiment 22. The recombinant polypeptide of embodiment 21, wherein the full CARD domain comprises a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to one of SEQ ID NO: 293-323.

Embodiment 23. The recombinant polypeptide of embodiment 21 or embodiment 22, wherein the full CARD domain comprises at least 90 amino acids.

Embodiment 24. The recombinant polypeptide of any one of embodiments 21-23, wherein the recombinant polypeptide comprises the full CARD domain.

Embodiment 25. The recombinant polypeptide of embodiments 21, wherein the recombinant polypeptide comprises a truncated portion of the CARD domain having no more than 85, no more than 80, no more than 75, no more than 70, no more than 60, no more than 50, or no more than 40, amino acids.

Embodiment 26. The recombinant polypeptide of embodiment 25, wherein the CARD domain comprises N-terminal and/or C-terminal truncation(s).

Embodiment 27. The recombinant polypeptide of embodiment 26, wherein the CARD domain comprises a truncation of at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, or at least 50 amino acids at the N-terminus.

Embodiment 28. The recombinant polypeptide of embodiment 26 or 27, wherein the CARD domain comprises a truncation of at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, or at least 50 amino acids at the C-terminus.

Embodiment 29. The recombinant polypeptide of any one of embodiments 25-28, wherein the CARD domain comprises a truncation of at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, or at least 50 amino acids in between the N-terminus and the C-terminus.

Embodiment 30. The recombinant polypeptide of embodiment 25, wherein the recombinant polypeptide does not comprise any full or truncated portion of CARD domain having at least 30 amino acids in length.

Embodiment 31. The recombinant polypeptide of embodiment 25-30, wherein the recombinant polypeptide comprises or consists of a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to any one of SEQ ID NO: 225-245.

Embodiment 32. The recombinant polypeptide of any one of embodiments 25-30, wherein the recombinant polypeptide does not comprise the polypeptide sequence “MDDY” within 10 amino acids before the N-term of the start of the CARD domain.

Embodiment 33. The recombinant polypeptide of any one of embodiments 21-32, wherein when the recombinant polypeptide is expressed in an immune cell and the immune cell binds a target antigen, the immune cell exhibits decreased:

(a) BCL10 binding,
(b) BCL10 and MALT1 complex formation,
(c) NFAT, NF-κB and/or AP-1 signaling,
(d) cytokine production,
(e) JAK/STAT signaling,
(f) co-stimulatory molecule signaling,
(g) phospholipase gamma signaling, and/or
(h) transcription factor activity,

compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1.

Embodiment 34. The recombinant polypeptide of any one of embodiments 21-33, wherein when the recombinant polypeptide is expressed in an immune cell and the immune cell binds a target antigen, the immune cell exhibits decreased IFNg and/or IL-2 production compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1.

Embodiment 35. The recombinant polypeptide of any one of embodiments 21-32, wherein when the recombinant polypeptide is expressed in an immune cell and the immune cell binds a target antigen, the immune cell exhibits equivalent or enhanced:

(a) BCL10 binding,

(b) BCL10 and MALT1 complex formation,

(c) NF-κB and/or AP-1 signaling,

(d) MALT1 paracaspase activity,

(e) cytokine production,

(f) peak tumor expansion,

(g) peak peripheral blood expansion,

(h) contraction following peak tumor expansion, and/or

(i) contraction following peak peripheral blood expansion,

compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1.

Embodiment 36. The recombinant polypeptide of any one of embodiments 21-32 and 35, wherein when the recombinant polypeptide is expressed in an immune cell and the immune cell binds a target antigen, the immune cell exhibits equivalent or higher IFNg and/or IL-2 production compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1.

Embodiment 37. The recombinant polypeptide of any one of embodiments 1-36, wherein the recombinant polypeptide comprises a full coiled-coil domain or a truncated portion thereof, wherein the start of the full coiled-coil domain corresponds to amino acid position 123 of SEQ ID NO: 1, and wherein the end of the full coiled-coil domain corresponds to amino acid position 442 of SEQ ID NO: 1; optionally, wherein the full coiled-coil domain comprises a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to amino acids 123-442 of SEQ ID NO: 1.

Embodiment 38. The recombinant polypeptide of embodiment 37, wherein the full coiled-coil domain comprises at least 310 amino acids.

Embodiment 39. The recombinant polypeptide of embodiment 37 or embodiment 38, wherein the recombinant polypeptide comprises the full coiled-coil domain.

Embodiment 40. The recombinant polypeptide of embodiment 37, wherein the recombinant polypeptide comprises a truncated portion of the coiled-coil domain having no more than 300, no more than 290, no more than 280, no more than 270, no more than 260, no more than 250, no more than 240, no more than 230, no more than 220, no more than 210, no more than 200, no more than 190, no more than 180, no more than 170, no more than 160, no more than 150, no more than 140, no more than 130, no mor than 120, no more than 110, no more than 100, no more than 95, no more than 90, no more than 85, no more than 80, no more than 75, no more than 70, no more than 60, no more than 50, or no more than 40 amino acids; preferably, wherein the truncated portion of the coiled-coil domain has no more than 260 amino acids.

Embodiment 41. The recombinant polypeptide of embodiment 40, wherein the coiled-coil domain comprises N-terminal and/or C-terminal truncation(s).

Embodiment 42. The recombinant polypeptide of embodiment 41, wherein the coiled-coil domain comprises a truncation of at least 5, at least 10, at least 15, 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, at least 110, at least 120, at least 130, at least 140, at least 150, at least 160, at least 170, at least 180, at least 190, at least 200, at least 210, at least 220, at least 230, at least 240, at least 250, at least 260, or at least 270 amino acids at the N-terminus.

Embodiment 43. The recombinant polypeptide of embodiment 41 or 42, wherein the coiled-coil domain comprises a truncation of at least 5, at least 10, at least 15, 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, at least 110, at least 120, at least 130, at least 140, at least 150, at least 160, at least 170, at least 180, at least 190, at least 200, at least 210, at least 220, at least 230, at least 240, at least 250, at least 260, or at least 270 amino acids at the C-terminus.

Embodiment 44. The recombinant polypeptide of any one of embodiments 40-43, wherein the coiled-coil domain comprises a truncation of at least 5, at least 10, at least 15, 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, at least 110, at least 120, at least 130, at least 140, at least 150, at least 160, at least 170, at least 180, at least 190, at least 200, at least 210, at least 220, at least 230, at least 240, at least

250, at least 260, or at least 270 amino acids in between the N-terminus and the C-terminus; optionally, the coiled-coil domain comprises a truncation of about 60 to about 80 amino acids in between the N-terminus and the C-terminus.

Embodiment 44.1 The recombinant polypeptide of embodiment 44, wherein a region within the coiled-coil domain corresponding to amino acids 253-324 of SEQ ID NO: 1 is removed or replaced by a polypeptide comprising no more than 20, 15, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acids.

Embodiment 44.2 The recombinant polypeptide of embodiment 44.1, wherein the region within the coiled-coil domain is replaced by a polypeptide comprising a sequence of SGGGGSGGGGS (SEQ ID NO: 431) or QAGKRSLPD (SEQ ID NO: 432), or a sequence having at most 1, 2, or 3 mutations thereto.

Embodiment 45. The recombinant polypeptide of any one of embodiments 1-36, wherein the recombinant polypeptide does not comprise any full or truncated portion of coiled-coil domain having at least 30 amino acids in length.

Embodiment 46. The recombinant polypeptide of embodiment 40-45, wherein the recombinant polypeptide comprises or consists of a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to any one of SEQ ID NO: 193-224 and 246-276.

Embodiment 47. The recombinant polypeptide of any one of embodiments 37-46, wherein when the recombinant polypeptide is expressed in an immune cell and the immune cell binds a target antigen, the immune cell exhibits decreased:

(a) BCL10 binding,
(b) BCL10 and MALT1 complex formation,
(c) NFAT, NF-κB and/or AP-1 signaling,
(d) cytokine production,
(e) JAK/STAT signaling,
(f) co-stimulatory molecule signaling,
(g) phospholipase gamma signaling, and/or
(h) transcription factor activity,

compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1.

Embodiment 48. The recombinant polypeptide of any one of embodiments 37-47, wherein when the recombinant polypeptide is expressed in an immune cell and the immune cell binds a target antigen, the immune cell exhibits decreased IFNg and/or IL-2 production compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1.

Embodiment 49. The recombinant polypeptide of any one of embodiments 37-46, wherein when the recombinant polypeptide is expressed in an immune cell and the immune cell binds a target antigen, the immune cell exhibits equivalent or enhanced:

(a) BCL10 binding,
(b) BCL10 and MALT1 complex formation,
(c) NF-κB and/or AP-1 signaling,
(d) MALT1 paracaspase activity,
(e) cytokine production,
(f) peak tumor expansion,
(g) peak peripheral blood expansion,
(h) contraction following peak tumor expansion, and/or
(i) contraction following peak peripheral blood expansion,

compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1.

Embodiment 50. The recombinant polypeptide of any one of embodiments 47-46 or 49, wherein when the recombinant polypeptide is expressed in an immune cell and the immune cell binds a target antigen, the immune cell exhibits equivalent or enhanced IFNg and/or IL-2 production compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1.

Embodiment 51. The recombinant polypeptide of any one of embodiments 1-54, wherein the recombinant polypeptide comprises an inhibitory domain or a truncated portion thereof, wherein the start of the inhibitory domain corresponds to amino acid position 443 of SEQ ID NO: 1, and wherein the end of the inhibitory domain corresponds to amino acid position 577 of SEQ ID NO: 1; optionally, wherein the inhibitory domain comprises a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to amino acids 443-577 of SEQ ID NO: 1.

Embodiment 51.1 The recombinant polypeptide of any one of embodiments 1-54, wherein the recombinant polypeptide comprises an inhibitory domain or a truncated portion thereof,

wherein the inhibitory domain comprises a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 441.

Embodiment 52. The recombinant polypeptide of embodiment 51 or 51.1, wherein the inhibitory domain comprises at least 130, or at least 125, amino acids.

Embodiment 53. The recombinant polypeptide of any one of embodiments 51-52, wherein the recombinant polypeptide comprises the full inhibitory domain.

Embodiment 54. The recombinant polypeptide of any one of embodiments 51-52, wherein the recombinant polypeptide comprises a truncated portion of the inhibitory domain having no more than 120, no more than 110, no more than 100, no more than 95, no more than 90, no more than 85, no more than 80, no more than 75, no more than 70, no more than 60, no more than 50, or no more than 40 amino acids.

Embodiment 55. The recombinant polypeptide of embodiment 54, wherein the inhibitory domain comprises N-terminal and/or C-terminal truncation(s).

Embodiment 56. The recombinant polypeptide of embodiment 55, wherein the inhibitory domain comprises a truncation of at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, or at least 90 amino acids at the N-terminus.

Embodiment 57. The recombinant polypeptide of embodiment 55 or 56, wherein the inhibitory domain comprises a truncation of at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, or at least 90 amino acids at the C-terminus.

Embodiment 58. The recombinant polypeptide of any one of embodiments 54-57, wherein the inhibitory domain comprises a truncation of at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, or at least 90 amino acids in between the N-terminus and the C-terminus.

Embodiment 59. The recombinant polypeptide of any one of embodiments 1-50, wherein the recombinant polypeptide does not comprise any full or truncated portion of inhibitory domain having at least 30 amino acids in length.

Embodiment 59.1. The recombinant polypeptide of any one of embodiments 51-59, wherein the inhibitory domain or the truncated portion thereof comprises at least 1, at least 2, at least 3, at least 4, at least 5, or at least 6 serines that are capable of being phosphorylated; optionally wherein the series are capable of being phosphorylated by a protein kinase C (PKC); optionally, wherein the PKC comprises PKCβ and/or PKCθ.

Embodiment 59.2. The recombinant polypeptide of embodiment 59.1, wherein the inhibitory domain or the truncated portion thereof comprises a sequence of DA**S**PRT (SEQ ID NO:425), RAK**S**PI (SEQ ID NO:426), DA**S**PSS (SEQ ID NO:427), SRS**S**IMS (SEQ ID NO: 428), RKF**S**LER (SEQ ID NO: 429), and/or FRP**S**VTS (SEQ ID NO: 430), or a sequence having at most 1, 2, or 3 mutations thereto; optionally, where the inhibitory domain or the truncated portion thereof comprises the sequence of RAK**S**PI (SEQ ID NO:426), DA**S**PSS (SEQ ID NO:427), and/or SRS**S**IMS (SEQ ID NO: 428).

Embodiment 59.3. The recombinant polypeptide of any one of embodiments 51-59.2, comprising, from N-term to C-term: the coiled-coil domain or the truncated portion thereof, and the inhibitory domain or the truncated portion thereof, without any additional amino acid in between.

Embodiment 60. The recombinant polypeptide of any one of embodiments 54-59.3, wherein the recombinant polypeptide comprises or consists of a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to any one of SEQ ID NO: 179-192, 277-288, and 289-292.

Embodiment 61. The recombinant polypeptide of any one of embodiments 51-60, wherein when the recombinant polypeptide is expressed in an immune cell and the immune cell binds a target antigen, the immune cell exhibits equivalent or enhanced:

(a) BCL10 binding,
(b) BCL10 and MALT1 complex formation,
(c) NF-κB and/or AP-1 signaling, and/or
(d) MALT1 paracaspase activity,
(e) cytokine production,
(f) peak tumor expansion,
(g) peak peripheral blood expansion,

(h) contraction following peak tumor expansion, and/or

(i) contraction following peak peripheral blood expansion,

compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1.

Embodiment 62. The recombinant polypeptide of any one of embodiments 51-61, wherein when the recombinant polypeptide is expressed in an immune cell, the immune cell exhibits equivalent or enhanced IFNg and/or IL-2 production compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1.

Embodiment 63. The recombinant polypeptide of any one of embodiments 1-63, wherein the recombinant polypeptide comprises (b) a recruitment domain or a functional fragment thereof, wherein the recruitment domain is capable of binding to (i) a substrate localized to the intracellular side of the plasma membrane of a cell and/or (ii) a target polypeptide comprising a phosphorylated tyrosine.

Embodiment 64. The recombinant polypeptide of embodiment 63, wherein the substrate localized to the intracellular side of the plasma membrane of a cell is a phosphoinositide; optionally, wherein the phosphoinositide is selected from phosphatidylinositol (3,4,5)-trisphosphate (PIP3) and phosphatidylinositol 4,5-bisphosphate (PI(4,5)P2).

Embodiment 65. The recombinant polypeptide of embodiment 64, wherein the recombinant polypeptide binds to the phosphoinositide with a Kd of less than 50 μM, 10 μM, 5 μM, 1μM, 0.5 μM, 0.1 μM, 0.05 μM, or 0.01 μM, and wherein the Kd is analyzed using SPR.

Embodiment 66. The recombinant polypeptide of embodiment 63, wherein the target polypeptide is derived from IGF-1R, CTLA-4, or CD28.

Embodiment 67. The recombinant polypeptide of embodiment 63 or embodiment 66, wherein the phosphorylated tyrosine is located at the position corresponding to pY1221 of SEQ ID NO: 324, or to pY1346 of SEQ ID NO: 325.

Embodiment 68. The recombinant polypeptide of any one of embodiments 63 and 66-67, wherein the polypeptide binds to the target polypeptide with a Kd of less than 10 μM, 5 μM, 1μM, 0.5 μM, 0.1 μM, 0.05 μM, or 0.01 μM, and wherein the Kd is analyzed by fluorescence polarization assay.

Embodiment 69. The recombinant polypeptide of any one of embodiments 63-68, wherein the recruitment domain is capable of binding to a substrate indirectly localized to the intracellular side of the plasma membrane, and wherin the recruitment domain binds a polypeptide or lipid that is directly localized to the intracellular side of the plasma membrane of a cell.

Embodiment 70. The recombinant polypeptide of any one of embodiments 63-69, wherein the recruitment domain is a Src Homology region 2 (SH2) domain, a Src Homology region 3 (SH3) domain, a phosphotyrosine-binding (PTB) domain, or a pleckstrin homology (PH) domain.

Embodiment 71. The recombinant polypeptide of any one of embodiments 63-70, wherein the recruitment domain is a Src Homology region 2 (SH2) domain.

Embodiment 72. The recombinant polypeptide of any one of embodiments 63-71, wherein the start of the recruitment domain corresponds to amino acid position 622 of SEQ ID NO: 1 and wherein the end of the recruitment domain corresponds to amino acid position 716 of SEQ ID NO: 1.

Embodiment 73. The recombinant polypeptide of embodiment 72, wherein the recruitment domain comprises at least 90 amino acids.

Embodiment 74. The recombinant polypeptide of embodiment 72 or embodiment 73, wherein the recombinant polypeptide comprises the functional fragment of the recruitment domain.

Embodiment 75. The recombinant polypeptide of embodiments 74, wherein the recombinant polypeptide comprises a truncated portion of the recruitment domain having no more than 85, no more than 80, no more than 75, no more than 70, no more than 60, no more than 50, or no more than 40, amino acids.

Embodiment 76. The recombinant polypeptide of embodiment 75, wherein the recruitment domain comprises N-terminal and/or C-terminal truncation(s).

Embodiment 77. The recombinant polypeptide of embodiment 76, wherein the recruitment domain comprises a truncation of at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, or at least 50 amino acids at the N-terminus.

Embodiment 78. The recombinant polypeptide of embodiment 76 or 77, wherein the recruitment domain comprises a truncation of at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, or at least 50 amino acids at the C-terminus.

Embodiment 79. The recombinant polypeptide of any one of embodiments 75-78, wherein the recruitment domain comprises a truncation of at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, or at least 50 amino acids in between the N-terminus and the C-terminus.

Embodiment 80. The recombinant polypeptide of any one of embodiments 75-79, wherein the recruitment domain or the functional fragment thereof comprises a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a segment of at least 30, 40, 50, 60, 70, 80, 90, or 97 amino acids within the region of amino acids 622-716 of SEQ ID NO: 1.

Embodiment 81. The recombinant polypeptide of any one of embodiments 1-62, wherein the recombinant polypeptide does not comprise any recruitment domain having at least 30 amino acids in length.

Embodiment 81.1. The recombinant polypeptide of any one of embodiments 1-81, comprising, from N-term to C-term: the inhibitory domain or the truncated portion thereof, and the recruitment domain or the functional fragment thereof, with no more than 5, no more than 10, no more than 15, no more than 20, or no more than 25, amino acids in between.

Embodiment 81.2 . The recombinant polypeptide of embodiment 81.1, comprising an amino acid sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to any one of SEQ ID NO: 433-438in between the inhibitory domain or the truncated portion thereof and the recruitment domain or the functional fragment thereof.

Embodiment 82. The recombinant polypeptide of any one of embodiments 1-81.2, wherein the recombinant polypeptide comprises, from N-terminus to C-terminus, the CARD domain, optionally the coiled-coil domain, optionally the inhibitory domain, and the recruitment domain.

Embodiment 83. The recombinant polypeptide of any one of embodiments 1-82, wherein the recombinant polypeptide comprises no more than 40, no more than 35, no more than 30, no

more than 25, no more than 20, no more than 15, no more than 10, or no more than 5, amino acids, in between the CARD containing protein or functional fragment thereof and the recruitment domain.

Embodiment 84. The recombinant polypeptide of any one of embodiments 1-83, wherein the recombinant polypeptide comprises or consists of a sequence having at least 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to any one of SEQ ID NO: 3-150.

Embodiment 85. The recombinant polypeptide of any one of embodiments 1-83, wherein the recombinant polypeptide comprises or consists of a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to any one of SEQ ID NO: 151-178.

Embodiment 86. The recombinant polypeptide of any one of embodiments 1-85, wherein the recombinant polypeptide comprises an aspartatic acid acid (D) at the position corresponding to amino acid 602 of SEQ ID NO: 1.

Embodiment 87. A nucleic acid construct comprising a polynucleotide sequence encoding the recombinant polypeptide of any one of embodiments 1-86.

Embodiment 88. The nucleic acid construct of embodiment 87, wherein the polynucleotide sequence encoding the recombinant polypeptide is operably linked to a promoter.

Embodiment 89. The nucleic acid construct of embodiment 88, wherein the promoter is a constitutive promoter or an inducible promoter.

Embodiment 90. The nucleic acid construct of embodiment 88 or embodiment 89, wherein the promoter is or comprises a minimal TATA promoter, pGK promoter, actin promoter, CD4 promoter, CD8a promoter, CD8b promoter, TCRa promoter, TCRb promoter, CD3d promoter, CD3g promoter, CD3e promoter, CD3z promoter, CARD9 promoter, CARD10 promoter, CARD11 promoter, CARD14 promoter, PIK3R3 promoter, CD25 promoter, IL2 promoter, IL7 promoter, IL15 promoter, KLRG-1 promoter, HLA-DR promoter, CD38 promoter, CD69 promoter, Ki-67 promoter, CD11a promoter, CD58 promoter, CD99 promoter, CD62L promoter, CD103 promoter, CCR4 promoter, CCR5 promoter, CCR6 promoter, CCR9 promoter, CCR10 promoter, CXCR3 promoter, CXCR4 promoter, CLA promoter, Granzyme A promoter, Granzyme B promoter, Perforin promoter, CD57

promoter, CD161 promoter, IL-18Ra promoter, CD69 promoter, GzmB promoter, T-bet promoter, IFNgamma promoter, TIM3 promoter, IL4 promoter, GATA3 promoter, IL1 promoter, IL5 promoter, IL6 promoter, IL13 promoter, IL10 promoter, IL17A promoter, IL6 promoter, IL21 promoter, IL23R promoter, FoxP3 promoter, CTLA4 promoter, CD25 promoter, PD1 promoter, CD45RO promoter, CCR7 promoter, CD28 promoter, CD95 promoter, CD28 promoter, CD27 promoter, CD127 promoter, PD-1 promoter, CD122 promoter, CD132 promoter, c-Kit promoter, nuclear factor of activated T cells (NFAT) promoter, programmed death 1 (PD-1) promoter, T cell immunoglobulin mucin-3 (TIM-3) promoter, cytotoxic T lymphocyte antigen-4 (CTLA4) promoter, lymphocyte-activation protein 3 (LAG-3) promoter, tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) promoter, B- and T-lymphocyte attenuator (BTLA) promoter, CD25 promoter, CD69 promoter, Fas ligand (FasL) promoter, TIGIT promoter, TGF-beta promoter, T-bet promoter, Eomes promoter, GATA3 promoter, CD45RA promoter, 2B4 promoter, Type I interferon (IFN) alpha, Type I IFN beta promoter, IFN gamma promoter, IRF3 promoter, IRF7 promoter, NFkB promoter, AP-1 promoter, TNF-alpha promoter, CD130 promoter, NR4A1 promoter, NR4A2, NR4A3 promoter, MND promoter, EF-1alpha promoter, short EF-1alpha promoter, CAG promoter, ubiquitin/S27a promoter, SV40 promoter, SV40 early promoter, adenovirus major late promoter, mouse metallothionein-I promoter, Moloney murine leukemia virus (MMLV) long terminal repeat (LTR) region, CMV promoter, immunoglobulin promoter, heat shock promoter, polyoma virus promoter, fowlpox virus promoter, bovine papilloma virus promoter, avian sarcoma virus promoter, retrovirus promoter, hepatitis-B virus promoter, PGK promoter, vaccinia virus 7.5K promoter, TK promoter of HSV, mouse mammary tumor virus (MMTV) promoter, LTR promoter of HIV, promoter of moloney virus, Epstein Barr virus (EBV) promoter, Rous sarcoma virus (RSV) promoter, U6 promoter, or UBC promoter.

Embodiment 91. The nucleic acid construct of embodiment 89, wherein the promoter is a constitutive promoter.

Embodiment 92. The nucleic acid construct of embodiment 91, wherein the constitutive promoter is or comprises a CD4 promoter, CD8a promoter, CD8b promoter, TCRa promoter, TCRb promoter, CD3d promoter, CD3g promoter, CD3e promoter, CD3z promoter, CARD9 promoter, CARD10 promoter, CARD11 promoter, CARD14 promoter, or PIK3R3 promoter.

Embodiment 93. The nucleic acid construct of embodiment 91, wherein the promoter is or comprises an MND promoter or a short EF1a promoter.

Embodiment 94. The nucleic acid construct of embodiment 87, wherein the polynucleotide sequence encoding the recombinant polypeptide is not operably linked to a promoter.

Embodiment 95. The nucleic acid construct of embodiments 94, wherein the nucleic acid construct further comprises a polynucleotide sequence encoding one or more cleavable linkers.

Embodiment 96. The nucleic acid construct of embodiment 95, wherein the one or more cleavable linkers comprise a P2A, E2A, F2A, or T2A self-cleaving peptide.

Embodiment 97. The nucleic acid construct of any one of embodiments 87-96, wherein the nucleic acid construct further comprises one or more nucleic acid coding or regulatory sequence.

Embodiment 98. The nucleic acid construct of embodiment 97, wherein the one or more nucleic acid coding or regulatory sequence is or comprises ribosomal binding sites, enhancer elements, activator elements, translational start sequences, translational termination sequences, transcription start sequences, transcription termination sequences, polyadenylation signal sequences, a 70 bp poly(A) tract, a 100 bp poly(A) tract, a 172 bp poly(A) tract, a 200 bp poly(A) tract, a 300 bp poly(A) tract, a 325 bp poly(A) tract, replication elements, RNA processing and export elements, transposon sequences, transposase sequences, insulator sequences, internal ribosome entry sites (IRES), 5'UTRs, 3'UTRs, mRNA 3' end processing sequences, boundary elements, locus control regions (LCR), matrix attachment regions (MAR), recombination or cassette exchange sequences, linker sequences, cleavable linker sequences, secretion signals, resistance markers, anchoring peptides, localization signals, fusion tags, affinity tags, chaperonins, proteases, or any combination thereof.

Embodiment 99. The nucleic acid construct of any one of embodiments 87-98, wherein the nucleic acid construct further comprises a polynucleotide sequence encoding one or more additional polypeptides and/or a non-coding RNA.

Embodiment 100. The nucleic acid construct of embodiment 99, wherein the non-coding RNA comprises a shRNA or a microRNA.

Embodiment 101. The nucleic acid construct of embodiment 99, wherein the one or more additional polypeptides comprise a chimeric antigen receptor (CAR), a recombinant T cell receptor (TCR), an additional potency enhancement polypeptide, a cytokine, a chemokine, a growth factor, a safety switch, or any combination thereof.

Embodiment 102. The nucleic acid construct of embodiment 101, wherein the one or more additional polypeptides comprise a CAR or a recombinant TCR.

Embodiment 103. The nucleic acid construct of embodiment 102, wherein the CAR or recombinant TCR has specificity for a target antigen selected from the group consisting of CD1, CD1a, CD1b, CD1c, CD1d, CD1e, CD2, CD3d, CD3e, CD3g, CD3ε, CD4, CD5, CD7, CD8a, CD8b, CD19, CD20, CD21, CD22, CD23, CD24, CD25, CD27, CD28, CD30, CD33, CD34, CD38, CD40, CD44v6, CD45, CD46, CD47 CD48, CD52, CD59, CD66, CD70, CD71, CD72, CD73, CD79A, CD79B, CD80 (B7.1), CD86 (B7.2), CD94, CD95, CD97, CD123, CD134, CD140 (PDGFR4), CD152, CD154, CD158, CD171, CD178, CD179, CD179a, CD181 (CXCR1), CD182 (CXCR2), CD183 (CXCR3), CD210, CD213A2, CD246, CD252, CD253, CD261, CD262, CD272, CD273 (PD-L2), CD274 (PD-L1), CD276 (B7H3), CD279, CD295, CD339 (JAG1), CD340 (HER2), CDH17, CEA, CLECL1, CLL-1, CLDN6, CLDN18.2, CS1, DLL3, LY6G6D, GCC, p53R175H, PRAME, EGFR, EGFRvIII, FGFR2, AFP, CA125, MUC-1, MAGE, ALPI, alkaline phosphatase placental-like 2 (ALPPL2), B-cell maturation antigen (BCMA), green fluorescent protein (GFP), enhanced green fluorescent protein (eGFP), KLK2, KLK3, Mesothelin, IL13Ra2, ggcc, signal regulatory protein α (SIRPα), TCRalpha, TCRbeta, TSHR, GD2, GD3, Tn Ag, cMET, Axl, ROR1, ROR2, GPC1, GPC2, GPC3, FLT3, TAG72, CEA, EPCAM, KIT (CD117), IL-13Ra2, IL-11Ra, PSCA, PRSS21, VEGFR2, LewisY, PDGFRβ, SSEA-4, folate receptor alpha, ERBB2 (Her2/neu), MUC1, MUC16, NCAM, prostase, PAP, ELF2M, Ephrin B2, IGF-I receptor, CAIX, LMP2, gp100, bcr-abl, tyrosinase, EphA2, STEAP1, STEAP2, fucosyl GM1, sLe, GM3, TGS5, HMWMAA, o-acetyl-GD2, folate receptor beta, TEM1/CD248, TEM7R, ROPN1, GPRC5D, GPA33, CXORF61, ALK, Polysialic acid, PLAC1, GloboH, NY-BR-1, UPK2, HAVCR1, ADRB3, PANX3, GPR20, LY6K, OR51E2, TARP, WT1, NY-ESO-1, LAGE-la, MAGE-Al, legumain, HPV E6,E7, MAGE-A4, ETV6-AML, sperm protein 17, XAGE1, Tie 2, MAD-CT-1, MAD-CT-2, Fos-related antigen 1,

p53, p53 mutant, p53R175H, KRAS, mutant KRAS, KRAS G12D, prostein, surviving, telomerase, PCTA-1/Galectin 8, MelanA/MART1, Ras mutant, hTERT, sarcoma translocation breakpoints, ML-IAP, ERG (TMPRSS2 ETS fusion gene), NA17, PAX3, androgen receptor, cyclin Bl, MYCN, RhoC, TRP-2, CYP1B1, BORIS, SART3, PAX5, OY-TES1, LCK, AKAP-4, SSX2, RAGE-1, human telomerase reverse transcriptase, RU1, RU2, intestinal carboxyl esterase, mut hsp70-2, LAIR1, FCAR, LILRA2, CD300LF, CLEC12A, BST2, EMR2, LY75, FCRL5, IGLL1, PSMA, TROP2, citrullinated vimentin, the extracellular portion of the APRIL protein, and any combinations thereof.

Embodiment 104. The nucleic acid construct of embodiment 102 or embodiment 103, wherein the CAR or recombinant TCR has specificity for a target antigen selected from the group consisting of B7H3, BCMA, CD19, CD20, CD22, CD70, CD79a, CD79b, CDH17, CLDN6, CLDN18.2, DLL3, GCC, GD2, GD3, GPC3, GPRC5D, GPA33, KLK3, LY6G6D, p53R175H, PRAME, ROPN1, STEAP1, and STEAP2.

Embodiment 105. The nucleic acid construct of any one of embodiments 102-104, wherein the CAR comprises one or more costimulatory domains.

Embodiment 106. The nucleic acid construct of embodiment 105, wherein the one or more costimulatory domains are derived from a protein selected from the group comprising CD3zeta, CD28, 4-1BB, OX40, IL2Rb turbodomains, MYD88/CD40, MYD88/CD40 inducible constimulatory domain, CD2, B7-1/CD80; B7-2/CD86; B7-H1/PD-L1; B7-H2; B7-H3; B7-H4; B7-H6; B7-H7; BTLA/CD272; CD28; CTLA-4; Gi24/VISTA/B7-H5; ICOS/CD278; PD-1; PD-L2/B7-DC; PDCD6); 4-1BB/TNFSF9/CD137; 4-1BB Ligand/TNFSF9; BAFF/BLyS/TNFSF13B; BAFF R/TNFRSF13C; CD27/TNFRSF7; CD27 Ligand/TNFSF7; CD30/TNFRSF8; CD30 Ligand/TNFSF8; CD40/TNFRSF5; CD40/TNFSF5; CD40 Ligand/TNFSF5; DR3/TNFRSF25; GITR/TNFRSF18; GITR Ligand/TNFSF18; HVEM/TNFRSF14; LIGHT/TNFSF14; Lymphotoxin-alpha/TNF-beta; OX40/TNFRSF4; 0X40 Ligand/TNFSF4; RELT/TNFRSF19L; TACI/TNFRSF13B; TL1A/TNFSF15; TNF-alpha; TNF RII/TNFRSF1B); 2B4/CD244/SLAMF4; BLAME/SLAMF8; CD2; CD2F-10/SLAMF9; CD48/SLAMF2; CD58/LFA-3; CD84/SLAMF5; CD229/SLAMF3; CRACC/SLAMF7; NTB- A/SLAMF6; SLAM/CD150); CD2; CD7; CD53; CD82/Kai-1; CD90/Thyl; CD96; CD160; CD200; CD300a/LMIRl; HLA Class I; HLA-DR; Ikaros; Integrin alpha 4/CD49d; Integrin alpha 4 beta 1; Integrin alpha 4 beta 7/LPAM-l; LAG-3; TCL1A; TCL1B; CRTAM; DAP12; Dectin- 1/CLEC7A;

DPPIV/CD26; EphB6; TIM-1/KIM-1/HAVCR; TIM-4; TSLP; TSLP R; lymphocyte function associated antigen-1 (LFA-1); NKG2C, an immunoreceptor tyrosinebased activation motif (ITAM), CD27, CD134/OX40, CD30, CD40, PD-1, ICOS, lymphocyte function-associated antigen-1 (LFA-1), CD7, LIGHT, NKG2C, and any combinations thereof.

Embodiment 107. The nucleic acid construct of embodiment 101, wherein the additional potency enhancement polypeptide is or comprises a dominant negative form of an inhibitor of a cell-mediated immune response of the immune cell (e.g., TGFβR2 DNR), c-Jun, CCL19, CCL21, IL2R, IL7, IL7Ralpha, IL15, IL15RA, IL18, decoy-resistant IL18 (DR-18), MyD88/CD40, PD1-CD28 switch receptor, PD1-41BB switch receptor, CD40L-CD28 switch receptor, CTBR12 switch receptor, or CD8alpha/beta.

Embodiment 108. The nucleic acid construct of any one of embodiments 99-107, wherein the polynucleotide sequence encoding the recombinant polypeptide and the polynucleotide sequence encoding the one or more additional polypeptides are separated by a polynucleotide sequence encoding one or more cleavable linkers, and wherein all the polynucleotide sequences form a continuous open reading frame.

Embodiment 109. The nucleic acid construct of embodiment108, wherein the one or more cleavable linkers comprise a P2A, E2A, F2A, or T2A self-cleaving peptide.

Embodiment 110. The nucleic acid construct of any one of embodiments 99-107, wherein the polynucleotide sequence encoding the recombinant polypeptide and the polynucleotide sequence encoding the one or more additional polypeptides are separated by an internal ribozyme entry site (IRES).

Embodiment 111. The nucleic acid construct of any one of embodiments 87-110, wherein the nucleic acid construct is a DNA construct.

Embodiment 112. The nucleic acid construct of any one of embodiments 87-110, wherein the nucleic acid construct is an RNA construct.

Embodiment 113. The nucleic acid construct of embodiment 110, wherein the RNA construct is an mRNA construct.

Embodiment 114. The nucleic acid construct of embodiment 112 or embodiment 113, wherein the nucleic acid construct comprises one or more modified nucleotides.

Embodiment 115. The nucleic acid construct of embodiment 113, wherein the one or more modified nucleotides are or comprise pseudouridine, N1-methylpseudouridine, 4'-thiouridine, 5-methylcytosine, 2-thio-l-methyl-1-deaza-pseudouridine, 2-thio-l-methyl-pseudouridine, 2-thio-5-aza-uridine, 2-thio-dihydropseudouridine, 2-thio-dihydrouridine, 2-thio-pseudouridine, 4-methoxy-2-thio-pseudouridine, 4-methoxy-pseudouridine, 4-thio-l-methyl- pseudouridine, 4-thio-pseudouridine, 5-aza-uridine, dihydropseudouridine, 5-methyluridine, 5-methyluridine, 5-methoxyuridine, 2'-O-methyl uridine, or any combination thereof.

Embodiment 116. The nucleic acid construct of embodiment 115, wherein one or more modified nucleotides are selected from the group consisting of pseudouridine, N1-methylpseudouridine, 5-methylcytosine, 5- methoxyuridine, and a combination thereof.

Embodiment 117. The nucleic acid construct of embodiment 115, wherein one or more modified nucleotides are N1- methylpseudouridines.

Embodiment 117.1 The nucleic acid construct of any one of embodiments 87-117, comprising a first homology arm before the 5' end of the nucleic acid sequence encoding the recombinant polypeptide and a second homology arm after the 3' end of the nucleic acid sequence encoding the recombinant polypeptide; optionally, wherein the lengths of the two homology arms are between about 150 bp and about 1500 bp.

Embodiment 117.2 The nucleic acid construct of embodiment 117.1, wherein the length of at least one of the homology arms is about 200bp, 225 bp, about 250 bp, about 275 bp, about 300 bp, about 325 bp, about 350 bp, about 375 bp, about 400 bp, about 425 bp, about 450 bp, about 475 bp, or about 500 bp; optionally, wherein the lengths of both of the homology arms are about 225 bp, about 250 bp, about 275 bp, about 300 bp, about 325 bp, about 350 bp, about 375 bp, about 400 bp, about 425 bp, about 450 bp, about 475 bp, about 500 bp, about 525 bp, about 550 bp, about 575 bp, or about 600 bp.

Embodiment 117.3 The nucleic acid construct of embodiment 117.1 or 117.2, wherein the total length of the homology arms is between about 400 bp and about 500 bp, about 500 bp and about 600 bp, about 600 bp and about 700 bp, about 700 bp and about 800 bp, about 800

bp and about 900 bp, about 900 bp and about 1000 bp, about 1000 bp and about 1100 bp, or about 1100 bp and about 1200 bp.

Embodiment 118. The nucleic acid construct of any one of embodiments 87-117.3, wherein the nucleic acid construct is less than 10 kb, less than 9 kb, less than 8 kb, less than 7 kb, less than 6 kb, less than 5 kb, less than 4 kb, or less than 3 kb in length.

Embodiment 119. The nucleic acid construct of embodiment 118, wherein the nucleic acid construct is less than about 5.0 kb in length.

Embodiment 119.1. The nucleic acid construct of embodiment 118, wherein the nucleic acid construct is less than about 4.7 kb in length.

Embodiment 119.2. The nucleic acid construct of any one of embodiments 87-119, wherein the length of the polynucleotide sequence encoding the one or more additional polypeptides is less than about 2100 bp, less than about 2150 bp, less than about 2200 bp, less than about 2250 bp, less than about 2300 bp, less than about 2350 bp, or less than about 2400 bp.

Embodiment 119.3. The nucleic acid construct of any one of embodiments 87-119, wherein the length of the one or more additional polypeptides is less than about 700 amino acids, less than about 725 amino acids, less than about 750 amino acids, less than about 775 amino acids, less than about 800 amino acids, less than about 825 amino acids, less than about 850 amino acids, less than about 875 amino acids, or less than about 900 amino acids.

Embodiment 120. A nucleic acid construct comprising, from 5' to 3':
(1) a promoter,
(2) a CAR encoding sequence,
(3) a cleavable linker encoding sequence, and
(4) a polynucleotide encoding the recombinant polypeptide of any one of embodiments 1-86.

Embodiment 121. A nucleic acid construct comprising, from 5' to 3':
(1) a promoter,
(2) a polynucleotide encoding the recombinant polypeptide of any one of embodiments 1-86,

(3) a cleavable linker encoding sequence, and

(4) a CAR encoding sequence.

Embodiment 122. A nucleic acid construct comprising, from 5' to 3':

(1) a promoter,

(2) a CAR encoding sequence,

(3) a cleavable linker encoding sequence,

(4) a polynucleotide encoding the recombinant polypeptide of any one of embodiments 1-86,

(5) a cleavable linker encoding sequence, and

(6) a polynucleotide encoding an additional potency enhancement polypeptide, a cytokine, a chemokine, or a growth factor.

Embodiment 123. A nucleic acid construct comprising, from 5' to 3':

(1) a promoter,

(2) a polynucleotide sequence encoding the recombinant polypeptide of any one of embodiments 1-86,

(3) a cleavable linker encoding sequence,

(4) a CAR encoding sequence,

(5) a cleavable linker encoding sequence, and

(6) a polynucleotide encoding an additional potency enhancement polypeptide, a cytokine, a chemokine, or a growth factor.

Embodiment 124. A nucleic acid construct comprising, from 5' to 3':

(1) a promoter,

(2) a polynucleotide sequence encoding the recombinant polypeptide of any one of embodiments 1-86,

(3) a cleavable linker encoding sequence,

(4) a polynucleotide encoding an additional potency enhancement polypeptide, a cytokine, a chemokine, or a growth factor

(5) a cleavable linker encoding sequence, and

(6) a CAR encoding sequence.

Embodiment 125. A nucleic acid construct comprising, from 5' to 3':

(1) a promoter,

(2) a polynucleotide encoding an additional potency enhancement polypeptide, a cytokine, a chemokine, or a growth factor,
(3) a cleavable linker encoding sequence,
(4) a polynucleotide sequence encoding the recombinant polypeptide of any one of embodiments 1-86,
(5) a cleavable linker encoding sequence, and
(6) a CAR encoding sequence.

Embodiment 126. A nucleic acid construct comprising, from 5' to 3':
(1) a promoter,
(2) a polynucleotide encoding an additional potency enhancement polypeptide, a cytokine, a chemokine, or a growth factor,
(3) a cleavable linker encoding sequence,
(4) a CAR encoding sequence,
(5) a cleavable linker encoding sequence, and
(6) a polynucleotide sequence encoding the recombinant polypeptide of any one of embodiments 1-86.

Embodiment 127. A nucleic acid construct comprising, from 5' to 3':
(1) a cleavable linker encoding sequence,
(2) a CAR encoding sequence,
(3) a cleavable linker encoding sequence,
(4) a polynucleotide sequence encoding the recombinant polypeptide of any one of embodiments 1-86, and
(5) a cleavable linker encoding sequence.

Embodiment 128. A nucleic acid construct comprising, from 5' to 3':
(1) a cleavable linker encoding sequence,
(2) a polynucleotide sequence encoding the recombinant polypeptide of any one of embodiments 1-86,
(3) a cleavable linker encoding sequence,
(4) a CAR encoding sequence, and
(5) a cleavable linker encoding sequence.

Embodiment 129. A nucleic acid construct comprising, from 5' to 3':

(1) a cleavable linker encoding sequence,

(2) a CAR encoding sequence,

(3) a cleavable linker encoding sequence,

(4) a polynucleotide sequence encoding the recombinant polypeptide of any one of embodiments 1-86,

(5) a cleavable linker encoding sequence,

(6) a polynucleotide encoding an additional potency enhancement polypeptide, a cytokine, a chemokine, or a growth factor,

(7) a cleavable linker encoding sequence.

Embodiment 130. A nucleic acid construct comprising, from 5' to 3':

(1) a cleavable linker encoding sequence,

(2) a CAR encoding sequence,

(3) a cleavable linker encoding sequence,

(4) a polynucleotide encoding an additional potency enhancement polypeptide, a cytokine, a chemokine, or a growth factor,

(5) a cleavable linker encoding sequence,

(6) a polynucleotide sequence encoding the recombinant polypeptide of any one of embodiments 1-86,

(7) a cleavable linker encoding sequence.

Embodiment 131. A nucleic acid construct comprising, from 5' to 3':

(1) a cleavable linker encoding sequence,

(2) a polynucleotide sequence encoding the recombinant polypeptide of any one of embodiments 1-86,

(3) a cleavable linker encoding sequence,

(4) a CAR encoding sequence,

(5) a cleavable linker encoding sequence,

(6) a polynucleotide encoding an additional potency enhancement polypeptide, a cytokine, a chemokine, or a growth factor,

(7) a cleavable linker encoding sequence.

Embodiment 132. A nucleic acid construct comprising, from 5' to 3':

(1) a cleavable linker encoding sequence,

(2) a polynucleotide sequence encoding the recombinant polypeptide of any one of embodiments 1-86,

(3) a cleavable linker encoding sequence,

(4) a polynucleotide encoding an additional potency enhancement polypeptide, a cytokine, a chemokine, or a growth factor,

(5) a cleavable linker encoding sequence,

(6) a CAR encoding sequence,

(7) a cleavable linker encoding sequence.

Embodiment 133. A nucleic acid construct comprising, from 5' to 3':

(1) a cleavable linker encoding sequence,

(2) a polynucleotide encoding an additional potency enhancement polypeptide, a cytokine, a chemokine, or a growth factor,

(3) a cleavable linker encoding sequence,

(4) a CAR encoding sequence,

(5) a cleavable linker encoding sequence,

(6) a polynucleotide sequence encoding the recombinant polypeptide of any one of embodiments 1-86,

(7) a cleavable linker encoding sequence.

Embodiment 134. A nucleic acid construct comprising, from 5' to 3':

(1) a cleavable linker encoding sequence,

(2) a polynucleotide encoding an additional potency enhancement polypeptide, a cytokine, a chemokine, or a growth factor,

(3) a cleavable linker encoding sequence,

(4) a polynucleotide sequence encoding the recombinant polypeptide of any one of embodiments 1-86,

(5) a cleavable linker encoding sequence,

(6) a CAR encoding sequence,

(7) a cleavable linker encoding sequence.

Embodiment 135. A nucleic acid construct comprising:

(1) a CAR encoding sequence, and

(2) a polynucleotide sequence encoding the recombinant polypeptide of any one of

embodiments 1-86,
wherein the nucleic acid construct is an RNA construct.

Embodiment 136. A composition comprising:
(1) a first nucleic acid construct encoding a CAR, and
(2) a second nucleic acid construct encoding the recombinant polypeptide of any one of embodiments 1-86,
wherein the first and the second nucleic acid constructs are both RNA constructs.

Embodiment 137. A composition comprising:
(1) a CAR encoding sequence,
(2) a polynucleotide sequence encoding the recombinant polypeptide of any one of embodiments 1-86, and
(3) a polynucleotide encoding an additional potency enhancement polypeptide, a cytokine, a chemokine, or a growth factor,
wherein the nucleic acid construct is an RNA construct.

Embodiment 138. A composition comprising:
(1) a first nucleic acid construct encoding a CAR,
(2) a second nucleic acid construct encoding the recombinant polypeptide of any one of embodiments 1-86, and
(3) a third nucleic acid construct encoding a polynucleotide encoding an additional potency enhancement polypeptide, a cytokine, a chemokine, or a growth factor, and
wherein the first, second, and third nucleic acid constructs are each RNA constructs

Embodiment 139. A vector comprising the nucleic acid construct of any one of embodiments 87-119.

Embodiment 140. The vector of embodiment 139, wherein the vector is a plasmid.

Embodiment 141. The vector of embodiment 139, wherein the vector is a synthetic DNA vector.

Embodiment 142. The vector of embodiment 139, wherein the vector is a linear DNA vector.

Embodiment 143. The vector of embodiment 139, wherein the vector is a closed linear DNA vector.

Embodiment 143.1. The vector of embodiment 139, wherein the vector is a synthetic RNA vector.

Embodiment 144. The vector of embodiment 139, wherein the vector is a phagemid vector.

Embodiment 145. The vector of embodiment 139, wherein the vector is a viral vector.

Embodiment 146. The vector of embodiment 145, wherein the viral vector is selected from a retrovirus vector, an adenovirus vector, and an adeno-associated virus (AAV) vector; optionally, wherein the viral vector is an AAV vector.

Embodiment 147. The vector of embodiment 146, wherein the retrovirus is a lentivirus.

Embodiment 148. The vector of embodiment 147, wherein the lentivirus is a VSV-G pseudotyped lentivirus.

Embodiment 149. The vector of embodiment 146, wherein the AAV vector is AAV6 vector.

Embodiment 150. The vector of embodiment 146, wherein the AAV vector is AAV9 vector.

Embodiment 151. The vector of embodiment 146, wherein the AAV vector is a split-intein dual AAV vector.

Embodiment 152. The vector of embodiment 145, wherein the vector is a redirected lentiviral vector.

Embodiment 153. The vector of embodiment 145, wherein the vector is a fusosome.

Embodiment 154. The vector of embodiment 145, wherein the vector is a lentiviral particle engineered with the anti-CD3 Cocal glycoprotein.

Embodiment 155. The vector of embodiment 145, wherein the vector is an enveloped delivery vehicle.

Embodiment 156. The vector of embodiment 145, wherein the vector is self-replicating RNA virus.

Embodiment 157. The vector of embodiment 139, wherein the vector is mRNA-packaging virus-like particle.

Embodiment 158. The vector of embodiment 139, wherein the vector is RNP-packaging virus-like particle.

Embodiment 159. A particle comprising the nucleic acid construct of any of embodiments 87-119.

Embodiment 160. The particle of embodiment 159, wherein the particle is a lipid nanoparticle (LNP).

Embodiment 161. The particle of embodiment 159, wherein the particle is a selective organ targeting (SORT) LNP.

Embodiment 162. The particle of embodiment 159, wherein the particle is an antibody targeted LNP.

Embodiment 163. The particle of any one of embodiments 160-162, wherein the LNP comprises: (i) an ionizable lipid (e.g., an amino lipid), (ii) a sterol or other structural lipid, (iii) a non-cationic helper lipid or phospholipid, and (iv) a PEG-lipid (e.g., a PEG-modified lipid).

Embodiment 164. The particle of embodiment 159, wherein the particle is a polymer nanoparticle.

Embodiment 165. The particle of embodiment 159, wherein the particle is a protein nanoparticle.

Embodiment 166. An engineered cell comprising the recombinant polypeptide of any one of embodiments 1-86 or the nucleic acid construct of any one of embodiments 87-119.

Embodiment 167. An engineered cell comprising SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 407, SEQ ID NO: 408, SEQ ID NO: 409, SEQ ID NO: 410, SEQ ID NO: 411, or SEQ ID NO: 412.

Embodiment 167.1 The engineered cell of embodiment 166 or 167, wherein activating the engineered cell results in the secretion of IFNg by the cell at a level that is at least 10%, at

least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold higher than the IFNg secretion level of a control cell lacking the recombinant polypeptide.

Embodiment 168. The engineered cell of any one of embodiments 166-167.1, wherein activating the engineered cell results in the secretion of IL-2 by the cell at a level that is at least 10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold higher than the IL-2 secretion level of a control cell lacking the recombinant polypeptide.

Embodiment 168.1. The engineered cell of embodiment 166 or 167, wherein activation of the engineered cell and subsequent removal of the target antigen results in CARD11-PIK3R3 signaling by the cell at a level that is at least 10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold lower than the CARD11-PIK3R3 signaling of a control cell comprising a CARD11-PIK3R3 fusion protein lacking the inhibitory domain.

Embodiment 168.2. The engineered cell of embodiment 166 or 167, wherein activation of the engineered cell and subsequent removal of the target antigen results in CARD11-PIK3R3 signaling by the cell at a level that is at least 10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold lower than the CARD11-PIK3R3 signaling of a control cell comprising a CARD11-PIK3R3 fusion protein lacking one of more of S466, S512, S535, S559, S644, and S652 according to SEQ ID NO: 518 in the inhibitory domain.

Embodiment 168.3. The engineered cell of any of embodiments 166-168.2, wherein CARD11-PIK3R3 signaling in the cell in the absence of a target antigen is at a level that is at least 10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold lower than the CARD11-PIK3R3 signaling of a control cell comprising a control CARD11-PIK3R3 fusion protein lacking the inhibitory domain.

Embodiment 168.4. The engineered cell of any of embodiments 166-168.2, wherein CARD11-PIK3R3 signaling in the cell in the absence of a target antigen is at a level that is at least 10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold lower than the CARD11-PIK3R3

signaling of a control cell comprising a control CARD11-PIK3R3 fusion protein lacking one of more of S466, S512, S535, S559, S644, and S652 according to SEQ ID NO: 518in the inhibitory domain.

Embodiment 168.5. The engineered cell of any of embodiments 168.1-168.4, wherein the CARD11-PIK3R3 signaling is measured by:

(a) BCL10 binding,

(b) BCL10 and MALT1 complex formation,

(c) NFAT, NF-κB and/or AP-1 signaling,

(d) cytokine production,

(e) JAK/STAT signaling,

(f) co-stimulatory molecule signaling,

(g) phospholipase gamma signaling, and/or

(h) transcription factor activity.

Embodiment 169. The engineered cell of any one of embodiments 166-168.5, wherein expression of the recombinant polypeptide is under the control of an endogenous promoter.

Embodiment 170. The engineered cell of embodiment 169, wherein the endogenous promoter is a TCRa promoter, a TCRb promoter, a CD3d promoter, a CD3g promoter, a CD3e promoter, a CD3z promoter, a CARD9 promoter, a CARD10 promoter, a CARD11 promoter, a CARD14 promoter, or a PIK3R3 promoter.

Embodiment 171. The engineered cell of any one of embodiments 166-168, wherein expression of the recombinant polypeptide is under the control of an exogenous promoter.

Embodiment 172. The engineered cell of embodiment 171, wherein the exogenous promoter is or comprises a minimal TATA promoter, pGK promoter, actin promoter, CD4 promoter, CD8a promoter, CD8b promoter, TCRa promoter, TCRb promoter, CD3d promoter, CD3g promoter, CD3e promoter, CD3z promoter, CARD9 promoter, CARD10 promoter, CARD11 promoter, CARD14 promoter, PIK3R3 promoter, CD25 promoter, IL2 promoter, IL7 promoter, IL15 promoter, KLRG-1 promoter, HLA-DR promoter, CD38 promoter, CD69 promoter, Ki-67 promoter, CD11a promoter, CD58 promoter, CD99 promoter, CD62L promoter, CD103 promoter, CCR4 promoter, CCR5 promoter, CCR6 promoter, CCR9 promoter, CCR10 promoter, CXCR3 promoter, CXCR4 promoter, CLA promoter, Granzyme A promoter, Granzyme B promoter, Perforin promoter, CD57 promoter, CD161

promoter, IL-18Ra promoter, CD69 promoter, GzmB promoter, T-bet promoter, IFNgamma promoter, TIM3 promoter, IL4 promoter, GATA3 promoter, IL1 promoter, IL5 promoter, IL6 promoter, IL13 promoter, IL10 promoter, IL17A promoter, IL6 promoter, IL21 promoter, IL23R promoter, FoxP3 promoter, CTLA4 promoter, CD25 promoter, PD1 promoter, CD45RO promoter, CCR7 promoter, CD28 promoter, CD95 promoter, CD28 promoter, CD27 promoter, CD127 promoter, PD-1 promoter, CD122 promoter, CD132 promoter, c-Kit promoter, nuclear factor of activated T cells (NFAT) promoter, programmed death 1 (PD-1) promoter, T cell immunoglobulin mucin-3 (TIM-3) promoter, cytotoxic T lymphocyte antigen-4 (CTLA4) promoter, lymphocyte-activation protein 3 (LAG-3) promoter, tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) promoter, B- and T-lymphocyte attenuator (BTLA) promoter, CD25 promoter, CD69 promoter, Fas ligand (FasL) promoter, TIGIT promoter, TGF-beta promoter, T-bet promoter, Eomes promoter, GATA3 promoter, CD45RA promoter, 2B4 promoter, Type I interferon (IFN) alpha, Type I IFN beta promoter, IFN gamma promoter, IRF3 promoter, IRF7 promoter, NFkB promoter, AP-1 promoter, TNF-alpha promoter, CD130 promoter, NR4A1 promoter, NR4A2, NR4A3 promoter, MND promoter, EF-1alpha promoter, short EF-1alpha promoter, CAG promoter, ubiquitin/S27a promoter, SV40 promoter, SV40 early promoter, adenovirus major late promoter, mouse metallothionein- I promoter, Moloney murine leukemia virus (MMLV) long terminal repeat (LTR) region, CMV promoter, immunoglobulin promoter, heat shock promoter, polyoma virus promoter, fowlpox virus promoter, bovine papilloma virus promoter, avian sarcoma virus promoter, retrovirus promoter, hepatitis-B virus promoter, PGK promoter, vaccinia virus 7.5K promoter, TK promoter of HSV, mouse mammary tumor virus (MMTV) promoter, LTR promoter of HIV, promoter of oloney virus, Epstein Barr virus (EBV) promoter, Rous sarcoma virus (RSV) promoter, U6 promoter, or UBC promoter.

Embodiment 173. The engineered cell of any one of embodiments 166-172, wherein the nucleic acid construct is inserted in a T-cell receptor (TCR) locus, a CD3 locus, a B2 microglobulin (B2M) locus, a class II transactivator (CIITA) locus, a CARD9 locus, a CARD10 locus, a CARD11 locus, a CARD14 locus, a PIK3R3 locus, or a safe harbor locus.

Embodiment 174. The engineered cell of embodiment 173, wherein the TCR locus is or comprises a TRAC locus, a TRBC1 locus, a TRBC2 locus, or a TRAJ locus.

Embodiment 174.1. The engineered cell of embodiment 174, wherein the TRAJ locus is or comprises a TRAJ intron splice acceptor locus.

Embodiment 175. The engineered cell of embodiment 173, wherein the cell does not express a gene product of an endogenous TRAC locus.

Embodiment 176. The engineered cell of embodiment 173, wherein the cell does not express a gene product of an endogenous TRBC1 locus.

Embodiment 177. The engineered cell of embodiment 173, wherein the cell does not express a gene product of an endogenous TRBC2 locus.

Embodiment 178. The engineered cell of embodiment 173, wherein the CD3 locus is or comprises: a CD3d locus, a CD3g locus, a CD3e locus, or CD3z locus.

Embodiment 179. The engineered cell of embodiment 178, wherein the cell does not express a gene product of an endogenous CD3d locus.

Embodiment 180. The engineered cell of embodiment 178, wherein the cell does not express a gene product of an endogenous CD3g locus.

Embodiment 181. The engineered cell of embodiment 178, wherein the cell does not express a gene product of an endogenous CD3e locus.

Embodiment 182. The engineered cell of embodiment 178, wherein the cell does not express a gene product of an endogenous CD3z locus.

Embodiment 183. The engineered cell of embodiment 173, wherein the safe harbor locus is or comprises an AAVS1, ABO, CCR5, CLYBL, CXCR4, F3, FUT1, HMGB1, KDM5D, LRP1, MICA, MICB, RHD, ROSA26, or SHS231 locus.

Embodiment 184. The engineered cell of any one of embodiments 166-183, wherein the nucleic acid construct is inserted is an exon, an intron, between an intron and an exon, or a regulatory region.

Embodiment 185. The engineered cell of any one of embodiments 166-184, wherein the cell is selected from the group of consisting of a T cell, a CD4+ T cell, a CD8+ T cell, a regulatory T cell (Treg), a gamma delta T cell (γδT), an invariant natural killer T (iNKT)

cell, a mucosal associated invariant T (MAIT) cell, a macrophage, a monocyte, a natural killer (NK) cell, a tumor infiltrating lymphocyte (TIL), a cytotoxic T cell, a T helper cell, a memory T cell, a central memory T (TCM) cell, a stem memory T (TSCM) cell, a stem-cell-like memory T cell (or stem-like memory T cells), an effector memory T (TEM) cell, a TEMRA (CD45RA+) cell, an effector T cell, a Th1 cell, a Th2 cell, a Th9 cell, a Th17 cell, a Th22 cell, a Tfh (follicular helper) cell, a natural killer T (NKT) cell, a transitional memory T (TTM) cell, a terminal effector T (TTE) cell, a naïve T (TN) cell, a hematopoietic stem cell, and a progenitor cell of the lymphoid lineage.

Embodiment 186. The engineered cell of embodiment 185, wherein the cell is selected from the group consisting of a T cell, a macrophage, a monocyte, and a natural killer (NK) cell.

Embodiment 187. The engineered cell of embodiment 186, wherein the cell is a T cell.

Embodiment 188. The engineered cell of embodiment 187, wherein the T cell is selected from the group consisting of a regulatory T cell (Treg), a gamma delta T cell, a CD8+ T cell, an invariant iNKT cell, a MAIT cell, a CAR T cell, a tumor-infiltrating lymphocyte, and an engineered T cell comprising a transcriptional receptor.

Embodiment 189. The engineered cell of any one of embodiments 166-188, wherein the cell is an autologous cell.

Embodiment 190. The engineered cell of any one of embodiments 166-188, wherein the cell is an allogeneic cell.

Embodiment 191. The engineered cell of any one of embodiments 166-190, wherein the cell is a primary cell.

Embodiment 192. The engineered cell of any one of embodiments 166-190, wherein the cell is derived from a stem cell.

Embodiment 193. The engineered cell of any one of embodiments 166-192, wherein the cell is genetically modified.

Embodiment 194. The engineered cell of any one of embodiments 166-193, wherein the cell has reduced or eliminated expression of an endogenous T cell receptor.

Embodiment 195. The engineered cell of any one of embodiments 166-194, wherein the cell is more prone to display central memory phenotype than a control cell lacking the recombinant polypeptide.

Embodiment 196. The engineered cell of any one of embodiments 166-195, wherein the cell further comprises:

(i) a chimeric antigen receptor (CAR) having specificity for a target antigen; or
(ii) a recombinant T cell receptor (TCR) having specificity for a target antigen.

Embodiment 197. The engineered cell of embodiment 196, wherein the engineered cell comprises a nucleic acid construct expressing the CAR or recombinant TCR; optionally, wherein the nucleic acid construct is an RNA construct.

Embodiment 198. The engineered cell of embodiment 196or 197, wherein the target antigen is CD1, CD1a, CD1b, CD1c, CD1d, CD1e, CD2, CD3d, CD3e, CD3g, CD3ε, CD4, CD5, CD7, CD8a, CD8b, CD19, CD20, CD21, CD22, CD23, CD24, CD25, CD27, CD28, CD30, CD33, CD34, CD38, CD40, CD44v6, CD45, CD46, CD47 CD48, CD52, CD59, CD66, CD70, CD71, CD72, CD73, CD79A, CD79B, CD80 (B7.1), CD86 (B7.2), CD94, CD95, CD97, CD123, CD134, CD140 (PDGFR4), CD152, CD154, CD158, CD171, CD178, CD179, CD179a, CD181 (CXCR1), CD182 (CXCR2), CD183 (CXCR3), CD210, CD213A2, CD246, CD252, CD253, CD261, CD262, CD272, CD273 (PD-L2), CD274 (PD-L1), CD276 (B7H3), CD279, CD295, CD339 (JAG1), CD340 (HER2), CDH17, CEA, CLECL1, CLL-1, CLDN6, CLDN18.2, CS1, DLL3, LY6G6D, GCC, p53R175H, PRAME, EGFR, EGFRvIII, FGFR2, AFP, CA125, MUC-1, MAGE, ALPI, alkaline phosphatase placental-like 2 (ALPPL2), B-cell maturation antigen (BCMA), green fluorescent protein (GFP), enhanced green fluorescent protein (eGFP), KLK2, KLK3, Mesothelin, IL13Ra2, ggcc, signal regulatory protein α (SIRPα), TCRalpha, TCRbeta, TSHR, GD2, GD3, Tn Ag, cMET, Axl, ROR1, ROR2, GPC1, GPC2, GPC3, FLT3, TAG72, CEA, EPCAM, KIT (CD117), IL-13Ra2, IL-11Ra, PSCA, PRSS21, VEGFR2, LewisY, PDGFRβ, SSEA-4, folate receptor alpha, ERBB2 (Her2/neu), MUC1, MUC16, NCAM, prostase, PAP, ELF2M, Ephrin B2, IGF-I receptor, CAIX, LMP2, gp100, bcr-abl, tyrosinase, EphA2, STEAP1, STEAP2, fucosyl GM1, sLe, GM3, TGS5, HMWMAA, o-acetyl-GD2, folate receptor beta, TEM1/CD248, TEM7R, ROPN1, GPRC5D, GPA33, CXORF61, ALK, Polysialic acid, PLAC1, GloboH, NY-BR-1, UPK2, HAVCR1, ADRB3, PANX3, GPR20, LY6K, OR51E2,

TARP, WT1, NY-ESO-1, LAGE-la, MAGE-Al, legumain, HPV E6,E7, MAGE-A4, ETV6-AML, sperm protein 17, XAGE1, Tie 2, MAD-CT-1, MAD-CT-2, Fos-related antigen 1, p53, p53 mutant, p53R175H, KRAS, mutant KRAS, KRAS G12D, prostein, surviving, telomerase, PCTA-1/Galectin 8, MelanA/MART1, Ras mutant, hTERT, sarcoma translocation breakpoints, ML-IAP, ERG (TMPRSS2 ETS fusion gene), NA17, PAX3, androgen receptor, cyclin Bl, MYCN, RhoC, TRP-2, CYP1B1, BORIS, SART3, PAX5, OY-TES1, LCK, AKAP-4, SSX2, RAGE-1, human telomerase reverse transcriptase, RU1, RU2, intestinal carboxyl esterase, mut hsp70-2,LAIR1, FCAR, LILRA2, CD300LF, CLEC12A, BST2, EMR2, LY75, FCRL5, IGLL1, PSMA, TROP2, citrullinated vimentin, the extracellular portion of the APRIL protein, and any combinations thereof.

Embodiment 199. The engineered cell of embodiment 198, wherein the target antigen is B7H3, BCMA, CD19, CD20, CD22, CD70, CD79a, CD79b, CDH17, CLDN6, CLDN18.2, DLL3, GCC, GD2, GD3, GPC3, GPRC5D, GPA33, KLK3, LY6G6D, p53R175H, PRAME, ROPN1, STEAP1, or STEAP2.

Embodiment 200. The engineered cell of embodiment 198 or embodiment 199, wherein the engineered cell has cell surface expression of the CAR.

Embodiment 201. The engineered cell of any one of embodiments 166-198, wherein the cell further comprises a chimeric auto antigen receptor (CAAR) having specificity for a target antigen.

Embodiment 202. The genetically engineered cell of embodiment 201, wherein the target antigen is pancreatic P-cell antigen, synovial joint antigen, myelin basic protein, proteolipid protein, myelin oligodendritic glycoprotein, MuSK, keratinocyte adhesion protein desmoglein 3 (Dsg3), Ro-RNP complex, La antigen, myeloperoxidase, proteinase 3, cardiolipin, citrullinated proteins, carbamylated proteins, or a3 chain of basement membrane collagen.

Embodiment 203. The engineered cell of embodiment 201 or embodiment 202, wherein the engineered cell has cell surface expression of the CAAR.

Embodiment 204. The engineered cell of any one of embodiments 166-195, wherein the cell further comprises a B-cell autoantibody receptor (BAR) having specificity for a target antigen.

Embodiment 205. The engineered cell of embodiment 204, wherein the engineered cell has cell surface expression of the BAR.

Embodiment 206. The engineered cell of any one of embodiments 166-205, wherein the cell further comprises a non-coding RNA.

Embodiment 207. The engineered cell of embodiment 206, wherein the non-coding RNA comprises a shRNA or a microRNA.

Embodiment 208. The engineered cell of any one of embodiments 166-204, wherein the cell further comprises an additional potency enhancement polypeptide, a cytokine, a chemokine, a growth factor, a tolerogenic factor, a safety switch, or any combination thereof.

Embodiment 209. The engineered cell of embodiment 208, wherein the additional potency enhancement polypeptide is or comprises a dominant negative form of an inhibitor of a cell-mediated immune response of the immune cell (e.g., TGFβR2 DNR), c-Jun, CCL19, CCL21, IL2R, IL7, IL7Ralpha, IL15, IL15RA, IL18, decoy-resistant IL18 (DR-18), MyD88/CD40, PD1-CD28 switch receptor, PD1-41BB switch receptor, CD40L-CD28 switch receptor, CTBR12 switch receptor, or CD8alpha/beta.

Embodiment 210. The engineered cell of embodiment 208, wherein the safety switch is or comprises a herpesvirus thymidine kinase (HSV-tk) gene, an Escherichia coli cytosine deaminase (EC-CD) gene, or an inducible Caspase9 (iCasp9) protein.

Embodiment 211. The engineered cell of any one of embodiments 166-210, wherein the cell further comprises one or more modifications that inactivate or disrupt one or more alleles of TGFbeta, Regnase-1, FAS, PTPN2, NR4A3, CD52, PD-1, B2M, CIITA, SOCS1, CBLB, DGKalpha, and/or DGKζ.

Embodiment 212. The engineered cell of any one of embodiments 166-211, wherein the cell is a mammalian cell.

Embodiment 213. The engineered cell of embodiment 212, wherein the cell is selected from the group consisting of a human cell, a mouse cell, and a canine cell.

Embodiment 214. The engineered cell of any one of embodiments 166-213, wherein the cell further comprises a sequence specific nuclease, a nucleic acid programmable DNA binding

protein, an RNA guided nuclease, an RNA-guided nuclease comprising a Cas nuclease and a guide RNA (CRISPR-Cas combination), a ribonucleoprotein (RNP) complex comprising a gRNA and a Cas nuclease, a homing endonuclease, a zinc finger nuclease (ZF) nucleic acid binding entity, a transcription activator-like effector (TALE) nucleic acid binding entity, a meganuclease, a Cas nuclease, a core Cas protein, a homing endonuclease, an endonuclease-deficient-Cas protein, an enzymatically inactive Cas protein, a CRISPR- associated transposase (CAST), a Type II or Type V Cas protein, or a functional portion thereof.

Embodiment 215. The engineered cell of embodiment 214, wherein the cell further comprises Casl, Cas2, Cas3, Cas4, Cas5, Cas6, Cas7, Cas8a, Cas8b, Cas8c, Cas9, CaslO, Casl2, Casl2a (Cpfl), Casl2b (C2cl), Casl2c (C2c3), Casl 2d (CasY), Casl2e (CasX), Casl2f (C2cl0), Cas 12g, Casl2h, Casl2i, Cas 12k (C2c5), Casl3, Casl3a (C2c2), Casl3b, Casl3c, Casl3d, C2c4, C2c8, C2c9, Cmrl, Cmr2, Cmr3, Cmr4, Cmr5, Cmr6, Csdl, Csd2, Cas5d, Csel, Cse2, Cse3, Cse4, Cas5e, Csfl, Csml, Csm2, Csm3, Csm4, Csm5, Csnl, Csn2, Cstl, Cst2, Cas5t, Cshl, Csh2, Cas5h, Csal, Csa2, Csa3, Csa4, Csa5, Cas5a, CsxlO, Csxl l, Csyl, Csy2, Csy3, Csy4, Mad7, SpCas9, eSpCas9, SpCas9-HFl, HypaSpCas9, HeFSpCas9, evoSpCas9 high-fidelity variants of SpCas9, SaCas9, NmeCas9, CjCas9, StCas9, TdCas9, LbCasl2a, AsCasl2a, AacCasl2b, BhCasl2b v4, TnpB, dCas (D10A), dCas (H840A), dCasl3a, dCasl3b, or a functional fragment thereof.

Embodiment 216. The method of embodiment 215, wherein the RNA-guided nuclease is a Life Edit nuclease (LEG), optionally a LEG14 nuclease.

Embodiment 217. An engineered cell comprising:
a recombinant nucleic acid sequence encoding a caspase-associated recruitment domain (CARD) containing protein or a functional fragment thereof,
wherein the recombinant nucleic acid sequence is integrated at exon 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 or intron 1, 2, 3, 4, 5, 6, 7, 8, or 9 of an endogenous PIK3R3 locus,
wherein the nucleic acid sequence encoding the CARD containing protein or the functional fragment thereof is expressed under the control of the endogenous PIK3R3 promoter, and
wherein integration of the nucleic acid sequence produces a fusion protein comprising (i) the CARD containing protein or the functional fragment thereof and (ii) a recruitment domain of PIK3R3.

Embodiment 218. The engineered cell of embodiment 217, wherein the recombinant nucleic acid sequence is integrated at exon 8 of an endogenous PIK3R3 locus.

Embodiment 219. The engineered cell of embodiment 217, wherein the recombinant nucleic acid sequence is integrated at intron 7 of an endogenous PIK3R3 locus.

Embodiment 220. An engineered cell comprising:
a recombinant nucleic acid sequence encoding a recruitment domain containing protein or a functional fragment thereof, wherein the recruitment domain is capable of binding to (i) a substrate localized to the intracellular side of the plasma membrane of a cell and/or (ii) a target polypeptide comprising a phosphorylated tyrosine,
wherein the recombinant nucleic acid is integrated at exon 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 or intron 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, or 24 of an endogenous CARD11 locus,
wherein the rectruitment domain containing protein or functional fragrment thereof is expressed under the control of the endogenous CARD11 promoter, and
wherein integration of the nucleic acid sequence produces a fusion protein comprising (i) the CARD containing protein or the functional fragment thereof and (ii) the recruitment domain containing protein or a functional fragment thereof.

Embodiment 221. The engineered cell of embodiment 220, wherein the recombinant nucleic acid sequence is integrated at exon 13 of an endogenous CARD11 locus.

Embodiment 222. The engineered cell of embodiment 220, wherein the recombinant nucleic acid sequence is integrated at intron 12 of an endogenous CARD11 locus.

Embodiment 222.1. The engineered cell of embodiment 220, wherein the recombinant nucleic acid sequence is integrated at exon 14 of an endogenous CARD11 locus.

Embodiment 222.2. The engineered cell of embodiment 220, wherein the recombinant nucleic acid sequence is integrated at intron 13 of an endogenous CARD11 locus.

Embodiment 223. The engineered cell of any one of embodiments 166-222, wherein the cell has reduced exhaustion, increased proliferative capacity, enhanced replicative lifespan, decreased replicative senescence, enhanced anti-tumor effect, reduced dysfunction, enhanced persistence, and/or increase intratumoral presence *in vivo*.

Embodiment 224. The engineered cell of any one of embodiments 166-223, wherein the cell has increased or decreased signaling through the CARD11-BCL10-MALT1 complex, NF-κB, AP-1, NFAT, JAK/STAT, and/or MEK/ERK pathways.

Embodiment 225. A composition comprising the engineered cell of any one of embodiments 166-224, or a plurality of the engineered cell of any one of embodiments 166-224.

Embodiment 226. The composition of embodiment 225, further comprising a pharmaceutically acceptable excipient.

Embodiment 227. The composition of embodiment 225 or embodiment 226, wherein at least at or about 70%, 75%, 80%, 85%, 90%, or 95% of the engineered cells, or of the total cells, in the composition express the recombinant polypeptide of any one of embodiments 1-86.

Embodiment 228. The composition of any one of embodiments 225-227, wherein at least at or about 70%, 75%, 80%, 85%, 90%, or 95% of the engineered cells, or of the total cells, in the composition express the CAR or recombinant TCR.

Embodiment 229. The composition of any one of embodiments 225-228, wherein at least at or about 70%, 75%, 80%, 85%, 90%, or 95% of the engineered cells, or of the total cells, in the composition do not express a gene product of an endogenous TRAC locus.

Embodiment 230. The composition of any one of embodiments 225-229, wherein: at least at or about 70%, 75%, 80%, 85%, 90%, or 95% of the engineered cells, or of the total cells, in the composition express the recombinant polypeptide of any one of embodiments 1-86; at least at or about 70%, 75%, 80%, 85%, 90%, or 95% of the engineered cells, or of the total cells, in the composition express the CAR or recombinant TCR; and/or at least at or about 70%, 75%, 80%, 85%, 90%, or 95% of the engineered cells, or of the total cells, in the composition do not express a gene product of an endogenous TRAC locus.

Embodiment 231. The composition of any one of embodiments 225-229, wherein the composition comprises CD4+ T cells and CD8+ T cells, and the percentage of CD4+ T cells in the composition is between at or about 20% and at or about 80%, or at or about 20%, 30%, 40%, 50%, 60%, 70%, or 80% of the total cells in the composition; and/or the percentage of CD8+ T cells in the composition is between at or about 20% and at or about 80%, or at or about 20%, 30%, 40%, 50%, 60%, 70%, or 80% of the total cells in the

composition; and/or the ratio of CD4+ T cells to CD8+ T cells is from at or about 1:3 to at or about 3:1, optionally at or about 1:1.

Embodiment 232. A method of preparing the engineered cell according to any one of embodiments 166-224, the method comprising expressing the recombinant polypeptide in the cell, thereby making the engineered cell.

Embodiment 233. A method of preparing an engineered cell, the method comprising expressing in the cell the recombinant polypeptide of any one of embodiments 1-86, thereby making the engineered cell.

Embodiment 233.1. The method of embodiment 232 or embodiment 233, comprising introducing into the cell the nucleic acid construct of any one of embodiments 87-135.

Embodiment 234. A method of preparing an engineered cell, the method comprising introducing into the cell the nucleic acid construct of any one of embodiments 87-135, thereby making the engineered cell.

Embodiment 235. The method of any one of embodiments 232-234, wherein the cell is *in vivo*.

Embodiment 236. The method of any one of embodiments 232-235, wherein the cell is prepared *in vivo*.

Embodiment 237. The method of any one of embodiments 232-236, wherein the cell is prepared in the body of a subject in need of treatment.

Embodiment 238. The method of any one of embodiments 235-237, wherein the recombinant polypeptide is expressed in the cell, and/or the nucleic acid construct is introduced into the cell, by contacting the cell with a vector or a particle.

Embodiment 239. The method of embodiment 238, wherein the vector is a plasmid, a synthetic DNA vector, a linear DNA vector, a closed linear DNA vector, a phagemid vector, an RNA vector, an mRNA vector, or a viral vector.

Embodiment 240. The method of embodiment 239, wherein the viral vector is selected from a retrovirus vector, an adenovirus vector, and an adeno-associated virus (AAV) vector.

Embodiment 241. The method of embodiment 239, wherein the retrovirus is a lentivirus, optionally a VSV-G pseudotyped lentivirus.

Embodiment 242. The method of embodiment 239, the AAV vector is an AAV6 vector or an AAV9 vector.

Embodiment 243. The method of embodiment 238, wherein the vector is a fusosome, a lentiviral particle engineered with the anti-CD3 Cocal glycoprotein, or an enveloped delivery vehicle.

Embodiment 244. The method of embodiment 238, wherein the vector is a self-replicating RNA virus, an mRNA-packaging virus-like particle, or a ribonucleoprotein (RNP)-packaging virus-like particle.

Embodiment 245. The method of embodiment 238, wherein the particle is a lipid nanoparticle (LNP), a selective organ targeting (SORT) LNP, or an antibody targeted LNP.

Embodiment 246. The method of embodiment 245, wherein the LNP comprises: (i) an ionizable lipid (e.g., an amino lipid), (ii) a sterol or other structural lipid, (iii) a non-cationic helper lipid or phospholipid, and (iv) a PEG-lipid (e.g., a PEG-modified lipid).

Embodiment 247. The method of embodiment 238, wherein the particle is a polymer nanoparticle or a protein nanoparticle.

Embodiment 248. The method of any one of embodiments 232-247, wherein the cell is *ex vivo*.

Embodiment 249. The method of any one of embodiments 232-248, wherein the cell is prepared *ex vivo*.

Embodiment 250. The method of any one of embodiments 232-247 and embodiment 249, wherein the cell is prepared outside of the body of a subject in need of treatment.

Embodiment 251. The method of any one of embodiments 248-250, wherein the nucleic acid construct is introduced into the cell by electroporation or transfection.

Embodiment 252. The method of embodiment 251, wherein the transfection is lipofection.

Embodiment 253. The method of embodiment 251, wherein the nucleic acid construct is a plasmid, a synthetic DNA vector, a linear DNA vector, a closed linear DNA vector, a phagemid vector, an RNA vector, or an mRNA vector.

Embodiment 254. The method of any one of embodiments 248-250, wherein the nucleic acid construct is introduced via a viral vector.

Embodiment 255. The method of embodiment 254, wherein the viral vector is selected from a retrovirus vector, an adenovirus vector, and an adeno-associated virus (AAV) vector.

Embodiment 256. The method of embodiment 255, wherein the retrovirus is a lentivirus, optionally a VSV-G pseudotyped lentivirus.

Embodiment 257. The method of embodiment 256, wherein the AAV vector is an AAV6 vector or an AAV9 vector.

Embodiment 258. The method of any one of embodiments 232-257, wherein the method comprises genetically modifying the cell for expression of the recombinant polypeptide.

Embodiment 259. The method of embodiment 258, wherein the method comprises introducing into the cell one or more nucleic acids encoding a sequence specific nuclease or a nucleic acid programmable DNA binding protein.

Embodiment 260. The method of embodiment 259, wherein the method additionally comprises introducing into the cell one or more guide RNAs.

Embodiment 261. The method of embodiment 259 or 260, wherein the sequence-specific nuclease is an RNA guided nuclease.

Embodiment 262. The method of any one of embodiments 259-261, wherein the sequence-specific nuclease or nucleic acid programmable DNA binding domain is a homing endonuclease, a zinc finger nuclease (ZF) nucleic acid binding entity, a transcription activator-like effector (TALE) nucleic acid binding entity, a meganuclease, a Cas nuclease, a core Cas protein, a homing endonuclease, an endonuclease-deficient-Cas protein, an enzymatically inactive Cas protein, a CRISPR- associated transposase (CAST), a Type II or Type V Cas protein, or a functional portion thereof.

Embodiment 263. The method of embodiment 262, wherein the Cas nuclease is Casl, Cas2, Cas3, Cas4, Cas5, Cas6, Cas7, Cas8a, Cas8b, Cas8c, Cas9, CaslO, Casl2, Casl2a (Cpfl), Casl2b (C2cl), Casl2c (C2c3), Casl 2d (CasY), Casl2e (CasX), Casl2f (C2cl0), Cas 12g, Casl2h, Casl2i, Cas 12k (C2c5), Casl3, Casl3a (C2c2), Casl3b, Casl3c, Casl3d, C2c4, C2c8, C2c9, Cmrl, Cmr2, Cmr3, Cmr4, Cmr5, Cmr6, Csdl, Csd2, Cas5d, Csel, Cse2, Cse3, Cse4, Cas5e, Csfl, Csml, Csm2, Csm3, Csm4, Csm5, Csnl, Csn2, Cstl, Cst2, Cas5t, Cshl, Csh2, Cas5h, Csal, Csa2, Csa3, Csa4, Csa5, Cas5a, CsxlO, Csxl l, Csyl, Csy2, Csy3, Csy4, Mad7, SpCas9, eSpCas9, SpCas9-HFl, HypaSpCas9, HeFSpCas9, and evoSpCas9 high-fidelity variants of SpCas9, SaCas9, NmeCas9, CjCas9, StCas9, TdCas9, LbCasl2a, AsCasl2a, AacCasl2b, BhCasl2b v4, TnpB, dCas (D10A), dCas (H840A), dCasl3a, dCasl3b, or a functional portion thereof.

Embodiment 264. The method of embodiment 262, wherein the RNA-guided nuclease is a Life Edit nuclease (LEG), optionally a LEG14 nuclease.

Embodiment 265. The method of any one of embodiments 259 - 264, wherein the method comprises introducing into the cell a ribonucleoprotein (RNP) complex comprising the sequence-specific nuclease or nucleic acid programmable DNA binding.

Embodiment 266. The method of embodiment 234, wherein introducing the nucleic acid construct comprises contacting the cell with the particle of any one of embodiments 159-165 or the vector of any one of embodiments 120-158.

Embodiment 267. The method of embodiment 234, wherein the nucleic acid construct is introduced via electroporation.

Embodiment 268. The method of embodiment 234, wherein the nucleic acid construct is introduced via AAV.

Embodiment 269. The method of embodiment 234, wherein the nucleic acid construct is introduced via lentivirus.

Embodiment 270. The method of embodiment 234, wherein the nucleic acid construct is introduced via LNP.

Embodiment 271. The method of any one of embodiments 232-110, wherein the method further comprises expressing in the cell an immune receptor that binds to an antigen target in a target cell.

Embodiment 272. The method of embodiment 271, wherein expressing the immune receptor comprises administering a nucleic acid construct encoding the immune receptor to the cell; optionally, wherein the nucleic construct is an RNA construct.

Embodiment 273. The method of embodiment 271, wherein the immune receptor and the recombinant polypeptide are encoded by the same nucleic acid construct.

Embodiment 274. The method of embodiment 271, wherein the immune receptor and the recombinant polypeptide are encoded by different nucleic acid constructs.

Embodiment 275. The method of embodiment 272 or embodiment 274, wherein expressing the immune receptor comprises introducing to the cell a nucleic acid construct encoding the immune receptor.

Embodiment 276. The method of embodiment 275, wherein the nucleic acid construct encoding the immune receptor is introduced via a viral vector.

Embodiment 276.1. The method of any one of embodiments 271-276.1, wherein activating the immune receptor results in the secretion of IFNg by the cell at a level that is at least 10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold higher than the IFNg secretion level of a control cell lacking the recombinant polypeptide.

Embodiment 277. The method of any one of embodiments 271-276.1, wherein activating the immune receptor results in the secretion of IL-2 by the cell at a level that is at least 10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold higher than the IL-2 secretion level of a control cell lacking the recombinant polypeptide.

Embodiment 278. The method of any one of embodiments 234-277, wherein the nucleic acid construct is inserted into an endogenous locus of the cell.

Embodiment 279. The method of embodiment 278, wherein the endogenous locus is or comprises a TRAC gene locus, a TRBC1 gene locus, a TRBC2 gene locus, a TRAJ locus, a CARD9 locus, a CARD10 locus, a CARD11 locus, a CARD14 locus, or a PIK3R3 locus.

Embodiment 280. The method of embodiment 279, wherein the endogenous locus is or comprises a TRAC gene locus.

Embodiment 280.1. The method of embodiment 279, wherein the endogenous locus is or comprises a TRAJ locus.

Embodiment 280.2. The method of embodiment 280.1, wherein the TRAJ locus is or comprises a TRAJ intron splice acceptor locus.

Embodiment 281. The method of embodiment 278, wherein the endogenous locus is or comprises a safe harbor locus.

Embodiment 282. The method of embodiment 280, wherein the safe harbor locus is an AAVS1, ABO, CCR5, CLYBL, CXCR4, F3, FUT1, HMGB1, KDM5D, LRP1, MICA, MICB, RHD, ROSA26, or SHS231 locus.

Embodiment 283. The method of any one of embodiments 278-282, wherein the first insertion site is an exon.

Embodiment 284. The method of any one of embodiments 278-282, wherein the first insertion site is an intron.

Embodiment 285. The method of any one of embodiments 278-282, wherein the first insertion site is between an intron and an exon.

Embodiment 286. The method of any one of embodiments 278-282, wherein the first insertion site is in a regulatory region.

Embodiment 287. The method of any one of embodiments 278-286, wherein the first insertion site is 25 nucleotides or less from a protospacer adjacent motif (PAM) sequence, wherein the PAM sequence is ngg, nag, ngrrt, ngrm, nnnngatt, nnnnryac, nnagaaw, naaaac, tttv, ttn, attn, tttn, gttn, or yttn and wherein:
(i) r = a or g,
(ii) y = c or t,

(iii) w = a or t,

(iv) v = a or c or g, and

(v) n = a, c, t, or g.

Embodiment 288. The method of any one of embodiments 278-280 or embodiments 283-287, wherein the cell comprises a modified TRAC gene locus.

Embodiment 289. The method of any one of embodiments 278-288, wherein the cell does not express an endogenous TCR.

Embodiment 290. The method of any one of embodiments 232-289, wherein the method further comprises introducing a gene editing machinery into the cell.

Embodiment 291. The method of any one of embodiments 232-290, wherein the step of introducing comprises homology-directed repair (HDR)-mediated insertion using the gene editing machinery.

Embodiment 291.1. The method of embodiment 291, where the HDR occurs in 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 95%, at least 98%, or at least 99%, of a population of the cells; optionally, wherein the HDR occurs in between about 20%-30%, about 30%-40%, about 40%-50%, about 50%-60%, about 60%-70%, about 70%-80%, about 80%-90%, or about 90%-100%, of the cells.

Embodiment 291.2. The method of embodiment 291 or embodiment 291.1, wherein the step of introducing comprises HDR-mediated insertion in the absence of an HDR enhancer; optionally, wherein the HDR enhancer is M3814 or AZD7648.

Embodiment 291.3. The method of any one of embodiments 291-291.2, wherein the nucleic acid construct comprises a first homology arm before the 5’ end of the nucleic acid sequence encoding the recombinant polypeptide and a second homology arm after the 3’ end of the nucleic acid sequence encoding the recombinant polypeptide; optionally, wherein the lengths of the two homology arms are between about 150 bp and about 1500 bp.

Embodiment 291.4. The method of embodiment 291.3, wherein the length of each of the homology arms is at least 200 bp.

Embodiment 291.5. The method of embodiment 291.3 or 291.4, wherein the length of at least one of the homology arms is about 200bp, 225 bp, about 250 bp, about 275 bp, about 300 bp, about 325 bp, about 350 bp, about 375 bp, about 400 bp, about 425 bp, about 450 bp, about 475 bp, or about 500 bp; optionally, wherein the lengths of both of the homology arms are about 225 bp, about 250 bp, about 275 bp, about 300 bp, about 325 bp, about 350 bp, about 375 bp, about 400 bp, about 425 bp, about 450 bp, about 475 bp, about 500 bp, about 525 bp, about 550 bp, about 575 bp, or about 600 bp.

Embodiment 291.6. The method of any one of embodiments 291-291.5, wherein the total length of the homology arms is between about 400 bp and about 500 bp, about 500 bp and about 600 bp, about 600 bp and about 700 bp, about 700 bp and about 800 bp, about 800 bp and about 900 bp, about 900 bp and about 1000 bp, about 1000 bp and about 1100 bp, or about 1100 bp and about 1200 bp.

Embodiment 291.7. The method of any one of embodiments 291-291.6, wherein the nucleic acid construct comprises, from 5' to 3', the first homology arm, a promoter, a CAR encoding sequence, a polynucleotide sequence encoding the recombinant polypeptide, and the second homology arm.

Embodiment 291.8. The method of any one of embodiments 291-291.6, wherein the nucleic acid construct comprises, from 5' to 3', the first homology arm, a cleavable linker encoding sequence, a CAR encoding sequence, a polynucleotide sequence encoding the recombinant polypeptide, and the second homology arm; optionally, wherein the cleavable linker comprises a 2A family (e.g., P2A, E2A, F2A, or T2A) self-cleaving peptide.

Embodiment 291.9. The method of any one of embodiments 291-291.6, wherein the nucleic acid construct comprises, from 5' to 3', the first homology arm, a promoter, a polynucleotide sequence encoding the recombinant polypeptide, a CAR encoding sequence, and the second homology arm.

Embodiment 291.10. The method of any one of embodiments 291-291.6, wherein the nucleic acid construct comprises, from 5' to 3', the first homology arm, a cleavable linker encoding sequence, a polynucleotide sequence encoding the recombinant polypeptide, a CAR encoding sequence, and the second homology arm; optionally, wherein the cleavable linker comprises a 2A family (e.g., P2A, E2A, F2A, or T2A) self-cleaving peptide.

Embodiment 292. The method of any one of embodiments 291-291.10, wherein the gene editing machinery is CRISPR/Cas9.

Embodiment 292.1. The method of any one of embodiments 291-291.10, wherein the gene editing machinery is prime-editing assisted site-specific integrase gene editing (PASSIGE) system.

Embodiment 293. The method of embodiment 292, wherein the gene editing machinery is introduced via electroporation.

Embodiment 294. The method of embodiment 292, wherein the nucleic acid construct is introduced via AAV.

Embodiment 295. The method of embodiment 292, wherein the nucleic acid construct is introduced via LNP.

Embodiment 296. The method of any one of embodiments 232-295, wherein the method thereby produces an engineered cell comprising: (i) the recombinant polypeptide; (ii) the immune receptor (e.g., CAR); and (iii) the modified TRAC gene locus.

Embodiment 296.1 The method of any one of embodiments 232-295, wherein the method thereby produces an engineered cell comprising: (i) the recombinant polypeptide; (ii) the immune receptor (e.g., CAR); and (iii) the modified TRAJ intron splice acceptor locus.

Embodiment 297. The method of any one of embodiments 232-296, wherein the cell is for use in a cell therapy.

Embodiment 298. A method of treating a subject, comprising administering to the subject the cell of any one of embodiments 166-224, or the cell prepared by the method of any one of embodiments 232-297.

Embodiment 299. The method of embodiment 298, wherein the subject has a cancer or an autoimmune disease.

Embodiment 300. The method of embodiment 299, wherein the autoimmune disease is lupus, systemic lupus erythematosus, rheumatoid arthritis, psoriasis, psoriatic arthritis, multiple sclerosis, Crohn's disease, ulcerative colitis, Addison's disease, Graves' disease, Sjogren's syndrome, Hashimoto's thyroiditis, or celiac disease.

Embodiment 301. The method of embodiment 299, wherein the cancer is a hematological cancer.

Embodiment 302. The method of embodiment 299, wherein the cancer is myeloid neoplasm, myelodysplastic syndromes (MDS), myeloproliferative/myelodysplastic syndromes, acute lymphoblastic leukemia (ALL), chronic lymphocytic leukemia (CLL), acute myeloid leukemia (AML), chronic myelogenous leukemia (CML), blast crisis chronic myelogenous leukemia (bcCML), B-cell acute lymphoid leukemia (B-ALL), T-cell acute lymphoid leukemia (T-ALL), T-cell lymphoma, or B-cell lymphoma.

Embodiment 303. The method of embodiment 299, wherein the cancer is a solid tumor.

Embodiment 304. The method of embodiment 299, wherein the cancer is small cell lung cancer, colorectal cancer, testicular cancer, ovarian cancer, melanoma, lymphoma, leukemia, multiple myeloma, prostate cancer, breast cancer, non-small cell lung cancer, gastric cancer, esophageal cancer, liver cancer, kidney cancer, head & neck cancer, glioblastoma, neuroblastoma, soft tissue sarcoma, uterine cancer, brain cancer, skin cancer, renal cancer, bladder cancer, pancreatic cancer, thyroid cancer, eye cancer, gastrointestinal cancer, carcinoma, or sarcoma.

Embodiment 305. The method of any one of embodiments 299-304, wherein the cancer expresses CD1, CD1a, CD1b, CD1c, CD1d, CD1e, CD2, CD3d, CD3e, CD3g, CD3ε, CD4, CD5, CD7, CD8a, CD8b, CD19, CD20, CD21, CD22, CD23, CD24, CD25, CD27, CD28, CD30, CD33, CD34, CD38, CD40, CD44v6, CD45, CD46, CD47 CD48, CD52, CD59, CD66, CD70, CD71, CD72, CD73, CD79A, CD79B, CD80 (B7.1), CD86 (B7.2), CD94, CD95, CD97, CD123, CD134, CD140 (PDGFR4), CD152, CD154, CD158, CD171, CD178, CD179, CD179a, CD181 (CXCR1), CD182 (CXCR2), CD183 (CXCR3), CD210, CD213A2, CD246, CD252, CD253, CD261, CD262, CD272, CD273 (PD-L2), CD274 (PD-L1), CD276 (B7H3), CD279, CD295, CD339 (JAG1), CD340 (HER2), CDH17, CEA, CLECL1, CLL-1, CLDN6, CLDN18.2, CS1, DLL3, LY6G6D, GCC, p53R175H, PRAME, EGFR, EGFRvIII, FGFR2, AFP, CA125, MUC-1, MAGE, ALPI, alkaline phosphatase placental-like 2 (ALPPL2), B-cell maturation antigen (BCMA), green fluorescent protein (GFP), enhanced green fluorescent protein (eGFP), KLK2, KLK3, Mesothelin, IL13Ra2, ggcc, signal regulatory protein α (SIRPα), TCRalpha, TCRbeta, TSHR, GD2, GD3, Tn Ag, cMET, Axl, ROR1, ROR2, GPC1, GPC2, GPC3, FLT3, TAG72, CEA, EPCAM, KIT

(CD117), IL-13Ra2, IL-11Ra, PSCA, PRSS21, VEGFR2, LewisY, PDGFRβ, SSEA-4, folate receptor alpha, ERBB2 (Her2/neu), MUC1, MUC16, NCAM, prostase, PAP, ELF2M, Ephrin B2, IGF-I receptor, CAIX, LMP2, gp100, bcr-abl, tyrosinase, EphA2, STEAP1, STEAP2, fucosyl GM1, sLe, GM3, TGS5, HMWMAA, o-acetyl-GD2, folate receptor beta, TEM1/CD248, TEM7R, ROPN1, GPRC5D, GPA33, CXORF61, ALK, Polysialic acid, PLAC1, GloboH, NY-BR-1, UPK2, HAVCR1, ADRB3, PANX3, GPR20, LY6K, OR51E2, TARP, WT1, NY-ESO-1, LAGE-la, MAGE-Al, legumain, HPV E6,E7, MAGE-A4, ETV6-AML, sperm protein 17, XAGE1, Tie 2, MAD-CT-1, MAD-CT-2, Fos-related antigen 1, p53, p53 mutant, p53R175H, KRAS, mutant KRAS, KRAS G12D, prostein, surviving, telomerase, PCTA-1/Galectin 8, MelanA/MART1, Ras mutant, hTERT, sarcoma translocation breakpoints, ML-IAP, ERG (TMPRSS2 ETS fusion gene), NA17, PAX3, androgen receptor, cyclin Bl, MYCN, RhoC, TRP-2, CYP1B1, BORIS, SART3, PAX5, OY-TES1, LCK, AKAP-4, SSX2, RAGE-1, human telomerase reverse transcriptase, RU1, RU2, intestinal carboxyl esterase, mut hsp70-2,LAIR1, FCAR, LILRA2, CD300LF, CLEC12A, BST2, EMR2, LY75, FCRL5, IGLL1, PSMA, TROP2, citrullinated vimentin, the extracellular portion of the APRIL protein, or any combination thereof.

Embodiment 306. The method of embodiment 305, wherein the cancer expresses B7H3, BCMA, CD19, CD20, CD22, CD70, CD79a, CD79b, CDH17, CLDN6, CLDN18.2, DLL3, GCC, GD2, GD3, GPC3, GPRC5D, GPA33, KLK3, LY6G6D, p53R175H, PRAME, ROPN1, STEAP1, or STEAP2.

Embodiment 307. The method of any one of embodiments 299-306, wherein the subject is not administered a lymphodepletive agent within 7 days prior to administration of the cell.

Embodiment 308. The method of embodiment 307, wherein the subject is not administered cyclophosphamide, fludarabine, or bendamustine within 7 days prior to administration of the cell.

Embodiment 309. The method of any one of embodiments 299-308, wherein the subject is not administered at least 600,000 IU/kg of IL-2 every 8 hours.

Embodiment 310. The method of any one of embodiments 299-309, wherein the method does not comprise a checkpoint therapy which blocks PD-1 or CTLA-4 signaling.

Embodiment 311. The method of any one of embodiments 299-310, wherein the cell has reduced exhaustion, increased proliferative capacity, enhanced replicative lifespan, decreased replicative senescence, enhanced anti-tumor effect, reduced dysfunction, enhanced persistence, and/or increase intratumoral presence *in vivo*.

Embodiment 312. The method of any one of embodiments 299-311, wherein the cell has increased or decreased signaling through the CARD11-BCL10-MALT1 complex, NF-κB, AP-1, NFAT, JAK/STAT, and/or MEK/ERK pathways.

Embodiment 313. The method of any one of embodiments 299-312, wherein the subject is administered one or more additional therapeutic agents.

Embodiment 314. The method of embodiment 313, wherein the one or more additional therapeutic agents are selected from the group consisting of a small molecule inhibitor, a chemotherapeutic agent, a cancer immunotherapy, an antibody, a cellular therapy, a nucleic acid, a surgery, a radiotherapy, an anti-angiogenic therapy, an anti-DNA repair therapy, an anti-inflammatory therapy, an anti-neoplastic agent, a growth inhibitory agent, a cytotoxic agent, a vaccine, a small molecule agonist, a virus-based therapy, an antibody-drug conjugate, a recombinant protein, a fusion protein, a natural compound, a peptide, a PROteolysis-TArgeting Chimera (PROTAC), and any combination thereof.

Embodiment 315. Use of the cell of any one of embodiments 166-224, or the cell prepared by the method of any one of embodiments 232-297, for the manufacture of a medicament for the treatment of a disease.

Embodiment 316. The use of embodiment 315, wherein the disease is a cancer or an autoimmune disease.

Embodiment 317. The use of embodiment 315, wherein the autoimmune disease is lupus, systemic lupus erythematosus, rheumatoid arthritis, psoriasis, psoriatic arthritis, multiple sclerosis, Crohn's disease, ulcerative colitis, Addison's disease, Graves' disease, Sjogren's syndrome, Hashimoto's thyroiditis, or celiac disease.

Embodiment 318. The use of embodiment 316, wherein the cancer is a hematological cancer.

Embodiment 319. The use of embodiment 316, wherein the cancer is a solid tumor.

Embodiment 320. The use of embodiment 316, wherein the cancer is small cell lung cancer, colorectal cancer, testicular cancer, ovarian cancer, melanoma, lymphoma, leukemia, multiple myeloma, prostate cancer, breast cancer, non-small cell lung cancer, gastric cancer, esophageal cancer, liver cancer, kidney cancer, head & neck cancer, glioblastoma, neuroblastoma, soft tissue sarcoma, uterine cancer, brain cancer, skin cancer, renal cancer, bladder cancer, pancreatic cancer, thyroid cancer, eye cancer, gastrointestinal cancer, carcinoma, or sarcoma.

Embodiment 321. The use of embodiment any one of embodiments 316-320, wherein the cancer expresses CD1, CD1a, CD1b, CD1c, CD1d, CD1e, CD2, CD3d, CD3e, CD3g, CD3ε, CD4, CD5, CD7, CD8a, CD8b, CD19, CD20, CD21, CD22, CD23, CD24, CD25, CD27, CD28, CD30, CD33, CD34, CD38, CD40, CD44v6, CD45, CD46, CD47 CD48, CD52, CD59, CD66, CD70, CD71, CD72, CD73, CD79A, CD79B, CD80 (B7.1), CD86 (B7.2), CD94, CD95, CD97, CD123, CD134, CD140 (PDGFR4), CD152, CD154, CD158, CD171, CD178, CD179, CD179a, CD181 (CXCR1), CD182 (CXCR2), CD183 (CXCR3), CD210, CD213A2, CD246, CD252, CD253, CD261, CD262, CD272, CD273 (PD-L2), CD274 (PD-L1), CD276 (B7H3), CD279, CD295, CD339 (JAG1), CD340 (HER2), CDH17, CEA, CLECL1, CLL-1, CLDN6, CLDN18.2, CS1, DLL3, LY6G6D, GCC, p53R175H, PRAME, EGFR, EGFRvIII, FGFR2, AFP, CA125, MUC-1, MAGE, ALPI, alkaline phosphatase placental-like 2 (ALPPL2), B-cell maturation antigen (BCMA), green fluorescent protein (GFP), enhanced green fluorescent protein (eGFP), KLK2, KLK3, Mesothelin, IL13Ra2, ggcc, signal regulatory protein α (SIRPα), TCRalpha, TCRbeta, TSHR, GD2, GD3, Tn Ag, cMET, Axl, ROR1, ROR2, GPC1, GPC2, GPC3, FLT3, TAG72, CEA, EPCAM, KIT (CD117), IL-13Ra2, IL-11Ra, PSCA, PRSS21, VEGFR2, LewisY, PDGFRβ, SSEA-4, folate receptor alpha, ERBB2 (Her2/neu), MUC1, MUC16, NCAM, prostase, PAP, ELF2M, Ephrin B2, IGF-I receptor, CAIX, LMP2, gp100, bcr-abl, tyrosinase, EphA2, STEAP1, STEAP2, fucosyl GM1, sLe, GM3, TGS5, HMWMAA, o-acetyl-GD2, folate receptor beta, TEM1/CD248, TEM7R, ROPN1, GPRC5D, GPA33, CXORF61, ALK, Polysialic acid, PLAC1, GloboH, NY-BR-1, UPK2, HAVCR1, ADRB3, PANX3, GPR20, LY6K, OR51E2, TARP, WT1, NY-ESO-1, LAGE-la, MAGE-Al, legumain, HPV E6,E7, MAGE-A4, ETV6-AML, sperm protein 17, XAGE1, Tie 2, MAD-CT-1, MAD-CT-2, Fos-related antigen 1, p53, p53 mutant, p53R175H, KRAS, mutant KRAS, KRAS G12D, prostein, surviving, telomerase, PCTA-1/Galectin 8, MelanA/MART1, Ras mutant, hTERT, sarcoma translocation breakpoints, ML-IAP, ERG (TMPRSS2 ETS fusion gene), NA17, PAX3,

androgen receptor, cyclin Bl, MYCN, RhoC, TRP-2, CYP1B1, BORIS, SART3, PAX5, OY-TES1, LCK, AKAP-4, SSX2, RAGE-1, human telomerase reverse transcriptase, RU1, RU2, intestinal carboxyl esterase, mut hsp70-2,LAIR1, FCAR, LILRA2, CD300LF, CLEC12A, BST2, EMR2, LY75, FCRL5, IGLL1, PSMA, TROP2, citrullinated vimentin, the extracellular portion of the APRIL protein, or any combination thereof.

Embodiment 322. The use of embodiment 321, wherein the cancer expresses B7H3, BCMA, CD19, CD20, CD22, CD70, CD79a, CD79b, CDH17, CLDN6, CLDN18.2, DLL3, GCC, GD2, GD3, GPC3, GPRC5D, GPA33, KLK3, LY6G6D, p53R175H, PRAME, ROPN1, STEAP1, or STEAP2.

Embodiment 323. The use of any one of embodiments 316-322, wherein the medicament is not for administration within 7 days after administration of a lymphodepletive agent.

Embodiment 324. The use of embodiment 323, wherein the medicament is not for administration within 7 days after administration of cyclophosphamide, fludarabine, or bendamustine.

Embodiment 325. The use of any one of embodiments 316-324, wherein the medicament is not for administration with at least 600,000 IU/kg of IL-2 every 8 hours.

Embodiment 326. The use of any one of embodiments 316-325, wherein the medicament is not for administration with a checkpoint therapy which blocks PD-1 or CTLA-4 signaling.

Embodiment 327. The use of any one of embodiments 316-326, wherein the cell has reduced exhaustion, increased proliferative capacity, enhanced replicative lifespan, decreased replicative senescence, enhanced anti-tumor effect, reduced dysfunction, enhanced persistence, and/or increase intratumoral presence *in vivo*.

Embodiment 328. The use of any one of embodiments 316-327, wherein the cell has increased or decreased signaling through the CARD11-BCL10-MALT1 complex, NF-κB, AP-1, NFAT, JAK/STAT, and/or MEK/ERK pathways.

Embodiment 329. The use of any one of embodiments 316-328, wherein the medicament is for administration with one or more additional therapeutic agents.

Embodiment 330. The use of embodiment 329, wherein the one or more additional therapeutic agents are selected from the group consisting of a small molecule inhibitor, a chemotherapeutic agent, a cancer immunotherapy, an antibody, a cellular therapy, a nucleic acid, a surgery, a radiotherapy, an anti-angiogenic therapy, an anti-DNA repair therapy, an anti-inflammatory therapy, an anti-neoplastic agent, a growth inhibitory agent, a cytotoxic agent, a vaccine, a small molecule agonist, a virus-based therapy, an antibody-drug conjugate, a recombinant protein, a fusion protein, a natural compound, a peptide, a PROteolysis-TArgeting Chimera (PROTAC), and any combination thereof.

## EXAMPLES

**[0867]** The practice of the present disclosure will employ, unless otherwise indicated, conventional techniques of molecular biology, microbiology, cell biology, biochemistry, nucleic acid chemistry, and immunology, which are well known to those skilled in the art. Such techniques are explained fully in the literature cited above.

**[0868]** Additional embodiments are disclosed in further detail in the following examples, which are provided by way of illustration and are not in any way intended to limit the scope of this disclosure or the claims.

### EXAMPLE 1

### Design of truncated CARD11-PIK3R3 fusion proteins

**[0869]** This Example describes the design of engineered CARD11-PIK3R3 fusion proteins with improved features compared to patient-derived CARD11-PIK3R3 fusion proteins. Specifically, this example describes engineered CARD11-PIK3R3 fusion proteins that, relative to patient-derived CARD11-PIK3R3 fusion proteins, are more compact in size and therefore offer greater compatibility with various viral and non-viral gene delivery methods, that may improve the efficiency and feasibility of manufacturing cell therapies, including T cell therapies. Suitable vectors include, for example, including AAV-mediated delivery (*e.g*, AAV6-mediated delivery), for example, as in Hiemenz et al. *Mol Ther Methods Clin Dev*. 2023 Nov 19;31:101162, which is incorporated by reference herein in its entirety To that end, this example describes engineered CARD11-PIK3R3 fusion proteins that, relative to patient-derived CARD11-PIK3R3 fusion proteins, are shorter in length enabling the user to choose the optimal gene delivery methods for generating cell therapies.

**[0870]** Without wishing to be bound by theory, the inventors hypothesized that the N terminal portion of the PIK3R3 protein (**FIGS. 1A and 1F**) could be manipulated to improve potential compatibility of the fusion with viral and non-viral gene delivery methods and/or provide fusion proteins with differential functional activity.

**[0871]** Wildtype CARD11 is restricted from oligomerizing with the CBM complex by its inhibitory domain (Jattani et al. *J. Biol. Chem.* 2016; 291:8338-8348; and Garcia et al. *Nature.* 2024; 626: 626-634), which has been shown to associate with other rgions of the CARD11 protein in a manner that autoinhibits downstream function (**FIG. 1B**). However, upon antigen receptor signaling, and the resulting downstream phosphorylation of specific sites within the inhibitory domain (**FIGS. 1C and 1D**), the inhibitory domain undergoes a conformational change, allowing CARD11 to initiate the CBM complex and downstream signaling (**FIG. 1D**). Further, upon elimination of the majority of tumor cells (and removal of the majority of tumor antigen), without wishing to be bound by theory, the CARD11-PIK3R3 fusion returns to its default state and downstream CARD11-PIK3R3 signaling is reduced. Without wishing to be bound by theory, the inventors hypothesized that the CARD11 inihibitory domain in a CARD11-PIK3R3 fusion protein could be manipulated while ensuring that critical regulatory portions of the inhibitory domain are maintained (**FIG. 1A**). Without wishing to be bound by theory, the inventors hypothesized that such a fusion would retain its auto regulation, a critical safety feature of the patient derived CARD11-PIK3R3 fusion protein, thus providing fusion signaling primarily in connection with TCR or TCR-like (e.g., CAR) signaling. Without wishing to be bound by theory, the inventors hypothesized that the engineered CARD11-PIK3R3 fusion proteins with a manipulated inhibitory domain included in Table 1 retain essential components of the inhibitory domain, such as the phosphorylation sites, to retain the mechanism of autoregulation while generating the most compact, yet effective, versions of the engineered CARD11-PIK3R3 fusion. Further, without wishing to be bound by theory, the inventors hypothesized that the C terminal portion of the CARD11 protein inhibitory domain and/or the N terminal portion of the CARD11 protein inhibitory domain could be manipulated, while either (1) maintaining the phosphorylation sites present in the inhibitory domain, or (2) altering the position of the phosphorylation sites within the inhibitory domain, to improve potential compatibility of the fusion with viral and non-viral gene delivery methods and/or provide fusion proteins with differential functional activity while also maintaining the CARD11-PIK3R3 auto inhibition function and TCR dependent activation. Additionally, without wishing to be bound by theory, the inventors hypothesized that the CARD11 coiled

coil domain could be manipulated, while maintaining most of the structured alpha helices, to improve potential compatibility of the fusion with viral and non-viral gene delivery methods without removing any portion of the CARD11 inhibitory domain sequence. With the above in mind, the inventors designed CARD11-PIK3R3 fusion proteins with different portions of the N terminal PIK3R3 protein removed, different portions of C terminal portion of the CARD11 inhibitory domain removed, and different portions of the CARD11 coiled coil domain removed (**FIG. 1F and Table 1**).

## EXAMPLE 2

## Testing truncated CARD11-PIK3R3 fusion proteins

**[0872]** This Example describes functional assessments of T cells modified to express engineered CARD11-PIK3R3 fusion proteins.

Methods:

*Cloning*

**[0873]** For non-targeted insertion, engineered CARD11-PIK3R3 fusion variants (with or without a CAR or TCR) were synthesized and cloned into lentiviral vectors containing an MND or sEF1a promoter. For targeted insertion into the TRAJ intron splice acceptor cut site locus (and expression controlled by the TRAC promoter), engineered CARD11-PIK3R3 fusion variants (with or without a CAR or TCR) were synthesized and cloned into AAV6 viral vectors.

*T Cell Manufacturing*

**[0874]** Pantropic VSV-G pseudotyped lentivirus was produced via transfection of Lenti-X 293T cells with the GOI transgene expression vector and viral packaging plasmids using Mirus TransIT-Lenti. Viral supernatants were harvested 48 hours post transfection, filtered, and mixed with activated T cells. Primary CD3+ T cells were sourced from donors. After thawing, T cells were cultured in human T cell medium consisting of X-VIVO 15, 5% Human AB serum, 10mM HEPEs, and 1x Glutamax, supplemented with 10ng/mL IL-7 and 5ng/mL IL-15. T cells were activated with TransACT (CD3 and CD28 activation) for 24 hours before being transduced with lentivirus. Virus and TransACT were removed 24 hours post transduction, and T cells were seeded to G-Rex plates to expand for 3-5 days before harvest. At harvest T cells were used immediately for co-culture assays or frozen in CryoStor10 and stored in liquid nitrogen. For TRAC editing, 24 hours after lentiviral transduction, T cells were electroporated with TRAC targeting CRISPR/Cas9 RNPs using a

Lonza Nucleofector. T cells were rested overnight, washed, and transferred to a G-Rex or 24 well plate for expansion.

**[0875]** For experiments including TRAJ intron splice acceptor cut site knock-in, T cells were electroporated with TRAJ intron splice acceptor cut site targeting CRISPR/Cas9 RNPs using a Lonza Nucleofector and gRNA targeting the TRAJ intron splice acceptor cut site locus (SEQ ID NO: 400 for gRNA). The cells were cultured in human T cell medium without serum (X-VIVO 15, 10mM HEPEs, and 1x Glutamax, supplemented with 10ng/mL IL-7 and 5ng/mL IL-15) and transduced with an AAV6 vector that provides the engineered CAR or TCR with or without CARD11-PIK3R3 fusion protein homology directed repair template. AAV6 transduced T cell cultures were supplemented with M3814 at a concentration of 1uM and rested overnight, then washed, resuspended in human T cell medium with serum (X-VIVO 15, 5% Human AB serum, 10mM HEPEs, and 1x Glutamax, supplemented with 10ng/mL IL-7 and 5ng/mL IL-15), and transferred to a G-Rex plate for expansion.

*In vitro assays*

**[0876]** For *in vitro* co-culture assays, transduced primary T cells were normalized by expression of CAR or TCR, then co-cultured with target cells. Supernatants were collected at 18-24 hours post co-culture and assessed for IFNgamma and IL-2 production via MSD using manufacturer protocols (e.g., https://www.mesoscale.com/~/media/files/product%20inserts/proinflammatory%20panel%201%20human%20insert.pdf, https://www.mesoscale.com/en/products/v-plex-human-il-2-kit-k151qqd, and/or https://www.mesoscale.com/en/products/v-plex-human-ifn-gamma-kit-k151qod).

*Flow Cytometry*

**[0877]** For all *in vitro* primary T cell assays, cells were washed with PBS 2% FBS, stained with surface staining markers at room temperature for 20 minutes, washed once, and resuspended in PBS 2% FBS before analysis on an Attune Flow Cytometer. The following antibodies and reagents were used: anti-human CD8 (RPA-T8), anti-human CD4 (SK3), anti-human CCR7 (3D12), anti-human CD45RA (HI100), anti-mouse TCRB (H57-597), and a human IgG Fc tagged-antigen (to detect CAR expression)

*Cell Lines*

**[0878]** Cancer cell lines that are antigen positive and antigen negative were used in the present experiments.

**Results:**

**[0879]** Primary human T cells from three healthy donors were transduced with a transgenic TCR and either the patient-derived CARD11-PIK3R3 fusion (C11P3) or an engineered CARD11-PIK3R3 fusion variant (either C11P3tr1 or C11P3tr2). Primary human T cells were also transduced with C11P3, C11P3tr1 or C11Ptr2 alone. At harvest the primary human T cells transduced with C11P3, C11P3tr1, or C113Ptr2 (alone or in combination with a transgenic TCR) exhibited a greater central memory phenotype than that of cells transduced with TCR alone (**FIGS. 2A and 2B**).

**[0880]** When co-cultured with cell lines expressing the TCR target antigen, the T cells expressing C11P3 variants and the TCR secreted more IFNgamma and IL-2 compared to T cells expressing the TCR alone (**FIGS. 3A and 3B**). When co-cultured with an antigen negative cell line there was minimal cytokine secretion from cells expressing either C11P3, C11P3tr1 or C11P3tr2, indicating that the engineered CARD11-PIK3R3 fusion proteins maintain antigen specific sensitivity (**FIGS. 3A and 3B**). Notably, transgenic TCR T cells engineered with C11P3tr1 and C11P3tr2 fusion proteins did not induce significantly different IFNgamma secretion as compared to C11P3 (**FIG. 3A**).

**[0881]** Primary human T cells from a single donor were transduced with lentiviral vectors encoding a CAR, the CAR followed by C11P3, or the CAR followed by C11P3tr1. After lentiviral transduction, these cells underwent electroporation to deliver CRISPR/Cas9 RNPs for editing of the TRAC locus (as described above). The CAR alone vectors were well expressed (**FIG. 4A**), however vectors containing CAR and a fusion had slightly lower CAR expression by percentage and MFI (**FIGS. 4A and 4B**). Expression of the CAR with the C11P3tr1 fusion variant was very similar to the expression of the CAR with C11P3, indicating the engineered fusion did not affect overall expression of the construct. All groups were successfully edited, with greater than 90% TCR knockout as measured by CD3e expression, indicating the expression of the C11P3 and C11P3tr1 had no effect on TCR knockout efficiency (**FIGS. 4C and 4D**).

**[0882]** At harvest the primary human T cells transduced with CAR+C11P3 or CAR+C11P3tr1 had greater central memory phenotype than that of cells transduced with CAR alone (**FIGS. 5A and 5B**).

**[0883]** When co-cultured with antigen positive and antigen negative cell lines the engineered primary human T cells transduced with CAR, CAR+C11P3, or CAR+C11P3tr1 secrete IL-2 and IFNgamma in an antigen dependent manner. Secretion of IL-2 and IFNgamma are not significantly different between CAR+C11P3 and CAR+C11P3tr1,

suggesting no significant difference in antigen-dependent cytokine secretion produced by the engineered C11P3tr1 fusion combined with a CAR (**FIGS. 6A and 6B**).

**[0884]** As described above, primary human T cells from a single donor were electroporated to deliver CRISPR/Cas9 RNPs targeting the TRAJ intron splice acceptor cut site locus and cells were transduced with AAV6 to provide a homology directed repair template encoding a CAR or the CAR+C11P3tr1. **FIG. 7A** demonstrates primary human T cells can be edited to knock-in the CAR or CAR+C11P3tr1 genetic cassette into the TRAJ intron splice acceptor cut site locus with expression controlled by the endogenous human TRAC promoter. Although the knock-in efficiency for the CAR+C11P3tr1 cassette is lower than the CAR only cassette, as is expected for genetic insertions of larger nucleic acid cassettes (**FIG. 7A**), importantly, the results demonstrate that the engineered CARD11-PIK3R3 fusion proteins, which are shorter than the patient-derived fusions, allow for delivery of both the CAR and fusion via a single AAV6 vector (which has a limited cargo capacity of ~4.7 Kb, inclusive of two ITR regions). The MFI of CAR expression is lower when delivered via TRAJ intron splice acceptor cut site knock-in as compared to lentiviral transduction, which, without wishing to be bound by theory, is likely a reflection of the number of copies integrated and the level of expression induced by the TRAC promoter as compared to the LVV MND promoter (**FIG. 7B**). However, CAR MFI was unaffected by the presence of C11P3tr1 (**FIG. 7B**).

**[0885]** Further, there is minimal difference in T cell phenotype when comparing AAV6 delivered CAR T cells and AAV6 delivered CAR+C11P3tr1 cells (**FIG. 8**). Finally, when co-cultured with antigen high, antigen low, and antigen negative cell lines, the engineered primary human T cells transduced with CAR or CAR+C11P3tr1 secrete IL-2 and IFNgamma in an antigen dependent manner (**FIGS. 9A and 9B**). Thus, incorporation of an engineered CARD11-PIK3R3 fusion (which is shorter in length than the patient-derived CARD11-PIK3R3 fusion) enables targeted CAR + Fusion gene delivery via AAV6.

**Conclusion:**

**[0886]** Engineered CARD11-PIK3R3 fusions can be delivered to cells via lentivirus (in single or dual vector formats) or via AAV6 to allow knock-in to the TRAJ intron splice acceptor cut site locus using CRISPR/Cas9 and homology directed repair. Two engineered CARD11-PIK3R3 fusion proteins, C11P3tr1 and C11P3tr2, induce a similar central memory phenotype as observed previously with full length C11P3, indicating that the engineered CARD11-PIK3R3 fusions and patient-derived CARD11-PIK3R3 all minimally effect T cell state in the absence of T cell activation. Further, T cells transduced with engineered

CARD11-PIK3R3 fusions (C11P3tr1 and C11P3tr2) and a transgenic TCR produce similar levels of cytokine compared to T cells transduced with C11P3 and a TCR when triggered by the corresponding antigen, indicating that, like the patient-derived CARD11-PIK3R3 fusion, the engineered CARD11-PIK3R3 fusion proteins can be used to develop potent cell therapies, including T cell therapies.

**[0887]** In addition, T cells transduced with engineered CARD11-PIK3R3 fusions (C11P3tr1) and a CAR produce similar levels of cytokine compared to T cells transduced with a patient-derived CARD11-PIK3R3 fusion when triggered by the corresponding antigen, suggesting that the engineered CARD11-PIK3R3 fusion proteins may be used in place of the patient derived sequence in cell therapies, including T cell therapies. This is especially important in the context of targeted insertion where the decreased size of the engineered CARD11-PIK3R3 fusions allow for the incorporation of both a CAR sequence and fusion sequence into AAV6 to enable targeted delivery and insertion into the TRAJ intron splice acceptor cut site locus. This targeted insertion manufacturing approach leads to more uniform CAR expression compared to random integration with lentiviral vectors, and furthermore, decreases risk of clonal expansion and/or oncogenic transformation.

**[0888]** Altogether, the data presented herein indicate that the engineered CARD11-PIK3R3 fusion proteins of the present invention are capable of enhancing the potency of CAR T cells and TCR T cells. Further, functional similarities in cytokine expression for T cells expressing the engineered CARD11-PIK3R3 fusion proteins of the present invention, as compared to a patient-derived CARD11-PIK3R3 fusion protein, described in the data above suggest the potential utility of engineered CARD11-PIK3R3 fusion proteins in developing potent cell therapies, including T cell therapies. Last, the data demonstrate that the reduced size of the engineered CARD11-PIK3R3 fusion proteins of the present invention enable delivery via AAV6 and targeted insertion via CRISPR and homology directed repair.

## EXAMPLE 3

## Testing truncated CARD11-PIK3R3 fusion proteins

**[0889]** This Example describes methods for assessing the impact of engineered CARD11-PIK3R3 fusion proteins on T cell function and activity.

**Methods:**

**[0890]** Engineered CARD11-PIK3R3 fusion proteins are cloned into lentiviral vectors, with or without a CAR or TCR, as described in Example 2. Primary human T cells are transduced

with the lentivirus carrying the engineered CARD11-PIK3R3 fusion proteins (+ or – a CAR or TCR) and the functionality of the engineered CARD11-PIK3R3 fusion proteins are assessed through co-culture assays with both cancer cell lines that are antigen negative and cancer cell lines that express a target antigen that is recognized by the CAR or TCR, as described in Example 2. Next, the supernatant from the co-culture assays is collected and analyzed via MSD for secretion of IL-2 and IFNgamma. Further, expression of the engineered CARD11-PIK3R3 fusion proteins is assessed by qPCR and other quantitative assays. Obtaining the expression characteristics of engineered CARD11-PIK3R3 fusion proteins provides additional insight regarding if, or how, variations in fusion length, or the specifics of particular fusion breakpoints, affect overall fusion expression and whether expression of the engineered CARD11-PIK3R3 fusion proteins affects T cell differentiation state and functional phenotypic outputs.

**[0891]** The impact of engineered CARD11-PIK3R3 fusion proteins on TCR signaling is investigated using a cell line that has fluorescent reporters downstream of key TCR signaling proteins. For example, engineered Jurkat lines have fluorescent reporters of NFAT, NFKB and AP-1 signaling (Jutz, S. et al. Assessment of costimulation and coinhibition in a triple parameter T cell reporter line: Simultaneous measurement of NF-κB, NFAT and AP-1. J Immunol Methods 430, 10-20, doi:10.1016/j.jim.2016.01.007 (2016).). The Jurkat signaling cell line is further modified to express a CAR and the engineered CARD11-PIK3R3 fusion proteins, and is then co-cultured with antigen positive targets. Finally, the expression of each fluorescent reporter is assessed by flow cytometry to determine how each engineered CARD11-PIK3R3 fusion impacts NFAT, NFKB, and AP-1 expression.

**[0892]** Finally, the anti-tumor efficacy of T cells expressing engineered CARD11-PIK3R3 fusion proteins is assessed by making primary human T cells that express the engineered CARD11-PIK3R3 fusion protein and a CAR or TCR. The T cells are further modified such that they do not express an endogenous TCR (for example via a knockout of the TRAC gene as described above). Then animals bearing tumors expressing the antigen recognized by the CAR or TCR are treated with the T cells and tumor volume and overall survival are measured over time.

**[0893]** Engineered CARD11-PIK3R3 fusion proteins with shorter overall lengths may exhibit improved knock-in efficiency when delivered via AAV or non-virally via ssDNA or dsDNA templates. Engineered CARD11-PIK3R3 fusion proteins with shorter overall lengths may be delivered (via AAV or non-virally via ssDNA or dsDNA templates) in combination with other T cell modifying payloads (e.g., cytokines, growth factors, and/or

shRNAs). Engineered CARD11-PIK3R3 fusion proteins with truncated CARD domains (SEQ ID NOs: 225-245) may lack the ability to bind BCL10 and thus provide no functional signaling output. These variants may have utility as dominant negative variants - for example, without wishing to be bound by theory, they could oligomerize with endogenous CARD11 and preventing binding with BCL10 to therefore prevent downstream activation of NFKB.

**[0894]** Further still, engineered CARD11-PIK3R3 fusion proteins with a truncated coiled coil domain (SEQ ID NOs: 193-224 and 246-276) may exhibit a decrease in signaling output relative to a patient-derived CARD11-PIK3R3 fusion protein, for example, due to, without wishing to be bound by theory, a decreased ability to oligomerize. As such, these variants may have utility in situations where the user wants to "tune down" fusion related signaling for a given antigen target or indication. In addition, engineered CARD11-PIK3R3 fusion proteins with a truncated coiled coil domain (SEQ ID NOs: 193-224 and 246-276) may act as dominant negative variants that are capable of reducing overall CARD signaling within the cell. These engineered CARD11-PIK3R3 fusion proteins may have utility in that they could provide varying signaling output, allowing for user determined tuning of signaling.

**[0895]** Next, engineered CARD11-PIK3R3 fusion proteins with a truncated inhibitory domain (SEQ ID NOs: 179-192, 277-288, and 289-292) may exhibit increased levels of background signaling relative to a patient-derived CARD11-PIK3R3 fusion protein, for example, due to, without wishing to be bound by theory, signaling from the fusion without regard to antigen specific activation. In addition, engineered CARD11-PIK3R3 fusion proteins with a truncated inhibitory domain (SEQ ID NOs: 179-192, 277-288, and 289-292) may exhibit a reduction in signaling output.

**[0896]** Finally, engineered CARD11-PIK3R3 fusion proteins with a truncated SH2 domain in PIK3R3 (SEQ ID NOs: 179-192, 277-288, and 289-292) may have decreased signaling, for example, due to, without wishing to be bound by theory, a reduction in SH2 mediated binding.

## EXAMPLE 4

## Delivery of CARD11-PIK3R3 fusion proteins via mRNA

**[0897]** This Example describes methods for assessing delivery of patient-derived CARD11-PIK3R3 and engineered CARD11-PIK3R3 fusion proteins to T cells via mRNA.

**Methods:**

**[0898]** mRNA encoding patient-derived or engineered CARD11-PIK3R3 fusion protein are synthesized using *in vitro* transcription methods known in the art. Next, the mRNA is delivered to target cells (e.g., T cells) via electroporation. After delivery, cells are collected from the culture dish and phenotype and functional responses, such as cytokine production and cytotoxicity following co-culture with target cells, are assessed by flow cytometry, ELISA, imaging, and other methods known in the art. Alternatively, in an *in vivo* setting, *in vitro* transcribed mRNA encoding patient-derived CARD11-PIK3R3 fusion protein or engineered CARD11-PIK3R3 fusion protein is delivered to T cells in a mouse via infusion or injection of a lipid nano-particle (LNP) mRNA complex (or other known methods). Optionally, cooperative biological agents (vaccines or T cell engagers, for example) are delivered contemporaneously to LNP-mRNA delivery. T cell responses to tumors or infectious agents are evaluated by imaging methods, tumor measurement, or other analytical methods known in the art, or *in vitro* as described in the Examples above following blood draw and isolation of PBMCs or T cells.

**[0899]** Both patient-derived and engineered CARD11-PIK3R3 fusion proteins can be delivered and expressed in T cells by mRNA via electroporation (*ex vivo*) and LNP delivery (*in vivo*). Modified T cells with engineered with CARD11-PIK3R3 fusion proteins made via mRNA based delivery methods may produce cytokines and carry out functional responses similar to T cells modified to constitutively express CARD11-PIK3R3 via gene editing based targeted insertion or via lentiviral insertion.

## EXAMPLE 5

## Assessment of *in vivo* generated CAR T cells expressing engineered CARD11-PIK3R3

**[0900]** This Example describes methods for assessing *in vivo* functionality of *in vivo* generated CAR T cells expressing patient-derived CARD11-PIK3R3 fusion proteins or engineered CARD11-PIK3R3 fusion proteins.

**Methods:**

**[0901]** mRNA encoding an antigen specific CAR (or TCR) and patient-derived or engineered CARD11-PIK3R3 fusion proteins are synthesized via *in vitro* transcription, either as independent mRNA species or together in a single open reading frame (ORF) with the CAR (or TCR) and CARD11-PIK3R3 fusion genes separated by an encoded self-cleaving 2A peptide. T cells transfected by electroporation (or via exposure to LNP-mRNA complexes) encoding combinations of the mRNA species described above are characterized

by flow cytometry and assessed for cytokine production and cytotoxicity. To assess delivery of the above described mRNA species to T cells *in vivo*, the mRNA encoding CAR (or TCR) alone, or combinations of CAR (or TCR) plus CARD11-PIK3R3 encoding mRNA species, or the linked CAR- (or TCR)-PIK3R3 encoding mRNA, are delivered to via LNP-mRNA complex infusion or injection (or other known methods) as described above. Finally, the efficacy of the modified T cells is assessed over time by imaging or measuring antigen-positive tumors engrafted into the mice, or by assessing the depletion of antigen-positive normal cells (such as B cells).

**[0902]** *In vivo* modified T cells expressing a CAR (or TCR) and patient-derived CARD11-PIK3R3 fusion proteins or engineered CARD11-PIK3R3 fusion proteins made via mRNA based delivery methods may demonstrate superior *in vivo* tumor control compared to T cells modified with mRNA encoding the CAR (or TCR) alone. This result would demonstrate the ability to generate potent antigen specific T cells *in vivo* with enhanced activity relative to T cells modified to only express the CAR (or TCR).

## EXAMPLE 6

## Materials and Methods

Receptor and Mutation Screening Construct Construction

**[0903]** Intracellular domains containing the appropriate costimulatory domain and CD3zeta domain were synthesized by Twist. Receptors were built by fusing the CD19 scFv1 to the corresponding receptor scaffold and intracellular tail. All receptors contain an n-terminal CD8α signal peptide (MALPVTALLLPLALLLHAARP) for membrane targeting and a flag-tag (DYKDDDDK) for easy determination of surface expression with α-flag PE or BV421 (Biolegend 637310, 637322). In some cases, the receptors additionally contained a T2A self-cleaving sequence followed by a tNGFR sequence, used in downstream applications for T cell isolations. The receptors were cloned into a modified pHR'SIN:CSW vector containing a PGK promoter for all primary T cell experiments.

Primary Human T cell Isolation and Culture

**[0904]** Primary CD3+, CD4+ and CD8+ T cells were isolated from anonymous donor blood after apheresis by negative selection (Biolegend Mojo Sort # 480129, 480130, 480131). Blood was obtained from Blood Centers of the Pacific (San Francisco, CA) as approved by the University Institutional Review Board. T cells were cryopreserved in RPMI-1640 (UCSF cell culture core) with 20% human AB serum (Valley Biomedical Inc., #HP1022) and 10% DMSO. After thawing, T cells were cultured in human T cell medium consisting of X-VIVO

15 (Lonza #04-418Q), 5% Human AB serum and 10 mM neutralized N-acetyl L-Cysteine (Sigma-Aldrich #A9165) supplemented with 30 units/mL IL-2 (NCI BRB Preclinical Repository) for all experiments unless otherwise noted. *In vivo* experiments were completed with bulk CD3+ cells isolated in a similar manner.

Lentiviral transduction of human T cells

**[0905]** Pantropic VSV-G pseudotyped lentivirus was produced via transfection of Lenti-X 293T cells (Clontech #11131D) with a pHR'SIN:CSW transgene expression vector and the viral packaging plasmids pCMVdR8.91 and pMD2.G using Mirus TransIT-Lenti (Mirus #MIR 6606). Primary T cells were thawed the same day, and after 24 hours in culture, were stimulated with Human T-Activator CD3/CD28 Dynabeads (Life Technologies #11131D) at a 1:3 cell:bead ratio. At 48 hours, viral supernatant was harvested and the primary T cells were exposed to the virus for 24 hours. At day 5 post T cell stimulation, the Dynabeads were removed, T cells were sorted, and the T cells expanded until day 10-14 when they were rested and could be used *in vitro* or *in vivo* assays. T cells were sorted for assays with a Beckton Dickinson (BD) FACs ARIA II.

TCR knockout and lentiviral transduction of human T cells

**[0906]** For TCR KO experiments, primary T cells were cultured in human T cell medium consisting of X-VIVO 15 (Lonza #04-418Q), 5% Human AB serum and 10 mM neutralized N-acetyl L-Cysteine (Sigma-Aldrich #A9165) supplemented with 100 units/mL IL-7 (Miltenyi #130-095-362) and 100 units/mL IL-15 (Miltenyi #130-095-765). Primary T cells were thawed, rested for 1 hour then stimulated with Human T-Activator CD3/CD28 Dynabeads (Life Technologies #11131D) at a 1:3 cell:bead ratio. One day after activation, T cells were transduced with concentrated virus. 24 hours later, virus and Dynabeads were removed, cells were rested for 24 hours, then resuspended at 1 x $100^6$ cells per mL in P3 electroporation buffer (Lonza #V4SP-3960) with gRNA (CAGGGTTCTGGATATCTGT) targeting the human TRAJ intron splice acceptor cut site locus and Cas9. 23 uL of this mixture was aliquoted to each well of a 96 well nucleofection plate (Lonza #V4SP-3960) and immediately electroporated using a 4-D Lonza Nucleofector with program EH115. Cells were resuspended in pre-warmed human T cell medium and recovered for 30 minutes in the incubator before being transferred to culture. Electroporated cells were assessed for TCR KO and lentiviral transduction via flow before injection to mice.

Cell lines

**[0907]** This Example describes the various cell lines used in the experiments of this study. The cancer cell lines used were K562 myelogenous leukemia cells (ATCC #CCL-24 3),

Jurkat cells (Clone E6-1, ATCC# TIB-152), B16-F10 melanoma cells (ATCC #CRL-6475) and A549 lung epithelial carcinoma cells (ATCC #CCL-18). K562s and A549s were lentivirally transduced to stably express human CD19. CD19 levels were determined by flow cytometry (Biolegend, clone HIB19). A549s were additionally transduced to express the nuclear stain mkate2. All cell lines were sorted for expression of the transgenes. Jurkat cells were transduced with retroviruses encoding fluorescent reporter constructs (Addgene 118095, 118094, and 118031) and stimulated with PMA/ionomycin to sort a line with high induction of each reporter. To enable screening with our mCherry expressing lentiviral constructs, an iRFP fluorescent reporter was subcloned to replace mCherry in the previously described AP1-mCherry construct. Triple reporter cells were then transduced with a CD19-CD28z CAR or CD19-BBz-CAR. Jurkat and K562 cells were cultured in RPMI + 10% FBS with penicillin/streptomycin and sodium pyruvate. B16-OVA and A549 cells were cultured in DMEM + 10% FBS with penicillin/streptomycin. All cell lines were routinely tested for mycoplasma contamination (Southern Biotech).

*In vitro* Primary T Cell Assays

**[0908]** For *in vitro* co-culture assays, transduced primary T cells were co-cultured with target cells at effector to target ratios and co-culture time courses. For Luminex assays, cells were co-cultured in media lacking exogenous IL-2, supernatants were collected 48 hours after start, frozen at -80C and sent for analysis to Eve Technologies. For IL-2 ELISA assays, supernatant was collected from 24-hour co-cultures of transduced T cells and target cells. Supernatants were assessed for IL-2 secretion using the Invitrogen Instant IL-2 ELISA kit (Invitrogen # BMS221INST). For repeat stimulation assay, transduced T cells were co-cultured on the adherent CD19 expressing A549 cell line, after 1 week T cells were removed from co-culture without disturbing adherent cells, counted and replated on CD19 expressing A549 cells seeded 24 hours prior. For co-culture assays performed over a period of days to weeks, media was supplemented regularly. For Mass cytometry by time of flight (CyTOF) transduced CD3+ T cells were co-cultured with CD19-K562s for 7.5, 15, 30, 60 or 120 minutes, then immediately fixed with 1.6% PFA for 10 minutes at room temperature. Cells were then pelleted, washed, and stored at -80C until ready for barcoding and staining. All primary T cell *in vitro* assays were performed with three donors.

Flow Cytometry

**[0909]** For all *in vitro* primary T cell assays, cells were washed with PBS 2% FBS twice, stained with surface staining markers at room temperature for 20 minutes, washed twice, and resuspended in PBS 2% FBS with DRAQ7 (diluted 1:1000) before analysis on a BD

FACSymphony X-50 Flow Cytometer. The following antibodies were used: CD8 (SK1), CD25 (M-A251), CD39 (A1), CD69 (FN50), CD271 (ME20.4), CD278 (C398.4A), CD279 (EH12.2H7)

K562 *In vivo* Xenograft Assays

**[0910]** NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice were dosed with 1 x $10^6$ CD19 expressing K562 cells via subcutaneous injection. 5 days post tumor injection, 3 to 6 x $10^6$ transduced or control T cells were dosed to tumor bearing animals via retro-orbital injection. Tumors were measured with calipers twice weekly, and tumor volume was calculated using the following formula: (length x width2)/2. Throughout experiment animal drinking water was supplemented with Clavomox to prevent bacterial infections.

Nalm6 *In vivo* Xenograft Assays

**[0911]** NOD.Cg-Prkdcscid Il2rgtm1Wjl/SzJ (NSG) mice were dosed with 5 x $10^5$ luciferase expressing Nalm6 cells via intravenous tail vein injection. 5 days post tumor injection, 1 x $10^6$ transduced or control T cells were dosed to tumor bearing animals via retro-orbital injection. Tumor burden was measured twice weekly via bioluminescence imaging after 200uL intraperontineal injection of D-Luciferin (15mg/mL, Goldbio), image analysis performed using Living Image (Perkin-Elmer). Animal drinking water was supplemented with Clavomox to prevent bacterial infections.

Jurkat reporter screening and analysis

**[0912]** CAR triple reporter Jurkat cells were transduced in 96 well plate format with individual T cell lymphoma mutation lentiviruses. 48 hours post-transduction, cells were plated at approximately a 1:1 ratio with K562 or K562-CD19 cells. Plates were spun at 300g for 2 minutes to promote interaction of CAR cells and target cells. Following 24 hours of co-culture, supernatants were removed for IL-2 ELISA (ELISA MAX™ Deluxe Set Human IL-2, Biolegend) and cells were washed in FACS buffer (PBS + 2% FBS) and analyzed by FACS. For the CD19-BBz CAR screen, cells were stained with anti-PD-1 (Biolegend, clone EH12.2H7) prior to FACS analysis. Each screen was performed in biological replicate with separate lentiviral transductions. For analysis, the percentage positive for each reporter in each condition (i.e. each CAR and co-culture cell type) was compared to the mCherry only controls and the corresponding wild-type gene control when available by T test followed by Bonferroni correction. Z scores were calculated for each condition as $(x - \mu) / \sigma$ (where x is the observed value, $\mu$ is the mean, and $\sigma$ is the standard deviation).

*In vivo* screening and analysis

**[0913]** Human CD3 T cells were lentivirally co-transduced with a CD19-BBZ CAR with tNGFR and the mutant construct library in an arrayed fashion. CD3+ T cells were assessed via flow for CD19-BBZ CAR (FLAG) and mutant construct (mCherry) expression. T cells were pooled based on mCherry expression and sorted for a purified dual positive population. 6 x 106 library T cells were injected to 15 CD19-K562 tumor bearing animals. T cells were isolated from the tumor and spleen by positive selection using the tNGFR (CELLection™ Biotin Binder Kit) at 7, 14, and 21 days post injection. gDNA was isolated from these T cells (NucleoSpin Tissue, Machery Nagel), and quantified by NanoDrop Spectrophotometry and adjusted to 8ng/uL. PCR amplification of the samples was performed to enrich for the mutations barcodes (PCR1). PCRs were performed with Kappa HiFi, in reaction volumes of 50 µL with between 10 and 1090 ng of gDNA, all reactions underwent 7 cycles of amplification. For the subsequent PCR to add Illumina barcodes and adapters to the products (PCR2), all products from PCR1 were quantified with a Qubit 1X dsDNA High Sensitivity Assay kit (Invitrogen) and up to 10ng of template was used in a 25 µL reaction with Kappa Hifi. Different forward and reverse primers were used for each sample for PCR2 to add unique custom Illumina I5 and I7 barcode sequences to each sample. Finally, PCR2 products were again quantified using Quibit, and these products were pooled at 1:1 molar ratio, diluted, loaded, and run on a MiniSeq 75 cycle cartridge using the standard manufacturer protocols.

Bulk RNAseq and analysis

**[0914]** For bulk RNAseq, T cells from 3 independent healthy donors transduced with the indicated constructs were either unstimulated or stimulated via co-culture with CD19 expressing A549 cells for 8 hours. RNA was then isolated (Nucleospin RNA XS, Machery-Nagel and cDNA libraries were constructed using SMART-Seqv4 Ultra Low Input RNA Kit (Takara Bio) and Nextera XT (Illumina). Sequencing reads were aligned using STAR, transcripts quantified using HT-Seq, and differentially expressed genes were identified using DESeq2 as previously described. (Love MI, Huber W, Anders S. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. Genome Biol. 2014;15(12):1-21.).

Immunoblot analysis

**[0915]** Jurkat cells (1-5 x $10^6$) were pre-treated for 30 minutes with the MALT1 inhibitor Z-VRPR-FMK (75 uM) or vehicle control. Cells were then treated with PMA/ionomycin as indicated for 2 hours. Whole-cell lysates were generated and analyzed by immunoblotting

with the following antibodies: anti-HOIL1 (Millipore Sigma, MABC576), anti-CYLD (Santa Cruz, sc-74435), and anti-β-actin (Cell Signaling Technologies, 4967).

Mass cytometry by time of flight (CyTOF)

**[0916]** Cells were thawed on ice for 15 minutes, washed, and barcoded with distinct combinations of stable Pd isotopes for 15 min at room temperature on a shaker in Maxpar Barcode Perm Buffer (Fluidigm, cat#201057) (Mass tag cellular barcodes; Bodenmiller B, Zunder ER, Finck R, et al. Multiplexed mass cytometry profiling of cellular states perturbed by small-molecule regulators. Nat Biotechnol 2012 309. 2012;30(9):858-867). Cells were washed twice with cell staining media (PBS with 0.5% BSA and 0.02% NaN3) and pooled into a single cluster tube. Cells were stained with surface staining antibodies for 30 min at room temperature on a shaker. After staining, cells were washed two times with cell staining media followed by permeabilization with methanol for 10 min at 4 °C. Permeabilized cells were washed twice in cell-staining media and stained with intracellular antibodies for 30 min at room temperature on a shaker. Cells were washed twice in cell-staining media and then stained with 1 ml of 1:4000 191/193Ir DNA intercalator (Fluidigm) diluted in PBS with 1.6% PFA overnight. Cells were diluted in Cell Acquisition Solution containing bead standards (Fluidigm, Cat#201078) to and analyzed on a Helios mass cytometer (Fluidigm) equilibrated with Cell Acquisition Solution. After acquisition, the data from the pooled samples underwent bead standard data normalization and de-barcoding using the R package from the PICI institute available at https://github.com/ParkerICI/premessa.

Mouse T cell purification and retroviral transduction

**[0917]** CD8+ T cells were isolated from the spleens of CD45.1+ or CD45.2 OT-I mice using a mouse CD8+ T cell isolation kit (Stemcell Technologies) or Mouse Pan CD3+ T cell isolation kit (Biolegend). T cells were then cultured with 100 U/mL recombinant human IL-2 (Peprotech), anti-CD3e (1 ug/mL) and anti-CD28 (0.5 ug/mL) overnight. For CAR experiments, T cells were stimulated overnight with anti-CD3/CD28 beads (ThermoFisher). Retroviral supernatants were added to T cells in Retronectin (Takara) coated plates and spinduction was performed for 1 hour at 2000 rpm at 30°C. Following transduction, T cells were resuspended and cultured in fresh media containing 100 U/mL IL-2 until adoptive transfer. Transduction efficiency was determined by flow cytometry prior to adoptive transfer.

B16-OVA melanoma tumor model

**[0918]** Female C57BL/6 mice age 6-8 weeks were injected subcutaneously with 5 x $10^5$ B16-OVA melanoma cells. Mice with similar sized tumors were randomized to receive

treatments of OT-I T cells. 100 uL of T cells (or PBS control) were retro-orbitally injected on day 8-12 post tumor inoculation. For dual-transfer competitive assay and tumor infiltrating lymphocyte analysis experiments, 1 x $10^6$ T cells were transferred. For anti-tumor efficacy, 2 x $10^6$ T cells were transferred. Tumor lengths and widths were determined every 2-3 days by digital caliper measurement and tumor volume was calculated as length × width × [(length × width)0.5] × $\pi/6$ as previously described (Wei, J., Long, L., Zheng, W. et al. Targeting REGNASE-1 programs long-lived effector T cells for cancer therapy. Nature 576, 471–476 (2019). https://doi.org/10.1038/s41586-019-1821-z).

**[0919]** Death was defined as a progressively growing tumor that reaches 15mm in its longest axis or developing ulceration or necrosis. For the anti-tumor efficacy experiment, measurement of tumors and determination of survival endpoint was blinded to experimental condition. Experiments were performed in accordance with Northwestern University Institutional Animal Care and Use Committee approved protocols.

B16-OVA tumor infiltrating lymphocyte (TIL) isolation and analysis

**[0920]** To isolate TILs, B16-OVA tumors were excised, minced, and digested using collagenase IV (1 mg/mL) and DNAse I (50 ug/mL) for 30 minutes at 37°C in a shaking incubator at 200 rpm. TILs were filtered through a 70 uM cell strainer and isolated over Percoll density centrifugation. For dual-transfer experiments, the isolated TILs were subjected to Fc receptor blocking, live/dead staining (Live/Dead Violet) and surface marker staining for flow cytometric analysis. For intracellular cytokine staining, cells were restimulated ex vivo in media containing brefeldin and monensin (Invitrogen), phorbol myristate acetate, and ionomycin for four hours. Following Fc blocking, live/dead staining, and cell surface marker staining, cells were fixed (BD CytoFix), permeabilized (Invitrogen 10x Perm), and stained for intracellular proteins.

CD19-B16 melanoma tumor model

**[0921]** Male B6.SJL-Ptprca Pepcb/BoyJ mice ages 6-12 weeks were injected subcutaneously with 1 x $10^5$ CD19-B16 melanoma cells. Mice with similar sized tumors were randomized to receive treatments of CAR T cells. 2 x $10^6$ CAR+ or untransduced (control) T cells were retro-orbitally injected on day 12 post tumor inoculation. Tumor lengths and widths were determined every 2-3 days by digital caliper measurement and tumor volume was calculated using the following formula: (length x width2)/2. Death was defined as a progressively growing tumor that reaches, 2000mm3 or 20mm in its longest axis, whichever comes first.

ALPPL2-40L mesothelioma tumor model

**[0922]** Male B6.SJL-Ptprca Pepcb/BoyJ mice ages 6-12 weeks were injected subcutaneously with 2 x $10^6$ ALPPL2-40L mesothelioma cells. Mice with similar sized tumors were randomized to receive treatments of CAR T cells. 2 x $10^6$ CAR+ or untransduced (control) T cells were retro-orbitally injected on day 12 post tumor inoculation. Tumor lengths and widths were determined every 2-3 days by digital caliper measurement and tumor volume was calculated using the following formula: (length x width2)/2. Death was defined as a progressively growing tumor that reaches, 2000mm3 or 20mm in its longest axis, whichever comes first.

**[0923]** While particular alternatives of the present disclosure have been disclosed, it is to be understood that various modifications and combinations are possible and are contemplated within the true spirit and scope of the appended claims. There is no intention, therefore, of limitations to the exact abstract and disclosure herein presented.

## Example 7
## T Cells Comprising An Engineered CARD11-PIK3R3 Fusion Protein Clear Multiple Tumor Challenges In Vivo

**[0924]** This Example describes *in vivo* functional assessments of T cells modified to express an engineered CARD11-PIK3R3 fusion protein compared to T cells modified to express a patient derived CARD11-PIK3R3 fusion protein.

**Methods:**

*Cloning*

**[0925]** A patient derived or an engineered CARD11-PIK3R3 fusion (with a CAR) was synthesized and cloned into lentiviral vectors containing an MND promoter.

*T Cell Manufacturing*

**[0926]** Pantropic VSV-G pseudotyped lentivirus was produced via transfection of Lenti-X 293T cells with the GOI transgene expression vector and viral packaging plasmids using Mirus TransIT-Lenti. Viral supernatants were harvested 48 hours post transfection, filtered, and mixed with activated T cells. Primary CD3+ T cells were sourced from donors. After thawing, T cells were cultured in human T cell medium consisting of X-VIVO 15, 5% Human AB serum, 10mM HEPEs, and 1x Glutamax, supplemented with 10ng/mL IL-7 and 5ng/mL IL-15. T cells were activated with TransACT (CD3 and CD28 activation) for 24 hours before being transduced with lentivirus. Virus and TransACT were removed 24 hours

post transduction, and T cells were seeded to G-Rex plates to expand for 3-5 days before harvest. At harvest T cells were used immediately for co-culture assays or frozen in CryoStor10 and stored in liquid nitrogen. For TRAC editing to knock out the endogenous TCR, 24 hours after lentiviral transduction, T cells were electroporated with TRAC targeting CRISPR/Cas9 RNPs using a Lonza Nucleofector. T cells were rested overnight, washed, and transferred to a G-Rex or 24 well plate for expansion.

*In vivo efficacy studies*

**[0927]** Tumor volumes were measured in mice bearing SHP77 small cell lung cancer (SCLC) tumors. Briefly, a subcutaneous inoculum of $10x10^6$ SHP77 cells were injected into 7-10 week old female NSG (JAX) mice. At day 10 post tumor injection (when the average tumor volume reached ~$100m^3$), mice were treated with an intraveneous dose of either $1x10^6$ untransduced T cells (UTD), $1x10^6$ T cells with a CAR alone (CAR), $1x10^6$ T cells with a CAR and the patient derived CARD11-PIK3R3 fusion (CAR.C11P3), or $1x10^6$ T cells with a CAR and an engineered CARD11-PIK3R3 fusion (CAR.C11P3tr1) via retro-orbital injection. At day 63 and 122, the surviving mice in the CAR, CAR.C11P3, and CAR.C11P3tr1 treatment groups were rechallenged with a subcutaneous inoculum of $10x10^6$ SHP77 cells.

**Results:**

**[0928]** At the $1x10^6$ CAR+ T cell dose, T cells with the engineered CARD11-PIK3R3 fusion (CAR.C11P3tr1) performed similarly to T cells with the patient derived CARD11-PIK3R3 fusion (CAR.C11P3) and were able to clear multiple tumor challenges (**FIGS. 10A-10D**). Of the mice infused with CAR only T cells (CAR), 2 out of 7 mice achieved full tumor regression (**FIG. 10B**), whereas 7 out of the 8 mice infused with CAR plus patient derived CARD11-PIK3R3 T cells (CAR.C11P3) achieved full tumor regression (**FIG. 10C**), and 6 out of 8 mice infused with CAR plus engineered CARD11-PIK3R3 T cells (CAR.C11P3tr1) achieved full tumor regression (**FIG. 10D**). Mice with completely regressed tumors were rechallenged twice, once on Day 63 and again on Day 122 post initial tumor challenge. Each rechallenge was cleared by all surviving mice, except 1 mouse in the CAR plus patient derived CARD11-PIK3R3 T cell cohort (**FIGS. 10C and D**).

## Example 8
## Low Doses of T Cells Comprising An Engineered CARD11-PIK3R3 Fusion Protein Clear Tumors In Vivo

**[0929]** This Example describes *in vivo* functional assessments of OT-I T cells modified to express an engineered CARD11-PIK3R3 fusion protein compared to OT-I T cells modified to express a patient derived CARD11-PIK3R3 fusion protein.

**Methods:**

*Cloning*

**[0930]** A patient derived CARD11-PIK3R3 fusion protein or an engineered CARD11-PIK3R3 fusion protein was synthesized and cloned into gammaretroviral vectors.

*T Cell Manufacturing*

**[0931]** Gammaretrovirus was produced via transfection of Lenti-X 293T cells with the GOI transgene expression vector and viral packaging plasmids using Fugene. Viral supernatants were harvested 48 hours post transfection and filtered. CD3+ murine T cells were isolated from harvested spleens of donor mice, activated with Dynabeads for 24 hours before activated T cells were spinoculated with fresh viral supernatants in retronectin-coated plates. T cells were cultured in murine T cell medium consisting of RPMI, 10% fetal bovine serum, 10mM HEPEs, 1x NEAA, 1x beta-mercaptoethanol, and 1x sodium pyruvate, supplemented with 100 U/mL IL-2. Virus and Dynabeads were removed 24 hours post transduction, and T cells were expanded for 2 days before harvest. At harvest, T cells were adoptively transferred immediately into tumor-bearing mice.

*In vivo efficacy studies*

**[0932]** A subcutaneous inoculum of $5x10^5$ B16-Ova cells were injected into 7-10 week old C57BL/6 mice. At day 8 post tumor injection (when the average tumor volume reached ~$50m^3$), mice were treated with an intraveneous dose of either 50,000, 10,000, or 2,000 untransduced OT-I T cells (UTD OT-I), OT-I T cells with a patient derived CARD11-PIK3R3 fusion (OT-I.C11P3), or OT-I T cells with an engineered CARD11-PIK3R3 fusion (OT-I.C11P3tr1) via retro-orbital injection.

**Results:**

**[0933]** OT-I T cells with the patient derived CARD11-PIK3R3 fusion were able to completely clear tumor at both the 50,000 and 10,000 and 2,000 cell dose (**FIGS. 11B-11D**), whereas untransduced OT-I T cells could not clear tumor (**FIG. 11A**). Further, while slightly less robust than the patient derived CARD11-PIK3R3 fusion, OT-I T cells with the engineered CARD11-PIK3R3 fusion were able to clear all tumors at the 50,000 cell dose, 7

of the 8 tumors at the 10,000 cell dose, and 6 of the 9 tumors at the 2,000 cell dose (**FIGS. 11E-11G**). This suggests that both engineered and patient derived CARD11-PIK3R3 cells have incredibly potency to clear tumors at extremely low doses.

## Example 9
## T Cells Comprising a Truncated CARD11-PIK3R3 Fusion Outperform a Clinical Comparator

**[0934]** The TGFbR2-DNR is a truncated version that has been reported to reduce the immunosuppressive effect of TGFβ signaling or convert the immunosuppressive TGFβ signaling to an immunostimulatory signal (for example, in WO2020043152A1 and WO2021008610A1). In some embodiments, expression of the TGFbR2-DNR in CAR T cells may improve the efficacy of adoptive T cell therapies. This Example describes *in vivo* functional assessments of T cells modified to express an engineered CARD11-PIK3R3 fusion protein compared to T cells modified to express a TGFbR2-DNR.

**Methods:**

*Cloning*

**[0935]** An engineered CARD11-PIK3R3 or TGFbR2-DNR together with a CAR was synthesized and cloned into a lentiviral vector containing a short EF1alpha promoter.

*T Cell Manufacturing*

**[0936]** Pantropic VSV-G pseudotyped lentivirus was produced via transfection of Lenti-X 293T cells with the GOI transgene expression vector and viral packaging plasmids using Mirus TransIT-Lenti. Viral supernatants were harvested 48 hours post transfection, filtered, and mixed with activated T cells. Primary CD3+ T cells were sourced from donors. After thawing, T cells were cultured in human T cell medium consisting of X-VIVO 15, 5% Human AB serum, 10mM HEPEs, and 1x Glutamax, supplemented with 10 ng/mL IL-7 and 5 ng/mL IL-15. T cells were activated with TransACT (CD3 and CD28 activation) for 24 hours before being transduced with lentivirus. Virus and TransACT were removed 24 hours post transduction, and T cells were seeded to G-Rex plates to expand for 3-5 days before harvest. At harvest T cells were used immediately for co-culture assays or frozen in CryoStor10 and stored in liquid nitrogen. For TRAC editing to knockout the endogenous TCR, 24 hours after lentiviral transduction, T cells were electroporated with TRAC targeting CRISPR/Cas9 RNPs using a Lonza Nucleofector. T cells were rested overnight, washed, and transferred to a G-Rex or 24 well plate for expansion.

**[0937]** For AAV6 mediated T cell manufacturing, T cells were electroporated with RNPs consisting of a site directed nuclease and a single guide RNA. The cells were cultured in human T cell medium without serum (X-VIVO 15,10 mM HEPEs, and 1x Glutamax, supplemented with 10 ng/mL IL-7 and 5 ng/mL IL-15) and transduced with an AAV6 vector at an MOI of $2.5x10^4$ (unless otherwise specified) providing the HDR template. AAV6 transduced T cell cultures were supplemented with a DNA-PK inhibitor (HDR enhancer/NHEJ inhibitor) rested overnight, then washed, and transferred to a G-Rex plate for expansion

*In vitro co-culture assays*

**[0938]** For *in vitro* co-culture assays, transduced primary T cells from healthy donors were normalized by CAR expression, then co-cultured with target tumor cells at an E:T ratio of 1:1 (unless otherwise specified). Supernatants were collected at 18-24 hours post co-culture set up and assessed for IFNγ and IL-2 secretion via MSD using manufacturer protocols.

*Flow cytometry*

**[0939]** For *in vitro* primary T cell assays, cells were washed with PBS supplemented with 2% FBS, stained with surface staining markers at room temperature for 20 minutes, washed once, and resuspended in PBS supplemented with 2% FBS before analysis on an Attune Flow Cytometer. A His tagged target protein was used to assess engineered anti-target CAR expression (Acro Biosystems).

*In vivo efficacy studies*

**[0940]** Tumor volumes were measured in mice bearing SHP77 small cell lung cancer (SCLC) tumors. Briefly, a subcutaneous inoculum of $10x10^6$ SHP77 cells were injected into 7-10 week old female NSG (JAX) mice. At day 14 post tumor injection (when the average tumor volume reached ~$100m^3$), mice were treated with an intravenous dose of either $1x10^6$ untransduced (UTD) T cells, $2x10^5$ or $1x10^6$ T cells with a CAR and an engineered CARD11-PIK3R3 fusion (CAR.C11P3tr1), or $2x10^5$ or $1x10^6$ T cells with a CAR and the TGFbR2-DNR (CAR.TGFbR2-DNR) via intravenous injection. The dose for the UTD treatment was matched to the highest total number of T cells infused in any given cohort.

**Results:**

**[0941]** In the in vitro experiments, T cells engineered to express the engineered CARD11-PIK3R3 fusion expressed higher levels IFNγ (**FIG. 12A**) and IL2 (**FIG. 12B**) upon co-culture with two different SCLC tumor cell lines relative to T cells engineered to express the TGFbR2-DNR. Further, T cells engineered to express the engineered CARD11-PIK3R3 fusion demonstrated a robust ability to control the in vitro growth of both an antigen high

expressing tumor line, SHP77 (**FIG. 12C**), and an antigen low expressing tumor line, DMS273 (**FIG.12D**), whereas T cells engineered to express the TGFbR2-DNR only controlled the SHP77 cell line. This suggests the engineered CARD11-PIK3R3 provides enhanced T cell potency against low and high antigen relative to the TGFbR2-DNR.

**[0942]** CAR T cells with the engineered CARD11-PIK3R3 fusion protein or CAR T cells with the TGFbR2-DNR were administered to immunodeficient mice bearing SHP77 SCLC tumors to assess *in vivo* anti-tumor control at two different doses $2x10^5$ CAR+ T cells (**FIGS. 12E** and **12F**) or $1x10^6$ CAR+ T cells (**FIGS. 12G-12J**). At both doses, T cells with the engineered CARD11-PIK3R3 fusion provided superior tumor control compared to T cells with the TGFbR2-DNR. At the high dose, 5 out of 6 mice treated with CAR plus engineered CARD11-PIK3R3 T cells had completely regressed tumors (**FIG. 12H**). In contrast, 0 out of 6 mice treated with CAR plus TGFbR2-DNR T cells were able to control tumor growth (**FIG. 12G**). In sum, CARD11-PIK3R3 dramatically enhances sustained anti-tumor activity of CAR T cells compared to TGFbR2-DNR.

## Example 10
## Testing the auto inhibition and TCR dependent activation of engineered CARD11-PIK3R3 fusion proteins

**[0943]** This Example describes methods for assessing the auto inhibition and TCR dependent activation of engineered CARD11-PIK3R3 fusion proteins.

**Methods:**

**[0944]** Constructs for co-expression of a CAR and the engineered CARD11-PIK3R3 fusion proteins with an altered inhibitory domain, as detailed in Table 1, are designed to enable targeted knock-in of the construct into the TRAJ intron splice acceptor locus (or, alternatively, the TRAC exon 1 locus) via AAV vectors or non-targeted integration via lentiviral vectors. Further, engineered CARD11-PIK3R3 fusion proteins with CARD11 gain of function mutations (G123S and R235Q) are used as positive controls with constitutively on CARD11-PIK3R3 signaling and engineered CARD11-PIK3R3 fusion proteins with S to A mutations at activating phosphorylation sites are used as negative controls with non-regulatable constitutively off CARD11-PIK3R3 signaling.

**[0945]** Primary T cells are engineered to express a CAR with the engineered CARD11-PIK3R3 fusion protein inhibitory domain variants or control variants and evaluated at three timepoints. The first timepoint, at baseline, evaluates basal CMB activation of the engineered primary T cells in the absence of antigen. Next, the second timepoint evaluates

the activation of the engineered primary T cells 24 hours after stimulation with an antigen-positive target will demonstrate the degree to which the engineered CARD11-PIK3R3 fusion protein variants exhibit decreased function relative to the patient derived CARD-PIK3R3 fusion protein. The final timepoint, 7 days post antigen stimulation, evaluates the degree to which primary T cells harboring the engineered CARD11-PIK3R3 fusion protein are able to return to homeostatic CMB signaling after activation. The primary T cells harboring the engineered CARD11-PIK3R3 fusion protein are compared to untransduced T cells, T cells with the CAR alone, T cells with the CAR and the patient derived CARD11-PIK3R3 fusion protein, and all T cell groups co-cultured with antigen negative targets.

**[0946]** In addition to the above, CBM signaling is assessed at the molecular, transcriptional and protein level. MALT1 activation is assessed through western blot analysis of downstream substrates including CYLD, HOIL1, Regnase 1, Roquin, A20, and BCL10. Further, signaling is further evaluated via phosph-flow using antibodies to detect levels of phosphorylated IKBa, which is inversely correlated to NFKb signaling. Next, RNA-sequencing is run on the respective samples to evaluate whether NFKb signaling is transcriptionally activated across timepoints in engineered CARD11-PIK3R3 fusion proteins and control variants. Lastly, proliferation, IFNgamma secretion, and IL-2 secretion is assessed via MSD of the T cell groups to determine the functional as well as the molecular characteristics of engineered CARD11-PIK3R3 fusion proteins with an altered inhibitory domain.

**[0947] Results:**

**[0948]** Prior to TCR or TCR-like stimulation, the inhibitory domain of CARD11 is in the constitutively off position (**FIG. 1B**), resulting in unstimulated T cells that are quiescent, have homeostatic but limited proliferation, have limited MALT1 substrate cleavage, have minimal NKFb signaling, have limited NFKb transcriptional signatures, and do not produce cytokines such as IFNgamma and IL-2 at steady state. Upon TCR or TCR-like stimulation, the inhibitory domain becomes phosphorylated and undergoes a conformational change allowing for oligomerization of the CBM complex (**FIGS. 1C and 1D**), cleavage of MALT1 targets, activation of downstream signaling, upregulation of transcriptional NFKb targets, and proliferation and secretion of cytokines such as IFNgamma and IL-2. Then, after elimination of the tumor cell (and thus the tumor antigen), the T cells return to a quiescent state (**FIG. 1E**).

**[0949]** Without wishing to be bound by theory, if the engineered CARD11-PIK3R3 fusion proteins maintained a functional and TCR inducible autoinhibitory domain like the patient

derived CARD11-PIK3R3 fusion protein, the primary T cell CMB signaling would remain quiescent without antigen stimulation, the primary T cells would have superior signaling and functional outputs after antigen stimulation, and then the primary T cells would return to baseline quiescent CMB signaling after a period without stimulation. Alternatively, if the autoinhibitory function of the engineered CARD11-PIK3R3 fusion proteins is destroyed primary T cells would exhibit basal CMB signaling, increased NFKb signaling, and effector functions in the absence of antigen stimulation (mirroring the gain of function positive control). Finally, if the potency enhancement capacity of CARD11-PIK3R3 is destroyed in the engineered CARD11-PIK3R3 fusion proteins, primary T cells would exhibit decreased functional outputs 24 hours after antigen positive stimulation relative to the patient derived CARD11-PIK3R3 fusion protein.

## Example 11
## Re-creating a CARD11-PIK3R3 Fusion Protein at the Endogenous CARD11 locus

**[0950]** There are a variety of gene delivery methods that can enable the expression of the CARD11-PIK3R3 fusion protein in human cells. Two broad categories are (i) the delivery of a contiguous open reading frame encoding the full sequence of the CARD11-PIK3R3 fusion protein, and (ii) re-creating one or more alleles in situ that leverage the endogenous CARD11 gene structure to locally insert the PIK3R3 SH2 domain encoding sequence and achieve expression of the full CARD11-PIK3R3 fusion protein. Nuclease mediated homology directed repair based insertion of the sequence encoding the PIK3R3 SH2 domain at the CARD11 locus is described in the Examples above. This example describes additional methods that could be used to insert the sequence minimally encoding the PIK3R3 SH2 domain into the appropriate region of the endogenous CARD11 gene on Chromosome 7 to re-create the expression of the CARD11-PIK3R3 fusion protein.

**Methods:**

**[0951]** As described in the Examples above, AAV6 mediated transgene delivery and RNA guided nuclease mediated insertion can be used to incorporate intended DNA sequences at target genomic locations. In this example, AAV6 mediated transgene delivery and RNA guided nuclease mediated insertion can be used to insert the nucleic acid sequence encoding the PIK3R3 SH2 domain (SEQ ID NO: 440) into the CARD11 gene locus.

**[0952]** Prime editing is a method wherein RNA-guided nucleases are modified such that one of the nuclease active sites is disabled, resulting in an enzyme with single-stranded DNA cleaving (nickase) activity (see, for example, Anzalone et al. *Nature Biotech.* 2021; 40: 731-

740, which is incorporated by reference herein in its entirely). The RNA-guided nickase is further fused to a reverse transcriptase (RT) and then complexed with a prime editing guide RNA (pegRNA) that comprises an additional RNA sequence that serves as a template for RT mediated DNA synthesis of the intended genetic outcome. The endogenous DNA repair machinery ultimately incorporates the intended DNA sequences at the targeted genomic location. In this example, PRIME editing is used to insert the nucleic acid sequence encoding the PIK3R3 SH2 domain (SEQ ID NO: 440) into the CARD11 gene locus.Twin (or dual) Prime Editing is a version of the Prime Editing method that employs two separate pegRNAs that simultaneously target both DNA strands in tandem, enabling larger edits than are possible with traditional Prime Editing by generating overlapping and complementary DNA flaps that can anneal (see, for example, Anzalone et al. *Nature Biotech.* 2021; 40: 731-740, which is incorporated by reference herein in its entirely). Then, endogenous DNA repair machinery ultimately resolves the final conversion of the endogenous sequence to the intended genetic outcome. While there are limitations to the size of the DNA insertions that can be achieved with this approach, without wishing to be bound by theory, novel pegRNA designs and chemical compositions could integrate a nucleic acid encoding the PIK3R3 SH2 domain (SEQ ID NO: 440) into the CARD11 gene locus at a site that recreates the patient derived, an engineered, or similar CARD11-PIK3R3 fusion protein. Without wishing to be bound by theory, such a strategy could include: (i) insertion of the coding sequence of the PIK3R3 SH2 domain into an existing CARD11 exon; or (ii) insertion of a synthetic intron comprising the coding sequence of the PIK3R3 SH2 domain. Without wishing to be bound by theory, in each case, additional sequences such as a 2A ribosomal skip peptide, splicing acceptor and donor sequences, and/or termination codons and polyadenylation signals, may be necessary to achieve expression of the desired CARD11-PIK3R3 protein.

**[0953]** An additional method for generating targeted insertion of sequences encoding PIK3R3 SH2 domain at the CARD11 gene is called Prime-Assisted Site-Specific Integrase Gene Editing (PASSIGE) (see, for example, Pandey et al, *Nature Biomed. Eng.* 2024; 9: 22-39, which is incorporated by reference herein in its entirety ). This method involves the delivery of three components, a Prime Editor, a DNA donor, and a site-specific recombinase, and operates via two sequential steps as follows: first, Prime Editing (as described above) is used to modify the target locus in a manner that inserts a landing pad sequence; second, the co-delivered recombinase recognizes the landing pad sequences and similar sequences present in the donor DNA and catalyzes a recombination event that places the cargo present in the donor DNA at the landing pad site. This method has the capacity to deliver much

larger DNA sequences than Twin Prime Editing. Therefore, strategies similar to this could be used to insert the coding sequence of the PIK3R3 SH2 domain as described for Twin Prime Editing, but could also be extended to the addition of other regulatory or functional sequence elements, such as genes and promoters to co-express CAR or TCR or other potency enhancement components.

# CLAIMS

1. A recombinant polypeptide comprising a caspase-associated recruitment domain (CARD domain) containing protein or a functional fragment thereof,
wherein the recombinant polypeptide comprises no more than 724 amino acids.

2. A recombinant polypeptide comprising:
(a) a CARD domain, and
(b) a recruitment domain or a functional fragment thereof, wherein the recruitment domain is capable of binding to (i) a substrate localized to the intracellular side of the plasma membrane of a cell and/or (ii) a target polypeptide comprising a phosphorylated tyrosine,
wherein the recombinant polypeptide comprises no more than 724 amino acids.

3. A recombinant polypeptide comprising:
(a) a first polypeptide capable of binding to a CARD domain on BCL10, and
(b) a recruitment domain or a functional fragment thereof, wherein the recruitment domain is capable of binding to (i) a substrate localized to the intracellular side of the plasma membrane of a cell and/or (ii) a target polypeptide comprising a phosphorylated tyrosine,
wherein the recombinant polypeptide comprises no more than 724 amino acids.

4. The recombinant polypeptide of any one of claims 1-3, wherein when the recombinant polypeptide is expressed in an immune cell and the immune cell binds a target antigen, the immune cell exhibits decreased Interferon gamma (IFNg) and/or Interleukin-2 (IL-2) production compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1.

5. The recombinant polypeptide of any one of claims 1-4, wherein when the recombinant polypeptide is expressed in an immune cell and the immune cell binds a target antigen, the immune cell exhibits equivalent or enhanced:
(a) BCL10 binding,
(b) BCL10 and MALT1 complex formation,
(c) NF-κB and/or AP-1 signaling,
(d) MALT1 paracaspase activity,
(e) cytokine production,
(f) peak tumor expansion,
(g) peak peripheral blood expansion,

(h) contraction following peak tumor expansion, and/or

(i) contraction following peak peripheral blood expansion,

compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1.

6. The recombinant polypeptide of any one of claims 1-5, wherein when the recombinant polypeptide is expressed in an immune cell and the immune cell binds a target antigen, the immune cell exhibits equivalent or higher IFNg and/or IL-2 production compared to an immune cell expressing a control polypeptide consisting of SEQ ID NO: 1.

7. The recombinant polypeptide of any one of claims 1-6, wherein the recombinant polypeptide comprises no more than 700, no more than 650, no more than 600, no more than 550, no more than 500, no more than 450, no more than 400, no more than 350, no more than 300, no more than 250, no more than 200, or no more than 150 amino acids.

8. The recombinant polypeptide of any one of claims 1-7, wherein the CARD domain-containing protein or the functional fragment thereof, the CARD domain, or the recombinant polypeptide, is capable of binding to a CARD domain on BCL10.

9. The recombinant polypeptide of any one of claims 1-8, wherein the CARD domain-containing protein or the functional fragment thereof, the CARD domain, or the recombinant polypeptide, is capable of forming a complex with BCL10 and MALT1.

10. The recombinant polypeptide of any one of claims 1-9, wherein the CARD domain-containing protein or the functional fragment thereof, or the CARD domain, comprises or consists of a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to any one of SEQ ID NO: 293-323.

11. The recombinant polypeptide of any one of claims 1-10, wherein the CARD domain-containing protein is selected from, or wherein the CARD domain is derived from a protein selected from, CARD9, CARD10, CARD11, and CARD14.

12. The recombinant polypeptide of claim 11, wherein the CARD domain-containing protein is, or wherein the CARD domain is derived from, CARD11.

13. The recombinant polypeptide of any one of claims 1-12, wherein the recombinant polypeptide comprises a full coiled-coil domain or a truncated portion thereof, wherein the

start of the full coiled-coil domain corresponds to amino acid position 123 of SEQ ID NO: 1, and wherein the end of the full coiled-coil domain corresponds to amino acid position 442 of SEQ ID NO: 1; optionally, wherein the full coiled-coil domain comprises a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to amino acids 123-442 of SEQ ID NO: 1.

14. The recombinant polypeptide of claim 13, wherein the full coiled-coil domain comprises at least 310 amino acids.

15. The recombinant polypeptide of claim 13 or claim 14, wherein the recombinant polypeptide comprises the full coiled-coil domain.

16. The recombinant polypeptide of claim 13, wherein the recombinant polypeptide comprises a truncated portion of the coiled-coil domain having no more than 300, no more than 290, no more than 280, no more than 270, no more than 260, no more than 250, no more than 240, no more than 230, no more than 220, no more than 210, no more than 200, no more than 190, no more than 180, no more than 170, no more than 160, no more than 150, no more than 140, no more than 130, no mor than 120, no more than 110, no more than 100, no more than 95, no more than 90, no more than 85, no more than 80, no more than 75, no more than 70, no more than 60, no more than 50, or no more than 40 amino acids; preferably, wherein the truncated portion of the coiled-coil domain has no more than 260 amino acids.

17. The recombinant polypeptide of claim 16, wherein a region within the coiled-coil domain corresponding to amino acids 253-324 of SEQ ID NO: 1 is removed or replaced by a polypeptide comprising no more than 20, 15, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, or 1 amino acids.

18. The recombinant polypeptide of claim 17, wherein the region within the coiled-coil domain is replaced by a polypeptide comprising a sequence of SGGGGSGGGGS (SEQ ID NO:431) or QAGKRSLPD (SEQ ID NO: 432), or a sequence having at most 1, 2, or 3 mutations thereto.

19. The recombinant polypeptide of any one of claims 1-18, wherein the recombinant polypeptide comprises an inhibitory domain or a truncated portion thereof, wherein the start of the inhibitory domain corresponds to amino acid position 443 of SEQ ID NO: 1, and

wherein the end of the inhibitory domain corresponds to amino acid position 577 of SEQ ID NO: 1; optionally, wherein the inhibitory domain comprises a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to amino acids 443-577 of SEQ ID NO: 1.

20. The recombinant polypeptide of any one of claims 1-19, wherein the recombinant polypeptide comprises an inhibitory domain or a truncated portion thereof, wherein the inhibitory domain comprises a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, or 100% identity to SEQ ID NO: 441.

21. The recombinant polypeptide of claim 19 or 20, wherein the inhibitory domain comprises at least 130, or at least 125, amino acids.

22. The recombinant polypeptide of any one of claims 19-21, wherein the recombinant polypeptide comprises the full inhibitory domain.

23. The recombinant polypeptide of any one of claims 19-22, wherein the recombinant polypeptide comprises a truncated portion of the inhibitory domain having no more than 120, no more than 110, no more than 100, no more than 95, no more than 90, no more than 85, no more than 80, no more than 75, no more than 70, no more than 60, no more than 50, or no more than 40 amino acids.

24. The recombinant polypeptide of claim 23, wherein the inhibitory domain comprises N-terminal and/or C-terminal truncation(s).

25. The recombinant polypeptide of claim 24, wherein the inhibitory domain comprises a truncation of at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, or at least 90 amino acids at the N-terminus.

26. The recombinant polypeptide of claim 24 or 25, wherein the inhibitory domain comprises a truncation of at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, or at least 90 amino acids at the C-terminus.

27. The recombinant polypeptide of any one of claims 24-26, wherein the inhibitory domain comprises a truncation of at least 5, at least 10, at least 15, at least 20, at least 30, at least 40, at least 50, at least 60, at least 70, at least 80, or at least 90 amino acids in between the N-terminus and the C-terminus.

28. The recombinant polypeptide of any one of claims 24-26, wherein the inhibitory domain or the truncated portion thereof comprises at least 1, at least 2, at least 3, at least 4, at least 5, or at least 6 serines that are capable of being phosphorylated; optionally wherein the serines are capable of being phosphorylated by a protein kinase C (PKC); optionally, wherein the PKC comprises PKCβ and/or PKCθ.

29. The recombinant polypeptide of claim 28, wherein the inhibitory domain or the truncated portion thereof comprises a sequence of DA**S**PRT (SEQ ID NO: 425), RAK**S**PI (SEQ ID NO: 426), DA**S**PSS (SEQ ID NO: 427), SRS**S**IMS (SEQ ID NO: 428), RKF**S**LER (SEQ ID NO: 429), and/or FRP**S**VTS (SEQ ID NO: 430), or a sequence having at most 1, 2, or 3 mutations thereto; optionally, where the inhibitory domain or the truncated portion thereof comprises the sequence of RAK**S**PI (SEQ ID NO: 426), DA**S**PSS (SEQ ID NO: 427), and/or SRS**S**IMS (SEQ ID NO: 428).

30. The recombinant polypeptide of any one of claims 20-29, comprising, from N-term to C-term: the coiled-coil domain or the truncated portion thereof, and the inhibitory domain or the truncated portion thereof, without any additional amino acid in between.

31. The recombinant polypeptide of any one of claims 1-30, wherein the recombinant polypeptide comprises (b) a recruitment domain or a functional fragment thereof, wherein the recruitment domain is capable of binding to (i) a substrate localized to the intracellular side of the plasma membrane of a cell and/or (ii) a target polypeptide comprising a phosphorylated tyrosine.

32. The recombinant polypeptide of claim 31, wherein the recruitment domain is capable of binding to a substrate indirectly localized to the intracellular side of the plasma membrane, and wherin the recruitment domain binds a polypeptide or lipid that is directly localized to the intracellular side of the plasma membrane of a cell.

33. The recombinant polypeptide of any one of claim 31 or 32, wherein the recruitment domain is a Src Homology region 2 (SH2) domain, a Src Homology region 3 (SH3) domain, a phosphotyrosine-binding (PTB) domain, or a pleckstrin homology (PH) domain.

34. The recombinant polypeptide of any one of claims 31-33, wherein the recruitment domain is a Src Homology region 2 (SH2) domain.

35. The recombinant polypeptide of any one of claims 31-34, wherein the recruitment domain or the functional fragment thereof comprises a sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a segment of at least 30, 40, 50, 60, 70, 80, 90, or 97 amino acids within the region of amino acids 622-716 of SEQ ID NO: 1.

36. The recombinant polypeptide of any one of claims 31-35, comprising, from N-term to C-term: the inhibitory domain or the truncated portion thereof, and the recruitment domain or the functional fragment thereof, with no more than 5, no more than 10, no more than 15, no more than 20, or no more than 25, amino acids in between.

37. The recombinant polypeptide of claim 36, comprising an amino acid sequence having at least 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to any one of SEQ ID NO: 433-438 in between the inhibitory domain or the truncated portion thereof and the recruitment domain or the functional fragment thereof.

38. The recombinant polypeptide of any one of claims 1-37, wherein the recombinant polypeptide comprises, from N-terminus to C-terminus, the CARD domain, optionally the coiled-coil domain, optionally the inhibitory domain, and the recruitment domain.

39. A nucleic acid construct comprising a polynucleotide sequence encoding the recombinant polypeptide of any one of claims 1-38.

40. The nucleic acid construct of claim 39, wherein the nucleic acid construct further comprises a polynucleotide sequence encoding one or more additional polypeptides and/or a non-coding RNA.

41. The nucleic acid construct of claim 40, wherein the one or more additional polypeptides comprise a chimeric antigen receptor (CAR), a recombinant T cell receptor (TCR), an additional potency enhancement polypeptide, a cytokine, a chemokine, a growth factor, a safety switch, or any combination thereof.

42. The nucleic acid construct of claim 41, wherein the one or more additional polypeptides comprise a CAR or a recombinant TCR.

43. The nucleic acid construct of any one of claims 39-42, comprising a first homology arm before the 5’ end of the nucleic acid sequence encoding the recombinant polypeptide

and a second homology arm after the 3' end of the nucleic acid sequence encoding the recombinant polypeptide; optionally, wherein the lengths of the two homology arms are between about 150 bp and about 1500 bp.

44. The nucleic acid construct of claim 43, wherein the length of at least one of the homology arms is about 200bp, 225 bp, about 250 bp, about 275 bp, about 300 bp, about 325 bp, about 350 bp, about 375 bp, about 400 bp, about 425 bp, about 450 bp, about 475 bp, or about 500 bp; optionally, wherein the lengths of both of the homology arms are about 225 bp, about 250 bp, about 275 bp, about 300 bp, about 325 bp, about 350 bp, about 375 bp, about 400 bp, about 425 bp, about 450 bp, about 475 bp, about 500 bp, about 525 bp, about 550 bp, about 575 bp, or about 600 bp.

45. The nucleic acid construct of claim 43 or 44, wherein the total length of the homology arms is between about 400 bp and about 500 bp, about 500 bp and about 600 bp, about 600 bp and about 700 bp, about 700 bp and about 800 bp, about 800 bp and about 900 bp, about 900 bp and about 1000 bp, about 1000 bp and about 1100 bp, or about 1100 bp and about 1200 bp.

46. The nucleic acid construct of any one of claims 39-45, wherein the nucleic acid construct is less than 10 kb, less than 9 kb, less than 8 kb, less than 7 kb, less than 6 kb, less than 5 kb, less than 4 kb, or less than 3 kb in length.

47. The nucleic acid construct of claim 46, wherein the nucleic acid construct is less than about 5.0 kb in length.

48. The nucleic acid construct of claim 46, wherein the nucleic acid construct is less than about 4.7 kb in length.

49. A vector comprising the nucleic acid construct of any one of claims 39-48.

50. The vector of claim 49, wherein the vector is a viral vector.

51. The vector of claim 50, wherein the viral vector is selected from a retrovirus vector, an adenovirus vector, and an adeno-associated virus (AAV) vector; optionally, wherein the viral vector is an AAV vector.

52. An engineered cell comprising the recombinant polypeptide of any one of claims 1-38 or the nucleic acid construct of any one of claims 39-48.

53. The engineered cell of claim 52, wherein activating the engineered cell results in the secretion of IFNg by the cell at a level that is at least 10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold higher than the IFNg secretion level of a control cell lacking the recombinant polypeptide.

54. The engineered cell of claim 52 or 53, wherein activating the engineered cell results in the secretion of IL-2 by the cell at a level that is at least 10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold higher than the IL-2 secretion level of a control cell lacking the recombinant polypeptide.

55. The engineered cell of claim 52, wherein activation of the engineered cell and subsequent removal of the target antigen results in CARD11-PIK3R3 signaling by the cell at a level that is at least 10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold lower than the CARD11-PIK3R3 signaling of a control cell comprising a CARD11-PIK3R3 fusion protein lacking the inhibitory domain.

56. The engineered cell of claim 52 or 55, wherein activation of the engineered cell and subsequent removal of the target antigen results in CARD11-PIK3R3 signaling by the cell at a level that is at least 10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold lower than the CARD11-PIK3R3 signaling of a control cell comprising a CARD11-PIK3R3 fusion protein lacking one of more of S466, S512, S535, S559, S644, and S652 according to SEQ ID NO: 518 in the inhibitory domain.

57. The engineered cell of any of claims 52-56, wherein CARD11-PIK3R3 signaling in the cell in the absence of a target antigen is at a level that is at least 10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold lower than the CARD11-PIK3R3 signaling of a control cell comprising a control CARD11-PIK3R3 fusion protein lacking the inhibitory domain.

58. The engineered cell of any of claims 52-57, wherein CARD11-PIK3R3 signaling in the cell in the absence of a target antigen is at a level that is at least 10%, at least 20%, at least 50%, at least 1-fold, at least 2-fold, at least 5-fold, at least 10-fold, at least 20-fold, least 30-fold, or at least 100-fold lower than the CARD11-PIK3R3 signaling of a control cell comprising a control CARD11-PIK3R3 fusion protein lacking one of more of S466, S512, S535, S559, S644, and S652 according to SEQ ID NO: 518 in the inhibitory domain.

59. The engineered cell of claim any one of claims 52-58, wherein the cell is selected from the group consisting of a T cell, a macrophage, a monocyte, and a natural killer (NK) cell.

60. The engineered cell of claim 59, wherein the cell is a T cell.

61. The engineered cell of any one of claims 52-60, wherein the cell further comprises:
(i) a chimeric antigen receptor (CAR) having specificity for a target antigen; or
(ii) a recombinant T cell receptor (TCR) having specificity for a target antigen.

62. A composition comprising the engineered cell of any one of claims 52-60, or a plurality of the engineered cell of any one of claims 52-60.

63. A method of preparing an engineered cell, the method comprising expressing in the cell the recombinant polypeptide of any one of claims 1-38, thereby making the engineered cell.

64. The method of claim 63, comprising introducing into the cell the nucleic acid construct of any one of claims 39-48.

65. A method of preparing an engineered cell, the method comprising introducing into the cell the nucleic acid construct of any one of claims 39-48, thereby making the engineered cell.

66. The method of any one of claims 63-65, wherein the step of introducing comprises homology-directed repair (HDR)-mediated insertion using a gene editing machinery.

67. The method of claim 66, where the HDR occurs in 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 95%, at least 98%, or at least 99%, of a population of the cells; optionally, wherein the HDR occurs

in between about 20%-30%, about 30%-40%, about 40%-50%, about 50%-60%, about 60%-70%, about 70%-80%, about 80%-90%, or about 90%-100%, of the cells.

68. The method of claim 66 or claim 67, wherein the step of introducing comprises HDR-mediated insertion in the absence of an HDR enhancer; optionally, wherein the HDR enhancer is M3814 or AZD7648.

69. The method of any one of claims 66-68, wherein the nucleic acid construct comprises a first homology arm before the 5' end of the nucleic acid sequence encoding the recombinant polypeptide and a second homology arm after the 3' end of the nucleic acid sequence encoding the recombinant polypeptide; optionally, wherein the lengths of the two homology arms are between about 150 bp and about 1500 bp.

70. The method of claim 69, wherein the length of each of the homology arms is at least 200 bp.

71. The method of claim 69 or 70, wherein the length of at least one of the homology arms is about 200bp, 225 bp, about 250 bp, about 275 bp, about 300 bp, about 325 bp, about 350 bp, about 375 bp, about 400 bp, about 425 bp, about 450 bp, about 475 bp, or about 500 bp; optionally, wherein the lengths of both of the homology arms are about 225 bp, about 250 bp, about 275 bp, about 300 bp, about 325 bp, about 350 bp, about 375 bp, about 400 bp, about 425 bp, about 450 bp, about 475 bp, about 500 bp, about 525 bp, about 550 bp, about 575 bp, or about 600 bp.

72. The method of any one of claims 66-71, wherein the total length of the homology arms is between about 400 bp and about 500 bp, about 500 bp and about 600 bp, about 600 bp and about 700 bp, about 700 bp and about 800 bp, about 800 bp and about 900 bp, about 900 bp and about 1000 bp, about 1000 bp and about 1100 bp, or about 1100 bp and about 1200 bp.

73. The method of any one of claims 66-72, wherein the nucleic acid construct comprises, from 5' to 3', the first homology arm, a promoter, a CAR encoding sequence, a polynucleotide sequence encoding the recombinant polypeptide, and the second homology arm.

74. The method of any one of claims 64-73, wherein the nucleic acid construct is introduced via AAV.

75. The method of any one of claims 63-74, wherein the method thereby produces an engineered cell comprising: (i) the recombinant polypeptide; (ii) the immune receptor (e.g., CAR); and (iii) the modified TRAC gene locus.

76. The method of any one of claims 63-74, wherein the method thereby produces an engineered cell comprising: (i) the recombinant polypeptide; (ii) the immune receptor (e.g., CAR); and (iii) the modified TRAJ intron splice acceptor locus.

77. A method of treating a subject, comprising administering to the subject the engineered cell of any one of claims 52-61, or the engineered cell prepared by the method of any one of claims 63-76.

78. The method of claim 77, wherein the subject has a cancer or an autoimmune disease.