METHODS AND COMPOSITIONS FOR TRANSDUCING LYMPHOCYTES AND REGULATED EXPANSION THEREOF

CROSS-REFERENCE TO RELATED APPLICATIONS

[1] This application claims benefit of U.S. Provisional Application No. 62/390,093, filed March 19, 2016; U.S. Provisional Application No. 62/360,041, filed July 8, 2016; and U.S. Provisional Application No. 62/467,039, filed March 3, 2017. These applications are incorporated by reference herein in their entirety.

SEQUENCE LISTING

[2] This application hereby incorporates by reference the material of the electronic Sequencing Listing filed concurrently herewith. The materials in the electronic Sequence Listing is submitted as a text (.txt) file entitled "F1_001_Sequence_listing.txt" created on March 16, 2017, which has a file size of 230 KB, and is herein incorporated by reference in its entirety.

FIELD OF INVENTION

[3] This disclosure relates to the field of immunology, or more specifically, to the genetic modification of T lymphocytes or other immune cells, and methods of making retroviruses and controlling the expression of genes.

BACKGROUND OF THE DISLOSURE

[4] Lymphocytes isolated from a subject (e.g. patient) can be activated *in vitro* and genetically modified to express synthetic proteins that enable redirected engagement with other cells and environments based upon the genetic programs incorporated. An example of such a synthetic protein is a chimeric antigen receptor (CAR). One CAR that is currently used is a fusion of an extracellular recognition domain (e.g., an antigen-binding domain), a transmembrane domain, and one or more intracellular signaling domains encoded by a replication incompetent retrovirus.

[5] While retroviruses have shown efficacy in infecting non-dividing cells, resting CD4 and CD8 lymphocytes are refractory to genetic transduction by these vectors. To overcome this difficulty, these cells are typically activated *in vitro* using stimulation reagents before genetic modification with the CAR gene vector can occur. Following stimulation and transduction, the genetically modified cells are expanded *in vitro* and subsequently reintroduced into a lymphodepleted patient. Upon antigen

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engagement *in vivo*, the intracellular signaling portion of the CAR can initiate an activation-related response in an immune cell and release of cytolytic molecules to induce tumor cell death.

[6] Such current methods require extensive manipulation and manufacturing of proliferating T cells outside the body prior to their reinfusion into the patient, as well as lymphodepleting chemotherapy to free cytokines and deplete competing receptors to facilitate T cell engraftment. Such CAR therapies further cannot be controlled for propagation rate *in vivo* once introduced into the body, nor safely directed towards targets that are also expressed outside the tumor. As a result, CAR therapies today are typically infused from cells expanded *ex vivo* from 12 to 28 days using doses from 1 x 10^5 to 1 x 10^8 cells/kg and are directed towards targets, for example tumor targets, for which off tumor on target toxicity is generally acceptable. These relatively long *ex vivo* expansion times create issues of cell viability and sterility, as well as sample identity in addition to challenges of scalability. Thus, there are significant needs for a safer, more effective scalable T cell or NK cell therapy.

SUMMARY

[7] In one aspect, provided herein is a method for genetically modifying and expanding lymphocytes of a subject, comprising:

A. contacting resting T cells and/or NK cells of the subject *ex vivo* without requiring prior *ex vivo* stimulation, with recombinant retroviruses comprising:

i. a pseudotyping element on its surface that is capable of binding to a T cell and/or NK cell and facilitating membrane fusion of the recombinant retrovirus thereto; and

ii. a polynucleotide comprising one or more transcriptional units operatively linked to a promoter active in T cells and/or NK cells, wherein the one or more transcriptional units encode a first engineered signaling polypeptide regulated by an *in vivo* control element, wherein said first engineered signaling polypeptide comprises a lymphoproliferative element,

wherein said contacting facilitates transduction of at least some of the resting T cells and/or NK cells by the recombinant retroviruses, thereby producing genetically modified T cells and/or NK cells;

B. introducing the genetically modified T cells and/or NK cells into the subject; and

C. exposing the genetically modified T cells and/or NK cells *in vivo* to a compound that binds the *in vivo* control element to affect expression of the first engineered signaling polypeptide and promote and/or potentiate expansion, engraftment, and/or persistence of the lymphocytes *in vivo*, thereby genetically modifying and expanding lymphocytes of the subject. In illustrative embodiments, the transduction is carried out without *ex vivo* stimulation.

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[8] In the above aspect and any of the method aspects for genetically modifying and expanding lymphocytes or for performing cellular therapy herein, if not recited in the broadest aspect, in certain embodiments the polynucleotide further comprises a transcriptional unit that encodes a second engineered signaling polypeptide comprising a first chimeric antigen receptor comprising an antigen-specific targeting region (ASTR), a transmembrane domain, and an intracellular activating domain.

[9] In another aspect, provided herein is a method for performing adoptive cell therapy on a subject, comprising:

A. collecting blood from the subject;

B. contacting resting T cells and/or NK cells from the blood of the subject *ex vivo* with recombinant retroviruses, wherein the recombinant retroviruses comprise

i. a pseudotyping element on their surface that is capable of binding to a T cell and/or NK cell and facilitating membrane fusion of the recombinant retroviruses thereto; and

ii. a polynucleotide comprising one or more transcriptional units operatively linked to a promoter active in T cells and/or NK cells, wherein the one or more transcriptional units encode a first engineered signaling polypeptide comprising a lymphoproliferative element whose expression is regulated by an *in vivo* control element, and a second engineered signaling polypeptide comprising a chimeric antigen receptor comprising an antigen-specific targeting region (ASTR), a transmembrane domain, and an intracellular activating domain,

wherein said contacting results in at least some of the resting T cells and/or NK cells becoming genetically modified; and

C. reintroducing the genetically modified T cells and/or NK cells into the subject, wherein expansion, engraftment, and/or persistence of the genetically modified T cells and/or NK cells occurs *in vivo* within the subject, and wherein the method between the collecting blood and the reintroducing the genetically modified T cells and/or NK cells is performed in no more than 24 hours, thereby performing adoptive cell therapy on the subject.

[10] Provided in another aspect herein is a method for performing adoptive cell therapy on a subject, comprising:

A. collecting blood from a subject;

B. isolating peripheral blood mononuclear cells (PBMCs) comprising resting T cells and/or resting NK cells;

C. contacting the resting T cells and/or resting NK cells of the subject *ex vivo*, with recombinant retroviruses, wherein the recombinant retroviruses comprise a pseudotyping element on their surface that is capable of binding a resting T cell and/or NK cell and facilitating membrane fusion

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of the recombinant retrovirus thereto, wherein said contacting facilitates transduction of the resting T cells and/or NK cells by the recombinant retroviruses, thereby producing genetically modified T cells and/or NK cells; and

D. reintroducing the genetically modified cells into the subject within 24 hours of collecting blood from the subject, thereby performing adoptive cell therapy in the subject.

[11] Provided in another aspect herein, is a method of transducing resting lymphocytes of a subject, comprising contacting resting T cells and/or resting NK cells of a subject *ex vivo*, with recombinant retroviruses, wherein the recombinant retroviruses comprise a pseudotyping element on their surface that is capable of binding a resting T cell and/or resting NK cell and facilitating membrane fusion of the recombinant retrovirus thereto, wherein said contacting facilitates transduction of the resting T cells and/or NK cells by the recombinant retroviruses, thereby producing genetically modified T cells and/or NK cells. In illustrative embodiments of this aspect, at least 10, 20, or 25% of the resting T cells and/or NK cells, or between 10% and 70%, or 20% and 50% of T cells and/or NK cells are transduced as a result of the process are transduced as a result of the process.

[12] Provided in another aspect herein is a method for transducing resting T cells and/or resting NK cells from isolated blood, comprising:

A. collecting blood from a subject;

B. isolating peripheral blood mononuclear cells (PBMCs) comprising resting T cells and/or resting NK cells;

C. contacting the resting T cells and/or resting NK cells of the subject *ex vivo*, with recombinant retroviruses, wherein the recombinant retroviruses comprise a pseudotyping element on their surface that is capable of binding a resting T cell and/or resting NK cell and facilitating membrane fusion of the recombinant retrovirus thereto, wherein said contacting facilitates transduction of at least 5% of the resting T cells and/or resting NK cells by the recombinant retroviruses, thereby producing genetically modified T cells and/or NK cells, thereby transducing resting T cells and/or NK cells.

[13] In one aspect, provided herein is a recombinant retrovirus, comprising:

A. one or more pseudotyping elements capable of binding to a T cell and/or an NK cell and facilitating membrane fusion of the recombinant retrovirus thereto;

B. a polynucleotide comprising one or more transcriptional units operatively linked to a promoter active in T cells and/or NK cells, wherein the one or more transcriptional units encode a first engineered signaling polypeptide comprising a chimeric antigen receptor comprising an antigen-specific targeting region, a transmembrane domain, and an intracellular activating domain, and a second engineered signaling polypeptide comprising a lymphoproliferative

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element; wherein expression of the first engineered signaling polypeptide and/or the second engineered signaling polypeptide are regulated by an *in vivo* control element; and

C. an activation element on its surface, wherein the activation element is capable of binding to a T cell and/or NK cell and is not encoded by a polynucleotide in the recombinant retrovirus.

[14] In another aspect, provided herein is a recombinant retrovirus, comprising:

A. a pseudotyping element on its surface that is capable of binding to a T cell and/or NK cell and facilitating membrane fusion of the recombinant retrovirus thereto, wherein said pseudotyping element comprises cytoplasmic domain deletion variants of a measles virus F polypeptide and/or a measles virus H polypeptide;

B. a polynucleotide comprising one or more transcriptional units operatively linked to a promoter active in T cells and/or NK cells, wherein the one or more transcriptional units encode a first engineered signaling polypeptide comprising a chimeric antigen receptor comprising an antigen-specific targeting region, a transmembrane domain, and an intracellular activating domain, and a second engineered signaling polypeptide comprising a constitutively active IL-7 receptor mutant; wherein expression of the IL-7 receptor mutant is regulated by a riboswitch that binds a nucleoside analog antiviral drug; and

C. a polypeptide capable of binding to CD3 and a polypeptide capable of binding to CD28, wherein said polypeptides are expressed on the surface of a recombinant retrovirus; are capable of binding to a T cell and/or NK cell; and are not encoded by a polynucleotide in the recombinant retrovirus. In illustrative embodiments of this aspect, binding of the nucleoside analog antiviral drug to the riboswitch increases expression of the IL-7 receptor mutant.

[15] In any of the method or composition aspects provided herein, if not already recited in the broadest aspect, the recombinant retrovirus or retroviruses comprises or further comprise an activation element on their surface that is capable of activating a resting T cell and/or a resting NK cell.

[16] In any of the methods or compositions herein that recite a T cell and/or a NK cell, or a resting T cell or a resting NK cell, in certain illustrative embodiments, the cell is a T cell.

[17] Typically, the recombinant retrovirus in any of the methods and compositions provided herein, is replication defective. That is, the virus cannot replicate. In illustrative embodiments, the retrovirus is a lentivirus, such as a replication defective HIV lentivirus.

[18] In illustrative embodiments of any of the methods aspects for genetically modifying and expanding lymphocytes or for performing adoptive cellular therapy herein, or similar methods, between 10% and 75%, or 10% and 70%, or 10% and 60%, or 10% and 50%, or 10% and 25%, or 20% and 75%, or 20% and 50%, or at least 10%, 20%, or 25% of resting T cells are transduced and between 0% and 75% of NK cells are transduced. In other embodiments, between 5% and 80%, or 10% and 80%, or 10% and 70%,

or 10% and 60%, or 10% and 50%, or 10% and 25%, or 10% and 20%, or 20% and 50% of resting NK cells are transduced.

[19] In illustrative embodiments of any of the methods aspects for genetically modifying and expanding lymphocytes or for performing adoptive cellular therapy herein, or similar methods or any compositions provided herein, if not explicitly recited in the broadest aspect, expression of said second engineered signaling polypeptide is regulated by the *in vivo* control element.

[20] In illustrative embodiments of any of the methods aspects for genetically modifying and expanding lymphocytes or for performing cellular therapy herein, or similar methods, if not explicitly recited in the broadest aspect the method, the contacting can be carried out for between 15, 30 or 45 minutes or 1, 2, 3, 4, 5, 6, 7, or 8 hours on the low end of the range, and between 6, 8, 10, 12, 18, 24, 36, , 48, and 72 hours on the high end of the range. For example, in illustrative embodiments, the contacting is carried out for between 2 and 24 hours, or between 4 and 12 hours, or between 4 and 8 hours.

[21] In illustrative embodiments of any of the methods aspects for genetically modifying and expanding lymphocytes or for performing adoptive cellular therapy herein, or similar methods, if not explicitly recited in the broadest aspect the method can further comprise exposing the genetically modified T cells and/or NK cells *in vivo* to a compound that binds the *in vivo* control element to affect expression of the first engineered signaling polypeptide and optionally the second engineered signaling polypeptide, and to promote expansion, engraftment, and/or persistence of the lymphocytes *in vivo*.

[22] In illustrative embodiments of any of the methods aspects for genetically modifying and expanding lymphocytes or for performing adoptive cellular therapy herein, or similar methods, if not explicitly recited in the broadest aspect, the genetically modified T cells and/or NK cells undergo 8, 7, 6, 5, 4, 3 or fewer cell divisions *ex vivo* prior to being introduced or reintroduced into the subject.

[23] In illustrative embodiments of any of the methods aspects for genetically modifying and expanding lymphocytes or for performing cellular therapy herein, or similar methods, if not explicitly recited in the broadest aspect, expansion, engraftment, and/or persistence of genetically modified T cells and/or NK cells *in vivo* is dependent on either the presence or absence of the compound that binds the *in vivo* control element, and in illustrative embodiments, is dependent on the presence of the compound that binds the *in vivo* control element.

[24] In illustrative embodiments of any of the methods aspects for genetically modifying and expanding lymphocytes or for performing adoptive cellular therapy herein, or similar methods, if not explicitly recited in the broadest aspect, the subject is not exposed to a lymphodepleting agent within 7, 14, or 21 days of performing the contacting, during the contacting, and/or within 7, 14, or 21 days after the modified T cells and/or NK cells are introduced into the subject. In other embodiments, the subject is not exposed to a lymphodepleting agent during the contacting.

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[25] In illustrative embodiments of any of the methods aspects for genetically modifying and expanding lymphocytes or for performing cellular therapy herein, or similar methods, if not explicitly recited in the broadest aspect, the resting T cells and/or resting NK cells are in contact with the recombinant retroviruses for between 15 minutes and 12 hours.

[26] In illustrative embodiments of any of the methods aspects for genetically modifying and expanding lymphocytes or for performing adoptive cellular therapy herein, or similar methods, if not explicitly recited in the broadest aspect, the method further includes the step of separating the recombinant retroviruses from the T cells and/or NK cells after the contacting but before the introducing.In illustrative embodiments of any of the methods aspects for genetically modifying and expanding lymphocytes or for performing cellular therapy herein, or similar methods, if not explicitly recited in the broadest aspect, said exposing step comprises administering a dose of the compound to the subject prior to or during the contacting, and/or after the genetically modified T cells and/or NK cells have been introduced into the subject.

[27] In illustrative embodiments of any of the methods aspects for genetically modifying and expanding lymphocytes or for performing adoptive cellular therapy herein, or similar methods, if not explicitly recited in the broadest aspect, the method comprises collecting blood comprising the T cells and/or the NK cells from the subject prior to contacting the T cells and/or NK cells *ex vivo* with the recombinant retroviruses, and wherein the introducing is reintroducing. For example, between 20 and 250 ml of blood are withdrawn from the subject.

[28] In illustrative embodiments of any of the methods aspects for genetically modifying and expanding lymphocytes or for performing cellular therapy herein, or similar methods, if not explicitly recited in the broadest aspect, no more than 8, 12, 24, or 48 hours pass between the time blood is collected from the subject and the time the modified T cells and/or NK cells are reintroduced into the subject.

[29] In illustrative embodiments of any of the methods aspects for genetically modifying and expanding lymphocytes or for performing cellular therapy herein, or similar methods, if not explicitly recited in the broadest aspect, between 4 or 8 hours on the low end and 12. 24, 36, or 48 hours on the high end of the range pass between the time blood is collected from the subject and the time the modified T cells and/or NK cells are reintroduced into the subject.

[30] In illustrative embodiments of any of the methods aspects for genetically modifying and expanding lymphocytes or for performing adoptive cellular therapy herein, or similar methods, if not explicitly recited in the broadest aspect, all steps after the blood is collected and before the blood is reintroduced, are performed in a closed system in which a person monitors the closed system throughout the processing. In another embodiment, after the blood is collected and before the blood is reintroduced, are performed in a closed system that remains in the same room with the subject.

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[31] In illustrative embodiments of any of the methods and compositions provided herein that include one or more engineered signaling polypeptides, if not recited in the broadest aspect, one of the engineered signaling polypeptide comprises or further comprises an antigen-specific targeting region (ASTR) and a transmembrane domain connecting the ASTR to the lymphoproliferative element. The ASTR of this engineered signaling polypeptide is capable of binding to a first tumor antigen and where present, the ASTR of the second engineered signaling polypeptide is capable of binding to a second tumor antigen. In illustrative embodiments, the first engineered signaling polypeptide and/or the second engineered signaling polypeptide further comprise a co-stimulatory domain. Furthermore, the first engineered signaling polypeptide and/or the second engineered signaling polypeptide further comprise a stalk. Furthermore, the first engineered signaling polypeptide further comprises an intracellular activating domain. The intracellular activating domain on the first engineered signaling polypeptide and/or the second engineered signaling polypeptide can be derived from CD3 zeta.

[32] In illustrative embodiments of any of the methods and compositions provided herein that include a lymphoproliferative element, the lymphoproliferative element can comprise a T cell survival motif. The T cell survival motif can comprise all or a functional fragment of IL-7 receptor, IL-15 receptor, or CD28. In other embodiments, the lymphoproliferative element can include a cytokine or cytokine receptor polypeptide, or a fragment thereof comprising a signaling domain. For example, the lymphoproliferative element can comprise an interleukin polypeptide covalently attached to its cognate interleukin receptor polypeptide via a linker. Alternatively, the lymphoproliferative element can be an intracellular signaling domain of an IL-7 receptor, an intracellular signaling domain of an IL-12 receptor, an intracellular signaling domain of IL-23, an intracellular signaling domain of IL-27, an intracellular signaling domain of an IL-15 receptor, an intracellular signaling domain of an IL-21 receptor, or an intracellular signaling domain of a transforming growth factor β (TGFβ) decoy receptor. In other illustrative embodiments, the lymphoproliferative element is constitutively active. Furthermore, the lymphoproliferative element can include a mutated IL-7 receptor or a fragment thereof, which can further include a constitutively active mutated IL-7 receptor or a constitutively active fragment thereof.

[33] In illustrative embodiments of any of the methods and compositions provided herein that include a recombinant retrovirus or retroviruses, if not explicitly recited in the broadest aspect, the recombinant retroviruses can comprise on their surface an activation element comprising:

A. a membrane-bound polypeptide capable of binding to CD3; and/or

B. a membrane-bound polypeptide capable of binding to CD28.

[34] Furthermore, the membrane-bound polypeptide capable of binding to CD3 is a polypeptide capable of binding to CD3 that can be fused to a heterologous GPI anchor attachment sequence and the membrane-bound polypeptide capable of binding to CD28 can be a polypeptide capable of binding to CD28

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that is fused to a heterologous GPI anchor attachment sequence. In some embodiments, the membrane-bound polypeptide capable of binding to CD28 is CD80, CD86, or a functional fragment thereof that is capable of inducing CD28-mediated activation of Akt, such as the extracellular domain of CD80.

[35] In illustrative embodiments of any of the methods and compositions provided herein that include a recombinant retrovirus, the membrane-bound polypeptide capable of binding CD3 can be an anti-CD3 scFv bound to a CD14 GPI anchor attachment sequence, and the membrane-bound polypeptide capable of binding to CD28 can be CD80, or the extracellular domain thereof, bound to a CD16B GPI anchor attachment sequence. In illustrative embodiments of any of the methods and compositions provided herein that include a recombinant retrovirus, the recombinant retroviruses can comprise on their surface, an anti-CD3 scFv bound to a CD14 GPI anchor attachment sequence, CD80, or the extracellular domain thereof, bound to a CD16B GPI anchor attachment sequence, and a fusion polypeptide of IL-7, or an active fragment thereof, and DAF comprising a GPI anchor attachment sequence. In illustrative embodiments of any of the methods and compositions provided herein that include a recombinant retrovirus, the IL-7, or an active fragment thereof, and DAF fusion, the anti-CD3 scFV, and the CD80, or extracellular domain thereof each comprises a DAF signal sequence.

[36] In illustrative embodiments of any of the methods and compositions provided herein that include a recombinant retrovirus or retroviruses, if not explicitly recited in the broadest aspect, the recombinant retroviruses can comprise on their surface a membrane-bound cytokine. The membrane-bound cytokine can be IL-7, IL-15, or an active fragment thereof. In other embodiments, the membrane-bound cytokine is a fusion polypeptide of IL-7, or an active fragment thereof, and DAF. For example, the fusion polypeptide can comprise the DAF signal sequence (nucleotides 1-31 of SEQ ID NO:107), IL-7 without its signal sequence (nucleotides 32-187 of SEQ ID NO:107), and a fragment of DAF that includes its GPI anchor attachment sequence (nucleotides 188-527 of SEQ ID NO:107).

[37] Illustrative embodiments of any of the method and composition aspects provided herein the pseudotyping element can comprise one or more heterologous envelope proteins. In other examples, the pseudotyping element can include one or more viral polypeptides recognized by T cells. The one or more pseudotyping elements can comprise a Measles Virus F polypeptide, a Measles Virus H polypeptide, and/or a fragment thereof. The one or more pseudotyping elements can be cytoplasmic domain deletion variants of a measles virus F polypeptide and/or a measles virus H polypeptide.

[38] In illustrative embodiments of any of the methods and compositions provided herein that include the *in vivo* control element the *in vivo* control element is the lymphoproliferative element, wherein the lymphoproliferative is inactive or less active at promoting proliferation of the T cells and/or NK cells in the absence of the compound, and wherein the compound is a molecular chaperone that binds the lymphoproliferative element and induces the activity of the lymphoproliferative element.

[39] In illustrative embodiments of any of the methods and compositions provided herein that include the *in vivo* control element, the *in vivo* control element can be a polynucleotide comprising a riboswitch. The riboswitch can be capable of binding a nucleoside analog and the compound that binds the *in vivo* control element is the nucleoside analog. The nucleoside analog can be an antiviral agent. The antiviral agent can be acyclovir or penciclovir.

[40] In illustrative embodiments of any of the methods and compositions provided herein that include an engineered signaling polypeptide, that includes an ASTR, the ASTR of either or both of the engineered signaling polypeptides can bind to a tumor associated antigen. In some illustrative embodiments, the antigen-specific targeting region of the second engineered polypeptide is a microenvironment restricted antigen-specific targeting region.

[41] In illustrative embodiments of any of the methods and compositions provided herein that include a recombinant retrovirus or retroviruses, if not explicitly recited in the broadest aspect, the recombinant retroviruses can encode a recognition domain for a monoclonal antibody approved biologic. In some embodiments, the recognition domain is expressed on the same transcript as the chimeric antigen receptor and wherein the recognition domain is separated from the chimeric antigen receptor by a ribosome skipping and/or cleavage signal. The ribosome skipping and/or cleavage signal can be 2A-1. The recognition domain can include a polypeptide that is recognized by an antibody that recognizes EGFR, or an epitope thereof. The recognition domain can be an EGFR mutant that is recognized by an EGFR antibody and expressed on the surface of transduced T cells and/or NK cells as another control mechanism provided herein. In related embodiments, the recognition domain can include a polypeptide that is recognized by an antibody that recognizes EGFR, or an epitope thereof.

[42] In any of the methods or compositions provided herein that include a lymphoproliferative element, the lymphoproliferative element can be a miRNA or shRNA that stimulates the STAT5 pathway or inhibits the SOCS pathway. For example, said miRNA or shRNA is a miRNA that binds to a nucleic acid encoding a protein selected from the group consisting of: ABCG1, SOCS, TGFbR2, SMAD2, cCBL, and PD1. In illustrative embodiments for any of the recombinant retroviruses, or transduced cells provided herein, or methods including the same, such recombinant retroviruses or transduced cells can encode an miRNA or shRNA, for example within an intron, in some embodiments, 1, 2, 3, or 4 embodiments that bind nucleic acids encoding one or more of the following target endogenous T cell expressed genes: PD-1; CTLA4; TCR alpha; TCR beta; CD3 zeta; SOCS; SMAD2; miR-155; IFN gamma; cCBL; TRAIL2; PP2A; or ABCG1. For example, in one embodiment, a combination of the following miRNAs can be included in a genome of a recombinant retrovirus or transduced cell: TCR alpha, CD3 zeta, IFN gamma, and PD-1; and in another embodiment SOCS 1, IFN gamma, TCR alpha, and CD3 zeta.

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[43] In illustrative embodiments of any of the methods and compositions provided herein, the recombinant retroviruses, mammalian cells, and/or packaging cells, can comprise a Vpx polypeptide. The Vpx polypeptide can be, for example, a fusion polypeptide, and in some examples, especially in packaging cells, a membrane bound Vpx polypeptide.

[44] In any of the methods or compositions provided herein, the one or more pseudotyping elements can include a vesicular stomatitis virus (VSV-G) envelope protein, a feline endogenous virus (RD114) envelope protein, an oncoretroviral amphotropic envelope protein, or an oncoretroviral ecotropic envelope protein, or functional fragments thereof.

[45] Provided herein in another aspect is a genetically modified T cell and/or NK cell comprising:

a. a first engineered signaling polypeptide comprising a lymphoproliferative element; and
b. a second engineered signaling polypeptide comprising a chimeric antigen receptor comprising an antigen-specific targeting region (ASTR), a transmembrane domain, and an intracellular activating domain.

[46] In any of the methods provided herein that include a mammalian packaging cell, including a recombinant retrovirus packaging system aspect, or a method for making a recombinant retrovirus, for example, the packagable RNA genome is encoded by a polynucleotide operably linked to a promoter, wherein said promoter is either constitutively active or inducible by either the first transactivator or the second transactivator. The packagable RNA genome can be encoded by a polynucleotide operably linked to a promoter, wherein said promoter is inducible by the second transactivator. A promoter used herein to drive expression of the first and/or second engineered signaling polypeptide, is typically active in target cells, for example lymphocytes, PBLs, T-cells and/or NK cells, but in illustrative embodiments, is not active in the packaging cell line. The second transactivator can regulate the expression of an activation element capable of binding to and activating the target cell. I any of the methods provided herein that include a mammalian packaging cell, including a recombinant retrovirus packaging system aspect, or a method for making a recombinant retrovirus, for example, the packagable RNA genome in some embodiments, expression of the packagable RNA genome can be regulated can be regulated by the second transactivator.

[47] Furthermore, the packagable RNA genome can comprise, from 5′ to 3′:

1.) a 5′ long terminal repeat, or active fragment thereof;
2.) a nucleic acid sequence encoding a retroviral cis-acting RNA packaging element;
3.) a nucleic acid sequence encoding a first target polypeptide;
4.) a promoter that is active in the target cell; and
5.) a 3′ long terminal repeat, or active fragment thereof.

[48] In some embodiments, the nucleic acid sequence encoding the first target polypeptide is in reverse orientation to an RNA encoding retroviral components for packaging and assembly.

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[49] In any of the methods provided herein that include a mammalian packaging cell, including a recombinant retrovirus packaging system aspect, or a method for making a recombinant retrovirus, for example, the first target polypeptide comprises a first engineered signaling polypeptide and wherein said first engineered signaling polypeptide comprises a lymphoproliferative element. The packagable RNA genome can further comprises a nucleic acid sequence encoding a second target polypeptide. The second target polypeptide can comprise a second engineered signaling polypeptide including a chimeric antigen receptor comprising:

1.) a first antigen-specific targeting region;

2.) a first transmembrane domain; and

3.) a first intracellular activating domain.

[50] In any of the methods provided herein that include a mammalian packaging cell, including a recombinant retrovirus packaging system aspect, or a method for making a recombinant retrovirus, for example, the mammalian cell, for example the packaging cell can include a nucleic acid sequence encoding Vpx, for example on the second or an optional third transcriptional unit, or on an additional transcriptional unit that is operably linked to the first inducible promoter. The mammalian cell, which can be a packaging cell, can be a 293 cell.

[51] In any of the methods provided herein that include a mammalian packaging cell, including a recombinant retrovirus packaging system aspect, or a method for making a recombinant retrovirus, a first ligand can be rapamycin and a second ligand can be tetracycline or doxorubicin or the first ligand can be tetracycline or doxorubicin and the second ligand can be rapamycin.

[52] In some aspects, provided herein is a cell that has been transduced with any of the recombinant retroviruses provided herein. The cell can be, for example, a lymphocyte, such as a T cell or NK cell. The cell in illustrative embodiments, is a human cell.

[53] In one aspect provided herein, is a method of expanding modified T cells and/or NK cells in a subject, said method comprising:

a.) contacting isolated resting T cells and/or resting NK cells obtained from said subject with the recombinant retrovirus of any of the embodiments disclosed herein;

b.) introducing the genetically modified T cells and/or NK cells into the subject; and

c.) providing an effective amount of acyclovir, an acyclovir prodrug, penciclovir, or a penciclovir prodrug to said subject, wherein said modified T cells and/or NK cells proliferate in said subject upon administration of acyclovir, an acyclovir prodrug, penciclovir, or a penciclovir prodrug, thereby expanding the modified T cells and/or NK cells in the subject.

[54] In another aspect, provided herein is a method of stopping the expansion, engraftment, and/or persistence of modified T cells and/or NK cells in a subject, said method comprising:

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a.) contacting isolated quiescent T cell and/or NK cells obtained from said subject with the recombinant retrovirus of any of the embodiments disclosed herein;

b.) introducing the modified T cell and/or NK cells into the subject;

c.) administering an effective amount of acyclovir, an acyclovir prodrug, penciclovir, or a penciclovir prodrug to said subject to expand the modified T cell and/or NK cells in the subject, wherein said modified T cell and/or NK cells proliferate in said subject upon administration of acyclovir, an acyclovir prodrug, penciclovir, or a penciclovir prodrug, thereby expanding the modified PBLs in the subject; and

d.) stopping administration of acyclovir, an acyclovir prodrug, penciclovir, or a penciclovir prodrug, wherein said modified T cell and/or NK cells stop proliferating in said subject upon stopping administration of acyclovir, an acyclovir prodrug, penciclovir, or a penciclovir prodrug, thereby controlling the expansion, engraftment, and/or persistence of the modified T cell and/or NK cells in the subject.

[55] In another aspect, provided herein is a method of treating cancer in a subject, said method comprising:

a. contacting isolated quiescent T cells and/or NK cells obtained from said subject with the recombinant vector according to any of the embodiments disclosed herein;
b. introducing the genetically modified T cells and/or NK cells into the subject; and
c. administering an effective amount of acyclovir, an acyclovir prodrug, penciclovir, or a penciclovir prodrug to said subject to expand the modified T cell and/or NK cells in the subject, wherein said modified T cell and/or NK cells proliferate in said subject upon administration of acyclovir, an acyclovir prodrug, penciclovir, or a penciclovir prodrug, and wherein the chimeric antigen receptor in said modified T cell and/or NK cells binds cancer cells in said subject, thereby treating cancer in the subject.

[56] In another aspect, provided herein is a transduced T cell and/or NK cell, comprising a recombinant polynucleotide comprising one or more transcriptional units operatively linked to a promoter active in T cells and/or NK cells, wherein the one or more transcriptional units encode a first engineered signaling polypeptide regulated by an *in vivo* control element, wherein said first engineered signaling polypeptide comprises a constitutively active IL-7 receptor mutant, and wherein the *in vivo* control element is capable of binding, and/or designed and/or configured to bind, to a compound *in vivo*.

[57] In another aspect, provided herein is a retroviral packaging system, comprising:

a mammalian cell comprising:

A. a first transactivator expressed from a constitutive promoter and capable of binding a first ligand and a first inducible promoter for affecting expression of a nucleic acid sequence

operably linked thereto in the presence versus absence of the first ligand;

B. a second transactivator capable of binding a second ligand and a second inducible promoter, and affecting expression of a nucleic acid sequence operably linked thereto in the presence versus absence of the second ligand; and

C. a packagable RNA genome for a retroviral particle,

wherein the first transactivator regulates expression of the second transactivator and a retroviral REV protein, wherein the second transactivator regulates expression of a gag polypeptide, a pol polypeptide, and one or more pseudotyping elements capable of binding to a target cell and facilitating membrane fusion thereto, and wherein the retroviral proteins are derived from a retrovirus. Embodiments of this aspect, can include any of the embodiments provided herein for the recited elements in other aspects.

[58] In another aspect, provided herein is a method for making a recombinant retrovirus, comprising:

A. culturing a population of packaging cells to accumulate a first transactivator, wherein the packaging cells comprise the first transactivator expressed from a first constitutive promoter, wherein the first transactivator is capable of binding a first ligand and a first inducible promoter for affecting expression of a nucleic acid sequence operably linked thereto in the presence versus absence of the first ligand, and wherein expression of a second transactivator and a retroviral REV protein is regulated by the first transactivator;

B. incubating the population of packaging cells comprising accumulated first transactivator in the presence of the first ligand to accumulate the second transactivator and the retroviral REV protein, wherein the second transactivator is capable of binding a second ligand and a second inducible promoter for affecting expression of a nucleic acid sequence operably linked thereto in the presence versus absence of the second ligand; and

C. incubating the population of packaging cells comprising accumulated second transactivator and retroviral REV protein in the presence of the second ligand thereby inducing expression of a gag polypeptide, a pol polypeptide, and one or more pseudotyping elements, thereby making the recombinant retrovirus,

wherein a packagable RNA genome is encoded by a polynucleotide operably linked to a third promoter, wherein said third promoter is either constitutively active or inducible by either the first transactivator or the second transactivator, and wherein the one or more pseudotyping elements are capable of binding to a target cell and/or facilitating membrane fusion of the recombinant retrovirus thereto.

[59] In some embodiments of the retroviral packaging system and method for for making a recombinant retrovirus aspects provided herein, the mammalian cell further comprises an activation element capable of binding to and activating a target cell, and the first transactivator regulates the expression of the

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activation element. The activation element is on the surface of the retrovirus and wherein the activation element can include: a membrane-bound polypeptide capable of binding to CD3; and/or a membrane-bound polypeptide capable of binding to CD28. The membrane-bound polypeptide capable of binding to CD3 is a polypeptide capable of binding to CD3 that is fused to a heterologous GPI anchor attachment sequence and the membrane-bound polypeptide capable of binding to CD28 is a polypeptide capable of binding to CD28 that is fused to a heterologous GPI anchor attachment sequence. The membrane-bound polypeptide capable of binding to CD28 in some embodiments comprises CD80, CD86, or a functional fragment thereof that is capable of inducing CD28-mediated activation of Akt, such as the extracullular domain of CD80. In other embodiments, membrane-bound polypeptide capable of binding CD3 is an anti-CD3 scFv bound to a CD14 GPI anchor attachment sequence, and wherein the membrane-bound polypeptide capable of binding to CD28 is CD80, or an extracellular fragment thereof, bound to a CD16B GPI anchor attachment sequence.

[60] In some embodiments of the retroviral packaging system and method for making a recombinant retrovirus aspects provided herein, the mammalian cell further comprises a membrane-bound cytokine, and the first transactivator regulates the expression of the membrane-bound cytokine. The membrane-bound cytokine can be, for example, IL-7, IL-15, or an active fragment thereof. The membrane-bound cytokine in embodiments can be a fusion polypeptide of IL-7, or an active fragment thereof, and DAF. For example, the fusion polypeptide can comprise the DAF signal sequence and IL-7 without its signal sequence, followed by residues 36-525 of DAF.

[61] In some embodiments of the retroviral packaging system and method for making a recombinant retrovirus aspects provided herein, the mammalian cell comprises associated with its membrane, an activation element comprising an anti-CD3 scFv bound to a CD14 GPI anchor attachment sequence and a CD80 bound, or an extracellular fragment thereof to a CD16B GPI anchor attachment sequence; and membrane-bound cytokine comprising a fusion polypeptide of IL-7, or an active fragment thereof, and DAF comprising a GPI anchor attachment sequence, and wherein the first transactivator regulates the expression of each of the activation element and membrane-bound cytokine. In some embodiments, the IL-7, or an active fragment thereof, and DAF fusion, the anti-CD3 scFV, and the CD80, or extracellular fragment thereof, each comprises a DAF signal sequence.

[62] In some embodiments of the retroviral packaging system and method for making a recombinant retrovirus aspects provided herein, the mammalian cell further comprises a Vpx polypeptide. In these or other embodiments, the one or more pseudotyping elements comprise one or more viral polypeptides recognized by T cells. The one or more pseudotyping elements can comprise a Measles Virus F polypeptide, a Measles Virus H polypeptide, and/or a fragment thereof. In certain illustrative embodiments, the one or more pseudotyping elements are cytoplasmic domain deletion variants of a

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measles virus F polypeptide and/or a measles virus H polypeptide.

[63] In some embodiments of the retroviral packaging system and method for making a recombinant retrovirus aspects provided herein, the packagable RNA genome is encoded by a polynucleotide operably linked to a third promoter, wherein said third promoter is either constitutively active or inducible by either the first transactivator or the second transactivator. In illustrative embodiments, the packagable RNA genome is encoded by a polynucleotide operably linked to a third promoter, wherein said third promoter is inducible by the second transactivator.

[64] In some embodiments of the retroviral packaging system and method for making a recombinant retrovirus aspects provided herein, the packagable RNA genome further comprises, from 5′ to 3′:

a) a 5′ long terminal repeat, or active fragment thereof;

b) a nucleic acid sequence encoding a retroviral cis-acting RNA packaging element;

c) a nucleic acid sequence encoding a first target polypeptide and an optional second target polypeptide;

d) a fourth promoter operably linked to the first target polypeptide and the optional second polypeptide, wherein said fourth promoter is active in the target cell but not active in the packaging cell line; and

e) a 3′ long terminal repeat, or active fragment thereof.

[65] In some embodiments of the retroviral packaging system and method for making a recombinant retrovirus aspects provided herein including the construct immediately above, the third promoter promotes transcription or expression in the opposite direction from transcription or expression promoted from the fourth promoter.

[66] In some embodiments of the retroviral packaging system and method for making a recombinant retrovirus aspects provided herein, the packagable RNA genome encodes the recombinant retrovirus of any embodiment disclosed in this disclosure, wherein the first target polypeptide and the second target polypeptide are the first engineered signaling polypeptide and the second engineered signaling polypeptide, respectively. In some embodiments, for example, the packagable RNA genome further comprises an *in vivo* control element operably linked to the nucleic acid encoding the first engineered signaling polypeptide or the second engineered signaling polypeptide. The *in vivo* control element in illustrative embodiments is a riboswitch. The riboswitch in illustrative embodiments is capable of binding a compound and the compound that binds the *in vivo* control element is a nucleoside analog, and the nucleoside analog can be an antiviral drug, for example acyclovir or penciclivir.

[67] In some embodiments of the retroviral packaging system and method for making a recombinant retrovirus aspects provided herein, the packagable RNA genome further comprises an intron

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comprising a polynucleotide encoding an miRNA or shRNA. The intron can be adjacent to and downstream of the fourth promoter.

[68] In some embodiments of the retroviral packaging system and method for making a recombinant retrovirus aspects provided herein, the target cell can be a T cell and/or an NK cell.

[69] In some embodiments of the retroviral packaging system and method for making a recombinant retrovirus aspects provided herein, the one or more pseudotyping elements comprise a vesicular stomatitis virus (VSV-G) envelope protein, a feline endogenous virus (RD114) envelope protein, an oncoretroviral amphotropic envelope protein, or an oncoretroviral ecotropic envelope protein, or functional fragments thereof.

[70] In some embodiments of the retroviral packaging system and method for making a recombinant retrovirus aspects provided herein, the packagable RNA genome is 11,000 KB or less or 10,000 KB or less in size. In some embodiments of the retroviral packaging system and method for making a recombinant retrovirus aspects provided herein, the first target polypeptide comprises a first engineered signaling polypeptide and wherein said first engineered signaling polypeptide comprises a lymphoproliferative element, and the second target polypeptide comprises a second engineered signaling polypeptide including a CAR.

[71] In one aspect, provided herein is an isolated polynucleotide for regulating expression of a target polynucleotide, comprising:

a polynucleotide encoding a target polynucleotide operably linked to a promoter and a riboswitch, wherein the riboswitch comprises:

a.) an aptamer domain capable of binding a nucleoside analogue antiviral drug and having reduced binding to guanine or 2′-deoxyguanosine relative to the nucleoside analogue antiviral drug; and

b.) a function switching domain capable of regulating expression of the target polynucleotide, wherein binding of the nucleoside analogue by the aptamer domain induces or suppresses the expression regulating activity of the function switching domain, thereby regulating expression of the target polynucleotide.

[72] In illustrative embodiments of any of the methods and compositions provided herein that include the *in vivo* control element can be a polynucleotide comprising a riboswitch. The riboswitch can be capable of binding a nucleoside analog and the compound that binds the *in vivo* control element is the nucleoside analog. The nucleoside analog can be an antiviral agent. The antiviral agent can be acyclovir or penciclovir. The riboswitch can preferentially bind acyclovir over penciclovir or preferentially bind penciclovir over acyclovir. The riboswitch can have reduced binding to the nucleoside analogue antiviral drug at temperatures above 37 °C, 37.5 °C, 38 °C, 38.5 °C, or 39 °C, for example, above 39 °C. The riboswitch

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can be between 35, 40, 45, and 50 nucleotides in length on the low end of the range and 60, 65, 70, 75, 80, 85, 90, 95, and 100 nucleotides in length on the high end of the range, for example, between 45 and 80 nucleotides in length. In illustrative embodiments of any of the methods and compositions provided herein that include the riboswitch, the target polynucleotide that is regulated by the riboswitch can include a region encoding a miRNA, an shRNA, and/or a polypeptide. The target polynucleotide can encode a lymphoproliferative element. The target polynucleotide can be operably linked to a promoter. The target polynucleotide can include a region encoding a polypeptide and the polypeptide can include a chimeric antigen receptor comprising an antigen-specific targeting region, a transmembrane domain, and an intracellular activating domain. In illustrative embodiments of any of the methods and compositions provided herein that include the riboswitch, the function switching domain can regulate an internal ribosome entry site, pre-mRNA splice donor accessibility in the viral gene construct, translation, termination of transcription, transcript degradation, miRNA expression, or shRNA expression, thereby regulating expression of the target polynucleotide. The riboswitch can include a ribozyme. In illustrative embodiments of any of the methods and compositions provided herein that include the riboswitch, the isolated polynucleotide can be a molecular cloning vector or an expression vector. In illustrative embodiments of any of the methods and compositions provided herein that include the riboswitch, the isolated polynucleotide can be integrated into a retroviral genome or into a mammalian chromosome, or fragment thereof.

[73] Another aspect provided herein, is a method for genetically modifying and expanding lymphocytes of a subject, comprising:

A. collecting blood from the subject;

B. contacting T cells and/or NK cells from the blood of the subject ex vivo with recombinant retroviruses comprising:

i. a pseudotyping element on its surface that is capable of binding to a T cell and/or NK cell and facilitating membrane fusion of the recombinant retrovirus thereto, wherein said pseudotyping element comprises cytoplasmic domain deletion variants of a measles virus F polypeptide and/or a measles virus H polypeptide;

ii. a polypeptide capable of binding to CD3 and a polypeptide capable of binding to CD28, wherein said polypeptides are expressed on the surface of a recombinant retrovirus and are capable of binding to a T cell and/or a NK cell and further wherein said polypeptides are not encoded by a polynucleotide in the recombinant retrovirus; and

iii. a polynucleotide comprising one or more transcriptional units operatively linked to a promoter active in T cells and/or NK cells,

wherein the one or more transcriptional units encode a first engineered signaling polypeptide

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comprising a constitutively active IL-7 receptor mutant and a second engineered signaling polypeptide comprising a chimeric antigen receptor comprising an antigen-specific targeting region (ASTR), a transmembrane domain, and an intracellular activating domain,

wherein expression of the IL-7 receptor mutant is regulated by a riboswitch that binds a nucleoside analog antiviral drug, wherein binding of the nucleoside analog antiviral drug to the riboswitch increases expression of the IL-7 receptor mutant, and

wherein said contacting results in at least some of the resting T cells and/or NK cells becoming genetically modified;

C. reintroducing the genetically modified T cells and/or NK cells into the subject; and

D. exposing the genetically modified T cells and/or NK cells *in vivo* to the nucleoside analog antiviral drug to promote expansion of the T cells and/or NK cells, wherein the method between the collecting blood and the reintroducing the genetically modified T cells and/or NK cells is performed in no more than 24 hours and/or without requiring prior ex vivo stimulation, thereby genetically modifying and expanding lymphocytes of the subject.

[74] In illustrative embodiments of this method aspect, the retrovirus is a lentivirus. In another illustrative embodiment, the recombinant retrovirus genetically modifies a T cell. In another illustrative embodiment, the polypeptide capable of binding to CD3 and the polypeptide capable of binding to CD28 are each fused to a heterologous GPI anchor attachment sequence. In some instances, the polypeptide capable of binding to CD3 can be anti-CD3 scFvFc or anti-CD3 scFv, and the polypeptide capable of binding to CD28 can be CD80. The anti-CD3 scFvFc or anti-CD3 scFv and CD80 can each be further fused to a DAF signal sequence. In another illustrative embodiment, the recombinant retroviruses further comprise on their surface a fusion polypeptide comprising a cytokine covalently attached to DAF. In some instances, the cytokine can be IL-7 or IL-15, and the fusion polypeptide can comprise the DAF signal sequence, IL-7 without its signal sequence, and a fragment of DAF comprising a GPI anchor attachment sequence.

[75] In another illustrative embodiment of this method aspect immediately above, the riboswitch further controls expression of the chimeric antigen receptor in a manner regulated by binding of the riboswitch to the nucleoside analog antiviral drug, which in some instances is acyclovir and/or penciclovir. In another embodiment, the constitutively active IL-7 can be replaced with a miRNA or shRNA. In some instances, the miRNA or shRNA can be encoded by nucleic acids within an intron.

[76] Another aspect provided herein is a recombinant retrovirus, comprising:

A. a pseudotyping element on its surface that is capable of binding to a T cell and/or NK cell and facilitating membrane fusion of the recombinant retrovirus thereto, wherein said pseudotyping element comprises cytoplasmic domain deletion variants of a measles virus F polypeptide and/or a measles virus

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H polypeptide;

B. a polynucleotide comprising one or more transcriptional units operatively linked to a promoter active in T cells and/or NK cells, wherein the one or more transcriptional units encode a first engineered signaling polypeptide comprising a chimeric antigen receptor comprising an antigen-specific targeting region, a transmembrane domain, and an intracellular activating domain, and a second engineered signaling polypeptide comprising a constitutively active IL-7 receptor mutant; wherein expression of the IL-7 receptor mutant is regulated by a riboswitch that binds a nucleoside analog antiviral drug, wherein binding of the nucleoside analog antiviral drug to the riboswitch increases expression of the IL-7 receptor mutant; and

C. a polypeptide capable of binding to CD3 and a polypeptide capable of binding to CD28, wherein said polypeptides are expressed on the surface of a recombinant retrovirus; are capable of binding to a T cell and/or NK cell; and are not encoded by a polynucleotide in the recombinant retrovirus.

[77] In illustrative embodiments of the recombinant retrovirus aspect immediately above, the retrovirus is a lentivirus. In other illustrative embodiments of the method, the polypeptide capable of binding to CD3 and the polypeptide capable of binding to CD28 are each fused to a heterologous GPI anchor attachment sequence. In some instances, the polypeptide capable of binding to CD3 can be anti-CD3 scFvFc or anti-CD3 scFv, and the polypeptide capable of binding to CD28 can be CD80. The anti-CD3 scFvFc or anti-CD3 scFv and CD80 can each be further fused to a DAF signal sequence. In another illustrative embodiment, the recombinant retroviruses further comprise on their surface a fusion polypeptide comprising a cytokine covalently attached to DAF. In some instances, the cytokine can be IL-7 or IL-15, and the fusion polypeptide can comprise the DAF signal sequence, IL-7 without its signal sequence, and a fragment of DAF comprising a GPI anchor attachment sequence.

[78] In another illustrative embodiment of the recombinant retrovirus aspect immediately above, the riboswitch further controls expression of the chimeric antigen receptor in a manner regulated by binding of the riboswitch to the nucleoside analog antiviral drug, which in some instances is acyclovir and/or penciclovir. In another embodiment, the constitutively active IL-7 can be replaced with a miRNA or shRNA. The miRNA or shRNA can be encoded by nucleic acids within an intron.

[79] Another aspect provided herein is a method for making a recombinant retrovirus, comprising:

A. culturing a population of packaging cells to accumulate a first transactivator, wherein the packaging cells comprise the first transactivator expressed from a constitutive promoter, wherein the first transactivator is capable of binding a first ligand and a first inducible promoter for affecting expression of a nucleic acid sequence operably linked thereto in the presence versus absence of the first ligand, and wherein expression of a second transactivator and a retroviral REV protein is regulated by the first transactivator;

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B. incubating the population of packaging cells comprising accumulated first transactivator in the presence of the first ligand to accumulate the second transactivator and the retroviral REV protein and an activation element typically on their surface, comprising a polypeptide capable of binding to CD3 and a polypeptide capable of binding to CD28, wherein the second transactivator is capable of binding a second ligand and a second inducible promoter for affecting expression of a nucleic acid sequence operably linked thereto in the presence versus absence of the second ligand; and

C. incubating the population of packaging cells comprising accumulated second transactivator and retroviral REV protein in the presence of the second ligand thereby inducing expression of a gag polypeptide, a pol polypeptide and a pseudotyping element capable of binding to a T cell and/or an NK cell and facilitating membrane fusion of the recombinant retrovirus thereto, wherein said pseudotyping element comprises cytoplasmic domain deletion variants of a measles virus F polypeptide and/or a measles virus H polypeptide,

wherein a packagable RNA genome is encoded by a polynucleotide operably linked to a third promoter and wherein said promoter is inducible by the second transactivator,

wherein the packagable RNA genome comprises, from 5′ to 3′:

i. a 5′ long terminal repeat, or active fragment thereof;

ii. a nucleic acid sequence encoding a retroviral cis-acting RNA packaging element;

iii. a nucleic acid sequence encoding a first engineered signaling polypeptide comprising a chimeric antigen receptor and a second engineered signaling polypeptide comprising a constitutively active IL-7 receptor mutant separated by a cleavage signal;

iv. a fourth promoter that is active in the T cell and/or the NK cell; and

v. a 3′ long terminal repeat, or active fragment thereof, and

wherein the packagable RNA genome further comprises a riboswitch that binds a nucleoside analog antiviral drug, wherein binding of the riboswitch to the nucleoside analog antiviral drug increases expression of the IL-7 receptor mutant,

thereby making the recombinant retrovirus.

[80] In an illustrative embodiment of the method, the riboswitch further controls expression of the chimeric antigen receptor in a manner regulated by binding of the riboswitch to the nucleoside analog antiviral drug. In another illustrative embodiment, the nucleoside analog antiviral drug is acyclovir and/or penciclovir. In another illustrative embodiment, the packagable RNA genome further comprises a recognition domain, wherein the recognition domain comprises a polypeptide that is recognized by an antibody that recognizes EGFR or an epitope thereof. In another illustrative embodiment, the first ligand is rapamycin and the second ligand is tetracycline or doxorubicin or the first ligand is tetracycline or doxorubicin and the second ligand is rapamycin. In another illustrative embodiment, the packaging

cell further comprises a nucleic acid sequence encoding Vpx on the second or an optional third transcriptional unit, or on an additional transcriptional unit that is operably linked to the first inducible promoter. In another illustrative embodiment, the polypeptide capable of binding to CD3 and the polypeptide capable of binding to CD28 are each fused to a heterologous GPI anchor attachment sequence. In some instances, the polypeptide capable of binding to CD3 can be anti-CD3 scFvFc or anti-CD3 scFv, or anti-CD3 scFv, and the polypeptide capable of binding to CD28 can be CD80. The anti-CD3 scFvFc or anti-CD3 scFv and CD80 can each be further fused to a DAF signal sequence. In another illustrative embodiment, expression of a fusion polypeptide comprising a cytokine covalently attached to DAF is also induced. In some instances, the cytokine can be IL-7 or IL-15, and the fusion polypeptide can comprise the DAF signal sequence, IL-7 without its signal sequence, and a fragment of DAF comprising a GPI anchor attachment sequence. In another illustrative embodiment, the riboswitch further controls expression of the chimeric antigen receptor in a manner regulated by binding of the riboswitch to the nucleoside analog antiviral drug, which in some instances is acyclovir and/or penciclovir. In another embodiment, the constitutively active IL-7 can be replaced with a miRNA or shRNA. The miRNA or shRNA can be encoded by nucleic acids within an intron. In an illustrative embodiment, the retrovirus that is made is a lentivirus.

[81] Provided in another aspect herein is a genetically modified lymphocyte comprising:

A. a first engineered signaling polypeptide comprising a constitutively active IL-7 receptor mutant; and

B. a second engineered signaling polypeptide comprising a chimeric antigen receptor comprising an antigen-specific targeting region (ASTR), a transmembrane domain, and an intracellular activating domain.

[82] In illustrative embodiments of the genetically modified lymphocyte aspect above, the genetically modified lymphocyte is a T cell and/or an NK cell. In certain embodiments, the lymphocyte is a T cell. In another illustrative embodiment, expression of said first engineered signaling polypeptide and/or said second engineered signaling polypeptide is regulated by a riboswitch that binds a nucleoside analog antiviral drug, wherein binding of the nucleoside analog antiviral drug to the riboswitch increases expression of the IL-7 receptor mutant. In another embodiment, the constitutively active IL-7 receptor may be replaced with a miRNA or an shRNA. The miRNA or shRNA can further be encoded by nucleic acids within an intron.

[83] Provided in another aspect herein is a genetically modified T cell and/or NK cell comprising:

a. a first engineered signaling polypeptide comprising a lymphoproliferative element; and

b. a second engineered signaling polypeptide comprising a chimeric antigen receptor comprising an antigen-specific targeting region (ASTR), a transmembrane domain, and an

intracellular activating domain.

[84] In illustrative embodiments of the genetically modified T cell and/or NK cell aspect, the lymphoproliferative element is constitutively active, and in some instances, is a constitutively active mutated IL-7 receptor or a fragment thereof. In another illustrative embodiment, expression of the first engineered signaling polypeptide and/or the second engineered signaling polypeptide is regulated by an *in vivo* control element. In some instances, the *in vivo* control element is a polynucleotide comprising a riboswitch. In some instances, the riboswitch is capable of binding a nucleoside analog and when the nucleoside analog is present, the first engineered signaling polypeptide and/or the second engineered polypeptide are expressed. In other illustrative embodiments, the genetically modified T cell and/or NK cell has on its surface an activation element, a pseudotyping element, and/or a membrane-bound cytokine. In some instances, the activation element comprises a membrane-bound polypeptide capable of binding to CD3; and/or a membrane-bound polypeptide capable of binding to CD28. In a certain embodiment, the activation element comprises anti-CD3 scFv fused to a heterologous GPI anchor attachment sequence and/or CD80 fused to a heterologous GPI anchor attachment sequence. In an illustrative embodiment, the pseudotyping element comprises a Measles Virus F polypeptide, a Measles Virus H polypeptide, and/or cytoplasmic domain deletion variants of a measles virus F polypeptide and/or a measles virus H polypeptide. In other embodiments, the membrane-bound cytokine is a fusion polypeptide comprising IL-7, or a fragment thereof, fused to DAF, or a fragment thereof comprising a GPI anchor attachment sequence.

[85] In one aspect, provided herein is a method for genetically modifying and expanding lymphocytes of a subject, comprising:

A. contacting resting T cells and/or NK cells of the subject *ex vivo,* typically without requiring prior *ex vivo* stimulation, with recombinant retroviruses comprising:

i. a pseudotyping element on its surface that is capable of binding to a T cell and/or NK cell and facilitating membrane fusion of the recombinant retrovirus thereto; and

ii. a polynucleotide comprising one or more transcriptional units operatively linked to a promoter active in T cells and/or NK cells, wherein the one or more transcriptional units encode a first engineered signaling polypeptide regulated by an *in vivo* control element, wherein said first engineered signaling polypeptide comprises a lymphoproliferative element and optionally encode a second engineered signaling polypeptide optionally regulated by an *in vivo* control element, wherein the second engineered signaling polypeptide comprises an intracellular activating domain and optionally other components of a CAR,

wherein said contacting facilitates transduction of at least some of the resting T cells and/or

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NK cells by the recombinant retroviruses, thereby producing genetically modified T cells and/or NK cells;

B. introducing the genetically modified T cells and/or NK cells into the subject; and

exposing the genetically modified T cells and/or NK cells *in vivo* to a compound that acts as the *in vivo* control element to affect expression of the first engineered signaling polypeptide and promote expansion, engraftment, and/or persistence of the lymphocytes *in vivo*, thereby genetically modifying and expanding lymphocytes of the subject.

[86] In illustrative embodiments, the transduction is carried out without *ex vivo* stimulation. In illustrative embodiments, the compound is a molecular chaperone, such as a small molecular chaperone. In illustrative embodiments, binding of the molecular chaperone to the lymphoproliferative element increases the proliferative activity of the lymphoproliferative element. The molecular chaperone can be administered to the subject before the blood is collected, during the contacting, and/or after the T cells and/or NK cells are introduced into the subject. It will be understood with this aspect where the compound is the *in vivo* control element, that such compound typically is capable of binding to a lymphoproliferative element and/or a component of a CAR, and does bind to such lymphoproliferative element or car component during performance of the method. Other embodiments and teaches related to methods provided herein that include transfecting a T cell and/or an NK cell with a recombinant retrovirus, apply to this aspect, including a molecular chaperone embodiment, as well.

[87] In another aspect, provided herein is a method for selecting a microenvironment restricted antigen-specific targeting region, comprising panning a polypeptide display library by:

a. subjecting polypeptides of the polypeptide display library to a binding assay under a normal physiological condition and a binding assay under an aberrant condition; and

b. selecting a polypeptide which exhibits an increase in binding activity at the aberrant condition compared to the physiological condition, thereby selecting the microenvironment restricted antigen specific targeting region.

[88] In another aspect, provided herein is a method for isolating a microenvironment restricted antigen-specific targeting region, comprising:

panning a polypeptide library by:

a) contacting the polypeptide library under aberrant conditions with a target antigen bound to a solid support, wherein clones expressing polypeptides that bind the target antigen remain bound to the solid support through the target antigen;

b) incubating the solid supports with bound polypeptides under physiological conditions; and

c) collecting clones that elute from the solid support under the physiological conditions, thereby isolating the microenvironment restricted antigen-specific targeting region.

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[89] In another aspect, provided herein is a chimeric antigen receptor for binding a target antigen, comprising:

a) at least one microenvironment restricted antigen specific targeting region selected by panning a polypeptide library and having an increase in activity in a binding assay at an aberrant condition compared to a normal physiological condition;

b) a transmembrane domain; and

c). an intracellular activating domain.

[90] In another aspect, provided herein is a chimeric antigen receptor for binding a target antigen, comprising:

a) a microenvironment restricted antigen-specific targeting region that exhibits an increase in binding to the target antigen in an aberrant condition compared to a normal physiological environment, wherein the antigen-specific targeting region binds to the target;

b) a transmembrane domain; and

c) an intracellular activating domain.

[91] In illustrative embodiments of any of the methods and compositions provided herein that include a microenvironment restricted antigen specific targeting region (ASTR), the ASTR can have at least a 5%, 10%, 15%, 20%, 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, or 100% increase in binding affinity to the target antigen in the assay at the aberrant condition compared to the normal condition. The aberrant conditions can be hypoxia, an acidic pH, a higher concentration of lactic acid, a higher concentration of hyaluronan, a higher concentration of albumin, a higher concentration of adenosine, a higher concentration of R-2-hydroxyglutarate, a higher concentration of PAD enzymes, a higher pressure, a higher oxidation, and a lower nutrient availability. The microenvironment restricted ASTR can exhibit an increase in antigen binding at a pH of 6.7 as compared to a pH of 7.4. The microenvironment restricted ASTR can exhibit an increase in antigen binding in a tumor environment and/or in an *in vitro* tumor surrogate assay condition, relative to a corresponding physiological condition. The target can be 4-1BB,ST4, adenocarcinoma antigen, alpha- fetoprotein, AXL, BAFF, B-lymphoma cell, C242 antigen, CA-125, carbonic anhydrase 9 (CA- IX), C-MET, CCR4, CD 152, CD 19, CD20, CD200, CD22, CD221, CD23 (IgE receptor), CD28, CD30 (TNFRSF8), CD33, CD4, CD40, CD44 v6, CD51, CD52, CD56, CD74, CD80, CEA, CNT0888, CTLA-4, DRS, EGFR, EpCAM, CD3, FAP, fibronectin extra domain-B, folate receptor 1, GD2, GD3 ganglioside, glycoprotein 75, GPNMB, HER2/neu, HGF, human scatter factor receptor kinase, IGF-1 receptor, IGF -I, IgG1, Ll- CAM, IL-13, IL-6, insulin- like growth factor I receptor, integrin nSP1, integrin nvP3, MORAb-009, MS4A1, MUC1, mucin CanAg, Nglycolylneuraminic acid, NPC-1C, PDGF-R a, PDL192, phosphatidylserine, prostatic carcinoma cells, RANKL, RON, ROR1, ROR2 SCH 900105, SDC1, SLAMF7, TAG-72, tenascin C, TGF beta 2, TGF-P, TRAIL-R1, TRAIL-R2, tumor antigen CTAA16. 88,

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VEGF-A, VEGFR-1, VEGFR2, and vimentin. The ASTR can be an antibody, an antigen, a ligand, a receptor binding domain of a ligand, a receptor, a ligand binding domain of a receptor, or an affibody. The ASTR can be a full-length antibody, a single-chain antibody, an Fab fragment, an Fab' fragment, an $(Fab')_2$ fragment, an Fv fragment, and a divalent single-chain antibody or a diabody. The ASTR can include a heavy chain and a light chain from an antibody. The antibody can be a single-chain variable fragment. In some embodiments, the heavy and light chains can be separated by a linker, wherein the linker is between 6 and 100 amino acids in length. In some embodiments, the heavy chain can be positioned N-terminal to the light chain on the chimeric antigen receptor and in some embodiments the light chain can be positioned N-terminal to the heavy chain on the chimeric antigen receptor.

[92] In illustrative embodiments of any of the methods that include a polypeptide display library, the polypeptide display library can be a phage display library or a yeast display library. The polypeptide display library can be an antibody display library. The antibody display library can be a human or humanized antibody display library. The antibody display library can be a naïve library. The methods can include infecting bacterial cells with the collected phage to generate a refined phage display library, and repeating the contacting, incubating, and collecting for 1 to 1000 cycles, using the refined phage display library generated from a previous cycle.

[93] In illustrative embodiments of any of the methods provided herein that include isolating or selecting a microenvironment restricted ASTR, the method can include determining the nucleotide sequence of a polynucleotide encoding the microenvironment restricted antigen-specific targeting region, thereby determining the polypeptide sequence of the microenvironment restricted ASTR. The methods can include making a microenvironment restricted biologic chimeric antigen receptor by generating a polynucleotide that encodes a polypeptide comprising the microenvironment restricted ASTR, a transmembrane domain, and an intracellular activating domain. The library can be a single chain antibody library.

[94] The methods for isolating a microenvironment restricted ASTR can include the panning is repeated for between 1 and 1000 times. The methods for isolating a microenvironment restricted ASTR can be performed without mutating polynucleotides encoding the isolated microenvironment restricted antigen-specific targeting region between rounds of panning. The methods for isolating a microenvironment restricted ASTR can be performed by culturing, high fidelity amplifying, and/or diluting polynucleotides encoding antigen-specific targeting regions, or host organisms including the same, between rounds of panning. The methods can include, prior to repeating, mutagenizing the selected and/or isolated microenvironment restricted antigen-specific targeting region. The methods can include determining the sequence of the selected and/or isolated microenvironment restricted antigen-specific targeting region, and/or a polynucleotide encoding the same after one or more round of panning via long read DNA sequencing. The methods can include determining the sequence before and after expansion of the isolated

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microenvironment restricted ASTR. The methods for isolating a microenvironment restricted ASTR can be performed without repeating the panning. The methods for isolating a microenvironment restricted ASTR can be performed without mutating a polynucleotide encoding the isolated microenvironment restricted ASTR after the microenvironment restricted ASTR is isolated.

[95] In illustrative embodiments of any of the compositions provided herein that include a chimeric antigen receptor with a microenvironment restricted ASTR, the microenvironment restricted ASTR can be identified by panning an antibody library. In some embodiments, the microenvironment restricted ASTR is identified by panning a phage display or a yeast display library. In some embodiments, the chimeric antigen receptor comprises a bispecific ASTR.

[96] Provided herein in another aspect is a transduced T cell and/or NK cell, comprising a recombinant polynucleotide comprising one or more transcriptional units operatively linked to a promoter active in T cells and/or NK cells, wherein the one or more transcriptional units encode a first engineered signaling polypeptide regulated by an *in vivo* control element, wherein said first engineered signaling polypeptide comprises a constitutively active IL-7 receptor mutant, and wherein the *in vivo* control element is capable of binding to a compound *in vivo* or is configured to bind a compound *in vivo*.

[97] Provided herein in another aspect is a recombinant retrovirus, comprising a recombinant polynucleotide comprising one or more transcriptional units operatively linked to a promoter active in T cells and/or NK cells, wherein the one or more transcriptional units encode a first engineered signaling polypeptide regulated by an *in vivo* control element, wherein said first engineered signaling polypeptide comprises a constitutively active IL-7 receptor mutant, and wherein the *in vivo* control element is capable of binding to a compound *in vivo* or is configured to bind a compound *in vivo*.

[98] Provided herein in another aspect is a method of transducing a T cell and/or NK cell, comprising contacting a T cell and/or NK cell, with a recombinant retrovirus comprising a recombinant polynucleotide comprising one or more transcriptional units operatively linked to a promoter active in T cells and/or NK cells, wherein the one or more transcriptional units encode a first engineered signaling polypeptide regulated by an *in vivo* control element, wherein said first engineered signaling polypeptide comprises a constitutively active IL-7 receptor mutant, and wherein the *in vivo* control element is capable of binding to a compound *in vivo*, under transduction conditions, thereby transducing the T cell and/or NK cell.

[99] In illustrative embodiments of the transduced T cell and/or NK cell aspects, the recombinant retrovirus aspects, and the method aspects, provided in the preceding paragraphs, the recombinant polynucleotide further comprises a transcriptional unit that encodes a second engineered signaling polypeptide comprising a first chimeric antigen receptor comprising an antigen-specific targeting region (ASTR), a transmembrane domain, and an intracellular activating domain. In other illustrative

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embodiments, the lymphoproliferative element comprises a mutated IL-7 receptor or a fragment thereof. In other illustrative embodiments, the *in vivo* control element is a polynucleotide comprising a riboswitch. In some instances, the riboswitch is capable of binding a nucleoside analog and the compound that binds the *in vivo* control element is the nucleoside analog. In some instances, the nucleoside analog is an antiviral agent such as for example acyclovir or penciclovir. In certain embodiments, the antiviral agent is acyclovir. In other illustrative embodiments, the constitutively active IL-7 receptor mutant is fused to EGFR or an epitope thereof. In other illustrative embodiments, the constitutively active IL-7 receptor mutant comprises an eTag. In other illustrative embodiments, the constitutively active IL-7 receptor mutant comprises a PPCL insertion. In other illustrative embodiments, the constitutively active IL-7 receptor mutant comprises a PPCL insertion at a position equivalent to position 243 in a wild-type human IL-8 receptor. In other illustrative embodiments, the transduced T cell or NK cell is a transduced T cell.

BRIEF DESCRIPTION OF THE DRAWINGS

[100] FIG. 1 shows a schematic of illustrative compositions including a packaging cell (100) and a recombinant retrovirus (200) produced by the packaging cell (100). In FIG. 1, various vectors (referred to as recombinant polynucleotides (110)) capable of encoding aspects of the invention are packaged into a recombinant retrovirus (200) that includes in its genome a first engineered signaling polypeptide that includes a lymphoproliferative element and in some embodiments, a second engineered signaling polypeptide that is a chimeric antigen receptor, or a CAR. The recombinant retrovirus expresses on its membrane, a pseudotyping element (in a non-limiting embodiment, a Measles Virus hemagglutinin (H) polypeptide and a Measles Virus fusion (F) polypeptide, or cytoplasmic domain deletion variants thereof) (240) that allows the retrovirus to bind to and fuse with a target cell; an activation element (in non-limiting embodiments an activation element that has a polypeptide capable of binding to CD28 and a polypeptide capable of binding to CD3) (210 and 220, respectively) that is capable of binding to and activating a resting T cell; and a membrane-bound cytokine (in a non-limiting embodiment, and IL-7 DAF fusion polypeptide) (230). Parts labeled as (250), (260), (270), (280), and (290) are the Src-FLAG-Vpx, HIV gag matrix, HIV gag capsid, RNA, and HIV pol, respectively.

[101] FIG. 2 shows a schematic of illustrative compositions including a recombinant retrovirus produced by a packaging cell (200) and a resting T cell (300) transfected by the recombinant retrovirus (200). The elements on the surface of the retrovirus bind to receptors and/or ligands on the surface of a resting T cell. The pseudotyping element can include a binding polypeptide and a fusogenic polypeptide (in non-limiting embodiments, a Measles Virus hemagglutinin (H) polypeptide and a Measles Virus fusion (F) polypeptide), or cytoplasmic domain deletion variants thereof) that facilitate the binding and

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fusion of the retrovirus to the T cell. In non-limiting embodiments, the retrovirus includes on its surface an activation element (in non-limiting embodiments an activation element that has a polypeptide capable of binding to CD28 and a polypeptide capable of binding to CD3) that is capable of activating the resting T cell by engaging the T-cell receptor complex and optionally a co-receptor (320). Furthermore, membrane-bound cytokines (in non-limiting embodiments, an IL-7 DAF fusion polypeptide) present on the surface of the retrovirus bind to IL-7Rα (310) on the surface of the resting T cell. The retrovirus fuses with the T cell, and polynucleotides that encode the first engineered signaling polypeptide that includes the lymphoproliferative element (in illustrative embodiments, a constitutively active IL-7Rα) (370), are reverse transcribed in the cytosol prior to migrating to the nucleus to be incorporated into the DNA of the activated T cell. In some embodiments, Src-FLAG-Vpx (250) packaged with the virus enters the cytosol of the resting T cells and promotes the degradation of SAMHD1 (350), resulting in an increased pool of cytoplasmic dNTPs available for reverse transcription. In some embodiments, the polynucleotides can also encode a second engineered signaling polypeptide that includes a CAR (360). In some embodiments, the lymphoproliferative element is expressed when a compound binds to an *in vivo* control element that regulates its expression (in non-limiting example, the *in vivo* control element is a riboswitch that binds a nucleoside analog). In some embodiments, expression of the CAR is also regulated by the *in vivo* control element. Part (330) is SLAM and CD46. Part (340) is CD3.

[102] FIGs. 3A-3E show schematics of vector systems. FIG. 3A shows a construct containing a polynucleotide sequence encoding an FRB domain fused to the NFκB p65 activator domain (p65 AD) and ZFHD1 DNA binding domain fused to three FKBP repeats that is constitutively expressed. The construct in FIG. 3A also includes HIV1 REV and Vpx as a SrcFlagVpx fusion under the rapamycin-inducible ZFHD1/p65 AD promoter. FIG. 3B shows a construct containing a polynucleotide encoding an rtTA sequence under the control of the ZFHD1/p65 AD promoter. FIG. 3C shows a construct containing a polynucleotide encoding a puromycin resistance gene flanked by loxP sites and the extracellular MYC tag flanked by lox2272 sites. Both selectable markers are under the control of a BiTRE promoter, which is flanked by FRT sites. FIG. 3D shows a construct that contains a polynucleotide encoding RFP flanked by loxP sites that is under the control of a TRE promoter and a single FRT site between the TRE promoter and the 5′ loxP site of RFP. FIG. 3E shows a construct containing a polynucleotide encoding GFP flanked by loxP sites that is under the control of the TRE promoter and a single FRT site between the TRE promoter and the 5′ loxP site of GFP. The constructs in FIGs. 3C-3E function as landing pads for other polynucleotide sequences to insert into the genome of the packaging cell line.

[103] Figs. 4A-4C show schematics of constructs. Fig. 4A shows a construct containing a tricistronic polynucleotide encoding anti-CD3 (clone UCHT1) scFvFc with a CD14 GPI anchor attachment site, CD80 extra cellular domain (ECD) capable of binding CD28 with a CD16B GPI anchor attachment site, and IL-

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7 fused to decay-accelerating factor (DAF) with transposon sequences flanking the polynucleotide region for integration into the HEK293S genome. FIG. 4B shows a construct containing a polynucleotide with a BiTRE promoter and a polynucleotide region encoding the gag and pol polypeptides in one direction and a polynucleotide region encoding the measles virus FΔx and HΔy proteins in the other direction. FIG. 4C shows a construct containing a polynucleotide sequence encoding a CAR and the lymphoproliferative element IL7Rα-insPPCL under the control of a CD3Z promoter which is not active in HEK293S cells, wherein the CAR and IL7Rα-insPPCL are separated by a polynucleotide sequence encoding a T2A ribosomal skip sequence and the IL7Rα-insPPCL has an acyclovir riboswitch controlled ribozyme. The CAR-containing construct further includes cPPT/CTS, an RRE sequence, and a polynucleotide sequence encoding HIV-1 Psi (Ψ). The entire polynucleotide sequence on the CAR-containing construct to be integrated into the genome is flanked by FRT sites.

[104] FIG. 5A-5C show molecular structures of acyclovir (FIG. 5A), penciclovir (FIG. 5B), and 2'-deoxyguanonsine (FIG. 5C) as representative nucleoside analogues for selective riboswitch control.

[105] FIG. 6 represents the *Mesoplasma florum* type I-A deoxyguanosine riboswitch regulatory region and associated gene product. The sequence is the reverse complement of *M. florum* L1 genomic DNA (AE017263.1) nt624396 to nt625670 which is same as *M. florum* W37 genomic DNA (CP006778.1) nt636277 to nt 637550. The deoxyguanosine binding aptamer sequence used for initial screen indicated in bold and underline. The downstream gene product (Ribonucleotide reductase of class Ib (aerobic), beta subunit) is indicated in capital letters.

[106] FIG. 7 represents the *M. florum* type I-A deoxyguanosine riboswitch aptamer regions targeted for directed evolution strategy. Nucleotides within empty ovals were targeted for randomization. Nucleotides within striped ovals were targeted for insertion/deletion and randomization.

[107] FIGs. 8A and 8B represent the *M. florum* type I-A deoxyguanosine riboswitch aptamer screening library. In FIG. 8A, nucleotides within boxes with solid lines are sequence regions targeted for randomization and nucleotides within boxes with dashed lines are sequence regions targeted for insertion/deletion and randomization. FIG. 8B shows possible sequences generated through mutation ("random nucleotides ("N")) and deletion/insertion.

[108] FIG. 9 represents the *M. florum* type I-A deoxyguanosine riboswitch aptamer oligo library synthesized as a reverse complement with additional base pairs added to allow for PCR amplification and T7 promoter addition for *in vitro* transcription for library screening. The corresponding T7 promoter amplification primer and reverse amplification primer are also shown.

[109] FIG. 10 represents the *Bacillus subtilis* guanosine xpt riboswitch regulatory region and associated gene product. The sequence is the reverse complement of *B. subtilis* subsp. *subtilis* 6051-HGW genomic DNA (CP003329.1) nt2319439 to nt2320353. The guanosine binding aptamer sequence used for initial

screen indicated in bold and underline. The downstream gene product (Xanthine phosphoribosyltransferase xpt) is indicated in capital letters.

[110] FIG. 11 represents the *B. subtilis* guanosine xpt riboswitch aptamer regions targeted for directed evolution strategy. Nucleotides within empty ovals were targeted for randomization. Nucleotides within striped ovals were targeted for insertion/deletion and randomization

[111] FIGs. 12A and 12B represent the *B. subtilis* guanosine xpt riboswitch aptamer screening library. In FIG. 12A, nucleotides within boxes with solid lines are sequence regions targeted for randomization and nucleotides within boxes with dashed lines are sequence regions targeted for insertion/deletion and randomization. FIG. 12B shows possible sequences generated through mutation (random nucleotides ("N")) and deletion/insertion.

[112] FIG. 13 represents the *B. subtilis* guanosine xpt riboswitch aptamer oligo library synthesized as a reverse complement with additional base pairs added to allow for PCR amplification and T7 promoter addition for *in vitro* transcription for library screening. The corresponding T7 promoter amplification primer and reverse amplification primer are also shown.

[113] FIG. 14 shows the selection library construction. The library was constructed on the basis of known guanosine- and deoxyguanosine-binding RNA (Pikovskaya, 2013).

[114] FIG. 15 shows an illustration of graphene oxide (GrO) aptamer selection. In step (1), RNA was transcribed and purified. In step (2), purified RNA was eluted. In step (3), aptamers were incubated with counter-targets and buffer. In step (4), sequences bound to counter-targets or buffer components were removed with graphene oxide. In step (5), centrifugation partitioned the non-specifically-responsive species within the supernatant, which is then discarded. Two additional 5-minute washes removed most of the residual counter-target-binding and buffer-binding sequences. In step (6), a solution of acyclovir in 1X selection buffer was added to the GrO-bound library for positive selection so potential aptamer sequences desorb from the GrO through interaction with the positive target. In step (7), a final centrifugation step separates the target-binding sequences in the supernatant from the non-responsive sequences still adsorbed to the GrO. In step (8) selected sequences were reverse-transcribed, then the library was amplified through PCR, then transcribed to generate library for the next selection round.

[115] FIG. 16 shows an illustration of graphene oxide parallel assessment. Enriched libraries undergoing parallel assessment were divided into four equal portions. Library samples were then added to graphene oxide and allowed to incubate to load the library on the graphene oxide. Two 5-minute washes were used to remove non-binding material. For the positive (acyclovir) and special target (penciclovir) sample, each target was prepared separately in 1X selection buffer to 1 μM; the counter target replaced the positive target with 10 μM of each counter-target in solution; the negative sample replaced the positive target with an equal volume of nuclease-free water. Samples were then combined with their

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respective graphene oxide preparations and incubated. Post-incubation, samples were centrifuged to recover their supernatants, and library recovery was determined by NanoDrop-1000 spectrophotometer reading (Thermo Fisher Scientific; Wilmington, DE). Remaining library sample was analyzed on denaturing PAGE. Images of the gels were taken after staining/destaining with Gel-Star. Bands corresponding to expected library size were recovered for a follow-up round of parallel assessment, with positive target acyclovir replacing counter-targets for the negative, counter, and special target samples' pre-loading incubation. Material recovered from the second parallel assessment was used for sequencing and analysis.

[116] FIG. 17 shows seven aptamer candidates against acyclovir. The free energy for each aptamer was computed at 37 °C and 1 M Na+ by Quikfold 3.0 (Zuker 2003). Sequences were identified using proprietary algorithms. The underlined regions in each sequence are the PCR primer annealing regions.

[117] FIG. 18 shows seven aptamer candidates against penciclovir. The free energy for each aptamer was computed at 37 °C and 1 M Na+ by Quikfold 3.0 (Zuker 2003). Sequences were identified using proprietary algorithms. The underlined regions in each sequence are the PCR primer annealing regions.

[118] FIG. 19A provides a schematic of IL7Rα variants tested for lymphoproliferative/survival activity when expressed in PBMCs. FIG. 19B provides a bar graph showing percent viability of PBMCs in the presence and absence of IL-2.

[119] FIG. 20 shows a plot of transduction efficiency against MOI for negatively selected and unstimulated T cells transduced using lentiviruses pseudotyped with either VSV-G or truncated versions of MV(Ed) F and H polypeptides. Symbols are staggered for improved clarity.

[120] FIG. 21 is a graph showing that the miRNAs targeting CD3zeta that are in the EF-1alpha promoter intron are able to knockdown expression of the CD3 complex.

DEFINITIONS

[121] As used herein, the term "chimeric antigen receptor" or "CAR" or "CARs" refers to engineered receptors, which graft an antigen specificity onto cells, for example T cells, NK cells, macrophages, and stem cells. The CARs of the invention include at least one antigen-specific targeting region (ASTR) and an intracellular activating domain (IAD) and can include a stalk, a transmembrane domain (TM), and one or more co-stimulatory domains (CSDs). In another embodiment, the CAR is a bispecific CAR, which is specific to two different antigens or epitopes. After the ASTR binds specifically to a target antigen, the IAD activates intracellular signaling. For example, the IAD can redirect T cell specificity and reactivity toward a selected target in a non-MHC-restricted manner, exploiting the antigen-binding properties of antibodies. The non-MHC-restricted antigen recognition gives T cells expressing the CAR the ability to recognize an antigen independent of antigen processing, thus bypassing a major mechanism of tumor

escape. Moreover, when expressed in T cells, CARs advantageously do not dimerize with endogenous T cell receptor (TCR) alpha and beta chains.

[122] As used herein, the term "microenvironment" means any portion or region of a tissue or body that has constant or temporal, physical, or chemical differences from other regions of the tissue or regions of the body. For example, a "tumor microenvironment" as used herein refers to the environment in which a tumor exists, which is the non-cellular area within the tumor and the area directly outside the tumorous tissue but does not pertain to the intracellular compartment of the cancer cell itself. The tumor microenvironment can refer to any and all conditions of the tumor milieu including conditions that create a structural and or functional environment for the malignant process to survive and/or expand and/or spread. For example, the tumor microenvironment can include alterations in conditions such as, but not limited to, pressure, temperature, pH, ionic strength, osmotic pressure, osmolality, oxidative stress, concentration of one or more solutes, concentration of electrolytes, concentration of glucose, concentration of hyaluronan, concentration of lactic acid or lactate, concentration of albumin, levels of adenosine, levels of R-2-hydroxyglutarate, concentration of pyruvate, concentration of oxygen, and/or presence of oxidants, reductants, or co-factors, as well as other conditions a skilled artisan will understand.

[123] As used interchangeably herein, the terms "polynucleotide" and "nucleic acid" refer to a polymeric form of nucleotides of any length, either ribonucleotides or deoxyribonucleotides. Thus, this term includes, but is not limited to, single-, double-, or multi-stranded DNA or RNA, genomic DNA, cDNA, DNA-RNA hybrids, or a polymer comprising purine and pyrimidine bases or other natural, chemically or biochemically modified, non-natural, or derivatized nucleotide bases.

[124] As used herein, the term "antibody" includes polyclonal and monoclonal antibodies, including intact antibodies and fragments of antibodies which retain specific binding to antigen. The antibody fragments can be, but are not limited to, fragment antigen binding (Fab) fragments, Fab' fragments, $F(ab')_2$ fragments, Fv fragments, Fab'-SH fragments, $(Fab')_2$ Fv fragments, Fd fragments, recombinant IgG (rIgG) fragments, single-chain antibody fragments, including single-chain variable fragments (scFv), divalent scFv's, trivalent scFv's, and single domain antibody fragments (e.g., sdAb, sdFv, nanobody). The term includes genetically engineered and/or otherwise modified forms of immunoglobulins, such as intrabodies, peptibodies, chimeric antibodies, single-chain antibodies, fully human antibodies, humanized antibodies, , fusion proteins including an antigen-specific targeting region of an antibody and a non-antibody protein, heteroconjugate antibodies, multispecific, e.g., bispecific, antibodies, diabodies, triabodies, and tetrabodies, tandem di-scFv's, and tandem tri-scFv's. Unless otherwise stated, the term "antibody" should be understood to include functional antibody fragments thereof. The term also includes

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intact or full-length antibodies, including antibodies of any class or sub-class, including IgG and sub-classes thereof, IgM, IgE, IgA, and IgD.

[125] As used herein, the term "antibody fragment" includes a portion of an intact antibody, for example, the antigen binding or variable region of an intact antibody. Examples of antibody fragments include Fab, Fab', $F(ab')_2$, and Fv fragments; diabodies; linear antibodies (Zapata et al., Protein Eng. 8(10): 1057-1062 (1995)); single-chain antibody molecules; and multispecific antibodies formed from antibody fragments. Papain digestion of antibodies produces two identical antigen-binding fragments, called "Fab" fragments, each with a single antigen-binding site, and a residual "Fe" fragment, a designation reflecting the ability to crystallize readily. Pepsin treatment yields an $F(ab')_2$ fragment that has two antigen combining sites and is still capable of cross-linking antigen.

[126] As used interchangeably herein, the terms "single-chain Fv," "scFv," or "sFv" antibody fragments include the V_H and V_L domains of antibody, wherein these domains are present in a single polypeptide chain. In some embodiments, the Fv polypeptide further includes a polypeptide linker or spacer between the V_H and V_L domains, which enables the sFv to form the desired structure for antigen binding. For a review of sFv, see *Pluckthun in The Pharmacology of Monoclonal Antibodies, vol. 113, Rosenburg and Moore eds., Springer-Verlag, New York, pp. 269-315 (1994).*

[127] As used herein, "naturally occurring" VH and VL domains refer to VH and VL domains that have been isolated from a host without further molecular evolution to change their affinities when generated in an scFv format under specific conditions such as those disclosed in US patent 8709755 B2 and application WO/2016/033331A1.

[128] As used herein, the term "affinity" refers to the equilibrium constant for the reversible binding of two agents and is expressed as a dissociation constant (Kd). Affinity can be at least I-fold greater, at least 2-fold greater, at least 3-fold greater, at least 4-fold greater, at least 5-fold greater, at least 6-fold greater, at least 7-fold greater, at least 8-fold greater, at least 9-fold greater, at least 10-fold greater, at least 20-fold greater, at least 30-fold greater, at least 40-fold greater, at least 50-fold greater, at least 60-fold greater, at least 70-fold greater, at least 80-fold greater, at least 90-fold greater, at least 100-fold greater, or at least 1000-fold greater, or more, than the affinity of an antibody for unrelated amino acid sequences. Affinity of an antibody to a target protein can be, for example, from about 100 nanomolar (nM) to about 0.1 nM, from about 100 nM to about 1 picomolar (pM), or from about 100 nM to about 1 femtomolar (fM) or more. As used herein, the term "avidity" refers to the resistance of a complex of two or more agents to dissociation after dilution. The terms "immunoreactive" and "preferentially binds" are used interchangeably herein with respect to antibodies and/or antigen-binding fragments.

[129] As used herein, the term "binding" refers to a direct association between two molecules, due to, for example, covalent, electrostatic, hydrophobic, and ionic and/or hydrogen-bond interactions, including

interactions such as salt bridges and water bridges. Non-specific binding would refer to binding with an affinity of less than about 10^{-7} M, e.g., binding with an affinity of 10^{-6} M, 10^{-5} M, 10^{-4} M, etc.

[130] As used herein, reference to a "cell surface expression system" or "cell surface display system" refers to the display or expression of a protein or portion thereof on the surface of a cell. Typically, a cell is generated that expresses proteins of interest fused to a cell-surface protein. For example, a protein is expressed as a fusion protein with a transmembrane domain.

[131] As used herein, the term "element" includes polypeptides, including fusions of polypeptides, regions of polypeptides, and functional mutants or fragments thereof and polynucleotides, including microRNAs and shRNAs, and functional mutants or fragments thereof.

[132] As used herein, the term "region" is any segment of a polypeptide or polynucleotide.

[133] As used herein, a "domain" is a region of a polypeptide or polynucleotide with a functional and/or structural property.

[134] As used herein, the terms "stalk" or "stalk domain" refer to a flexible polypeptide connector region providing structural flexibility and spacing to flanking polypeptide regions and can consist of natural or synthetic polypeptides. A stalk can be derived from a hinge or hinge region of an immunoglobulin (e.g., IgGl) that is generally defined as stretching from Glu216 to Pro230 of human IgGl (Burton (1985) *Molec. Immunol.,* 22:161-206). Hinge regions of other IgG isotypes may be aligned with the IgG1 sequence by placing the first and last cysteine residues forming inter-heavy chain disulfide (S-S) bonds in the same positions. The stalk may be of natural occurrence or non-natural occurrence, including but not limited to an altered hinge region, as disclosed in U.S. Pat. No. 5,677,425. The stalk can include a complete hinge region derived from an antibody of any class or subclass. The stalk can also include regions derived from CD8, CD28, or other receptors that provide a similar function in providing flexibility and spacing to flanking regions.

[135] The term "isolated" as used herein means that the material is removed from its original environment (e.g., the natural environment if it is naturally occurring). For example, a naturally-occurring polynucleotide or polypeptide present in a living animal is not isolated, but the same polynucleotide or polypeptide, separated from some or all of the coexisting materials in the natural system, is isolated. Such polynucleotides could be part of a vector and/or such polynucleotides or polypeptides could be part of a composition, and still be isolated in that such vector or composition is not part of its natural environment.

[136] As used herein, a "polypeptide" is a single chain of amino acid residues linked by peptide bonds. A polypeptide does not fold into a fixed structure nor does it have any posttranslational modification. A "protein" is a polypeptide that folds into a fixed structure. "Polypeptides" and "proteins" are used interchangeably herein.

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[137] As used herein, a polypeptide may be "purified" to remove contaminant components of a polypeptide's natural environment, e.g. materials that would interfere with diagnostic or therapeutic uses for the polypeptide such as, for example, enzymes, hormones, and other proteinaceous or nonproteinaceous solutes. A polypeptide can be purified (1) to greater than 90%, greater than 95%, or greater than 98%, by weight of antibody as determined by the Lowry method, for example, more than 99% by weight, (2) to a degree sufficient to obtain at least 15 residues of N-terminal or internal amino acid sequence by use of a spinning cup sequenator, or (3) to homogeneity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing or nonreducing conditions using Coomassie blue or silver stain.

[138] As used herein, the term "immune cells" generally includes white blood cells (leukocytes) which are derived from hematopoietic stem cells (HSC) produced in the bone marrow. "Immune cells" includes, e.g., lymphocytes (T cells, B cells, natural killer (NK) cells) and myeloid-derived cells (neutrophil, eosinophil, basophil, monocyte, macrophage, dendritic cells).

[139] As used herein, "T cell" includes all types of immune cells expressing CD3 including T-helper cells ($CD4^+$ cells), cytotoxic T cells ($CD8^+$ cells), T-regulatory cells (Treg) and gamma-delta T cells.

[140] As used herein, a "cytotoxic cell" includes $CD8^+$ T cells, natural-killer (NK) cells, NK-T cells, $\gamma\delta$ T cells, a subpopulation of $CD4^+$ cells, and neutrophils, which are cells capable of mediating cytotoxicity responses.

[141] As used herein, the term "stem cell" generally includes pluripotent or multipotent stem cells. "Stem cells" includes, e.g., embryonic stem cells (ES); mesenchymal stem cells (MSC); induced-pluripotent stem cells (iPS); and committed progenitor cells (hematopoeitic stem cells (HSC); bone marrow derived cells, etc.).

[142] As used herein, the terms "treatment," "treating," and the like, refer to obtaining a desired pharmacologic and/or physiologic effect. The effect may be prophylactic in terms of completely or partially preventing a disease or symptom thereof and/or may be therapeutic in terms of a partial or complete cure for a disease and/or adverse effect attributable to the disease. "Treatment," as used herein, covers any treatment of a disease in a mammal, e.g., in a human, and includes: (a) preventing the disease from occurring in a subject which may be predisposed to the disease but has not yet been diagnosed as having it; (b) inhibiting the disease, i.e., arresting its development; and (c) relieving the disease, i.e., causing regression of the disease.

[143] As used interchangeably herein, the terms "individual", "subject", "host", and "patient" refer to a mammal, including, but not limited to, humans, murines (e.g., rats, mice), lagomorphs (e.g., rabbits), non-human primates, humans, canines, felines, ungulates (e.g., equines, bovines, ovines, porcines, caprines), etc.

[144] As used herein, the terms "therapeutically effective amount" or "efficacious amount" refers to the amount of an agent, or combined amounts of two agents, that, when administered to a mammal or other subject for treating a disease, is sufficient to affect such treatment for the disease. The "therapeutically effective amount" will vary depending on the agent(s), the disease and its severity and the age, weight, etc., of the subject to be treated.

[145] As used herein, the term "evolution" or "evolving" refers to using one or more methods of mutagenesis to generate a different polynucleotide encoding a different polypeptide, which is itself an improved biological molecule and/or contributes to the generation of another improved biological molecule. "Physiological" or "normal" or "normal physiological" conditions are conditions such as, but not limited to, pressure, temperature, pH, ionic strength, osmotic pressure, osmolality, oxidative stress, concentration of one or more solutes, concentration of electrolytes, concentration of glucose, concentration of hyaluronan, concentration of lactic acid or lactate, concentration of albumin, levels of adenosine, levels of R-2-hydroxyglutarate, concentration of pyruvate, concentration of oxygen, and/or presence of oxidants, reductants, or co-factors, as well as other conditions, that would be considered within a normal range at the site of administration, or at the tissue or organ at the site of action, to a subject.

[146] As used herein, a "genetically modified cell" includes cells that contain exogenous nucleic acids whether or not the exogenous nucleic acids are integrated into the genome of the cell.

[147] A "polypeptide" as used herein can include part of or an entire protein molecule as well as any posttranslational or other modifications.

[148] A pseudotyping element as used herein can include a "binding polypeptide" that includes one or more polypeptides, typically glycoproteins, that identify and bind the target host cell, and one or more "fusogenic polypeptides" that mediate fusion of the retroviral and target host cell membranes, thereby allowing a retroviral genome to enter the target host cell. The "binding polypeptide" as used herein, can also be referred to as a "T cell and/or NK cell binding polypeptide" or a "target engagement element," and the "fusogenic polypeptide" can also be referred to as a "fusogenic element".

[149] A "resting" lymphocyte, such as for example, a resting T cell, is a lymphocyte in the G0 stage of the cell cycle that does not express activation markers such as Ki-67. Resting lymphocytes can include naïve T cells that have never encountered specific antigen and memory T cells that have been altered by a previous encounter with an antigen. A "resting" lymphocyte can also be referred to as a "quiescent" lymphocyte.

[150] As used herein, "lymphodepletion" involves methods that reduce the number of lymphocytes in a subject, for example by administration of a lymphodepletion agent. Lymphodepletion can also be attained by partial body or whole body fractioned radiation therapy. A lymphodepletion agent can be a chemical

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compound or composition capable of decreasing the number of functional lymphocytes in a mammal when administered to the mammal. One example of such an agent is one or more chemotherapeutic agents. Such agents and dosages are known, and can be selected by a treating physician depending on the subject to be treated. Examples of lymphodepletion agents include, but are not limited to, fludarabine, cyclophosphamide, cladribine, denileukin diftitox, or combinations thereof.

[151] It is to be understood that the present disclosure and the aspects and embodiments provided herein, are not limited to particular examples disclosed, as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of disclosing particular examples and embodiments only, and is not intended to be limiting, since the scope of the present disclosure will be limited only by the appended claims.

[152] 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 invention, 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 invention. When multiple low and multiple high values for ranges are given, a skilled artisan will recognize that a selected range will include a low value that is less than the high value. All headings in this specification are for the convenience of the reader and are not limiting.

[153] Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any methods and materials similar or equivalent to those described herein can also be used in the practice or testing of the present invention, the preferred methods and materials are now described. All publications mentioned herein are incorporated herein by reference to disclose and describe the methods and/or materials in connection with which the publications are cited.

[154] It must be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a chimeric antigen receptor" includes a plurality of such chimeric antigen receptors and equivalents thereof known to those skilled in the art, and so forth. It is further noted that the claims may be drafted to exclude any optional element. As such, this statement is intended to serve as antecedent basis for use of such exclusive terminology as "solely," "only" and the like in connection with the recitation of claim elements, or use of a "negative" limitation.

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[155] It is appreciated that certain features of the invention, 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 invention, 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 invention are specifically embraced by the present invention 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 invention and are disclosed herein just as if each and every such sub-combination was individually and explicitly disclosed herein.

DETAILED DESCRIPTION

[156] The present disclosure overcomes these prior art challenges by providing methods and compositions for genetically modifying lymphocytes and methods for performing adoptive cellular therapy that include transducing T cells and/or NK cells, that requires far less time *ex vivo*, for example, 24, 12, or 8 hours or less, and in some embodiments without prior *ex vivo* stimulation. These methods are well-suited for closed system *ex vivo* processing of blood from a subject, and can be performed with the subject present in the same room as and/or in some embodiments, within their line of sight of their blood or isolated blood cells thereof at all times during performance of the method. More specifically, the aspects and embodiments of the disclosure herein overcome problems associated with current adoptive cellular therapies by providing methods for transducing resting T cells and/or resting NK cells, that typically utilize a pseudotyping element that facilitates binding and fusion of a recombinant retrovirus to a resting T cell and/or a resting NK cell, to facilitate genetic modification of the resting T cells and/or NK cells by the recombinant retroviruses. Furthermore, methods provided herein overcome problems of the art by utilizing in illustrative embodiments, a chimeric antigen receptor and a lymphoproliferative element whose expression is under the control of an *in vivo* control element, such that exposure of the subject to a compound that binds the *in vivo* control element, or termination of such exposure, promotes expansion of the genetically modified T cells and/or NK cells *in vivo*.

[157] As a result of these and other improvements disclosed in detail herein, in one aspect, provided herein is a method for modifying resting T cells and/or resting NK cells of a subject, such as a patient having a disease or disorder, wherein blood from the subject is collected; resting T cells and/or NK cells are genetically modified by contacting them with a recombinant retrovirus; and the genetically modified cells are reintroduced into the subject typically within a shorter period of time than prior methods, for example within 24 hours and in some non-limiting embodiments, within 12 hours and/or without further expanding the population of genetically modified T cells and/or NK cells *ex vivo,* for example such that

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the genetically modified resting T cells and/or NK cells do not undergo more than 4 cell divisions *ex vivo*. Thus, methods provided herein can be performed in much less time than current CAR therapies, thereby providing processes by which a subject can remain in a clinic for the entire time of the *ex vivo* steps. This facilitates performance of the *ex vivo* steps in a closed system, which reduces the chances for contamination and mixing of patient samples and can be performed more readily by clinical labs.

[158] Accordingly, FIGs. 1 and 2 provide schematic diagrams of illustrative compositions used in methods provided herein. FIG. 1 provides a diagram of a packaging cell (100) and a recombinant retrovirus produced by such a packaging cell (200). The packaging cell (100) includes recombinant polynucleotides (110) incorporated into its genome that include recombinant transcriptional elements that express retroviral proteins and various different membrane-bound polypeptides under the control of inducible promoters that are regulated by transactivators, which bind and are activated by ligands. These transactivators, inducible promoters, and ligands are used to induce the sequential expression and accumulation of cell membrane-bound polypeptides that will be incorporated into the membrane of the recombinant retrovirus as well as retroviral components necessary for packaging and assembly of the retrovirus.

[159] As a result of the sequential induced expression of the various polynucleotides as discussed in detail herein below, the illustrative packaging cell (100) illustrated in FIG. 1 is produced, and can be used in illustrative methods to produce recombinant retroviruses used in methods of transfecting resting T cells and/or NK cells ((300) in FIG. 2) provided herein. The packaging cell (100), in non-limiting illustrative embodiments, includes in its genome nucleic acids encoding a packagable retroviral RNA genome that includes at least some of the elements of a retroviral genome necessary for packaging and assembly of the retrovirus (as non-limiting illustrative examples, a retroviral psi element, a retroviral gag polypeptide and a retroviral pol polypeptide).

[160] Some membrane bound polypeptides incorporated or associated with the cell membrane of the packaging cell will become incorporated or associated into the retrovirus, but are not encoded by the retroviral genome. For example, the packaging cell and recombinant retrovirus formed therefrom, can include a retroviral Vpx polypeptide (250), which in non-limiting illustrative examples can be expressed as a membrane associated fusion protein, for example a Src-Flag-Vpx polypeptide; a pseudotyping element that can include a binding polypeptide and a fusogenic polypeptide (240), which in a non-limiting embodiment includes a Measles Virus hemagglutinin (H) polypeptide and a Measles Virus fusion (F) polypeptide, or cytoplasmic domain deletion variants thereof; optionally, one or more activation elements (210, 220), which in a non-limiting embodiment includes a membrane-bound polypeptide capable of binding to CD3 and a membrane-bound polypeptide capable of binding to CD28; and/or optionally a membrane-bound cytokine (230), a non-limiting embodiment of which is a fusion polypeptide that

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includes IL-7 fused to DAF, or a fragment thereof. Various other specific types of these membrane bound polypeptides are provided herein.

[161] As a result of the sequential expression of the transcriptional elements by the packaging cell, a recombinant retrovirus is produced. The RNA retroviral genome inside of and typically integrated into the genome of the packaging cell that becomes the genome of the recombinant retrovirus, includes retroviral components (as non-limiting illustrative examples, retroviral Gag and Pol polynucleotides) that are necessary for retroviral production, infection and integration into the genome of a host cell, which is typically a resting T cell and/or NK cell. Furthermore, the retroviral genome furthermore includes polynucleotides encoding one or typically two engineered signaling polypeptides provided herein. One of the engineered signaling polypeptides typically encodes a lymphoproliferative element (in non-limiting examples a constitutive interleukin 7 receptor mutant) and the other engineered signaling polypeptide typically encodes a chimeric antigen receptor.

[162] The recombinant retrovirus (200) is then used to transduce a resting T cell and/or resting NK cell (300) in methods provided herein. As shown in FIG. 2, after the resting T cell and/or NK cell (300) is contacted with the recombinant retrovirus (200), membrane polypeptides discussed above on the surface of the retrovirus bind to receptors and/or ligands on the surface of the resting T cell and/or NK cell (300). For example, the pseudotyping element, which as indicated above can include a binding polypeptide that binds to molecules on the surface of resting T cells and/or resting NK cells and a fusogenic polypeptide, facilitates the binding and fusion of the retrovirus (200) to the T cell and/or NK cell membrane. The activation element(s) (210, 220) activate the resting T cell and/or NK cell (300) by engaging the T-cell receptor complex, a process which occurs over the time course of the contacting or an incubation thereafter. Furthermore, the membrane-bound cytokines (230) can be present on the surface of the retrovirus and bind cytokine receptors (310) on the surface of the resting T cell and/or NK cell (300), thus further promoting binding and activation. Thus, not to be limited by theory, in illustrative embodiments provided herein, as a result of one or more of these recombinant retrovirus (200) components, *ex vivo* stimulation or activation by an element that is not already in or on the retrovirus (200) is not required. This in turn, helps to cut down the *ex vivo* time that is required for completion of the methods in these illustrative methods provided herein.

[163] Upon binding to the T cell and/or NK cell (200), the retrovirus then fuses with the T cell and/or NK cell (300), and polypeptides and nucleic acids in the retrovirus enter the T cell and/or NK cell (300). As indicated above, one of these polypeptides in the retrovirus is the Vpx polypeptide (250). The Vpx polypeptide (250) binds to and induces the degradation of the SAMHD1 restriction factor (350), which degrades free dNTPs in the cytoplasm. Thus, the concentration of free dNTPs in the cytoplasm increases

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as Vpx degrades SAMHD1, and reverse transcription activity is increased, thus facilitating reverse transcription of the retroviral genome and integration into the T cell and/or NK cell genome.

[164] After integration of the retroviral genome into the T cell and/or NK cell (200), the T cell and/or NK cell genome includes nucleic acids encoding the signaling polypeptide encoding the lymphoproliferative element (370) and optionally the signaling polypeptide encoding the CAR (360). Expression of the lymphoproliferative element and optionally the CAR are under the control of an *in vivo* control element. Exposure to a compound that binds the *in vivo* control element, which occurs *in vivo* by administering it to a subject whose T cell and/or NK cell (300) was transduced, promotes proliferation of the T cell and/or NK cell (300) *in vivo* by expressing the lymphoproliferative element and optionally as a result of expression of the CAR and binding of the CAR to its target cell. Thus, T cells and/or NK cells that are transduced with recombinant retroviruses herein, have one or more signals that drive proliferation and/or inhibit cell death, which in turn in illustrative embodiments, avoids the requirements of prior methods to lymphodeplete a host before returning transduced T cells and/or NK cells back into the subject. This in turn, in illustrative embodiments, further reduces the requirement for days of processing before transduced T cells and/or NK cells are reintroduced into a subject. Thus, in illustrative embodiments, no more than 36 hours, 24 hours, 12 hours, or in some instances even 8 hours, of time is required from collection of blood from the subject to reintroduction of the blood to the subject, which fundamentally changes the CAR-T process from prior methods. Furthermore, the *in vivo* control element provides one of the safety mechanisms provided herein as well. For example, ceasing administration of the compound can down-regulate or even terminate expression of the lymphoproliferative element and optionally the CAR, thus ending a proliferation and/or survival signal to the transduced T cell and/or NK cell and its progeny.

METHODS FOR PERFORMING ADOPTIVE CELL THERAPY

[165] In certain aspects, provided herein are methods for performing adoptive cell therapy on a subject, As an illustrative example, the method can include the following:

A. collecting blood from a subject;

B. isolating peripheral blood mononuclear cells (PBMCs) comprising resting T cells and/or resting NK cells;

C. contacting the resting T cells and/or resting NK cells of the subject *ex vivo*, with recombinant retroviruses, wherein the recombinant retroviruses comprise a pseudotyping element on their surface that is capable of binding a resting T cell and/or NK cell and facilitating membrane fusion of the recombinant retrovirus thereto, wherein said contacting facilitates transduction of the resting

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T cells and/or NK cells by the recombinant retroviruses, thereby producing genetically modified T cells and/or NK cells; and

D. reintroducing the genetically modified cells into the subject within 36, 24, 12, or even 8 hours of collecting blood from the subject, thereby performing adoptive cell therapy in the subject.

[166] In some aspects provided herein, methods with similar steps are referred to as methods for genetically modifying and expanding lymphocytes of a subject. A skilled artisan will understand that the discussion herein as it applies to methods and compositions for performing adoptive cell therapy apply to methods for genetically modifying and expanding lymphocytes of a subject as well.

[167] Typically, the adoptive cell therapy methods of the present disclosure are carried out by autologous transfer, in which the cells are isolated and/or otherwise prepared from the subject who is to receive the cell therapy, or from a sample derived from such a subject. Thus, in some aspects, the cells are derived from a subject, e.g., patient, in need of a treatment and the cells, following isolation and processing are administered to the same subject. In some embodiments of the methods and compositions disclosed herein, a subject having a disease or disorder enters a medical facility where the subject's blood is drawn using known methods, such as venipuncture. In certain embodiments, the volume of blood drawn from a subject is between 10, 15, 20, 25, 30, 35, 40, 50, 75, or 100 ml on the low end of the range and 200, 250, 300, 350, 400, 500, 750, 1000, 2000, or 2500 ml on the high end of the range. In some embodiments, between 10 and 400 ml are drawn from the subject. In some embodiments, between 20 and 250 ml of blood are drawn from the subject. In some embodiments, the blood is fresh when it is processed. In any of the embodiments disclosed herein, fresh blood can be blood that was withdrawn from a subject less than 15, 30, 45, 60, 90, 120, 150, or 180 minutes prior. In some embodiments, the blood is processed in the methods provided herein without storage.

[168] Contact between the T cells and/or NK cells and the recombinant retroviruses typically facilitates transduction of the T cells and/or NK cells by the recombinant retrovirus. Throughout this disclosure, a transduced T cell and/or NK cell includes progeny of *ex vivo* transduced cells that retain at least some of the nucleic acids or polynucleotides that are incorporated into the cell during the *ex vivo* transduction. In methods herein that recite "reintroducing" a transduced cell, it will be understood that such cell is typically not in a transduced state when it is collected from the blood of a subject. A subject in any of the aspects disclosed herein can be for example, an animal, a mammal, and in illustrative embodiments a human.

[169] Not to be limited by theory, in non-limiting illustrative methods, the delivery of a polynucleotide encoding a lymphoproliferative element, such as an IL7 constitutively active mutant, to a resting T cell and/or NK cell *ex vivo*, which can integrate into the genome of the T cell or NK cell, provides that cell with a driver for *in vivo* expansion without the need for lymphodepleting the host. Thus, in illustrative

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embodiments, the subject is not exposed to a lymphodepleting agent within 1, 2, 3, 4, 5, 6, 7, 10, 14, 21, or 28 days, or within 1 month, 2 months, 3 months or 6 months of performing the contacting, during the contacting, and/or within 1, 2, 3, 4, 5, 6, 7, 10, 14, 21, or 28 days, or within 1 month, 2 months, 3 months or 6 months after the modified T cells and/or NK cells are reintroduced back into the subject. Furthermore, in non-limiting illustrative embodiments, methods provided herein can be performed without exposing the subject to a lymphodepleting agent during a step wherein a recombinant retrovirus is in contact with resting T cells and/or resting NK cells of the subject and/or during the entire *ex vivo* method.

[170] Hence, methods of expanding genetically modified T cells and/or NK cells in a subject in a vivo is a feature of some embodiments of the present disclosure. In illustrative embodiments, such methods are ex vivo propagation-free or substantially propagation-free.

[171] This entire method/process from blood draw from a subject to reintroduction of blood back into the subject after *ex vivo* transduction of T cells and/or NK cells, in non-limiting illustrative embodiments herein, can occur over a time period less than 48 hours, less than 36 hours, less than 24 hours, less than 12 hours, less than 11 hours, less than 10 hours, less than 9 hours, less than 8 hours, less than 7 hours, less than 6 hours, less than 5 hours, less than 4 hours, less than 3 hours, or less than 2 hours. In other embodiments, the entire method/process from blood draw/collection from a subject to reintroduction of blood back into the subject after *ex vivo* transduction of T cells and/or NK cells, in non-limiting illustrative embodiments herein, occurs over a time period between 1 hour and 12 hours, or between 2 hours and 8 hours, or between 4 hours and 12 hours, or between 4 hours and 24 hours, or between 8 hours and 24 hours, or between 8 hours and 36 hours, or between 8 hours and 48 hours, or between 12 hours and 24 hours, or between 12 hours and 36 hours, or between 12 hours and 48 hours, or over a time period between 15, 30, 60, 90, 120, 180, and 240 minutes on the low end of the range, and 120, 180, and 240, 300, 360, 420, and 480 minutes on the high end of the range. In other embodiments, the entire method/process from blood draw/collection from a subject to reintroduction of blood back into the subject after *ex vivo* transduction of T cells and/or NK cells, occurs over a time period between 1, 2, 3, 4, 6, 8, 10, and 12 hours on the low end of the range, and 8, 9, 10, 11, 12, 18, 24, 36, or 48 hours on the high end of the range. In some embodiments, the genetically modified T cells and/or NK cells are separated from the recombinant retroviruses after the time period in which contact occurs.

[172] Because methods provided herein for adoptive cell therapy and related methods for modifying resting T cells and/or resting NK cells *ex vivo* before expanding them *in vivo*, can be performed in significantly less time than prior methods, fundamental improvements in patient care and safety as well as product manufacturability are made possible. Therefore, such processes are expected to be favorable in the view of regulatory agencies responsible for approving such processes when carried out *in vivo* for

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therapeutic purposes. For example, the subject in non-limiting examples, can remain in the same building (e.g. infusion clinic) or room as the instrument processing their blood or sample for the entire time that the sample is being processed before modified T cells and/or NK cells are reintroduced into the patient. In non-limiting illustrative embodiments, a subject remains within line of site and/or within 100, 50, 25, or 12 feet or arm's distance of their blood or cells that are being processed, for the entire method/process from blood draw/collection from the subject to reintroduction of blood to the subject after *ex vivo* transduction of T cells and/or NK cells. In other non-limiting illustrative embodiments, a subject remains awake and/or at least one person can continue to monitor the blood or cells of the subject that are being processed, throughout and/or continuously for the entire method/process from blood draw/collection from the subject to reintroduction of blood to the subject after *ex vivo* transduction of T cells and/or NK cells. Because of improvements provided herein, the entire method/process for adoptive cell therapy and/or for transducing resting T cells and/or NK cells from blood draw/collection from the subject to reintroduction of blood to the subject after *ex vivo* transduction of T cells and/or NK cells can be performed with continuous monitoring by a human. In other non-limiting illustrative embodiments, at no point the entire method/process from blood draw/collection from the subject to reintroduction of blood to the subject after *ex vivo* transduction of T cells and/or NK cells, are blood cells incubated in a room that does not have a person present. In other non-limiting illustrative embodiments, the entire method/process from blood draw/collection from the subject to reintroduction of blood to the subject after *ex vivo* transduction of T cells and/or NK cells, is performed next to the subject and/or in the same room as the subject and/or next to the bed or chair of the subject. Thus, sample identity mix-ups can be avoided, as well as long and expensive incubations over periods of days or weeks. This is further provided by the fact that methods provided herein are readily adaptable to closed and automated blood processing systems, where a blood sample and its components that will be reintroduced into the subject, only make contact with disposable, single-use components.

[173] Methods for performing adoptive cell therapy provided herein, typically include methods of transducing resting T cells and/or NK cells, which themselves form distinct aspects of the present disclosure. A skilled artisan will recognize that details provided herein for transducing T cells and/or NK cells can apply to any aspect that includes such step(s). Accordingly, provided herein in certain aspects, is a method of transducing a T cell and/or an NK cell, typically a resting T cell and/or resting NK cell, that includes contacting the resting T cell and/or resting NK cell with a recombinant retrovirus, wherein the recombinant retrovirus typically comprises a pseudotyping element on its surface that is capable of binding the resting T cell and/or NK cell and facilitating membrane fusion of the recombinant retrovirus thereto, wherein said contacting (and incubation under contacting conditions) facilitates transduction of the resting T cell and/or NK cell by the recombinant retroviruses, thereby producing the genetically

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modified T cell and/or NK cell. Further embodiments of such a method can include any of the embodiments of retroviruses, lymphoproliferative elements, CARs, pseudotyping elements, riboswitches, activation elements, membrane-bound cytokines, miRNAs, and/or other elements disclosed herein. Such a method for transduding a T cell and/or NK cell can be performed *in vitro* or *ex vivo*.

[174] In methods for adoptive cell therapy and any method provided herein that include transducing resting T cells and/or resting NK cells *ex vivo*, typically, neutrophils/granulocytes are separated away from the blood cells before the cells are contacted with recombinant retrovirus. In some embodiments, peripheral blood mononuclear cells (PBMCs) including peripheral blood lymphocytes (PBLs) such as T cell and/or NK cells, are isolated away from other components of a blood sample using for example, apheresis, and/or density gradient centrifugation. In some embodiments, neutrophils are removed before PBMCs and/or T cells and/or NK cells are processed, contacted with a recombinant retrovirus, transduced, or transfected. With reference to the subject to be treated, the cells may be allogeneic and/or autologous.

[175] As non-limiting examples, in some embodiments, for performing the PBMCs are isolated using a Sepax or Sepax 2 cell processing system (BioSafe). In some embodiments, the PBMCs are isolated using a CliniMACS Prodigy cell processor (Miltenyi Biotec). In some embodiments, an automated apheresis separator is used which takes blood from the subject, passes the blood through an apparatus that sorts out a particular cell type (such as, for example, PBMCs), and returns the remainder back into the subject. Density gradient centrifugation can be performed after apheresis. In some embodiments, the PBMCs are isolated using a leukoreduction filter device. In some embodiments, magnetic bead activated cell sorting is then used for purifying a specific cell population from PBMCs, such as, for example, PBLs or a subset thereof, according to a cellular phenotype (i.e. positive selection). Other methods for purification can also be used, such as, for example, substrate adhesion, which utilizes a substrate that mimics the environment that a T cell encounters during recruitment, allowing them to adhere and migrate, or negative selection, in which unwanted cells are targeted for removal with antibody complexes that target the unwanted cells. In some embodiments, red blood cell rosetting can be used to purify cells.

[176] In some illustrative embodiments of any of the relevant aspects herein, the PBLs include T cells and/or NK cells. The T cells and/or NK cells that are contacted by recombinant retroviruses of the present disclosure during certain embodiments herein, for example in methods of modifying lymphocytes and methods of performing adoptive cellular therapy, are mainly resting T cells. In some embodiments, the T cells and/or NK cells consist of between 95 and 100% resting cells (Ki-67$^-$). In some embodiments, the T cell and/or NK cells that are contacted by recombination retroviruses include between 90, 91, 92, 93, 94, and 95% resting cells on the low end of the range and 96, 97, 98, 99, or 100% resting cells on the high end of the range. In some embodiments, the T cells and/or NK cells include naïve cells.

[177] In some embodiments of the methods and compositions disclosed herein, T cells and/or NK cells are contacted *ex vivo* with recombinant retroviruses to genetically modify T cells and/or NK cells to illicit a targeted immune response in the subject when reintroduced into the subject. During the period of contact, the recombinant retroviruses identify and bind to T cells and/or NK cells at which point the retroviral and host cell membranes start to fuse. Then, through the process of transduction, genetic material from the recombinant retroviruses enters the T cells and/or NK cells and is incorporated into the host cell DNA. 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.

[178] Many of the methods provided herein include transduction of T cells and/or NK cells. Methods are known in the art for transducing T cells and/or NK cells *ex vivo* with retroviruses, such as lentiviruses. Methods provided herein, in illustrative embodiments, do not require *ex vivo* stimulation or activation. Thus, this common step in prior methods can be avoided in the present method, although *ex vivo* stimulatory molecule(s) such as anti-CD3 and/or anti-CD28 beads, can be present during the transduction. However, with illustrative methods provided herein, *ex vivo* stimulation is not required. In certain exemplary methods, between 3 and 10 multiplicity of infection (MOI), and in some embodiments, between 5 and 10 MOI units of retrovirus, for example lentivirus, can be used.

[179] The transduction reaction can be carried out in a closed system, such as a Sepax system, as discussed herein, wherein the transduction reaction can be carried out in disposable bags loaded on the system. Blood cells, such as PBMCs, from the collected blood sample from the subject, can be contacted with recombinant retroviruses disclosed herein, in a bag as soon as these blood cells are separated, isolated, and/or purified away from granulocytes, including neutrophils, which are typically not present during the contacting step (i.e. the transduction reaction).

[180] The retrovirus can be introduced into the bag that contains the isolated PBMCs, thereby contacting the PBMCs. The time from blood collection from the subject to the time when blood cells, such as PBMCs are added to the transduction reaction bag, can be between 30 minutes and 4 hours, between 30 minutes and 2 hours, or around 1 hour, in some examples. Additives such as media, human serum albumin, human AB+ serum, and/or serum derived from the subject can be added to the transduction reaction mixture. Media is typically present, such as those known in the art for *ex vivo* processes (as non-limiting examples, X-VIVO 15 (Lonza) or CTS media (Thermo Fisher Scientific). Supportive cytokines can be added to the transduction reaction mixture, such as IL2, IL7, or IL15, or those found in HSA.

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[181] The transduction reaction mixture can be incubated at between 23 and 39 °C, and in some illustrative embodiments at 37 °C. In certain embodiments, the transduction reaction can be carried out at 37-39 °C for faster fusion/transduction. dGTP can be added to the transduction reaction. The transduction reaction mixture can be incubated for 1 to 12 hours, and in some embodiments, 6 to 12 hrs. After transduction, before the transduced T cells and/or NK cells are infused back into the subject, the cells are washed out of the transduction reaction mixture. For example, the system, such as a Sepax instrument, can be used to wash cells, for example with 10-50 ml of wash solution, before the transduced cells are infused back into the subject. In some embodiments, neutrophils are removed before PBMCs and/or T cells and/or NK cells are processed, contacted with a recombinant retrovirus, transduced, or transfected.

[182] In an illustrative embodiment for performing adoptive cell therapy, blood is collected from a subject into a blood bag and the blood bag is attached to a cell processing system such as a Sepax cell processing system. PBMCs isolated using the cell processing system are collected into a bag, contacted with the recombinant retrovirus in conditions sufficient to transduce T cells and/or NK cells, and incubated. After incubation, the bag containing the mixture of PBMCs and recombinant retrovirus is attached to a cell processing system and the PBMCs are washed. The washed PBMCs are collected into a bag and reinfused into the subject. In some embodiments, the entire method, from collecting blood to reinfusing transduced T and/or NK cells, is performed within 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 18, or 24 hours. In illustrative embodiments, the entire method is performed within 12 hours.

[183] In some embodiments, the target cells for the recombinant retroviruses are PBLs. In some embodiments, the target cells are T cells and/or NK cells. In some embodiments, the T cells are helper T cells and/or killer T cells.

[184] In some embodiments, the recombinant retroviruses provided herein have pseudotyping elements on their surface that are capable of binding to T cells and/or NK cells and facilitating membrane fusion of the recombinant retroviruses thereto. In other embodiments, the recombinant retroviruses have activation elements on their surface that are capable of binding to resting T cells and/or NK cells. In still other embodiments, the recombinant retroviruses have membrane-bound cytokines on their surface. In some embodiments, the recombinant retroviruses include a polynucleotide having one or more transcriptional units encoding one or more engineered signaling polypeptides, one or more of which includes a lymphoproliferative element. In other embodiments, when two signaling polypeptides are utilized, one includes a lymphoproliferative element and the other is typically a chimeric antigen receptor (CAR) that includes an antigen-specific targeting region (ASTR), a transmembrane domain, and an intracellular activating domain. As indicated herein, an activation element(s) that is typically associated with the surface of a recombinant retrovirus provided herein, is capable of, and as a resulting of contacting resting T cells and/or NK cells for a sufficient period of time and under appropriate conditions, activates resting

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T cells and/or NK cells. It will be understood that such activation occurs over time during a contacting step of methods herein. Furthermore, it will be understood that in some embodiments where a pseudotyping element is found on the surface of a recombinant retrovirus, that binds a T cell and/or an NK cell, in methods herein, activation can be induced by binding of the pseudotyping element. An activation element is optional in those embodiments.

[185] Further details regarding a pseudotyping element, an activation element, a membrane-bound cytokine, an engineered signaling polypeptide, a lymphoproliferative element, and a CAR are provided in other sections herein.

[186] In some embodiments of the methods and compositions disclosed herein, between 5% and 90% of the total lymphocytes collected from the blood are transduced. In some embodiments, the percent of lymphocytes that are transduced is between 5, 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, and 60% on the low end of the range, and 50, 55, 60, 65, 70, 75, 80, 85, and 90% on the high end of the range. In some embodiments, the percent of lymphocytes that are transduced is at least 5%, at least 10%, at least 15%, at least 20%, at least 25%, at least 30%, at least 35%, at least 40%, at least 45%, at least 50%, at least 55%, or at least 60%.

[187] In some embodiments of the methods and compositions disclosed herein, the genetically modified T cells and/or NK cells are introduced back, reintroduced, or reinfused into the subject without additional *ex vivo* manipulation, such as stimulation and/or activation of T cells and/or NKs. In the prior art methods, *ex vivo* manipulation is used for stimulation/activation of T cells and/or NK cells and for expansion of genetically modified T cells and/or NK cells prior to introducing the genetically modified T cells and/or NK cells into the subject. In prior art methods, this generally takes days or weeks and requires a subject to return to a clinic for a blood infusion days or weeks after an initial blood draw. In some embodiments of the methods and compositions disclosed herein, T cells and/or NK cells are not stimulated *ex vivo* by exposure to anti-CD3/anti-CD28 solid supports such as, for example, beads coated with anti-CD3/anti-CD28, prior to contacting the T cells and/or NK cells with the recombinant retroviruses. As such provided herein is an *ex vivo* propagation-free method. In other embodiments, genetically modified T cells and/or NK cells are not expanded *ex vivo*, or only expanded for a small number of cell divisions (e.g. 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 rounds of cell division), but are rather expanded, or predominantly expanded, *in vivo*, i.e. within the subject. In some embodiments, no additional media is added to allow for further expansion of the cells. In some embodiments, no cell manufacturing of the PBLs occurs while the PBLs are contacted with the recombinant retrovirus. In illustrative embodiments, no cell manufacturing of the PBLs occurs while the PBLs are *ex vivo*. In previous methods of adoptive cell therapy, subjects were lymphodepleted prior to reinfusion with genetically modified T cells and or NK cells. In some embodiments, patients or subjects are not

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lymphodepleted prior to blood being withdrawn. In some embodiments, patients or subjects are not lymphodepleted prior to reinfusion with genetically modified T cells and or NK cells.

[188] In any of the embodiments disclosed herein, the number of T cells and/or NK cells to be reinfused into a subject can be between 1 x 10^3, 2.5 x 10^3, 5 x 10^3, 1 x 10^4, 2.5 x 10^4, 5 x 10^4, 1 x 10^5, 2.5 x 10^5, 5 x 10^5, 1 x 10^6, 2.5 x 10^6, 5 x 10^6, and 1 x 10^7 cells/kg on the low end of the range and 5 x 10^4, 1 x 10^5, 2.5 x 10^5, 5 x 10^5, 1 x 10^6, 2.5 x 10^6, 5 x 10^6, 1 x 10^7, 2.5 x 10^7, 5 x 10^7, and 1 x 10^8 cells/kg on the high end of the range. In illustrative embodiments, the number of T cells and/or NK cells to be reinfused into a subject can be between 1 x 10^4, 2.5 x 10^4, 5 x 10^4, and 1 x 10^5 cells/kg on the low end of the range and 2.5 x 10^4, 5 x 10^4, 1 x 10^5, 2.5 x 10^5, 5 x 10^5, and 1 x 10^6 cells/kg on the high end of the range. In some embodiments, the number of PBLs to be reinfused into a subject can be fewer than 5 x 10^5, 1 x 10^6, 2.5 x 10^6, 5 x 10^6, 1 x 10^7, 2.5 x 10^7, 5 x 10^7, and 1 x 10^8 cells and the low end of the range and 2.5 x 10^6, 5 x 10^6, 1 x 10^7, 2.5 x 10^7, 5 x 10^7, 1 x 10^8, 2.5 x 10^8, 5 x 10^8, and 1 x 10^9 cells on the high end of the range. In some embodiments, the number of T cells and/or NK cells available for reinfusion into a 70 kg subject or patient is between 7 x 10^5 and 2.5 x 10^8 cells. In other embodiments, the number of T cells and/or NK cells available for transduction is approximately 7 x 10^6 plus or minus 10%.

[189] In the methods disclosed herein, the entire adoptive cell therapy procedure, from withdrawing blood to the reinfusion of genetically modified T cells and/or NK cells, can advantageously be performed in a shorter time than previous methods. In some embodiments, the entire adoptive cell therapy procedure can be performed in less than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 18, or 24 hours. In illustrative embodiments, the entire adoptive cell therapy procedure can be performed in less than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, or 12 hours. In some embodiments, the entire adoptive cell therapy procedure can be performed in between 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or 15 hours on the low end of the range and 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 18, or 24 hours on the high end of the range.

[190] In some embodiments provided herein, the steps of withdrawing a blood sample from a subject, contacting T cells and/or NK cells with recombinant retroviruses, and/or introducing genetically modified T cells and/or NK cells into the subject, occur in a closed system. A closed system is a culture process that is generally closed or fully closed to contamination. An advantage of the present invention, is that provided herein are methods for performing CAR therapy in a closed system. One of the greatest risks to safety and regulatory control in the cell processing procedure is the risk of contamination through frequent exposure to the environment as is found in traditional open cell culture systems. To mitigate this risk, particularly in the absence of antibiotics, some commercial processes have been developed that focus on the use of disposable (single-use) equipment. However even with their use under aseptic conditions, there is always a risk of contamination from the opening of flasks to sample or add additional growth media. To overcome this problem, provided herein is a closed-system process, a process that is designed

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and can be operated such that the product is not exposed to the outside environment. This is important because the outside environment is typically not sterile. Material transfer occurs via sterile connections or tube welding. Air for gas exchange occurs via a gas permeable membrane or like other additions, via 0.2 μm filter to prevent environmental exposure.

[191] In some embodiments, the closed system includes an *ex vivo* circulating system connected to the *in vivo* circulatory system of the subject such that blood is drawn and then circulated to the *ex vivo* circulatory system before being introduced back into the subject. In some embodiments, the *ex vivo* circulatory system includes a system or apparatus for isolating PBLs and/or a system or apparatus for isolating T cells and/or NK cells, in combination with the system or apparatus for exposing the cells to the recombinant retrovirus. In some embodiments, the closed system does not allow the T cells and/or NK cells to be exposed to air.

[192] Such closed system methods can be performed with commercially available devices. For example, the method can be carried out in devices adapted for closed system T cell production. Such devices include a G-Rex™, a WAVE Bioreactor™, an OriGen PermaLife™ bags, and a VueLife® bags.

[193] In some embodiments of the methods and compositions disclosed herein, genetically modified T cells and/or NK cells within a subject are exposed to a compound that binds to an *in vivo* control element present therein, in which the *in vivo* control element is a part of the genetic material introduced by the recombinant retroviruses. In some embodiments, the *in vivo* control element can be a riboswitch and the compound can bind the aptamer domain of the riboswitch. In some embodiments, the *in vivo* control element can be a molecular chaperone. In any of the embodiments disclosed herein, the compound can be a nucleoside analogue. In some embodiments, the nucleoside analogue can be a nucleoside analogue antiviral drug, wherein an antiviral drug is a compound approved by the Food and Drug Administration for antiviral treatment or a compound in an antiviral clinical trial in the United States. In illustrative embodiments, the compound can be acyclovir or penciclovir. In some embodiments, the compound can be famciclovir, the oral prodrug of penciclovir, or valaciclovir, the oral prodrug of acyclovir. Binding of the compound to the *in vivo* control element affects expression of the introduced genetic material and hence, propagation of genetically modified T cells and/or NK cells.

[194] In some embodiments, the nucleoside analogue antiviral drug or prodrug, for example acyclovir, valaciclovir, penciclovir or famciclovir, is administered to the subject prior to, concurrent with, and/or following PBLs being isolated from the blood of the subject and before T cells and/or NK cells are contacted with a recombinant retrovirus. In some embodiments, the nucleoside analogue antiviral drug or prodrug is administered to the subject for between 5, 10, 15, 30, and 60 minutes on the low end of the range and 1.5, 2, 3, 4, 5, 6, 8, 12, or 24 hours on the high end of the range prior to PBLs being isolated from the blood or prior to T cells and/or NK cells being contacted with a recombinant retrovirus. In other

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embodiments, the nucleoside analogue antiviral drug or prodrug is administered to the subject for between 1.5, 2, 3, 4, 5, 6, 8, 12, or 24 hours on the low end of the range and ½, 1, 2, 3, 4, 5, 6, 7, 10, 14, 21, or 28 days on the high end of the range after PBLs are isolated from the blood and T cells and/or NK cells are contacted with a recombinant retrovirus in methods provided herein. In some embodiments, the nucleoside analogue antiviral drug or prodrug is administered to the subject for at least 1.5, 2, 3, 4, 5, 6, 8, 12, or 24 hours, or at least 2, 3, 4, 5, 6, 7, 10, 14, 21, or 28 days after PBLs are isolated from the blood and T cells and/or NK cells are contacted with a recombinant retrovirus in methods provided herein. In some embodiments, the nucleoside analogue antiviral drug or prodrug is administered to the subject for at least 1, 2, 3, 4, 5, 7, 10, 14, 21, 28, 30, 60, 90, or 120 days or 5, 6, 9, 12, 24, 36, 48, 60, 72, 84, 96, 120 months or indefinitely after the PBLs have been reinfused into the subject. In any of the embodiments disclosed herein, the nucleoside analogue antiviral drug or prodrug can be administered before and/or during the reinfusion of the PBLs and/or after the PBLs have been reinfused.

[195] In some embodiments, the compound that binds to the *in vivo* control element is administered once, twice, three times, or four times daily to the subject. In some embodiments, daily doses of the compound are provided for 1 week, 2 weeks, 4 weeks, 3 months, 6 months, 1 year, until a subject is disease free, such as cancer free, or indefinitely. The drug, in illustrative embodiments is a nucleoside analogue antiviral drug that binds to a nucleoside analog, such as a riboswitch, as disclosed in further detail herein.

[196] Methods are known in the art for delivering drugs, whether small molecules or biologics, and can be used in methods provided herein. Any such methods can be used to deliver drugs or candidate compounds or antibodies for use in methods of the present invention. For example, common routes of administration include non-invasive peroral (through the mouth), topical (skin), transmucosal (nasal, buccal/sublingual, vaginal, ocular and rectal) and inhalation routes. Many protein and peptide drugs, such as monoclonal antibodies, have to be delivered by injection or a nanoneedle array. For example, many immunizations are based on the delivery of protein drugs and are often done by injection.

ENGINEERED SIGNALING POLYPEPTIDE(S)

[197] In some embodiments, the recombinant retroviruses used to contact T cells and/or NK cells have a polynucleotide having one or more transcriptional units that encode one or more engineered signaling polypeptides, one or more of which includes a lymphoproliferative element. In some embodiments, a signaling polypeptide includes any combination of the following: an extracellular antigen-binding domain (or antigen-specific targeting region or ASTR), a stalk, a transmembrane domain, an intracellular activating domain, a lymphoproliferative element, a modulatory domain (such as a co-stimulatory domain), and a T cell survival motif. In illustrative embodiments, at least one, two, or all of the

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engineered signaling polypeptides is a CAR. In some embodiments, when two signaling polypeptides are utilized, one encodes a lymphoproliferative element and the other encodes a chimeric antigen receptor (CAR) that includes an antigen-specific targeting region (ASTR), a transmembrane domain, and an intracellular activating domain. In other embodiments, a CAR can include a lymphoproliferative element fused to an antigen-specific targeting region. In other embodiments, when the lymphoproliferative element is a constitutively active interleukin receptor, such as a known variant of IL-7Rα, no antigen-specific targeting region is needed because binding is not dependent on the presence of the ligand. One of ordinary skill in the art would be able to reconfigure the system to put the lymphoproliferative element and the CAR on distinct polynucleotides with similar or dissimilar control elements for the methods and compositions disclosed herein. A skilled artisan will recognize that such engineered polypeptides can also be referred to as recombinant polypeptides.

Antigen-specific targeting region

[198] In some embodiments, an engineered signaling polypeptide includes a member of a specific binding pair, which is typically an ASTR, sometimes called an antigen binding domain herein. Specific binding pairs include, but are not limited to, antigen-antibody binding pairs; ligand-receptor binding pairs; and the like. Thus, a member of a specific binding pair suitable for use in an engineered signaling polypeptide of the present disclosure includes an ASTR that is an antibody, an antigen, a ligand, a receptor binding domain of a ligand, a receptor, a ligand binding domain of a receptor, and an affibody.

[199] An ASTR suitable for use in an engineered signaling polypeptide of the present disclosure can be any antigen-binding polypeptide. In certain embodiments, the ASTR is an antibody such as a full-length antibody, a single-chain antibody, an Fab fragment, an Fab' fragment, an (Fab')2 fragment, an Fv fragment, and a divalent single-chain antibody or a diabody.

[200] In some embodiments, the ASTR is a single chain Fv (scFv). In some embodiments, the heavy chain is positioned N-terminal of the light chain in the engineered signaling polypeptide. In other embodiments, the light chain is positioned N-terminal of the heavy chain in the engineered signaling polypeptide. In any of the disclosed embodiments, the heavy and light chains can be separated by a linker as discussed in more detail herein. In any of the disclosed embodiments, the heavy or light chain can be at the N-terminus of the engineered signaling polypeptide and is typically C-terminal of another domain, such as a signal sequence or peptide.

[201] Other antibody-based recognition domains (cAb VHH (camelid antibody variable domains) and humanized versions, IgNAR VII (shark antibody variable domains) and humanized versions, sdAb VII (single domain antibody variable domains) and "camelized" antibody variable domains are suitable for use with the engineered signaling polypeptides and methods using the engineered signaling polypeptides

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of the present disclosure. In some instances, T cell receptor (TCR) based recognition domains such as single chain TCR (scTv, single chain two-domain TCR containing VαVβ) are also suitable for use.

[202] In some embodiments, the ASTR can be multispecific, e.g. bispecific antibodies. Multispecific antibodies have binding specificities for at least two different sites. In certain embodiments, one of the binding specificities is for one target antigen and the other is for another target antigen. In certain embodiments, bispecific antibodies may bind to two different epitopes of ta target antigen. Bispecific antibodies may also be used to localize cytotoxic agents to cells which express a target antigen. Bispecific antibodies can be prepared as full length antibodies or antibody fragments.

[203] An ASTR suitable for use in an engineered signaling polypeptide of the present disclosure can have a variety of antigen-binding specificities. In some cases, the antigen-binding domain is specific for an epitope present in an antigen that is expressed by (synthesized by) a target cell. In one example, the target cell is a cancer cell associated antigen. The cancer cell associated antigen can be an antigen associated with, e.g., a breast cancer cell, a B cell lymphoma, a Hodgkin lymphoma cell, an ovarian cancer cell, a prostate cancer cell, a mesothelioma, a lung cancer cell (e.g., a small cell lung cancer cell), a non-Hodgkin B-cell lymphoma (B-NHL) cell, an ovarian cancer cell, a prostate cancer cell, a mesothelioma cell, a lung cancer cell (e.g., a small cell lung cancer cell), a melanoma cell, a chronic lymphocytic leukemia cell, an acute lymphocytic leukemia cell, a neuroblastoma cell, a glioma, a glioblastoma, a medulloblastoma, a colorectal cancer cell, etc. A cancer cell associated antigen may also be expressed by a non-cancerous cell.

[204] Non-limiting examples of antigens to which an ASTR of an engineered signaling polypeptide can bind include, e.g., CD19, CD20, CD38, CD30, ERBB2, CA125, MUC-1, prostate-specific membrane antigen (PSMA), CD44 surface adhesion molecule, mesothelin, carcinoembryonic antigen (CEA), epidermal growth factor receptor (EGFR), EGFRvIII, vascular endothelial growth factor receptor-2 (VEGFR2), high molecular weight-melanoma associated antigen (HMW-MAA), MAGE-Al, IL-13R-a2, GD2, Axl, Ror2, and the like.

[205] In some cases, a member of a specific binding pair suitable for use in an engineered signaling polypeptide is an ASTR that is a ligand for a receptor. Ligands include, but are not limited to, cytokines (e.g., IL-13, etc.); growth factors (e.g., heregulin; vascular endothelial growth factor (VEGF); and the like); an integrin-binding peptide (e.g., a peptide comprising the sequence Arg-Gly-Asp); and the like.

[206] Where the member of a specific binding pair in an engineered signaling polypeptide is a ligand, the engineered signaling polypeptide can be activated in the presence of a second member of the specific binding pair, where the second member of the specific binding pair is a receptor for the ligand. For example, where the ligand is VEGF, the second member of the specific binding pair can be a VEGF receptor, including a soluble VEGF receptor.

[207] As noted above, in some cases, the member of a specific binding pair that is included in an engineered signaling polypeptide is an ASTR that is a receptor, e.g., a receptor for a ligand, a co-receptor, etc. The receptor can be a ligand-binding fragment of a receptor. Suitable receptors include, but are not limited to, a growth factor receptor (e.g., a VEGF receptor); a killer cell lectin-like receptor subfamily K, member 1 (NKG2D) polypeptide (receptor for MICA, MICB, and ULB6); a cytokine receptor (e.g., an IL-13 receptor; an IL-2 receptor; etc.); CD27; a natural cytotoxicity receptor (NCR) (e.g., NKP30 (NCR3/CD337) polypeptide (receptor for HLA-B-associated transcript 3 (BAT3) and B7-H6); etc.); etc.

Stalk

[208] In some embodiments, the engineered signaling polypeptide includes a stalk which is located in the portion of the engineered signaling polypeptide lying outside the cell and interposed between the ASTR and the transmembrane domain. In some cases, the stalk has at least 85, 90, 95, 96, 97, 98, 99, or 100% identity to a wild-type CD8 stalk region (TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGG AVHTRGLDFA (SEQ ID NO:79), has at least 85, 90, 95, 96, 97, 98, 99, or 100% identity to a wild-type CD28 stalk region (FCKIEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPSKP (SEQ ID NO:80)), or has at least 85, 90, 95, 96, 97, 98, 99, or 100% identity to a wild-type immunoglobulin heavy chain stalk region. In an engineered signaling polypeptide, the stalk employed allows the antigen-specific targeting region, and typically the entire engineered signaling polypeptide, to retain increased binding to a target antigen.

[209] The stalk region can have a length of from about 4 amino acids to about 50 amino acids, e.g., from about 4 aa to about 10 aa, from about 10 aa to about 15 aa, from about 15 aa to about 20 aa, from about 20 aa to about 25 aa, from about 25 aa to about 30 aa, from about 30 aa to about 40 aa, or from about 40 aa to about 50 aa.

[210] In some cases, the stalk of an engineered signaling polypeptide includes at least one cysteine. For example, in some cases, the stalk can include the sequence Cys-Pro-Pro-Cys (SEQ ID NO:62). If present, a cysteine in the stalk of a first engineered signaling polypeptide can be available to form a disulfide bond with a stalk in a second engineered signaling polypeptide.

[211] Stalks can include immunoglobulin hinge region amino acid sequences that are known in the art; see, e.g., Tan et al. (1990) *Proc. Natl. Acad. Sci. USA* 87:162; and Huck et al. (1986) *Nucl. Acids Res.* 14:1779. As non-limiting examples, an immunoglobulin hinge region can include a domain with at least 50, 60, 70, 75, 80, 85, 90, 95, 96, 97, 98, 99 or 100% sequence identity to a stretch of at least 10, 15, 20, or all of the amino acids of any of the following amino acid sequences: DKTHT (SEQ ID NO:63); CPPC (SEQ ID NO:62); CPEPKSCDTPPPCPR (SEQ ID NO:64) (see, e.g., Glaser et al. (2005) *J. Biol. Chem.* 280:41494); ELKTPLGDTTHT (SEQ ID NO:65); KSCDKTHTCP (SEQ ID NO:66); KCCVDCP (SEQ

ID NO:67); KYGPPCP (SEQ ID NO:68); EPKSCDKTHTCPPCP (SEQ ID NO:69) (human IgGl hinge); ERKCCVECPPCP (SEQ ID NO:70) (human IgG2 hinge); ELKTPLGDTTHTCPRCP (SEQ ID NO:71) (human IgG3 hinge); SPNMVPHAHHAQ (SEQ ID NO:72) (human IgG4 hinge); and the like. The stalk can include a hinge region with an amino acid sequence of a human IgG1, IgG2, IgG3, or IgG4, hinge region. The stalk can include one or more amino acid substitutions and/or insertions and/or deletions compared to a wild-type (naturally-occurring) hinge region. For example, His229 of human IgG 1 hinge can be substituted with Tyr, so that the stalk includes the sequence EPKSCDKTYTCPPCP (see, e.g., Yan et al. (2012) *J. Biol. Chem.* 287:5891). The stalk can include an amino acid sequence derived from human CD8; e.g., the stalk can include the amino acid sequence: TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD (SEQ ID NO:73), or a variant thereof.

Transmembrane domain

[212] An engineered signaling polypeptide of the present disclosure can include transmembrane domains for insertion into a eukaryotic cell membrane. The transmembrane domain can be interposed between the ASTR and the co-stimulatory domain. The transmembrane domain can be interposed between the stalk and the co-stimulatory domain, such that the chimeric antigen receptor includes, in order from the amino terminus (N-terminus) to the carboxyl terminus (C-terminus): an ASTR; a stalk; a transmembrane domain; and an activating domain.

[213] Any transmembrane (TM) domain that provides for insertion of a polypeptide into the cell membrane of a eukaryotic (e.g., mammalian) cell is suitable for use in aspects and embodiments disclosed herein. Non-limiting examples of TM domains suitable for any of the aspects or embodiments provided herein, include a domain with at least 50, 60, 70, 75, 80, 85, 90, 95, 96, 97, 98, 99 or 100% sequence identity to a stretch of at least 10, 15, 20, or all of the amino acids of any of the following TM domains: a) CD* alpha (IYIWAPLAGTCGVLLLSLVITLYC (SEQ ID NO:46)); b) CD8 beta (LGLLVAGVLVLLVSLGVAIHLCC (SEQ ID NO:47)); c) CD4 (ALIVLGGVAGLLLFIGLGIFFCVRC (SEQ ID NO:48)); d) CD3Z (LCYLLDGILFIYGVILTALFLRV (SEQ ID NO:49); e) CD28 (FWVLVVVGGVLACYSLLVTVAFIIFWV (SEQ ID NO:50)); f) CD134 (OX40): (VAAILGLGLVLGLLGPLAILLALYLL (SEQ ID NO:51)); g) CD7 (ALPAALAVISFLLGLGLGVACVLA (SEQ ID NO:52)), h) CD8 TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITL YC (SEQ ID NO:75), and i) CD28 IEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPSKPFWVLVVVGGVLACYSLLVTVAFIIFWV (SEQ ID NO:76).

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[214] As non-limiting examples, a transmembrane domain of an aspect of the invention can have at least 80, 90, or 95% sequence identity to the SEQ ID NO:46 transmembrane domain, the CD8 beta transmembrane domain, the CD4 transmembrane domain, the CD3 zeta transmembrane domain, the CD28 transmembrane domain, the CD134 transmembrane domain, or the CD7 transmembrane domain.

Intracellular activating domain

[215] Intracellular activating domains suitable for use in an engineered signaling polypeptide of the present disclosure when activated, typically induce the production of one or more cytokines; increased cell death; and/or increased proliferation of $CD8^+$ T cells, $CD4^+$ T cells, natural killer T cells, γδ T cells, and/or neutrophils. Activating domains can also be referred to as activation domains herein.

[216] In some embodiments, the intracellular activating domain includes at least one (e.g., one, two, three, four, five, six, etc.) ITAM motifs as described below. In some embodiments, the intracellular activating domain includes DAP10/CD28 type signaling chains. In some embodiments, the intracellular activating domain is not covalently attached to the membrane bound engineered signaling polypeptide, but is instead diffused in the cytoplasm. As non-limiting examples, an intracellular activating domain of an aspect of the invention can have at least 80%, 90%, or 95% sequence identity to the CD3Z, CD3D, CD3E, CD3G, CD79A, DAP12, FCERlG, DAP10/CD28, or ZAP70 domains as described below.

[217] Intracellular activating domains suitable for use in an engineered signaling polypeptide of the present disclosure include immunoreceptor tyrosine-based activation motif (ITAM)-containing intracellular signaling polypeptides. An ITAM motif is YX_1X_2L/I, where X_1 and X_2 are independently any amino acid. In some cases, the intracellular activating domain of an engineered signaling polypeptide includes 1, 2, 3, 4, or 5 ITAM motifs. In some cases, an ITAM motif is repeated twice in an intracellular activating domain, where the first and second instances of the ITAM motif are separated from one another by 6 to 8 amino acids, e.g., $(YX_1X_2L/I)(X_3)_n(YX_1X_2L/I)$, where n is an integer from 6 to 8, and each of the 6-8 X_3 can be any amino acid. In some cases, the intracellular activating domain of an engineered signaling polypeptide includes 3 ITAM motifs.

[218] A suitable intracellular activating domain can be an ITAM motif-containing portion that is derived from a polypeptide that contains an ITAM motif. For example, a suitable intracellular activating domain can be an ITAM motif-containing domain from any ITAM motif-containing protein. Thus, a suitable intracellular activating domain need not contain the entire sequence of the entire protein from which it is derived. Examples of suitable ITAM motif-containing polypeptides include, but are not limited to: CD3Z (CD3 zeta); CD3D (CD3 delta); CD3E (CD3 epsilon); CD3G (CD3 gamma); CD79A (antigen receptor complex-associated protein alpha chain); DAP12; and FCERlG (Fc epsilon receptor I gamma chain).

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[219] In some cases, the intracellular activating domain is derived from T cell surface glycoprotein CD3 zeta chain (also known as CD3Z, T cell receptor T3 zeta chain, CD247, CD3-ZETA, CD3H, CD3Q, T3Z, TCRZ, etc.). For example, a suitable intracellular activating domain can include a domain with at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a stretch of at least 10, 15, 20, or all amino acids in the following sequences or to a contiguous stretch of from about 100 amino acids to about 110 amino acids (aa), from about 110 aa to about 115 aa, from about 115 aa to about 120 aa, from about 120 aa to about 130 aa, from about 130 aa to about 140 aa, from about 140 aa to about 150 aa, or from about 150 aa to about 160 aa, of either of the following amino acid sequences (2 isoforms):
MKWKALFTAAILQAQLPITEAQSFGLLDPKLCYLLDGILFIYGVILTALFLRVKFSRSADAPAYQQ GQNQL**YNEL**NLGRREE**YDVL**DKRRGRDPEMGGKPRRKNPQEGL**YNEL**QKDKMAEA**YSEI**GM KGERRRGKGHDGL**YQGL**STATKDT**YDAL**HMQALPPR (SEQ ID NO:11) or
MKWKALFTAAILQAQLPITEAQSFGLLDPKLCYLLDGILFIYGVILTALFLRVKFSRSADAPAYQQ GQNQL**YNEL**NLGRREE**YDVL**DKRRGRDPEMGGKPQRRKNPQEGL**YNEL**QKDKMAEA**YSEI**G MKGERRRGKGHDGL**YQGL**STATKDT**YDAL**HMQALPPR (SEQ ID NO:12), where the ITAM motifs are in bold and are underlined.

[220] Likewise, a suitable intracellular activating domain polypeptide can include an ITAM motif-containing a portion of the full length CD3 zeta amino acid sequence. Thus, a suitable intracellular activating domain can include a domain with at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a stretch of at least 10, 15, 20, or all amino acids in the following sequences or to a contiguous stretch of from about 100 amino acids to about 110 amino acids (aa), from about 110 aa to about 115 aa, from about 115 aa to about 120 aa, from about 120 aa to about 130 aa, from about 130 aa to about 140 aa, from about 140 aa to about 150 aa, or from about 150 aa to about 160 aa, of either of the following amino acid sequences:
RVKFSRSADAPAYQQGQNQL**YNEL**NLGRREE**YDVL**DKRRGRDPEMGGKPRRKNPQEGL**YNEL** QKDKMAEA**YSEI**GMKGERRRGKGHDGL**YQGL**STATKDT**YDAL**HMQALPPR (SEQ ID NO:13); RVKFSRSADAPAYQQGQNQL**YNEL**NLGRREE**YDVL**DKRRGRDPEMGGKPQRRKNPQEGL**YNE L**QKDKMAEA**YSEI**GMKGERRRGKGHDGL**YQGL**STATKDT**YDAL**HMQALPPR (SEQ ID NO:81); NQL**YNEL**NLGRREE**YDVL**DKR SEQ ID NO:14); EGL**YNEL**QKDKMAEA**YSEI**GMK (SEQ ID NO:15); or DGL**YQGL**STATKDT**YDAL**HMQ (SEQ ID NO:16), where the ITAM motifs are in bold and are underlined.

[221] In some cases, the intracellular activating domain is derived from T cell surface glycoprotein CD3 delta chain (also known as CD3D; CD3-DELTA; T3D; CD3 antigen, delta subunit; CD3 delta; CD3d antigen, delta polypeptide (TiT3 complex); OKT3, delta chain; T cell receptor T3 delta chain; T

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cell surface glycoprotein CD3 delta chain; etc.). Thus, a suitable intracellular activating domain can include a domain with at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a stretch of at least 10, 15, 20, or all amino acids in the following sequences or to a contiguous stretch of from about 100 amino acids to about 110 amino acids (aa), from about 110 aa to about 115 aa, from about 115 aa to about 120 aa, from about 120 aa to about 130 aa, from about 130 aa to about 140 aa, from about 140 aa to about 150 aa, or from about 150 aa to about 160 aa, of either of the following amino acid sequences:

MEHSTFLSGLVLATLLSQVSPFKIPIEELEDRVFVNCNTSITWVEGTVGTLLSDITRLDLGKRILDP RGIYRCNGTDIYKDKESTVQVHYRMCQSCVELDPATVAGIIVTDVIATLLLALGVFCFAGHETGR LSGAADTQALLRNDQV**YQPL**RDRDDAQ**YSHL**GGNWARNK (SEQ ID NO:17) or

MEHSTFLSGLVLATLLSQVSPFKIPIEELEDRVFVNCNTSITWVEGTVGTLLSDITRLDLGKRILDP RGIYRCNGTDIYKDKESTVQVHYRTADTQALLRNDQV**YQPL**RDRDDAQ**YSHL**GGNWARNK (SEQ ID NO:18), where the ITAM motifs are in bold and are underlined.

[222] Likewise, a suitable intracellular activating domain polypeptide can comprise an ITAM motif-containing portion of the full length CD3 delta amino acid sequence. Thus, a suitable intracellular activating domain can include a domain with at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a stretch of at least 10, 15, 20, or all amino acids in the following sequence: DQV**YQPL**RDRDDAQ**YSHL**GGN (SEQ ID NO:19), where the ITAM motifs are in bold and are underlined.

[223] In some cases, the intracellular activating domain is derived from T cell surface glycoprotein CD3 epsilon chain (also known as CD3e, T cell surface antigen T3/Leu-4 epsilon chain, T cell surface glycoprotein CD3 epsilon chain, AI504783, CD3, CD3epsilon, T3e, etc.). Thus, a suitable intracellular activating domain can include a domain with at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a stretch of at least 10, 15, 20, or all amino acids in the following sequences or to a contiguous stretch of from about 100 amino acids to about 110 amino acids (aa), from about 110 aa to about 115 aa, from about 115 aa to about 120 aa, from about 120 aa to about 130 aa, from about 130 aa to about 140 aa, from about 140 aa to about 150 aa, or from about 150 aa to about 160 aa, of the following amino acid sequence:

MQSGTHWRVLGLCLLSVGVWGQDGNEEMGGITQTPYKVSISGTTVILTCPQYPGSEILWQHNDK NIGGDEDDKNIGSDEDHLSLKEFSELEQSGYYVCYPRGSKPEDANFYLYLRARVCENCMEMDMS VATIVIVDICITGGLLLLVYYWSKNRKAKAKPVTRGAGAGGRQRGQNKERPPPVPNPD**YEPI**RK GQRDL**YSGL**NQRRI (SEQ ID NO:20), where the ITAM motifs are in bold and are underlined.

[224] Likewise, a suitable intracellular activating domain polypeptide can comprise an ITAM motif-containing portion of the full length CD3 epsilon amino acid sequence. Thus, a suitable intracellular

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activating domain can include a domain with at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a stretch of at least 10, 15, 20, or all amino acids in the following sequence: NPD**YEPI**RKGQRDL**YSGL**NQR (SEQ ID NO:21), where the ITAM motifs are in bold and are underlined.

[225] In some cases, the intracellular activating domain is derived from T cell surface glycoprotein CD3 gamma chain (also known as CD3G, T cell receptor T3 gamma chain, CD3-GAMMA, T3G, gamma polypeptide (TiT3 complex), etc.). Thus, a suitable intracellular activating domain can include a domain with at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a stretch of at least 10, 15, 20, or all amino acids in the following sequences or to a contiguous stretch of from about 100 amino acids to about 110 amino acids (aa), from about 110 aa to about 115 aa, from about 115 aa to about 120 aa, from about 120 aa to about 130 aa, from about 130 aa to about 140 aa, from about 140 aa to about 150 aa, or from about 150 aa to about 160 aa, of the following amino acid sequence:

MEQGKGLAVLILAIILLQGTLAQSIKGNHLVKVYDYQEDGSVLLTCDAEAKNITWFKDGKMIGF LTEDKKKWNLGSNAKDPRGMYQCKGSQNKSKPLQVYYRMCQNCIELNAATISGFLFAEIVSIFV LAVGVYFIAGQDGVRQSRASDKQTLLPNDQLYQPLKDREDDQYSHLQGNQLRRN (SEQ ID NO:22), where the ITAM motifs are in bold and are underlined.

[226] Likewise, a suitable intracellular activating domain polypeptide can comprise an ITAM motif-containing portion of the full length CD3 gamma amino acid sequence. Thus, a suitable intracellular activating domain can include a domain with at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a stretch of at least 10, 15, 20, or all amino acids in the following sequence: DQL**YQPL**KDREDDQ**YSHL**QGN (SEQ ID NO:23), where the ITAM motifs are in bold and are underlined.

[227] In some cases, the intracellular activating domain is derived from CD79A (also known as B-cell antigen receptor complex-associated protein alpha chain; CD79a antigen (immunoglobulin-associated alpha); MB-1 membrane glycoprotein; Ig-alpha; membrane-bound immunoglobulin-associated protein; surface IgM-associated protein; etc.). Thus, a suitable intracellular activating domain can include a domain with at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a stretch of at least 10, 15, 20, or all amino acids in the following sequences or to a contiguous stretch of from about 100 amino acids to about 110 amino acids (aa), from about 110 aa to about 115 aa, from about 115 aa to about 120 aa, from about 120 aa to about 130 aa, from about 130 aa to about 140 aa, from about 140 aa to about 150 aa, or from about 150 aa to about 160 aa, of either of the following amino acid sequences:

MPGGPGVLQALPATIFLLFLLSAVYLGPGCQALWMHKVPASLMVSLGEDAHFQCPHNSSNNAN

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VTWWRVLHGNYTWPPEFLGPGEDPNGTLIIQNVNKSHGGIYVCRVQEGNESYQQSCGTYLRVR QPPPRPFLDMGEGTKNRIITAEGIILLFCAVVPGTLLLFRKRWQNEKLGLDAGDEYEDENL**YEGL** NLDDCSM**YEDI**SRGLQGTYQDVGSLNIGDVQLEKP (SEQ ID NO:24) or MPGGPGVLQALPATIFLLFLLSAVYLGPGCQALWMIIKVPASLMVSLGEDAIIFQCPIINSSNNAN VTWWRVLHGNYTWPPEFLGPGEDPNEPPPRPFLDMGEGTKNRIITAEGIILLFCAVVPGTLLLFRK RWQNEKLGLDAGDEYEDENLYEGLNLDDCSMYEDISRGLQGTYQDVGSLNIGDVQLEKP (SEQ ID NO:25), where the ITAM motifs are in bold and are underlined.

[228] Likewise, a suitable intracellular activating domain polypeptide can comprise an ITAM motif-containing portion of the full length CD79A amino acid sequence. Thus, a suitable intracellular activating domain can include a domain with at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a stretch of at least 10, 15, 20, or all amino acids in the following sequence: ENL**YEGL**NLDDCSM**YEDI**SRG (SEQ ID NO:26), where the ITAM motifs are in bold and are underlined.

[229] In some cases, the intracellular activating domain is derived from DAP12 (also known as TYROBP; TYRO protein tyrosine kinase binding protein; KARAP; PLOSL; DNAX-activation protein 12; KAR-associated protein; TYRO protein tyrosine kinase-binding protein; killer activating receptor associated protein; killer-activating receptor-associated protein; etc.). For example, a suitable intracellular activating domain can include a domain with at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a stretch of at least 10, 15, 20, or all amino acids in the following sequences or to a contiguous stretch of from about 100 amino acids to about 110 amino acids (aa), from about 110 aa to about 115 aa, from about 115 aa to about 120 aa, from about 120 aa to about 130 aa, from about 130 aa to about 140 aa, from about 140 aa to about 150 aa, or from about 150 aa to about 160 aa, of either of the following amino acid sequences (4 isoforms): MGGLEPCSRLLLLPLLLAVSGLRPVQAQAQSDCSCSTVSPGVLAGIVMGDLVLTVLIALAVYFLG RLVPRGRGAAEAATRKQRITETESP**YQEL**QGQRSDV**YSDL**NTQRPYYK (SEQ ID NO:27), MGGLEPCSRLLLLPLLLAVSGLRPVQAQAQSDCSCSTVSPGVLAGIVMGDLVLTVLIALAVYFLG RLVPRGRGAAEATRKQRITETESP**YQEL**QGQRSDV**YSDL**NTQ (SEQ ID NO:28), MGGLEPCSRLLLLPLLLAVSDCSCSTVSPGVLAGIVMGDLVLTVLIALAVYFLGRLVPRGRGAAE AATRKQRITETESP**YQEL**QGQRSDV**YSDL**NTQRPYYK (SEQ ID NO:29), or MGGLEPCSRLLLLPLLLAVSDCSCSTVSPGVLAGIVMGDLVLTVLIALAVYFLGRLVPRGRGAAE ATRKQRITETESP**YQEL**QGQRSDV**YSDL**NTQRPYYK (SEQ ID NO:30), where the ITAM motifs are in bold and are underlined.

[230] Likewise, a suitable intracellular activating domain polypeptide can comprise an ITAM motif-containing portion of the full length DAP12 amino acid sequence. Thus, a suitable intracellular activating

domain can include a domain with at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a stretch of at least 10, 15, 20, or all amino acids in the following sequence: ESP**YQEL**QGQRSDV**YSDL**NTQ (SEQ ID NO:31), where the ITAM motifs are in bold and are underlined.

[231] In some cases, the intracellular activating domain is derived from FCERlG (also known as FCRG; Fc epsilon receptor I gamma chain; Fc receptor gamma-chain; fc-epsilon RI-gamma; fcRgamma; fceRI gamma; high affinity immunoglobulin epsilon receptor subunit gamma; immunoglobulin E receptor, high affinity, gamma chain; etc.). For example, a suitable intracellular activating domain can include a domain with at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a stretch of at least 10, 15, 20, or all amino acids in the following sequences or to a contiguous stretch of from about 50 amino acids to about 60 amino acids (aa), from about 60 aa to about 70 aa, from about 70 aa to about 80 aa, or from about 80 aa to about 88 aa, of the following amino acid sequence:

MIPAVVLLLLLLVEQAAALGEPQLCYILDAILFLYGIVLTLLYCRLKIQVRKAAITSYEKSDGV**YT GL**STRNQET**YETL**KHEKPPQ (SEQ ID NO:32), where the ITAM motifs are in bold and are underlined.

[232] Likewise, a suitable intracellular activating domain polypeptide can comprise an ITAM motif-containing portion of the full length FCER1G amino acid sequence. Thus, a suitable intracellular activating domain can include a domain with at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a stretch of at least 10, 15, 20, or all amino acids in the following sequence: DGV**YTGL**STRNQET**YETL**KHE (SEQ ID NO:33), where the ITAM motifs are in bold and are underlined.

[233] Intracellular activating domains suitable for use in an engineered signaling polypeptide of the present disclosure include a DAP10/CD28 type signaling chain. An example of a DAP10 signaling chain is the amino acid sequence is: RPRRSPAQDGKV**YINM**PGRG (SEQ ID NO:34). In some embodiments, a suitable intracellular activating domain includes a domain with at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a stretch of at least 10, 15, 20, or all amino acids in the following sequence: RPRRSPAQDGKV**YINM**PGRG (SEQ ID NO:34).

[234] An example of a CD28 signaling chain is the amino acid sequence is FWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSD**YMNM**TPRRPGPTRKHYQPYAPPRDF AAYRS (SEQ ID NO:35). In some embodiments, a suitable intracellular domain includes a domain with at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a stretch of at least 10, 15, 20, or all amino acids in the following sequence:

FWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSD**YMNM**TPRRPGPTRKHYQPYAPPRDF AAYRS (SEQ ID NO:35).

[235] Intracellular activating domains suitable for use in an engineered signaling polypeptide of the present disclosure include a ZAP70 polypeptide, For example, a suitable intracellular activating domain can include a domain with at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a stretch of at least 10, 15, 20, or all amino acids in the following sequences or to a contiguous stretch of from about 300 amino acids to about 400 amino acids, from about 400 amino acids to about 500 amino acids, or from about 500 amino acids to 619 amino acids, of the following amino acid sequence:

MPDPAAHLPFFYGSISRAEAEEHLKLAGMADGLFLLRQCLRSLGGYVLSLVHDVRFHHFPIERQL NGTYAIAGGKAHCGPAELCEFYSRDPDGLPCNLRKPCNRPSGLEPQPGVFDCLRDAMVRDYVR QTWKLEGEALEQAIISQAPQVEKLIATTAHERMPWYHSSLTREEAERKLYSGAQTDGKFLLRPR KEQGTYALSLIYGKTVYHYLISQDKAGKYCIPEGTKFDTLWQLVEYLKLKADGLIYCLKEACPN SSASNASGAAAPTLPAHPSTLTHPQRRIDTLNSDGYTPEPARITSPDKPRPMPMDTSVYESPYSDP EELKDKKLFLKRDNLLIADIELGCGNFGSVRQGVYRMRKKQIDVAIKVLKQGTEKADTEEMMR EAQIMHQLDNPYIVRLIGVCQAEALMLVMEMAGGGPLHKFLVGKREEIPVSNVAELLHQVSMG MKYLEEKNFVHRDLAARNVLLVNRHYAKISDFGLSKALGADDSYYTARSAGKWPLKWYAPECI NFRKFSSRSDVWSYGVTMWEALSYGQKPYKKMKGPEVMAFIEQGKRMECPPECPPELYALMSD CWIYKWEDRPDFLTVEQRMRACYYSLASKVEGPPGSTQKAEAACA (SEQ ID NO:36).

Lymphoproliferative elements

[236] Peripheral T lymphocyte numbers are maintained at remarkably stable levels throughout adulthood, despite the continuing addition of cells, due to emigration from the thymus and proliferation in response to antigen encounter, and loss of cells owing to the removal of antigen-specific effectors after antigen clearance (Marrak, P. et al. 2000. *Nat Immunol* 1:107-111; Freitas, A.A. et al. 2000. *Annu Rev Immunol* 18:83-111). The size of the peripheral T cell compartment is regulated by multiple factors that influence both proliferation and survival. However, in a lymphopenic environment, T lymphocytes divide independently of cognate antigen, due to "acute homeostatic proliferation" mechanisms that maintain the size of the peripheral T cell compartment. Conditions for lymphopenia have been established in subjects or patients during adoptive cell therapy by proliferating T cells *in vitro* and introducing them into lymphodepleted subjects, resulting in enhanced engraftment and antitumor function of transferred T cells. However, lymphodepletion of a subject is not desirable because it can cause serious side effects, including immune dysfunction and death.

[237] Studies have shown that lymphodepletion removes endogenous lymphocytes functioning as cellular sinks for homeostatic cytokines, thereby freeing cytokines to induce survival and proliferation of adoptively transferred cells. Some cytokines, such as for example, IL-7 and IL-15, are known to mediate antigen-independent proliferation of T cells and are thus capable of eliciting homeostatic proliferation in non-lymphopenic environments. However, these cytokines and their receptors have intrinsic control mechanisms that prevent lymphoproliferative disorders at homeostasis.

[238] Many of the aspects provided herein include a lymphoproliferative element, or a nucleic acid encoding the say, typically as part of an engineered signaling polypeptide. In illustrative embodiments herein, a lymphoproliferative element is introduced into a resting T cell and/or resting NK cell, typically by transducing the resting T cell and/or resting NK cell with a retrovirus whose genome encodes the lymphoproliferative element as part of an engineered signaling polypeptide. The lymphoproliferative element can be a cytokine or in further illustrative embodiments, a cytokine receptor, or a fragment that includes a signaling domain thereof, that activates a STAT3 pathway, a STAT4 pathway, or in even further illustrative embodiments, a Jak/STAT5 pathway. As such, a lymphoproliferative element, can be, in a non-limiting example, a cytokine receptor, or active fragment that includes a signaling domain thereof, such as an interleukin receptor, or an active fragment that includes a signaling domain thereof, that activates STAT5. Thus, a lymphoproliferative element is a polypeptide that induces proliferation of a T cell and/or NK cell. Illustrative lymphoproliferative elements induce proliferation by activating STAT5. Thus, fragments of such lymphoproliferative elements retain the ability to induce proliferation of T cells and/or NK cells, in illustrative embodiments, by activating STAT5.

[239] In some of the methods and compositions presented herein, a lymphoproliferative element is used to promote proliferation or expansion of genetically modified T cells *in vivo* without having to lymphodeplete subjects. As such, non-limiting illustrative embodiments of methods provided herein that include inserting a lymphoproliferative element into a resting T cell and/or NK cell of a subject, typically by transducing such T cell and/or NK cell can be performed without lymphodepleting the subject before, during and/or after performing the method, or without lymphodepleting the subject before, during and/or after collecting blood from a subject before performing such method, or without lymphodepleting the subject before, during, and/or after genetically modifying T cells or NK cells *ex vivo* from the subject, and/or before, during, or after reintroducing the genetically modified T cells and/or NK cells into the subject. Factors that promote proliferation of T cells *in vivo* include cytokines and their receptors, in which a receptor typically includes a ligand binding domain and a signaling domain. In some embodiments, the lymphoproliferative element used in the methods and compositions disclosed herein is a cytokine and/or a cytokine receptor. The cytokine can be an interleukin, and the cytokine receptor can be an interleukin receptor. The lymphoproliferative element can be a functional fragment of a cytokine

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and/or a functional fragment of a cytokine receptor, such as a signaling domain thereof, wherein the fragment is capable of promoting proliferation of T cells, for example by activating STAT5.

[240] In some embodiments, the cytokine lymphoproliferative element in the methods and compositions herein include one or more of the following: Interleukin-7 (IL-7) or its receptor (IL-7R), or a signaling domain thereof; Interleukin-12 (IL-12) or its receptor (IL-12R), or a signaling domain thereof; Interleukin-23 (IL-23) or its receptor composed of IL-12R β1 and IL-23R, or a signaling domain thereof; Interleukin-27 (IL-27) or its receptor (IL-27R), or a signaling domain thereof; Interleukin-15 (IL-15) or its receptor (IL-15R), or a signaling domain thereof; Interleukin-21 (IL-21) or its receptor (IL-21R), or a signaling domain thereof; or transforming growth factor β (TGFβ) or its receptor (TGFβR) or a signaling domain thereof; or the TGFβ decoy receptor (TGF-β—dominant-negative receptor II (DNRII)). In some embodiments, the lymphoproliferative element is the IL-12R or the TGFβ decoy receptor (TGF-β—dominant-negative receptor II (DNRII)).

[241] IL-7 binds to the IL-7 receptor, a heterodimer consisting of IL-7R alpha and common gamma chain receptor. Binding results in a cascade of signals important for T cell development within the thymus and survival within the periphery. Binding of IL-7 to the IL-7 receptor is known to activate the Jak/STAT5 pathway.

[242] IL-12 is involved in the differentiation of naïve T cells into Th1 cells (Hsieh CS et al. 1993. *Science*. 260(5107):547-9) and is known as a T cell-stimulating factor. IL-12 binds to the IL-12 receptor, which is a heterodimeric receptor formed by IL-12R-β1 and IL-12R-β2. IL12 can act by activating STAT4, but has been shown to activate STAT5 in T cells as well (Ahn, H., et al. 1998. *J. Immun.* 161:5893-5900). The IL-12 family is composed of the cytokines IL-12, IL-23, and IL-27. The receptor for IL-23 is composed of IL-12R β1 and IL-23R. IL-27 is a heterodimeric cytokine that is composed of two distinct genes, Epstein-Barr virus-induced gene 3(EBI3) and IL-27p28. IL-27 interacts with IL-27 receptor.

[243] IL-15 is a T and NK cell stimulatory factor that is similar in structure and function to IL-2. Both cytokines induce proliferation of T cells; and their shared functions are thought to result from both receptors using the IL-2/IL-15Rβ and common γ chains. Signaling pathway of IL-15 begins with binding to IL-15Rα receptor, with subsequent presentation to surrounding cells bearing IL-15Rβγc complex on their cell surface. Upon binding IL-15β subunit activates Janus kinase 1 (Jak1) and γc subunit Janus kinase 3 (Jak3), which leads to phosphorylation and activation of STAT3 and STAT5.

[244] IL-21 is expressed in activated human CD4+ T cells and in NK T cells, and IL-21 expression is up-regulated in Th2 and Th17 subsets of T helper cells. The IL-21 receptor (IL-21R) is expressed on the surface of T, B and NK cells and is similar in structure to the receptors for other type I cytokines like IL-2R or IL-15. IL-21R requires dimerization with the common gamma chain (γc) in order to bind IL-21.

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When bound to IL-21, the IL-21 receptor acts through the Jak/STAT pathway, activating STAT1, STAT3, and STAT5.

[245] TGFβ decoy receptors (TGF-β—dominant-negative receptor II (DNRII)) block TGFβ signaling by competing with the natural receptors for TGFβ binding. TGFβ-DNRII is a kinase-dead truncated form of RII that contains the extracellular TGFβ binding domain and the transmembrane domain of RII. TGFβ-DNRII binds the ligand but does not phosphorylate and activate RI, which thereby diminishes or eliminates Smad phosphorylation.

[246] Gain-of-function mutations in IL-7Rα have been identified in subjects with B and T cell acute lymphoblastic leukemias (B-ALL and T-ALL) (Zenatti PP, et al. 2011. *Nat Genet* 43:932-939; Snochat, C. et al. 2011. *J Exp Med* 208:901-908; McElroy, C.A. et al. 2012. *PNAS* 109(7):2503-2508). The mutations included insertions and deletions in the N-terminal region of the IL-7Rα TMD, with nearly all of the sequences containing an extra Cys residue, and an S165-to-C165 mutation. The cysteine resulted in constitutive activation of the receptor. Some of the mutations in the T-all group activated JAK1. These gain-of-function IL-7R mutants can be used in any of the aspects provided herein as one of the lymphoproliferative element(s).

[247] Accordingly, in some embodiments, the lymphoproliferative element is a mutated IL-7 receptor. In other embodiments, the mutated IL-7 receptor is constitutively active, activating the JAK-STAT5 pathway in the absence of the cytokine ligand. In still other embodiments, the mutated IL-7 receptor comprises a 1 to 10 amino acid insertion at a position between 237 and 254 that includes a cysteine residue that includes the ability to constitutively activate the STAT5 pathway. In some embodiments, the mutated IL-7 receptor is IL-7Rα-insPPCL (represented by SEQ ID NO:82).

[248] In some embodiments, the lymphoproliferative element is a chimeric cytokine receptor such as but not limited to a cytokine tethered to its receptor that typically constitutively activates the same STAT pathway as a corresponding activated wild-type cytokine receptor such as STAT3, STAT4, and in illustrative embodiments, STAT5. In some embodiments, the chimeric cytokine receptor is an interleukin, or a fragment thereof, tethered to or covalently attached to its cognate receptor, or a fragment thereof, via a linker. In some embodiments, the chimeric cytokine receptor is IL-7 tethered to IL-7Rα. In other embodiments, the chimeric cytokine receptor is IL-7 tethered to a domain of IL-7Rα, such as for example, the extracellular domain of IL-7Rα and/or the transmembrane domain of IL-7Rα. In some embodiments, the lymphoproliferative element is a cytokine receptor that is not tethered to a cytokine, and in fact in illustrative embodiments, provided herein a lymphoproliferative element is a constitutively active cytokine receptor that is not tethered to a cytokine. These chimeric IL-7 receptors typically constitutively activate STAT5 when expressed.

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[249] In some embodiments, the lymphoproliferative element is not a cytokine or a cytokine receptor but is a miRNA that stimulates the STAT5 pathway typically by potentiating activation of STAT5 by degrading a negative regulator in the SOCS pathway. In some embodiments, the miRNA is to proteins that affect proliferation such as but not limited to ABCG1, SOCS1, TGFbR2, SMAD2, cCBL, and PD1. In illustrative embodiments, as exemplified herein, such miRNAs can be located in introns in a packaging cells and/or a recombinant retrovirus genome, typically with expression driven by a promoter that is active in a T cell and/or NK cell. Not to be limited by theory, inclusion of introns in transcription units are believed to result in higher expression and/or stability of transcripts. As such, the ability to place miRNAs within introns of a retroviral genome adds to the teachings of the present disclosure that overcome challenges in the prior art of trying to get maximum activities into the size restrictions of a retroviral, such as a lentivirus genome. In some embodiments, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 miRNAs, in illustrative embodiments between 2 and 5, for example 4 miRNAs, one or more of which each bind nucleic acids encoding one or more of ABCG1, SOCS1, TGFbR2, SMAD2, cCBL, and PD1, can be included in the recombinant retroviral genome and delivered to a target cell, for example T cells and/or NK cells, using methods provided herein. In fact, as provided herein 1, 2, 3, or 4 miRNAs can be delivered in a single intron such as the EF1a intron.

[250] ABCG1 is an ATP-binding cassette transporter that negatively regulates thymocyte and peripheral lymphocyte proliferation (Armstrong et al. 2010. *J Immunol* 184(1):173-183).

[251] SOCS1 is a member of the SOCS (Suppressor of cytokine signaling) family of negative regulators of cytokine signal transduction that inhibit the Jak/Stat pathway such as STAT5. SOCS1 is also known as JAB (Janus Kinase binding protein), SSI-1 (Stat-induced Stat inhibitor-1), and TIP3 (Tec-interacting protein).

[252] TGFbR2 is a member of the serine/threonine protein kinase family that binds TGF-β, forming a complex that phosphorylates proteins that then enter the nucleus and regulate transcription of genes related to proliferation.

[253] SMAD2 mediates the signal of the transforming growth factor (TGF)-β and regulates multiple cellular processes, such as cell proliferation, apoptosis, and differentiation.

[254] cCBL is an E3 ubiquitin ligase that inhibits TCR signaling by dephosphorylation and inactivation of ZAP-70 and through internalization of the TCR.

[255] PD1 (CD279) is a cell surface receptor expressed on T cells and ProB cells. PD-1 binds two ligands, PD-L1 and PD-L2. Signaling through PD-1 functions to prevent activation of cells.

[256] In some of the methods and compositions disclosed herein, expression of the lymphoproliferative element is induced by and can even dependent on binding of a compound to an *in vivo* control element (as discussed elsewhere herein), which in non-limiting embodiments is a ribowsitch. In some embodiments,

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the lymphoproliferative element is expressed from a promoter active in a T cell and/or an NK cell. For methods and compositions provided herein, a skilled artisan will recognize that promoters are known that are active in T cells and/or NK cells and can be used to express a first engineered signaling polypeptide or a second engineered signaling polypeptide, or any component thereof. In illustrative embodiments, such a promoter is not active in a packaging cell line, such as the packaging lines disclosed herein. In some embodiments, the promoter is the EF1a promoter or the murine stem cell virus (MSCV) promoter (Jones et al., *Human Gene Therapy* (2009) 20: 630-40). In illustrative embodiments, the promoter is the T cell specific CD3 zeta promoter.

[257] In some embodiments, the lymphoproliferative element is microenvironment restricted. For example, the lymphoproliferative element can be a mutated receptor that binds its respective cytokine differentially in aberrant versus physiological conditions. For example, an IL-7R that can bind IL7 more strongly in a tumor environment than in a normal physiological environment can be used.

[258] In some embodiments, the lymphoproliferative element is fused to a recognition or elimination domain. Such recognition or elimination domains are disclosed in more detail herein. Such fusion provides the advantage, especially when a truncated or other mutated lymphoproliferative element is used, of requiring less polynucleotides in the retroviral genome. This is important in illustrative embodiments provided herein, because it helps to permit more nucleic acids encoding functional elements to be included in the retroviral genome. In other embodiments, the lymphoproliferative element is fused to a co-stimulatory domain and/or an intracellular activating domain. A lymphoproliferative element as disclosed herein, is not a chimeric antigen receptor (CAR) or an intracellular activating domain or co-stimulating domain thereof. However, in some embodiments, a lymphoproliferative element can be fused to an antigen-specific targeting region (ASTR) and activated by binding of the ASTR to its antigen. In still other embodiments, an engineered signaling polypeptide can include an ASTR, an intracellular activation domain (such as a CD3 zeta signaling domain), a co-stimulatory domain, and a lymphoproliferative domain. Further details regarding co-stimulatory domains, intracellular activating domains, ASTRs and other CAR domains, are disclosed elsewhere herein.

[259] In illustrative embodiments herein, a T cell and/or NK cell survival element is introduced into a resting T cell and/or resting NK cell, typically by transducing the resting T cell and/or resting NK cell with a retrovirus whose genome encodes the T cell and/or NK cell survival element as part of an engineered signaling polypeptide. In some embodiments, a lymphoproliferative element is also a T cell and/or NK cell survival element. As discussed above, some of the lymphoproliferative elements not only promote proliferation, but they promote cell survival as well. In some embodiments, the T cell and/or NK survival motif is not a lymphoproliferative element. For example, the T cell and/or NK cell survival motif can be a CD28 T cell survival motif or a CD137 cell survival motif. Such T cell survival motifs can be

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found on engineered signaling polypeptides that include an ASTR, such as an scFV. In an illustrative embodiment, the T cell survival motif is a CD28 T cell survival motif or a CD137 motif connected to an scFv through a CD8a transmembrane domain or a CD28 transmembrane domain. In certain embodiments, said intracellular signaling domain comprises a polypeptide sequence comprising an immunoreceptor tyrosine-based activation motif (ITAM). In a certain embodiment, said polypeptide sequence is a CD3ζ signaling domain.

Modulatory domains

[260] Modulatory domains can change the effect of the intracellular activating domain in the engineered signaling polypeptide, including enhancing or dampening the downstream effects of the activating domain or changing the nature of the response. Modulatory domains suitable for use in an engineered signaling polypeptide of the present disclosure include co-stimulatory domains. A modulatory domain suitable for inclusion in the engineered signaling polypeptide can have a length of from about 30 amino acids to about 70 amino acids (aa), e.g., a modulatory domain can have a length of from about 30 aa to about 35 aa, from about 35 aa to about 40 aa, from about 40 aa to about 45 aa, from about 45 aa to about 50 aa, from about 50 aa to about 55 aa, from about 55 aa to about 60 aa, from about 60 aa to about 65 aa, or from about 65 aa to about 70 aa. In other cases, modulatory domain can have a length of from about 70 aa to about 100 aa, from about 100 aa to about 200 aa, or greater than 200 aa.

[261] Co-stimulatory domains typically enhance and/or change the nature of the response to an activation domain. Co-stimulatory domains suitable for use in an engineered signaling polypeptide of the present disclosure are generally polypeptides derived from receptors. In some embodiments, co-stimulatory domains homodimerize. A subject co-stimulatory domain can be an intracellular portion of a transmembrane protein (i.e., the co-stimulatory domain can be derived from a transmembrane protein). Non-limiting examples of suitable co-stimulatory polypeptides include, but are not limited to, 4-1BB (CD137), CD27, CD28, CD28 deleted for Lck binding (ICΔ), ICOS, OX40, BTLA, CD27, CD30, GITR, and HVEM. For example, a co-stimulatory domain of an aspect of the invention can have at least 80%, 90%, or 95% sequence identity to the co-stimulatory domain of 4-1BB (CD137), CD27, CD28, CD28 deleted for Lck binding (ICΔ), ICOS, OX40, BTLA, CD27, CD30, GITR, or HVEM. For example, a co-stimulatory domain of an aspect of the invention can have at least 80%, 90%, or 95% sequence identity to the co-stimulatory domain of Non-limiting examples of suitable co-stimulatory polypeptides include, but are not limited to, 4-1BB (CD137), CD27, CD28, CD28 deleted for Lck binding (ICΔ), ICOS, OX40, BTLA, CD27, CD30, GITR, and HVEM. For example, a co-stimulatory domain of an aspect of the invention can have at least 80%, 90%, or 95% sequence identity to the co-stimulatory domain of 4-1BB

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(CD137), CD27, CD28, CD28 deleted for Lck binding (ICΔ), ICOS, OX40, BTLA, CD27, CD30, GITR, or HVEM.

[262] A co-stimulatory domain suitable for inclusion in an engineered signaling polypeptide can have a length of from about 30 amino acids to about 70 amino acids (aa), e.g., a co-stimulatory domain can have a length of from about 30 aa to about 35 aa, from about 35 aa to about 40 aa, from about 40 aa to about 45 aa, from about 45 aa to about 50 aa, from about 50 aa to about 55 aa, from about 55 aa to about 60 aa, from about 60 aa to about 65 aa, or from about 65 aa to about 70 aa. In other cases, the co-stimulatory domain can have a length of from about 70 aa to about 100 aa, from about 100 aa to about 200 aa, or greater than 200 aa.

[263] In some cases, the co-stimulatory domain is derived from an intracellular portion of the transmembrane protein CD137 (also known as TNFRSF9; CD137; 4-1BB; CDw137; ILA; etc.). For example, a suitable co-stimulatory domain can include a domain with at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a stretch of at least 10, 15, 20, or all of the amino acids in the following amino acid sequence: KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL (SEQ ID NO:1). In some of these embodiments, the co-stimulatory domain has a length of from about 30 aa to about 35 aa, from about 35 aa to about 40 aa, from about 40 aa to about 45 aa, from about 45 aa to about 50 aa, from about 50 aa to about 55 aa, from about 55 aa to about 60 aa, from about 60 aa to about 65 aa, or from about 65 aa to about 70 aa.

[264] In some cases, the co-stimulatory domain is derived from an intracellular portion of the transmembrane protein CD28 (also known as Tp44). For example, a suitable co-stimulatory domain can include a domain with at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a stretch of at least 10, 15, 20, or all of the amino acids in the following amino acid sequence: RSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRS (SEQ ID NO:2). In some of these embodiments, the co-stimulatory domain has a length of from about 30 aa to about 35 aa, from about 35 aa to about 40 aa, from about 40 aa to about 45 aa, from about 45 aa to about 50 aa, from about 50 aa to about 55 aa, from about 55 aa to about 60 aa, from about 60 aa to about 65 aa, or from about 65 aa to about 70 aa.

[265] In some cases, the co-stimulatory domain is derived from an intracellular portion of the transmembrane protein CD28 deleted for Lck binding (ICΔ). For example, a suitable co-stimulatory domain can include a domain with at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a stretch of at least 10, 15, 20, or all of the amino acids in the following amino acid sequence: RSKRSRLLHSDYMNMTPRRPGPTRKHYQAYAAARDFAAYRS (SEQ ID NO:3). In some of these embodiments, the co-stimulatory domain has a length of from about 30

aa to about 35 aa, from about 35 aa to about 40 aa, from about 40 aa to about 45 aa, from about 45 aa to about 50 aa, from about 50 aa to about 55 aa, from about 55 aa to about 60 aa, from about 60 aa to about 65 aa, or from about 65 aa to about 70 aa.

[266] In some cases, the co-stimulatory domain is derived from an intracellular portion of the transmembrane protein ICOS (also known as AILIM, CD278, and CVIDl). For example, a suitable co-stimulatory domain can include a domain with at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a stretch of at least 10, 15, 20, or all of the amino acids in the following amino acid sequence: TKKKYSSSVHDPNGEYMFMRAVNTAKKSRLTDVTL (SEQ ID NO:4). In some of these embodiments, the co-stimulatory domain has a length of from about 30 aa to about 35 aa, from about 35 aa to about 40 aa, from about 40 aa to about 45 aa, from about 45 aa to about 50 aa, from about 50 aa to about 55 aa, from about 55 aa to about 60 aa, from about 60 aa to about 65 aa, or from about 65 aa to about 70 aa.

[267] In some cases, the co-stimulatory domain is derived from an intracellular portion of the transmembrane protein OX40 (also known as TNFRSF4, RP5-902P8.3, ACT35, CD134, OX-40, TXGPlL). For example, a suitable co-stimulatory domain can include a domain with at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a stretch of at least 10, 15, 20, or all of the amino acids in the following amino acid sequence: RRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKI (SEQ ID NO:5). In some of these embodiments, the co-stimulatory domain has a length of from about 30 aa to about 35 aa, from about 35 aa to about 40 aa, from about 40 aa to about 45 aa, from about 45 aa to about 50 aa, from about 50 aa to about 55 aa, from about 55 aa to about 60 aa, from about 60 aa to about 65 aa, or from about 65 aa to about 70 aa.

[268] In some cases, the co-stimulatory domain is derived from an intracellular portion of the transmembrane protein CD27 (also known as S 152, T 14, TNFRSF7, and Tp55). For example, a suitable co-stimulatory domain can include a domain with at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a stretch of at least 10, 15, 20, or all of the amino acids in the following amino acid sequence: HQRRKYRSNKGESPVEPAEPCRYSCPREEEGSTIPIQEDYRKPEPACSP (SEQ ID NO:6). In some of these embodiments, the co-stimulatory domain has a length of from about 30 aa to about 35 aa, from about 35 aa to about 40 aa, from about 40 aa to about 45 aa, from about 45 aa to about 50 aa, from about

[269] In some cases, the co-stimulatory domain is derived from an intracellular portion of the transmembrane protein BTLA (also known as BTLAl and CD272). For example, a suitable co-stimulatory domain can include a domain with at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a stretch of at least 10, 15, 20, or all of the amino

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acids in the following amino acid sequence:
CCLRRHQGKQNELSDTAGREINLVDAHLKSEQTEASTRQNSQVLLSETGIYDNDPDLCFRMQEG
SEVYSNPCLEENKPGIVYASLNHSVIGPNSRLARNVKEAPTEYASICVRS (SEQ ID NO:7).

[270] In some cases, the co-stimulatory domain is derived from an intracellular portion of the transmembrane protein CD30 (also known as TNFRSF8, DlS166E, and Ki-1). For example, a suitable co-stimulatory domain can include a domain with at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a stretch of from about 100 amino acids to about 110 amino acids (aa), from about 110 aa to about 115 aa, from about 115 aa to about 120 aa, from about 120 aa to about 130 aa, from about 130 aa to about 140 aa, from about 140 aa to about 150 aa, from about 150 aa to about 160 aa, or from about 160 aa to about 185 aa of the following amino acid sequence:
RRACRKRIRQKLHLCYPVQTSQPKLELVDSRPRRSSTQLRSGASVTEPVAEERGLMSQPLMETCH
SVGAAYLESLPLQDASPAGGPSSPRDLPEPRVSTEHTNNKIEKIYIMKADTVIVGTVKAELPEGRG
LAGPAEPELEEELEADHTPHYPEQETEPPLGSCSDVMLSVEEEGKEDPLPTAASGK (SEQ ID NO:8).

[271] In some cases, the co-stimulatory domain is derived from an intracellular portion of the transmembrane protein GITR (also known as TNFRSF18, RP5-902P8.2, AITR, CD357, and GITR-D). For example, a suitable co-stimulatory domain can include a domain with at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a stretch of at least 10, 15, 20, or all of the amino acids in the following amino acid sequence:
HIWQLRSQCMWPRETQLLLEVPPSTEDARSCQFPEEERGERSAEEKGRLGDLWV (SEQ ID NO:9). In some of these embodiments, the co-stimulatory domain has a length of from about 30 aa to about 35 aa, from about 35 aa to about 40 aa, from about 40 aa to about 45 aa, from about 45 aa to about 50 aa, from about 50 aa to about 55 aa, from about 55 aa to about 60 aa, from about 60 aa to about 65 aa, or from about 65 aa to about 70 aa.

[272] In some cases, the co-stimulatory domain derived from an intracellular portion of the transmembrane protein HVEM (also known as TNFRSF14, RP3-395M20.6, ATAR, [SEP] CD270, HVEA, HVEM, LIGHTR, and TR2). For example, a suitable co-stimulatory domain can include a domain with at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to a stretch of at least 10, 15, 20, or all of the amino acids in the following amino acid sequence:
CVKRRKPRGDVVKVIVSVQRKRQEAEGEATVIEALQAPPDVTTVAVEETIPSFTGRSPNH (SEQ ID NO:10). In some of these embodiments, the co-stimulatory domain of both the first and the second polypeptide has a length of from about 30 aa to about 35 aa, from about 35 aa to about 40 aa, from about 40 aa to about 45 aa, from about 45 aa to about 50 aa, from about 50 aa to about 55 aa, from about 55 aa to about 60 aa, from about 60 aa to about 65 aa, or from about 65 aa to about 70 aa.

Linker

[273] In some cases, the engineered signaling polypeptide includes a linker between any two adjacent domains. For example, a linker can be between the transmembrane domain and the first co-stimulatory domain. As another example, the ASTR can be an antibody and a linker can be between the heavy chain and the light chain. As another example, a linker can be between the ASTR and the transmembrane domain and a co-stimulatory domain. As another example, a linker can be between the co-stimulatory domain and the intracellular activating domain of the second polypeptide. As another example, the linker can be between the ASTR and the intracellular signaling domain.

[274] The linker peptide may have any of a variety of amino acid sequences. Proteins can be joined by a spacer peptide, generally of a flexible nature, although other chemical linkages are not excluded. A linker can be a peptide of between about 1 and about 100 amino acids in length, or between about 1 and about 25 amino acids in length. These linkers can be produced by using synthetic, linker-encoding oligonucleotides to couple the proteins. Peptide linkers with a degree of flexibility can be used. The linking peptides may have virtually any amino acid sequence, bearing in mind that suitable linkers will have a sequence that results in a generally flexible peptide. The use of small amino acids, such as glycine and alanine, are of use in creating a flexible peptide. The creation of such sequences is routine to those of skill in the art.

[275] Suitable linkers can be readily selected and can be of any of a suitable of different lengths, such as from 1 amino acid (e.g., Gly) to 20 amino acids, from 2 amino acids to 15 amino acids, from 3 amino acids to 12 amino acids, including 4 amino acids to 10 amino acids, 5 amino acids to 9 amino acids, 6 amino acids to 8 amino acids, or 7 amino acids to 8 amino acids, and may be 1, 2, 3, 4, 5, 6, or 7 amino acids.

[276] Exemplary flexible linkers include glycine polymers $(G)_n$, glycine-serine polymers (including, for example, $(GS)_n$, $GSGGS_n$, $GGGS_n$, and $GGGGS_n$ where n is an integer of at least one), glycine-alanine polymers, alanine-serine polymers, and other flexible linkers known in the art. Glycine and glycine-serine polymers are of interest since both of these amino acids are relatively unstructured, and therefore may serve as a neutral tether between components. Glycine polymers are of particular interest since glycine accesses significantly more phi-psi space than even alanine, and is much less restricted than residues with longer side chains (see Scheraga, *Rev. Computational Chem.* 11173-142 (1992)). Exemplary flexible linkers include, but are not limited GGGGSGGGGSGGGGS (SEQ ID NO:53), GGGGSGGGGSGGGGSGGGGSGGGGSGGGGS (SEQ ID NO:54), GGGGSGGGGSGGGGS (SEQ ID NO:55), GGSG (SEQ ID NO:56), GGSGG (SEQ ID NO:57), GSGSG (SEQ ID NO:58), GSGGG (SEQ ID NO:59), GGGSG (SEQ ID NO:60), GSSSG (SEQ ID NO:61), and the like. The ordinarily skilled

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artisan will recognize that design of a peptide conjugated to any elements described above can include linkers that are all or partially flexible, such that the linker can include a flexible linker as well as one or more portions that confer less flexible structure.

Chimeric antigen receptor

[277] In some aspects of the present invention, an engineered signaling polypeptide is a chimeric antigen receptor (CAR) or a polynucleotide encoding a CAR, which, for simplicity, is referred to herein as "CAR." In some embodiments, a CAR of the present disclosure includes: a) at least one antigen-specific targeting region (ASTR) ; b) a transmembrane domain; and c) an intracellular activating domain. In illustrative embodiments, the antigen-specific targeting region of the CAR is a scFv portion of an antibody to the target antigen.

[278] A CAR of the present disclosure can be present in the plasma membrane of a eukaryotic cell, e.g., a mammalian cell, where suitable mammalian cells include, but are not limited to, a cytotoxic cell, a T lymphocyte, a stem cell, a progeny of a stem cell, a progenitor cell, a progeny of a progenitor cell, and an NK cell, an NK-T cell, and a macrophage. When present in the plasma membrane of a eukaryotic cell, a CAR of the present disclosure is active in the presence of one or more target antigens that, in certain conditions, binds the ASTR. The target antigen is the second member of the specific binding pair. The target antigen of the specific binding pair can be a soluble (e.g., not bound to a cell) factor; a factor present on the surface of a cell such as a target cell; a factor presented on a solid surface; a factor present in a lipid bilayer; and the like. Where the ASTR is an antibody, and the second member of the specific binding pair is an antigen, the antigen can be a soluble (e.g., not bound to a cell) antigen; an antigen present on the surface of a cell such as a target cell; an antigen presented on a solid surface; an antigen present in a lipid bilayer; and the like.

[279] In some instances, a CAR of the present disclosure, when present in the plasma membrane of a eukaryotic cell, and when activated by one or more target antigens, increases expression of at least one nucleic acid in the cell. For example, in some cases, a CAR of the present disclosure, when present in the plasma membrane of a eukaryotic cell, and when activated by the one or more target antigens, increases expression of at least one nucleic acid in the cell by at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 40%, at least about 50%, at least about 75%, at least about 2-fold, at least about 2.5-fold, at least about 5-fold, at least about 10-fold, or more than 10-fold, compared with the level of transcription of the nucleic acid in the absence of the one or more target antigens.

[280] As an example, the CAR of the present disclosure can include an immunoreceptor tyrosine-based activation motif (ITAM)-containing intracellular signaling polypeptide.

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[281] A CAR of the present disclosure, when present in the plasma membrane of a eukaryotic cell, and when activated by one or more target antigens, can, in some instances, result in increased production of one or more cytokines by the cell. For example, a CAR of the present disclosure, when present in the plasma membrane of a eukaryotic cell, and when activated by the one or more target antigens, can increase production of a cytokine by the cell by at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 40%, at least about 50%, at least about 75%, at least about 2-fold, at least about 2.5-fold, at least about 5-fold, at least about 10-fold, or more than 10-fold, compared with the amount of cytokine produced by the cell in the absence of the one or more target antigens. Cytokines whose production can be increased include, but are not limited to interferon gamma (IFN-γ), tumor necrosis factor-alpha (TNF-a), IL-2, IL-15, IL-12, IL-4, IL-5, IL-10; a chemokine; a growth factor; and the like.

[282] In some cases, a CAR of the present disclosure, when present in the plasma membrane of a eukaryotic cell, and when activated by one or more target antigens, can result in both an increase in transcription of a nucleic acid in the cell and an increase in production of a cytokine by the cell.

[283] In some instances, a CAR of the present disclosure, when present in the plasma membrane of a eukaryotic cell, and when activated by one or more target antigens, results in cytotoxic activity by the cell toward a target cell that expresses on its cell surface an antigen to which the antigen-binding domain of the first polypeptide of the CAR binds. For example, where the eukaryotic cell is a cytotoxic cell (e.g., an NK cell or a cytotoxic T lymphocyte), a CAR of the present disclosure, when present in the plasma membrane of the cell, and when activated by the one or more target antigens, increases cytotoxic activity of the cell toward a target cell that expresses on its cell surface the one or more target antigens. For example, where the eukaryotic cell is an NK cell or a T lymphocyte, a CAR of the present disclosure, when present in the plasma membrane of the cell, and when activated by the one or more target antigens, increases cytotoxic activity of the cell by at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 40%, at least about 50%, at least about 75%, at least about 2-fold, at least about 2.5-fold, at least about 5-fold, at least about 10-fold, or more than 10-fold, compared to the cytotoxic activity of the cell in the absence of the one or more target antigens.

[284] In some cases, a CAR of the present disclosure, when present in the plasma membrane of a eukaryotic cell, and when activated by one or more target antigens, can result in other CAR activation related events such as proliferation and expansion (either due to increased cellular division or anti-apoptotic responses).

[285] In some cases, a CAR of the present disclosure, when present in the plasma membrane of a eukaryotic cell, and when activated by one or more target antigens, can result in other CAR activation related events such as intracellular signaling modulation, cellular differentiation, or cell death.

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[286] A CAR of the present disclosure can be present in a eukaryotic cell membrane, where the first and second polypeptides of the CAR are not covalently linked to one another. A CAR of the present disclosure can be present in a eukaryotic cell membrane as a single heterodimer that is not covalently linked to any other polypeptide in the membrane. Alternatively, a first CAR of the present disclosure can be present in a eukaryotic cell membrane as a heterodimer that is covalently or non-covalently linked to a second CAR of the present disclosure. In some cases, the first and the second CAR are covalently linked via a disulfide bond formed between cysteines present in a stalk present in both the first polypeptide of the first CAR and the first polypeptide of the second CAR.

[287] In some cases, a CAR of the present disclosure can be present in a eukaryotic cell membrane, where the first polypeptides of the CAR include an antibody fragment and the second polypeptides of the CAR include a signal transducing domain derived from a cytokine receptor, such that, upon dimerization, the CAR may represent a heterodimeric-signalobody CAR, e.g., a signalobody composed of at least two independent polypeptides. A "signalobody", as it is known in the art, is a single chimeric macromolecule composed of an antibody fragment and a signal transduction domain derived from a cytokine receptor. In certain instances, a heterodimeric-signalobody CAR of the present disclosure, when present in the cell membrane of a eukaryotic cell, dimerized by a dimerizer, and activated by an antigen, e.g., an oligomerized antigen, may induce the oligomerization of the heterodimeric-signalobody CAR. Such ligand-induced oligomerization of a heterodimeric-signalobody CAR may activate, e.g., increase, or perpetuate, e.g., maintain, signal transduction, e.g., ligand-induced oligomerization of a heterodimeric-signalobody CAR may transmit a signal eliciting a cellular response. In some instances, a plurality of heterodimeric-signalobody CARs may be utilized combinatorially to elicit a desired cellular response.

[288] In some embodiments, CARs of the present disclosure are microenvironment restricted. This property is typically the result of the microenvironment restricted nature of the ASTR domain of the CAR. Thus, CARs of the present disclosure can have a lower binding affinity or, in illustrative embodiments, can have a higher binding affinity to one or more target antigens under a condition(s) in a microenvironment than under a condition in a normal physiological environment.

Recombination of sequences

[289] In certain instances, sequences of the engineered signaling polypeptides, which can be referred to herein as recombinant polypeptides, may be rearranged or deleted in a cell through the use of site-specific recombination technology. In certain embodiments, the cellular activation-related response to a particular engineered signaling polypeptide can be changed by site-specific recombination, e.g., a first intracellular activating domain of an engineered signaling polypeptide eliciting a first activation-related response may be exchanged for a second intracellular activating domain eliciting a second activation-related response.

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As will be clear to one skilled in the art, site-specific recombination can be used in a cell to exchange any domain or sequence of an engineered signaling polypeptide with any other domain or sequence as disclosed herein. As will also be clear to one skilled in the art, site-specific recombination can be used in a cell to delete any domain or sequence of an engineered signaling polypeptide. Such exchange and excision of sequences and domains is known in the art, see, e.g., domain switching in signalobodies as described in Tone et al. (2013) *Biotechnology and Bioengineering,* 3219-3226, the disclosure of which is disclosed herein by reference. Mechanisms and requirements for performing site-specific recombination *in vivo* are also well known in the art, see, e.g., Grindley et al. (2006) *Annual Review of Biochemistry,* 567-605 and Tropp (2012) Molecular Biology (Jones & Bartlett Publishers, Sudbury, MA), the disclosures of which are incorporated herein by reference.

[290] In some embodiments, the engineered signaling polypeptides are generated by fusing all the different domains discussed above together to form a fusion protein. The engineered signaling polypeptide is typically generated by a transcriptional unit comprising polynucleotide sequences that encode the different domains of the engineered signaling polypeptides as discussed herein. In some embodiments, the ASTR of the present invention, which functions to recognize and bind with an antigen on target cells, is microenvironment restricted.

[291] The wild-type or native protein that is suitable to be used in whole or in part for at least its binding domain for the target antigen, as an ASTR in the present invention may be discovered by generating a protein library and screening the library for a protein with a desired binding affinity to the target antigen. The wild-type protein may be discovered by screening a cDNA library. A cDNA library is a combination of cloned cDNA (complementary DNA) fragments inserted into a collection of host cells, which together constitute some portion of the transcriptome of the organism. cDNA is produced from fully transcribed mRNA and therefore contains the coding sequence for expressed proteins of an organism. The information in cDNA libraries is a powerful and useful tool for discovery of proteins with desired properties by screening the libraries for proteins with the desired binding affinity to the target antigen.

Combinations

In some embodiments, a polynucleotide provided by the recombinant retroviruses has one or more transcriptional units that encode certain combinations of the one or more engineered signaling polypeptides. In some methods and compositions provided herein, genetically modified T cells include the combinations of the one or more engineered signaling polypeptides after transduction of T cells by the recombinant retroviruses. It will be understood that the reference of a first polypeptide, a second polypeptide, a third polypeptide, etc. is for convenience and elements on a "first polypeptide" and those

on a "second polypeptide" means that the elements are on different polypeptides that are referenced as first or second for reference and convention only, typically in further elements or steps to that specific polypeptide.

[292] In some embodiments, the first engineered signaling polypeptide includes an extracellular antigen binding domain, which is capable of binding an antigen, and an intracellular signaling domain. In other embodiments, the first engineered signaling polypeptide also includes a T cell survival motif and/or a transmembrane domain. In some embodiments, the first engineered signaling polypeptide does not include a co-stimulatory domain, while in other embodiments, the first engineered signaling polypeptide does include a co-stimulatory domain.

[293] In some embodiments, a second engineered signaling polypeptide includes a lymphoproliferative gene product and optionally an extracellular antigen binding domain. In some embodiments, the second engineered signaling polypeptide also includes one or more of the following: a T cell survival motif, an intracellular signaling domain, and one or more co-stimulatory domains. In other embodiments, when two engineered signaling polypeptides are used, at least one is a CAR.

[294] In one embodiment, the one or more engineered signaling polypeptides are expressed under a T cell specific promoter or a general promoter under the same transcript wherein in the transcript, nucleic acids encoding the engineered signaling polypeptides are separated by nucleic acids that encode one or more internal ribosomal entry sites (IREs) or one or more protease cleavage peptides.

[295] In certain embodiments, the polynucleotide encodes two engineered signaling polypeptides wherein the first engineered signaling polypeptide includes a first extracellular antigen binding domain, which is capable of binding to a first antigen, and a first intracellular signaling domain but not a co-stimulatory domain, and the second polypeptide includes a second extracellular antigen binding domain, which is capable of binding VEGF, and a second intracellular signaling domain, such as for example, the signaling domain of a co-stimulatory molecule. In a certain embodiment, the first antigen is PSCA, PSMA, or BCMA. In a certain embodiment, the first extracellular antigen binding domain comprises an antibody or fragment thereof (e.g., scFv), e.g., an antibody or fragment thereof specific to PSCA, PSMA, or BCMA. In a certain embodiment, the second extracellular antigen binding domain that binds VEGF is a receptor for VEGF, i.e., VEGFR. In certain embodiments, the VEGFR is VEGFR1, VEGFR2, or VEGFR3. In a certain embodiment, the VEGFR is VEGFR2.

[296] In certain embodiments, the polynucleotide encodes two engineered signaling polypeptides wherein the first engineered signaling polypeptide includes an extracellular tumor antigen binding domain and a CD3ζ signaling domain, and the second engineered signaling polypeptide includes an antigen-binding domain, wherein the antigen is an angiogenic or vasculogenic factor, and one or more co-stimulatory molecule signaling domains. The angiogenic factor can be, e.g., VEGF. The one or more co-

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stimulatory molecule signaling motifs can comprise, e.g., co-stimulatory signaling domains from each of CD27, CD28, OX40, ICOS, and 4-1BB.

[297] In certain embodiments, the polynucleotide encodes two engineered signaling polypeptides wherein the first engineered signaling polypeptide includes an extracellular tumor antigen-binding domain and a CD3ζ signaling domain, the second polypeptide comprises an antigen-binding domain, which is capable of binding to VEGF, and co-stimulatory signaling domains from each of CD27, CD28, OX40, ICOS, and 4-1BB. In a further embodiment, the first signaling polypeptide or second signaling polypeptide also has a T cell survival motif. In some embodiments, the T cell survival motif is, or is derived from, an intracellular signaling domain of IL-7 receptor (IL-7R), an intracellular signaling domain of IL-12 receptor, an intracellular signaling domain of IL-15 receptor, an intracellular signaling domain of IL-21 receptor, or an intracellular signaling domain of transforming growth factor β (TGFβ) receptor or the TGFβ decoy receptor (TGF-β—dominant-negative receptor II (DNRII)).

[298] In certain embodiments, the polynucleotide encodes two engineered signaling polypeptides wherein the first engineered signaling polypeptide includes an extracellular tumor antigen-binding domain and a CD3ζ signaling domain, and the second engineered signaling polypeptide includes an antigen-binding domain, which is capable of binding to VEGF, an IL-7 receptor intracellular T cell survival motif, and co-stimulatory signaling domains from each of CD27, CD28, OX40, ICOS, and 4-1BB.

[299] In some embodiments, more than two signaling polypeptides are encoded by the polynucleotide. In certain embodiments, only one of the engineered signaling polypeptides includes an antigen binding domain that binds to a tumor-associated antigen or a tumor-specific antigen; each of the remainder of the engineered signaling polypeptides comprises an antigen binding domain that binds to an antigen that is not a tumor-associated antigen or a tumor-specific antigen. In other embodiments, two or more of the engineered signaling polypeptides include antigen binding domains that bind to one or more tumor-associated antigens or tumor-specific antigens, wherein at least one of the engineered signaling polypeptides comprises an antigen binding domain that does not bind to a tumor-associated antigen or a tumor-specific antigen.

[300] In some embodiments, the tumor-associated antigen or tumor-specific antigen is Her2, prostate stem cell antigen (PSCA), PSMA (prostate-specific membrane antigen), B cell maturation antigen (BCMA), alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA), cancer antigen-125 (CA-125), CA19-9, calretinin, MUC-1, epithelial membrane protein (EMA), epithelial tumor antigen (ETA), tyrosinase, melanoma-associated antigen (MAGE), CD34, CD45, CD99, CD117, chromogranin, cytokeratin, desmin, glial fibrillary acidic protein (GFAP), gross cystic disease fluid protein (GCDFP-15), HMB-45 antigen, protein melan-A (melanoma antigen recognized by T lymphocytes; MART-1), myo-

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D1, muscle-specific actin (MSA), neurofilament, neuron-specific enolase (NSE), placental alkaline phosphatase, synaptophysin, thyroglobulin, thyroid transcription factor-1, the dimeric form of the pyruvate kinase isoenzyme type M2 (tumor M2-PK), CD19, CD22, CD27, CD30, CD70, GD2 (ganglioside G2), EphA2, CSPG4, CD138, FAP (Fibroblast Activation Protein), CD171, kappa, lambda, 5T4, αvβ6 integrin, integrin αvβ3 (CD61), galactin, K-Ras (V-Ki-ras2 Kirsten rat sarcoma viral oncogene), Ral-B, B7-H3, B7-H6, CAIX, CD20, CD33, CD44, CD44v6, CD44v7/8, CD123, EGFR, EGP2, EGP40, EpCAM, fetal AchR, FRα, GD3, HLA-A1+MAGE1, HLA-A1+NY-ESO-1, IL-11Rα, IL-13Rα2, Lewis-Y, Muc16, NCAM, NKG2D Ligands, NY-ESO-1, PRAME, ROR1, Survivin, TAG72, TEMs, VEGFR2, EGFRvIII (epidermal growth factor variant III), sperm protein 17 (Sp17), mesothelin, PAP (prostatic acid phosphatase), prostein, TARP (T cell receptor gamma alternate reading frame protein), Trp-p8, STEAP1 (six-transmembrane epithelial antigen of the prostate 1), an abnormal ras protein, or an abnormal p53 protein.

[301] In some embodiments, the first engineered signaling polypeptide includes a first extracellular antigen binding domain that binds a first antigen, and a first intracellular signaling domain; and a second engineered signaling polypeptide includes a second extracellular antigen binding domain that binds a second antigen, or a receptor that binds the second antigen; and a second intracellular signaling domain, wherein the second engineered signaling polypeptide does not comprise a co-stimulatory domain. In a certain embodiment, the first antigen-binding domain and the second antigen-binding domain are independently an antigen-binding portion of a receptor or an antigen-binding portion of an antibody. In a certain embodiment, either or both of the first antigen binding domain or the second antigen binding domain are scFv antibody fragments. In certain embodiments, the first engineered signaling polypeptide and/or the second engineered signaling polypeptide additionally comprises a transmembrane domain. In a certain embodiment, the first engineered signaling polypeptide or the second engineered signaling polypeptide comprises a T cell survival motif, e.g., any of the T cell survival motifs described herein.

[302] In another embodiment, the first engineered signaling polypeptide includes a first extracellular antigen binding domain that binds HER2 and the second engineered signaling polypeptide includes a second extracellular antigen binding domain that binds MUC-1.

[303] In another embodiment, the second extracellular antigen binding domain of the second engineered signaling polypeptide binds an interleukin.

[304] In another embodiment, the second extracellular antigen binding domain of the second engineered signaling polypeptide binds a damage associated molecular pattern molecule (DAMP; also known as an alarmin). In other embodiments, a DAMP is a heat shock protein, chromatin-associated protein high mobility group box 1 (HMGB1), S100A8 (also known as MRP8, or calgranulin A), S100A9

(also known as MRP14, or calgranulin B), serum amyloid A (SAA), deoxyribonucleic acid, adenosine triphosphate, uric acid, or heparin sulfate.

[305] In certain embodiments, said second antigen is an antigen on an antibody that binds to an antigen presented by a tumor cell.

[306] In some embodiments, signal transduction activation through the second engineered signaling polypeptide is non-antigenic, but is associated with hypoxia. In certain embodiments, hypoxia is induced by activation of hypoxia-inducible factor-1α (HIF-1α), HIF-1β, HIF-2α, HIF-2β, HIF-3α, or HIF-3β.

[307] In some embodiments, expression of the one or more engineered signaling polypeptides is regulated by an *in vivo* control element, which is disclosed in more detail herein.

Additional sequences

[308] The engineered signaling polypeptides, such as CARs, can further include one or more additional polypeptide domains, where such domains include, but are not limited to, a signal sequence; an epitope tag; an affinity domain; and a polypeptide that produces a detectable signal. Non-limiting examples of additional domains for any of the aspects or embodiments provided herein, include a domain with at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99% or 100% sequence identity to any of the following sequences as described below: a signal sequence, an epitope tag, an affinity domain, or a polypeptide that produces a detectable signal.

[309] Signal sequences that are suitable for use in a subject CAR, e.g., in the first polypeptide of a subject CAR, include any eukaryotic signal sequence, including a naturally-occurring signal sequence, a synthetic (e.g., man-made) signal sequence, etc. In some embodiments, for example, the signal sequence can be the CD8 signal sequence MALPVTALLLPLALLLHAARP (SEQ ID NO:74).

[310] Suitable epitope tags include, but are not limited to, hemagglutinin (HA; e.g., YPYDVPDYA; SEQ ID NO:37); FLAG (e.g.,DYKDDDDK; SEQ ID NO:38); c-myc (e.g., EQKLISEEDL; SEQ ID NO:39), and the like.

[311] Affinity domains include peptide sequences that can interact with a binding partner, e.g., such as one immobilized on a solid support, useful for identification or purification. DNA sequences encoding multiple consecutive single amino acids, such as histidine, when fused to the expressed protein, may be used for one-step purification of the recombinant protein by high affinity binding to a resin column, such as nickel sepharose. Exemplary affinity domains include His5 (HHHHH; SEQ ID NO:40), HisX6 (HHHHHH; SEQ ID NO:41), c-myc (EQKLISEEDL; SEQ ID NO:39), Flag (DYKDDDDK; SEQ ID NO:38), Strep Tag (WSHPQFEK; SEQ ID NO:42), hemagglutinin, e.g., HA Tag (YPYDVPDYA; SEQ ID NO:37), GST, thioredoxin, cellulose binding domain, RYIRS (SEQ ID NO:43), Phe-His-His-Thr (SEQ ID NO:44), chitin binding domain, S-peptide, T7 peptide, SH2 domain, C-end RNA tag,

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WEAAAREACCRECCARA (SEQ ID NO:45), metal binding domains, e.g., zinc binding domains or calcium binding domains such as those from calcium-binding proteins, e.g., calmodulin, troponin C, calcineurin B, myosin light chain, recoverin, S-modulin, visinin, VILIP, neurocalcin, hippocalcin, frequenin, caltractin, calpain large-subunit, S100proteins, parvalbumin, calbindin D9K, calbindin D28K, and calretinin, inteins, biotin, streptavidin, MyoD, Id, leucine zipper sequences, and maltose binding protein.

[312] Suitable detectable signal-producing proteins include, e.g., fluorescent proteins; enzymes that catalyze a reaction that generates a detectable signal as a product; and the like.

[313] Suitable fluorescent proteins include, but are not limited to, green fluorescent protein (GFP) or variants thereof, blue fluorescent variant of GFP (BFP), cyan fluorescent variant of GFP (CFP), yellow fluorescent variant of GFP (YFP), enhanced GFP (EGFP), enhanced CFP (ECFP), enhanced YFP (EYFP), GFPS65T, Emerald, Topaz (TYFP), Venus, Citrine, mCitrine, GFPuv, destabilized EGFP (dEGFP), destabilized ECFP (dECFP), destabilized EYFP (dEYFP), mCFPm, Cerulean, T-Sapphire, CyPet, YPet, mKO, HcRed, t-HcRed, DsRed, DsRed2, DsRed-monomer, J-Red, dimer2, t-dimer2(12), mRFPl, pocilloporin, Renilla GFP, Monster GFP, paGFP, Kaede protein and kindling protein, Phycobiliproteins and Phycobiliprotein conjugates including B-Phycoerythrin, R-Phycoerythrin and Allophycocyanin. Other examples of fluorescent proteins include mHoneydew, mBanana, mOrange, dTomato, tdTomato, mTangerine, mStrawberry, mCherry, mGrapel, mRaspberry, mGrape2, mPlum (Shaner et al. (2005) *Nat. Methods* 2:905-909), and the like. Any of a variety of fluorescent and colored proteins from Anthozoan species, as described in, e.g., Matz et al. (1999) *Nature Biotechnol.* 17:969-973, is suitable for use.

[314] Suitable enzymes include, but are not limited to, horse radish peroxidase (HRP), alkaline phosphatase (AP), beta-galactosidase (GAL), glucose-6-phosphate dehydrogenase, beta-N-acetylglucosaminidase, β-glucuronidase, invertase, Xanthine Oxidase, firefly luciferase, glucose oxidase (GO), and the like.

Recognition and/or elimination domain

[315] Any of the recombinant retroviruses provided herein can include nucleic acids that encode a recognition or elimination domain as part of, or separate from, nucleic acids encoding any of the engineered signaling polypeptides provided herein. Thus, any of the engineered signaling polypeptides provided herein, can include a recognition or elimination domain. For example, any of the CARs disclosed herein can include a recognition or elimination domain. Moreover, a recognition or elimination domain can be expressed together with, or even fused with any of the lymphoproliferative elements

disclosed herein. The recognition or elimination domains are expressed on the T cell and/or NK cell but are not expressed on the retrovirus.

[316] In some embodiments, the recognition or elimination domain can be derived from herpes simplex virus–derived enzyme thymidine kinase (HSV-tk) or inducible caspase-9. In some embodiments, the recognition or elimination domain can include a modified endogenous cell-surface molecule, for example as disclosed in U.S. Patent 8,802,374. The modified endogenous cell-surface molecule can be any cell-surface related receptor, ligand, glycoprotein, cell adhesion molecule, antigen, integrin, or cluster of differentiation (CD) that is modified. In some embodiments, the modified endogenous cell-surface molecule is a truncated tyrosine kinase receptor. In one aspect, the truncated tyrosine kinase receptor is a member of the epidermal growth factor receptor (EGFR) family (e.g., ErbB1, ErbB2, ErbB3, ErbB4. In some embodiments, the recognition domain can be a polypeptide that is recognized by an antibody that recognizes the extracellular domain of an EGFR member. In some embodiments, the recognition domain can be at least 20 contiguous amino acids of an EGFR family member, or for example, between 20 and 50 contiguous amino acids of an EGFR family member. For example, SEQ ID NO:78, is an exemplary polypeptide that is recognized by, and under the appropriate conditions bound by an antibody that recognizes the extracellular domain of an EGFR member. Such extracellular EGFR epitopes are sometimes referred to herein as eTags. In illustrative embodiments, such epitopes are recognized by commercially available anti-EGFR monoclonal antibodies.

[317] Epidermal growth factor receptor, also known as EGFR, ErbB1 and HER1, is a cell-surface receptor for members of the epidermal growth factor family of extracellular ligands. Alterations in EGFR activity have been implicated in certain cancers. In some embodiments, a gene encoding an EGFR polypeptide including human epidermal growth factor receptor (EGFR) is constructed by removal of nucleic acid sequences that encode polypeptides including the membrane distal EGF-binding domain and the cytoplasmic signaling tail, but retains the extracellular membrane proximal epitope recognized by an anti-EGFR antibody. Preferably, the antibody is a known, commercially available anti-EGFR monoclonal antibody, such as cetuximab, matuzumab, necitumumab or panitumumab.

[318] Others have shown that application of biotinylated-cetuximab to immunomagnetic selection in combination with anti-biotin microbeads successfully enriches T cells that have been lentivirally transduced with EGFRt-containing constructs from as low as 2% of the population to greater than 90% purity without observable toxicity to the cell preparation. Furthermore, others have shown that constitutive expression of this inert EGFR molecule does not affect T cell phenotype or effector function as directed by the coordinately expressed chimeric antigen receptor (CAR), CD19R. In addition, others have shown that through flow cytometric analysis, EGFR was successfully utilized as an *in vivo* tracking marker for T cell engraftment in mice. Furthermore, EGFR was demonstrated to have suicide gene

potential through Erbitux® mediated antibody dependent cellular cytotoxicity (ADCC) pathways. The inventors of the present disclosure have successfully expressed eTag in PBMCs using lentiviral vectors, and have found that expression of eTag *in vitro* by PBMCs exposed to Cetuximab, provided an effective elimination mechanism for PBMCs. Thus, EGFR may be used as a non-immunogenic selection tool, tracking marker, and suicide gene for transduced T cells that have immunotherapeutic potential. The EGFR nucleic acid may also be detected by means well known in the art.

[319] In some embodiments provided herein, EGFR is expressed as part of a single polypeptide that also includes the CAR or as part of a single polypeptide that includes the lymphoproliferative element. In some embodiments, the amino acid sequence encoding the EGFR recognition domain can be separated from the amino acid sequence encoding the chimeric antigen receptor by a cleavage signal and/or a ribosomal skip sequence. The ribosomal skip and/or cleavage signal can be any ribosomal skip and/or cleavage signal known in the art. Not to be limited by theory, the ribosomal skip sequence can be, for example 2A-1 with amino acid sequence GSGEGRGSLLTCGDVEENPGP (SEQ ID NO:77). Not to be limited by theory, other examples of cleavage signals and ribosomal skip sequences include FMDV 2A (F2A); equine rhinitis A virus 2A (abbreviated as E2A); porcine teschovirus-1 2A (P2A); and *Thoseaasigna* virus 2A (T2A). In some embodiments, the polynucleotide sequence encoding the recognition domain can be on the same transcript as the CAR or lymphoproliferative element but separated from the polynucleotide sequence encoding the CAR or lymphoproliferative element by an internal ribosome entry site.

[320] In other embodiments as exemplified empirically herein, a recognition domain can be expressed as part of a fusion polypeptide, fused to a lymphoproliferative element. Such constructs provide the advantage, especially in combination with other "space saving" elements provided herein, of taking up less genomic space on an RNA genome compared to separate polypeptides. In one illustrative embodiment, an eTag is expressed as a fusion polypeptide, fused to an IL7Rα mutant, as experimentally demonstrated herein.

PSEUDOTYPING ELEMENTS

[321] Many of the methods and compositions provided herein include pseudotyping elements. The pseudotyping of retroviruses with heterologous envelope glycoproteins typically alters the tropism of a virus and facilitates the transduction of host cells. A pseudotyping element as used herein can include a "binding polypeptide" that includes one or more polypeptides, typically glycoproteins, that identify and bind the target host cell, and one or more "fusogenic polypeptides" that mediate fusion of the retroviral and target host cell membranes, thereby allowing a retroviral genome to enter the target host cell. In

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some embodiments provided herein, pseudotyping elements are provided as polypeptide(s)/protein(s), or as nucleic acid sequences encoding the polypeptide(s)/protein(s).

[322] In some embodiments, the pseudotyping element is the feline endogenous virus (RD114) envelope protein, the oncoretroviral amphotropic envelope protein, the oncoretroviral ecotropic envelope protein, the vesicular stomatitis virus (VSV-G) envelope protein, and/or the paramyxovirus Measles envelope proteins H and F.

[323] In some embodiments, the pseudotyping elements include a binding polypeptide and a fusogenic polypeptide derived from different proteins. For example, the recombinant retroviruses of the methods and compositions disclosed herein can be pseudotyped with the fusion (F) and hemagglutinin (H) polypeptides of the measles virus (MV), as non-limiting examples, clinical wildtype strains of MV, and vaccine strains including the Edmonston strain (MV-Edm) or fragments thereof. Not to be limited by theory, both hemagglutinin (H) and fusion (F) polypeptides are believed to play a role in entry into host cells wherein the H protein binds MV to receptors CD46, SLAM, and Nectin-4 on target cells and F mediates fusion of the retroviral and host cell membranes. In an illustrative embodiment, especially where the target cell is a T cell and/or NK cell, the binding polypeptide is a Measles Virus H polypeptide and the fusogenic polypeptide is a Measles Virus F polypeptide.

[324] In some studies, lentiviral particles pseudotyped with truncated F and H polypeptides had a significant increase in titers and transduction efficiency (Funke et al. 2008. *Molecular Therapy.* 16(8):1427-1436), (Frecha et al. 2008. *Blood.* 112(13):4843-4852). The highest titers were obtained when the F cytoplasmic tail was truncated by 30 residues (referred to as MV(Ed)-FΔ30 (SEQ ID NO:105)). For the H variants, optimal truncation occurred when 18 or 19 residues were deleted (MV(Ed)-HΔ18 (SEQ ID NO:106) or MV(Ed)-HΔ19), although variants with a truncation of 24 residues with and without replacement of deleted residues with alanine (MV(Ed)-HΔ24 (SEQ ID NO:235) and MV(Ed)-HΔ24+A) also resulted in optimal titers.

[325] In some embodiments, including those directed to transducing T cells and/or NK cells, the recombinant retroviruses of the methods and compositions disclosed herein are pseudotyped with mutated or variant versions of the measles virus fusion (F) and hemagglutinin (H) polypeptides, in illustrative examples, cytoplasmic domain deletion variants of measles virus F and H polypeptides. In some embodiments, the mutated F and H polypeptides are "truncated H" or "truncated F" polypeptides, whose cytoplasmic portion has been truncated, i.e. amino acid residues (or coding nucleic acids of the corresponding nucleic acid molecule encoding the protein) have been deleted. "HΔY" and "FΔX" designate such truncated H and F polypeptide, respectively, wherein "Y" refers to 1-34 residues that have been deleted from the amino termini and "X" refers to 1-35 residues that have been deleted from the carboxy termini of the cytoplasmic domains. In a further embodiment, the "truncated F polypeptide" is

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FΔ24 or FΔ30 and/or the "truncated H protein" is selected from the group consisting of HΔ14, HΔ15, HΔ16, HΔ17, HΔ18, HΔ19, HΔ20, HΔ21+A, HΔ24 and HΔ24+4A, more preferably HΔ18 or HΔ24. In an illustrative embodiment, the truncated F polypeptide is MV(Ed)-FΔ30 and the truncated H polypeptide is MV(Ed)-HΔ18.

[326] In some embodiments, the fusogenic polypeptide includes multiple elements expressed as one polypeptide. In some embodiments, the binding polypeptide and fusogenic polypeptide are translated from the same transcript but from separate ribosome binding sites; in other embodiments, the binding polypeptide and fusogenic polypeptide are separated by a cleavage peptide site, which not to be bound by theory, is cleaved after translation, as is common in the literature, or a ribosomal skip sequence. In some embodiments, the translation of the binding polypeptide and fusogenic polypeptide from separate ribosome binding sites results in a higher amount of the fusogenic polypeptide as compared to the binding polypeptide. In some embodiments, the ratio of the fusogenic polypeptide to the binding polypeptide is at least 2:1, at least 3:1, at least 4:1, at least 5:1, at least 6:1, at least 7:1, or at least 8:1. In some embodiments, the ratio of the fusogenic polypeptide to the binding polypeptide is between 1.5:1, 2:1, or 3:1, on the low end of the range, and 3:1, 4:1, 5:1, 6:1, 7:1, 8:1. 9:1 or 10:1 on the high end of the range.

ACTIVATION ELEMENTS

[327] Many of the methods and composition aspects of the present disclosure include an activation element, or a nucleic acid encoding an activation element. The restrictions associated with lentiviral (LV) transduction into resting T cells are attributed to a series of pre-entry and post-entry barriers as well as cellular restrictive factors (Strebel et al 2009. *BMC Medicine* 7:48). One restriction is the inability for the envelope pseudotyped-LV particles to recognize potential receptors and mediate fusion with the cellular membrane. However, under certain conditions, the transduction of resting T cells with HIV-1-based lentiviral vectors is possible mostly upon T cell receptor (TCR) CD3 complex and CD28 co-stimulation (Korin & Zack. 1998. *Journal of Virology*. 72:3161-8, Maurice et al. 2002. *Blood* 99:2342-50), as well as through exposure to cytokines (Cavalieri et al 2003).

[328] Cells of the immune system such as T lymphocytes recognize and interact with specific antigens through receptors or receptor complexes which, upon recognition or an interaction with such antigens, cause activation of the cell and expansion in the body. An example of such a receptor is the antigen-specific T lymphocyte receptor complex (TCR/CD3). The T cell receptor (TCR) is expressed on the surface of T lymphocytes. One component, CD3, is responsible for intracellular signaling following occupancy of the TCR by ligand. The T lymphocyte receptor for antigen-CD3 complex (TCR/CD3) recognizes antigenic peptides that are presented to it by the proteins of the major histocompatibility complex (MHC). Complexes of MHC and peptide are expressed on the surface of antigen presenting cells

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and other T lymphocyte targets. Stimulation of the TCR/CD3 complex results in activation of the T lymphocyte and a consequent antigen-specific immune response. The TCR/CD3 complex plays a central role in the effector function and regulation of the immune system.

[329] T lymphocytes also require a second, co-stimulatory signal to become fully active. Without such a signal, T lymphocytes are either non-responsive to antigen binding to the TCR, or become anergic. Such a co-stimulatory signal, for example, is provided by CD28, a T lymphocyte protein, which interacts with CD80 and CD86 on antigen-producing cells. ICOS (Inducible COStimulator), another T lymphocyte protein, provides a co-stimulatory signal when bound to ICOS ligand.

[330] Activation of the T cell receptor (TCR) CD3 complex and co-stimulation with CD28 can occur by *ex vivo* exposure to solid surfaces (e.g. beads) coated with anti-CD3 and anti-CD28. In some embodiments of the methods and compositions disclosed herein, resting T cells are activated by exposure to solid surfaces coated with anti-CD3 and anti-CD28 *ex vivo*.

[331] In certain illustrative embodiments of the methods and compositions provided herein, polypeptides that are capable of binding CD3 and/or CD28, are presented as "activation elements" on the surface of recombinant retroviruses of the methods and compositions disclosed herein, which are also aspects of the invention. Polypeptides that bind CD3 and/or CD28 are referred to as "activation elements" because of their ability to activate resting T cells.

[332] In some embodiments, the activation element is a polypeptide capable of binding to CD3. In some embodiments, the polypeptide capable of binding to CD3 is an anti-CD3 antibody, or a fragment thereof that retains the ability to bind to CD3. In illustrative embodiments, the anti-CD3 antibody or fragment thereof is a single chain anti-CD3 antibody, such as but not limited to, an anti-CD3 scFv. In another illustrative embodiment, the polypeptide capable of binding to CD3 is anti-CD3scFvFc.

[333] A number of anti-human CD3 monoclonal antibodies and antibody fragments thereof are available, and can be used in the present invention, including but not limited to UCHT1, OKT-3, HIT3A, TRX4, X35-3, VIT3, BMA030 (BW264/56), CLB-T3/3, CRIS7, YTH12.5, F111409, CLB-T3.4.2, TR-66, WT31, WT32, SPv-T3b, 11D8, XIII-141, XIII46, XIII-87, 12F6, T3/RW2-8C8, T3/RW24B6, OKT3D, M-T301, SMC2 and F101.01.

[334] In some embodiments, the activation element is a polypeptide capable of binding to CD28. In some embodiments, the polypeptide capable of binding to CD28 is an anti-CD28 antibody, or a fragment thereof that retains the ability to bind to CD28. In other embodiments, the polypeptide capable of binding to CD28 is CD80, CD86, or a functional fragment thereof that is capable of CD28 and inducing CD28-mediated activation of Akt, such as an external fragment of CD80. In illustrative embodiments, the anti-CD28 antibody or fragment thereof is a single chain anti-CD28 antibody, such as, but not limited to, an

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anti-CD28 scFv. In another illustrative embodiment, the polypeptide capable of binding to CD28 is CD80, or a fragment of CD80 such as an external fragment of CD80.

[335] Anti-CD28 antibodies are known in the art and can include, as non-limiting examples, monoclonal antibody 9.3, an IgG2a antibody (Dr. Jeffery Ledbetter, Bristol Myers Squibb Corporation, Seattle, Wash.), monoclonal antibody KOLT-2, an IgG1 antibody, 15E8, an IgG1 antibody, 248.23.2, an IgM antibody and EX5.3D10, an IgG2a antibody.

[336] In an illustrative embodiment, an activation element includes two polypeptides, a polypeptide capable of binding to CD3 and a polypeptide capable of binding to CD28.

[337] In certain embodiments, the polypeptide capable of binding to CD3 or CD28 is an antibody, a single chain monoclonal antibody or an antibody fragment, for example a single chain antibody fragment. Accordingly, the antibody fragment can be, for example, a single chain fragment variable region (scFv), a antibody binding (Fab) fragment of an antibody, a single chain antigen-binding fragment (scFab), a single chain antigen-binding fragment without cysteines (scFabΔC), a fragment variable region (Fv), a construct specific to adjacent epitopes of an antigen (CRAb), or a single domain antibody (VH or VL).

[338] In some embodiments, an activation element is fused to a heterologous signal sequence and/or a heterologous membrane attachment sequence, both of which help direct the activation element to the membrane. The heterologous signal sequence targets the activation element to the endoplasmic reticulum, where the heterologous membrane attachment sequence covalently attaches to one or several fatty acids (also known as posttranslational lipid modification) such that the activation elements that are fused to the heterologous membrane attachment sequence are anchored in the lipid rafts of the plasma membrane. In some embodiments, posttranslational lipid modification can occur via myristoylation, palmitoylation, or GPI anchorage. Myristoylation is a post-translational protein modification which corresponds to the covalent linkage of a 14-carbon saturated fatty acid, the myristic acid, to the N-terminal glycine of a eukaryotic or viral protein. Palmitoylation is a post-translational protein modification which corresponds to the covalent linkage of a C16 acyl chain to cysteines, and less frequently to serine and threonine residues, of proteins. GPI anchorage refers to the attachment of glycosylphosphatidylinositol, or GPI, to the C-terminus of a protein during posttranslational modification.

[339] In some embodiments, the heterologous membrane attachment sequence is a GPI anchor attachment sequence. The heterologous GPI anchor attachment sequence can be derived from any known GPI-anchored protein (reviewed in Ferguson MAJ, Kinoshita T, Hart GW. Glycosylphosphatidylinositol Anchors. In: Varki A, Cummings RD, Esko JD, et al., editors. *Essentials of Glycobiology. 2nd edition.* Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2009. Chapter 11). In some embodiments, the heterologous GPI anchor attachment sequence is the GPI anchor attachment sequence from CD14, CD16, CD48, CD55 (DAF), CD59, CD80, and CD87. In some embodiments, the

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heterologous GPI anchor attachment sequence is derived from CD16. In illustrative embodiments, the heterologous GPI anchor attachment sequence is derived from Fc receptor FcγRIIIb (CD16b) or decay accelerating factor (DAF), otherwise known as complement decay-accelerating factor or CD55.

[340] In some embodiments, one or both of the activation elements include a heterologous signal sequence to help direct expression of the activation element to the cell membrane. Any signal sequence that is active in the packaging cell line can be used. In some embodiments, the signal sequence is a DAF signal sequence. In illustrative embodiments, an activation element is fused to a DAF signal sequence at its N terminus and a GPI anchor attachment sequence at its C terminus.

[341] In an illustrative embodiment, the activation element includes anti-CD3 scFvFc fused to a GPI anchor attachment sequence derived from CD14 and CD80 fused to a GPI anchor attachment sequence derived from CD16b; and both are expressed on the surface of a recombinant retrovirus provided herein. In some embodiments, the anti-CD3 scFvFc is fused to a DAF signal sequence at its N terminus and a GPI anchor attachment sequence derived from CD14 at its C terminus and the CD80 is fused to a DAF signal sequence at its N terminus and a GPI anchor attachment sequence derived from CD16b at its C terminus; and both are expressed on the surface of a recombinant retrovirus provided herein. In some embodiments, the DAF signal sequence includes amino acid residues 1-30 of the DAF protein.

MEMBRANE-BOUND CYTOKINES

[342] Some embodiments of the method and composition aspects provided herein, include a membrane-bound cytokine, or polynucleotides encoding a membrane-bound cytokine. Ctyokines are typically, but not always, secreted proteins. Cytokines that are naturally secreted can be engineered as fusion proteins to be membrane-bound. Membrane-bound cytokine fusion polypeptides are included in methods and compositions disclosed herein, and are also an aspect of the invention. In some embodiments, recombinant retroviruses have a membrane-bound cytokine fusion polypeptide on their surface that is capable of binding a T cell and/or NK cell and promoting proliferation and/or survival thereof. Typically, membrane-bound polypeptides are incorporated into the membranes of recombinant retroviruses, and when a cell is transduced by the recombinant retrovirus, the fusion of the retroviral and host cell membranes results in the polypeptide being bound to the membrane of the transduced cell.

[343] In some embodiments, the cytokine fusion polypeptide includes IL-7, IL-15, or an active fragment thereof. The membrane-bound cytokine fusion polypeptides are typically a cytokine fused to heterologous signal sequence and/or a heterologous membrane attachment sequence. In some embodiments, the heterologous membrane attachment sequence is a GPI anchor attachment sequence. The heterologous GPI anchor attachment sequence can be derived from any known GPI-anchored protein (reviewed in Ferguson MAJ, Kinoshita T, Hart GW. Glycosylphosphatidylinositol Anchors. In: Varki A,

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Cummings RD, Esko JD, et al., editors. *Essentials of Glycobiology. 2nd edition*. Cold Spring Harbor (NY): Cold Spring Harbor Laboratory Press; 2009. Chapter 11). In some embodiments, the heterologous GPI anchor attachment sequence is the GPI anchor attachment sequence from CD14, CD16, CD48, CD55 (DAF), CD59, CD80, and CD87. In some embodiments, the heterologous GPI anchor attachment sequence is derived from CD16. In an illustrative embodiment, the heterologous GPI anchor attachment sequence is derived from Fc receptor FcγRIIIb (CD16b). In some embodiments, the GPI anchor is the GPI anchor of DAF.

[344] In illustrative embodiments, the membrane-bound cytokine is a fusion polypeptide of a cytokine fused to DAF. DAF is known to accumulate in lipid rafts that are incorporated into the membranes of retroviruses budding from packaging cells. Accordingly, not to be limited by theory, it is believed that DAF fusion proteins are preferentially targeted to portions of membranes of packaging cells that will become part of a recombinant retroviral membrane.

[345] In non-limiting illustrative embodiments, the cytokine fusion polypeptide is an IL-7, or an active fragment thereof, fused to DAF. In a specific non-limiting illustrative embodiment, the fusion cytokine polypeptide includes in order: the DAF signal sequence (residues 1-31 of DAF), IL-7 without its signal sequence, and residues 36-525 of DAF.

***IN VIVO* CONTROL ELEMENT**

Riboswitches

[346] Some of the compositions and methods provided herein include one or more riboswitches or polynucleotides that include one or more riboswitch, which themselves form distinct aspects of the present disclosure. Riboswitches are a common feature in bacteria to regulate gene expression and are a means to achieve RNA control of biological functions. Riboswitches are polynucleotides that can be present in the 5′-untranslated region of mRNAs and allow for regulatory control over gene expression through binding of a small molecule ligand that induces or suppresses a riboswitch activity. Typically, the riboswitch controls a gene product involved in the generation of the small molecule ligand, thus forming a feedback loop. Riboswitches typically act in a cis-fashion, although riboswitches have been identified that act in a trans-fashion. Natural riboswitches consist of two domains: an aptamer domain that binds the ligand through a three-dimensional folded RNA structure and a function switching domain that induces or suppresses an activity in the riboswitch based on the absence or presence of the ligand. Thus, there are two ligand sensitive conformations achieved by the riboswitch, representing on and off states (Garst et al., 2011). The function switching domain can affect the expression of a polynucleotide by regulating: an internal ribosome entry site, pre-mRNA splice donor accessibility in the retroviral gene construct, translation, termination of transcription, transcript degradation, miRNA expression, or shRNA expression

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(Dambach and Winkler 2009). The aptamer and function switching domains can be used as modular components allowing for synthetic RNA devices to control gene expression either as native aptamers, mutated/evolved native aptamers, or totally synthetic aptamers that are identified from screening random RNA libraries (McKeague et al 2016).

[347] The purine riboswitch family represents one of the largest families with over 500 sequences found (Mandal et al 2003; US20080269258; and WO2006055351). The purine riboswitches share a similar structure consisting of three conserved helical elements/stem structures (P1, P2, P3) with intervening loop/junction elements (J1-2, L2, J2-3, L3, J3-1). The aptamer domains of the purine family of riboswitches naturally vary in their affinity/regulation by various purine compounds such as adenine, guanine, adenosine, guanosine, deoxyadenosine, deoxyguanosine (FIG. 5), etc. due to sequence variation (Kim et al. 2007).

[348] In one aspect, provided herein is an isolated polynucleotide for regulating expression of a target polynucleotide, including: a polynucleotide encoding the target polynucleotide operably linked to a promoter and a riboswitch, wherein the riboswitch includes: a.) an aptamer domain capable of binding a nucleoside analogue antiviral drug and having reduced binding to guanine or 2′-deoxyguanosine relative to the nucleoside analogue antiviral drug; and b.) a function switching domain capable of regulating expression of the target polynucleotide, wherein binding of the nucleoside analogue by the aptamer domain induces or suppresses the expression regulating activity of the function switching domain, thereby regulating expression of the target gene. In some embodiments, the target polynucleotide can be a polypeptide encoding region, an miRNA, or an shRNA. In a non-limiting example, the riboswitch is operably linked to a nucleic acid encoding a polypeptide, miRNA, or shRNA with *in vivo* activity, for example that is effective at treating a disease. For example, in such a non-limiting example, the riboswitch is operably linked to a nucleic acid encoding a chimeric antigen receptor. In non-limiting illustrative examples provided herein, the target polynucleotide encodes one or more engineered signaling polypeptides included in various other aspects of the present disclosure. In these non-limiting illustrative examples, the riboswitch and the target polynucleotide encoding one or more engineered signaling polypeptides can be found in the genome of a packaging cell, a recombinant retrovirus, a T cell and/or an NK cell.

[349] In some embodiments, the aptamer domain can be between 30, 35, 40, 45, 50, 55, 60, 65, and 70 nucleotides in length on the low end of the range and 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, and 100 nucleotides in length on the high end of the range, for example between 45 and 80 nucleotides in length, between 45 and 60 nucleotides in length, or between 45 and 58 nucleotides in length. In illustrative embodiments, the nucleoside analogue antiviral drug can be the pharmaceutical ligand acyclovir (also known as aciclovir and acycloguanosine) or penciclovir (FIG. 5). In some embodiments,

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the aptamer domain can have a binding affinity to the nucleoside analogue antiviral drug greater than, for example at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100-fold greater than the binding affinity to the nucleoside or nucleotide.

[350] The *in vivo* control element promotes expansion of transduced T cells *in vivo*. In some embodiments, expansion is dependent on the presence of the control element. However, in other embodiments, expansion of the transduced T cells can be at least partially driven by other factors such as the presence of interleukins within the subject and binding of the ASTR of a CAR on the recombinant T cell to its ligand.

[351] In some embodiments, a nucleoside analogue antiviral drug, for example acyclovir or penciclovir, is administered to a subject before, during, and/or after PBLs are isolated from the blood and before T cells and/or NK cells are contacted with a recombinant retrovirus that includes an *in vivo* control element, which in illustrative non-limiting examples is a riboswitch, that binds to the nucleoside analogue antiviral drug and regulates expression of one or more target polynucleotides. The one or more target polynucleotides can encode one or more polypeptides that in non-limiting illustrative examples are one or more engineered signaling polypeptides, at least one of which encodes a lymphoproliferative element. In some embodiments, the nucleoside analogue antiviral drug, for example acyclovir or penciclovir, is administered to the subject for between 5, 10, 15, 30, and 60 minutes on the low end of the range, and 1.5, 2, 3, 4, 5, 6, 8, 12, 24, 48, or 72 hours on the high end of the range, before PBLs are isolated from the blood or before T cells and/or NK cells are contacted with a recombinant retrovirus. In some embodiments, the nucleoside analogue antiviral drug, for example acyclovir or penciclovir, is administered to the subject for between 1.5, 2, 3, 4, 5, 6, 8, 12, or 24 hours on the low end of the range, ½, 1, 2, 3, 4, 5, 6, 7, 10, 14, 21, or 28 days on the high end of the range, after PBLs are isolated from the blood or after T cells and/or NK cells are contacted with a recombinant retrovirus in methods provided herein. In some embodiments, the nucleoside analogue antiviral drug, for example acyclovir or penciclovir, is administered to the subject for at least 1.5, 2, 3, 4, 5, 6, 8, 12, or 24 hours, or at least 2, 3, 4, 5, 6, 7, 10, 14, 21, or 28 days after PBLs are isolated from the blood or after T cells and/or NK cells are contacted with a recombinant retrovirus in methods provided herein. In some embodiments, the nucleoside analogue antiviral drug, for example acyclovir or penciclovir, is administered to the subject for at least 1, 2, 3, 4, 5, 7, 10, 14, 21, 28, 30, 60, 90, or 120 days or 5, 6, 9, 12, 24, 36, 48, 60, 72, 84, 96, 120 months or indefinitely after the PBLs have been reinfused into the subject. In any of the embodiments disclosed herein, the nucleoside analogue antiviral drug can be administered before and/or during the reinfusion of the PBLs and/or after the PBLs have been reinfused. In some embodiments, the nucleoside analogue antiviral drug is administered until a subject no longer experiences symptoms of, or is afflicted by, a disease for which the target polynucleotide is related.

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[352] In some embodiments, the aptamer domain can preferentially bind penciclovir over acyclovir or alternatively another antiviral agent, such that concomitant antiviral therapy may be utilized without affecting the riboswitch. In some embodiments, the aptamer domain can bind penciclovir with a binding affinity greater than, for example at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100-fold greater than the aptamer domain binds acyclovir or another antiviral agent. In some embodiments, the aptamer domain can preferentially bind acyclovir over penciclovir or alternatively another antiviral agent, such that concomitant antiviral therapy may be utilized without affecting the riboswitch. In some embodiments, the aptamer domain can bind acyclovir with a binding affinity greater than, for example at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100-fold greater than the aptamer domain binds penciclovir or another antiviral agent. In some embodiments, the oral prodrugs of penciclovir (famciclovir) and acyclovir (valaciclovir) can be given to a subject.

[353] In some embodiments, the aptamer domain of an isolated polynucleotide can share at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity or be identical to any one of the sequences of SEQ ID NOs:87-93 and retain the ability to bind acyclovir and a reduced ability to bind to guanine or 2′-deoxyguanosine relative to the nucleoside analogue antiviral drug, and wherein the aptamer domain retains the ability to induce or suppress the expression regulating activity of the function switching domain when bound by acyclovir. In some embodiments, the aptamer domain of an isolated polynucleotide can share at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity or be identical to the aptamer domain of SEQ ID NOs:94-100 and retain the ability to bind penciclovir and a reduced ability to bind to guanine or 2′-deoxyguanosine relative to the nucleoside analogue antiviral drug, and wherein the aptamer domain retains the ability to induce or suppress the expression regulating activity of the function switching domain when bound by penciclovir. In some embodiments, a region of an isolated polynucleotide or a region of a riboswitch can share at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity or be identical to any one of the sequences of SEQ ID NOs:87-100.

[354] In some embodiments, a DNA sequence containing a region of an aptamer domain of an isolated polynucleotide can share at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity or be identical to any one of the sequences of SEQ ID NOs:108-221. In some embodiments, a region of an isolated polynucleotide can share at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity or be identical to any one of the sequences of SEQ ID NOs:108-221.

[355] In some embodiments, a DNA sequence containing a region of an aptamer domain of an isolated polynucleotide can share at least 80%, 85%, 90%, 91%, 91.84%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity or be identical to SEQ ID NO:108. In some embodiments, a DNA sequence

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containing a region of an aptamer domain of an isolated polynucleotide can share at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 95.83%, 96%, 97%, 98%, or 99% sequence identity or be identical to SEQ ID NO:147. In some embodiments, a DNA sequence containing a region of an aptamer domain of an isolated polynucleotide can share at least 80%, 85%, 90%, 91%, 92%, 93%, 93.88%, 94%, 95%, 96%, 97%, 98%, or 99% sequence identity or be identical to SEQ ID NO:164. In some embodiments, a DNA sequence containing a region of an aptamer domain of an isolated polynucleotide can share at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 95.83%96%, 97%, 98%, or 99% sequence identity or be identical to SEQ ID NO:183. In some embodiments, a DNA sequence containing a region of an aptamer domain of an isolated polynucleotide can share at least 80%, 85%, 90%, 91%, 91.84%, 92%, 93%, 94%, 95%, 95.83%96%, 97%, 98%, or 99% sequence identity or be identical to SEQ ID NO:198.

[356] In some embodiments, a region of an isolated polynucleotide can include any one of the consensus sequences of SEQ ID NOs:222-226. In some embodiments, a region of an isolated polynucleotide can share at least 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 95.83%, 96%, 97%, 98%, or 99% sequence identity or be identical to any one of the sequences of SEQ ID NOs:222-226.

[357] In any of the embodiments disclosed herein, the isolated polynucleotide can retain the ability to bind acyclovir and/or penciclovir. In any of the embodiments disclosed herein, an isolated polynucleotide can be the reverse complement of any one of the sequences of SEQ ID NOs: 87-100 or SEQ ID NOs:108-221. In any of the embodiments disclosed herein, an isolated polynucleotide can be a transcription or RNA version of either the DNA sequences of SEQ ID NOs:108-221 or the DNA sequences complementary to SEQ ID NOs:108-221. In any of the embodiments disclosed herein, an isolated polynucleotide can be a reverse transcription or DNA version of any one of the RNA sequences of SEQ ID NOs:87-100 or the DNA strand complementary to a reverse transcription of any one of the RNA sequences of SEQ ID NOs:87-100.

[358] In some embodiments provided herein, riboswitch scaffolds can be used for mutational analysis or molecular evolution. The riboswitches selected for mutational analysis or molecular evolution can be from any known organism, for example, bacteria. In some embodiments, the type I-A deoxyguanosine riboswitch from *Mesoplasma florum* can be used for molecular evolution. In some embodiments, the derived aptamer domain can be at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, or 95% identical to the aptamer domain from the type I-A deoxyguanosine riboswitch from *Mesoplasma florum* (SEQ ID NO:237). In other embodiments, the xpt riboswitch from *Bacillus subtilis* can be used. In some embodiments, the derived aptamer domain can be at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, or 95% identical to the aptamer domain from the xpt riboswitch from *Bacillus subtilis* (SEQ ID NO:243).

[359] The aptamer domains can be used as modular components and combined with any of the function switching domains to affect the RNA transcript. In any of the embodiments disclosed herein, the

riboswitch can affect the RNA transcript by regulating any of the following activities: internal ribosomal entry site (IRES), pre-mRNA splice donor accessibility, translation, termination of transcription, transcript degradation, miRNA expression, or shRNA expression. In some embodiments, the function switching domain can control binding of an anti-IRES to an IRES (see, e.g. Ogawa, *RNA* (2011), 17:478-488, the disclosure of which is incorporated by reference herein in its entirety). In any of the embodiments disclosed herein, the presence or absence of the small molecule ligand can cause the riboswitch to affect the RNA transcript. In some embodiments, the riboswitch can include a ribozyme. Riboswitches with ribozymes can inhibit or enhance transcript degradation of target polynucleotides in the presence of the small molecule ligand. In some embodiments, the ribozyme can be a pistol class of ribozyme, a hammerhead class of ribozyme, a twisted class of ribozyme, a hatchet class of ribozyme, or the HDV (hepatitis delta virus) ribozyme.

[360] In any of the embodiments disclosed herein, the riboswitch can be located in various positions relative to the target polynucleotide, as is known generally for riboswitches. In some embodiments, the riboswitch can regulate pre-mRNA splice donor accessibility and be located before the target polynucleotide. In some embodiments, the riboswitch can regulate the inclusion of a poly(A) tail and be located after the target polynucleotide. In some embodiments, the riboswitch can regulate an anti-IRES and be located upstream of an IRES. In non-limiting illustrative embodiments, a riboswitch provided herein can be located in any of these positions relative to a nucleic acid encoding one or more engineered signaling polypeptides provided herein.

[361] In some embodiments, the riboswitch can be destabilized at temperatures above 37.5 °C, 38 °C, 38.5 °C, 39 °C, 39.5 °C, or 40 °C such that the riboswitch is no longer responsive to the ligand. In some embodiments, molecular evolution can be used to select riboswitches that are destabilized at temperatures above 37.5 °C, 38 °C, 38.5 °C, 39 °C, 39.5 °C, or 40 °C.

[362] In some embodiments, the target polynucleotide can encode a miRNA, shRNA, and/or a polypeptide, wherein the target polynucleotide is operably linked to a promoter. In some embodiments, the target polynucleotide can encode a lymphoproliferative element. In some embodiments, the target polynucleotide can be an miRNA or shRNA. In some embodiments, the miRNA or shRNA can potentiate the STAT5 pathway or inhibit the SOCS pathway. In some embodiments, the miRNA or shRNA can target transcripts from SOCS1, SMAD2, TGFb, or PD-1. In some embodiments, the miRNA is miR-155. In some embodiments, the target polynucleotide encodes a polypeptide and the polypeptide can include a CAR including an antigen-specific targeting region, a transmembrane domain, and an intracellular activating domain.

[363] In another aspect, provided herein is an isolated polynucleotide for regulating expression of a target polynucleotide, including: a polynucleotide encoding the target polynucleotide operably linked to a

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promoter and a riboswitch, wherein the riboswitch includes: a.) an aptamer domain capable of binding a nucleoside analogue antiviral drug with a binding affinity at least two-fold greater affinity than the aptamer domain binds guanine or 2′-deoxyguanosine; and b.) a function switching domain capable of regulating expression of the target polynucleotide, wherein binding of the nucleoside analogue by the aptamer domain induces or suppresses the expression regulating activity of the function switching domain. In some embodiments, the aptamer domain can bind the nucleoside analogue antiviral drug with a binding affinity at least 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100-fold greater affinity than the aptamer domain binds guanine or 2′-deoxyguanosine. In some embodiments, the aptamer domain can be between 30, 35, 40, 45, 50, 55, 60, 65, and 70 nucleotides in length on the low end of the range and 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, and 100 nucleotides in length on the high end of the range, for example between 45 and 80 nucleotides in length or between 45 and 58 nucleotides in length. In illustrative embodiments, the nucleoside analogue antiviral drug can be the pharmaceutical ligand acyclovir (also known as aciclovir and acycloguanosine) or penciclovir. In some embodiments, the aptamer domain can have a binding affinity to the nucleoside analogue antiviral drug that is greater than, for example at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100-fold greater than the binding affinity to the nucleoside or nucleotide. In some embodiments, binding of the nucleoside analogue by the aptamer domain can induce an activity in the riboswitch.

[364] In some embodiments, the aptamer domain can be specific for penciclovir and lack reactivity to acyclovir or alternatively another antiviral agent, such that concomitant antiviral therapy may be utilized without affecting the riboswitch. In some embodiments, the aptamer domain can bind penciclovir with a binding affinity at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100-fold greater than the aptamer domain binds acyclovir or another antiviral agent. In some embodiments, the aptamer domain can be specific for acyclovir and lack reactivity to penciclovir or alternatively another antiviral agent, such that concomitant antiviral therapy may be utilized without affecting the riboswitch. In some embodiments, the aptamer domain can bind acyclovir with a binding affinity at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 20, 30, 40, 50, 60, 70, 80, 90, or 100-fold greater than the aptamer domain binds penciclovir or another antiviral agent. In some embodiments, the oral prodrugs of penciclovir (famciclovir) and acyclovir (valaciclovir) can be given to a subject. In some embodiments, the derived aptamer domain can be at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, or 95% identical to the aptamer domain from the type I-A deoxyguanosine riboswitch from *Mesoplasma florum*. In some embodiments, the derived aptamer domain can be at least 50%, 60%, 70%, 75%, 80%, 85%, 90%, or 95% identical to the aptamer domain from the xpt riboswitch from *Bacillus subtilis*. In any of the embodiments disclosed herein, the riboswitch can affect the RNA transcript by regulating any of the following activities: internal ribosomal entry site, pre-mRNA splice donor accessibility in the retroviral gene construct, translation, termination of

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transcription, transcript degradation, miRNA expression, or shRNA expression. In some embodiments, the function switching domain can control binding of an anti-IRES to an IRES. In any of the embodiments disclosed herein, the presence or absence of the small molecule ligand can cause the riboswitch to affect the RNA transcript. In some embodiments, the riboswitch can include a ribozyme. Riboswitches with ribozymes can inhibit or enhance transcript degradation of genes of interest in the presence of the small molecule ligand. In some embodiments, the ribozyme can be a pistol class of ribozyme, a hammerhead class of ribozyme, a twisted class of ribozyme, a hatchet class of ribozyme, or the HDV (hepatitis delta virus) ribozyme. In some embodiments, the riboswitch can be destabilized at temperatures above 37.5 °C, 38 °C, 38.5 °C, 39 °C, 39.5 °C, or 40 °C such that the riboswitch is no longer responsive to the ligand. In some embodiments, molecular evolution can be used to select riboswitches that are destabilized at temperatures above 37.5 °C, 38 °C, 38.5 °C, 39 °C, 39.5 °C, or 40 °C. In some embodiments, the target polynucleotide can encode a miRNA, shRNA, and/or a polypeptide, wherein the target polynucleotide is operably linked to a promoter. In some embodiments, the target polynucleotide can encode a lymphoproliferative element. In some embodiments, the target polynucleotide can be an miRNA and, optionally, the miRNA can stimulate the STAT5 pathway or inhibit the SOCS pathway. In some embodiments, the miRNA can target transcripts from SOCS1, SHP, SMAD2, TGFb, or PD-1. In these embodiments, the miRNA can be miR-155. In some embodiments, the target polynucleotide encodes a polypeptide and the polypeptide can include a CAR including an antigen-specific targeting region, a transmembrane domain, and an intracellular activating domain. Further embodiments of CARs are disclosed elsewhere herein.

[365] In some embodiments, the evolution of aptamers can be performed via aptamer selection from randomized native purine or guanine aptamer libraries using SELEX (Systematic Evolution of Ligands by EXponential enrichment) methods including, but not limited to, those methods that employ graphene oxide in the selection process and screening. In other embodiments, random mutagenesis methodology such as error prone PCR can be used to evolve aptamer constructs or riboswitch constructs where the aptamer is incorporated in the context of any of the riboswitch activities described herein by screening *in vitro* or in mammalian cells. In other embodiments, random libraries of nucleotides can be used in the evolution of the riboswitch. In any of the embodiments disclosed herein, riboswitches can be identified from screening such libraries *in vitro* or in mammalian cells.

[366] In some embodiments, the evolved or derived aptamer domain can have increased binding to analogues of the native ligand and decreased binding to the native ligand. In some embodiments, the aptamer domain can be configured to have increased binding to analogues of the native ligand and decreased binding to the native ligand. In some embodiments, the aptamer domain can be derived from the purine riboswitch family. In some embodiments, the native ligand can be a nucleoside or nucleotide

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and the analogue can be a nucleoside analogue or nucleotide analogue. In some embodiments, the nucleoside analogue is an antiviral drug. In illustrative embodiments, the aptamer domains can be derived from 2′-deoxyguanosine and guanine riboswitch scaffolds and the derived aptamer domains can show reduced binding to 2′-deoxyguanosine and guanine relative to the wild-type riboswitch.

[367] In some embodiments, the riboswitch can regulate pre-mRNA splice donor accessibility in the retroviral gene construct, wherein the retroviral construct drives the CAR genes or other genes of interest from the reverse strand under a general promoter or a T cell specific promoter. In other embodiments, the riboswitch can regulate an IRES in the retroviral gene construct, wherein the retroviral construct drives the translation of CAR genes or other genes of interest. In other embodiments, the riboswitch can control transcription termination of the RNA, miRNA, or gene transcripts or can control translation of the transcript. In other embodiments, the nucleoside analogue riboswitch can be integrated with a ribozyme to inhibit or enhance transcript degradation of the CAR genes or other genes of interest in the presence of the nucleoside analogue.

[368] In some embodiments, the isolated polynucleotide for regulating expression of a target polynucleotide that includes a polynucleotide encoding the target polynucleotide operably linked to a promoter and a riboswitch that binds a nucleoside analogue antiviral drug, is a molecular cloning vector. The molecular cloning vector can be any type of molecular cloning vector known in the art. As non-limiting examples, the vector can be a plasmid, a virus, or a retrovirus, any of which can be an expression vector. Such an expression vector can encode any of the target polynucleotides provided hereinabove. One or more restriction and/or multiple cloning sites can be included on a molecular cloning vector 5' or 3' to a riboswitch provided herein such that the riboswitch is operably linked to a target polynucleotide inserted into the restriction and/or multiple cloning site.

Molecular Chaperones

[369] In one aspect, provided herein is a method for genetically modifying and expanding lymphocytes of a subject, comprising:

A. contacting resting T cells and/or NK cells of the subject *ex vivo*, typically without requiring prior *ex vivo* stimulation, with recombinant retroviruses comprising:

i. a pseudotyping element on its surface that is capable of binding to a T cell and/or NK cell and facilitating membrane fusion of the recombinant retrovirus thereto; and

ii. a polynucleotide comprising one or more transcriptional units operatively linked to a promoter active in T cells and/or NK cells, wherein the one or more transcriptional units encode a first engineered signaling polypeptide regulated by an *in vivo* control element, wherein said first engineered signaling polypeptide comprises a lymphoproliferative

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element and/or a chimeric antigen receptor,

wherein said contacting facilitates transduction of at least some of the resting T cells and/or NK cells by the recombinant retroviruses, thereby producing genetically modified T cells and/or NK cells;

B. introducing the genetically modified T cells and/or NK cells into the subject; and

C. exposing the genetically modified T cells and/or NK cells *in vivo* to a compound that acts as the *in vivo* control element to affect expression of the first engineered signaling polypeptide and promote expansion of the lymphocytes *in vivo*, thereby genetically modifying and expanding lymphocytes of the subject.

[370] In illustrative embodiments, the transduction is carried out without *ex vivo* stimulation. In illustrative embodiments, the compound is a molecular chaperone, such as a small molecule molecular chaperone. In illustrative embodiments, binding of the molecular chaperone to the lymphoproliferative element and/or CAR component increases the proliferative activity of the lymphoproliferative element and/or the CAR. The molecular chaperone can be administered to the subject before the blood is collected, during the contacting, and/or after the T cells and/or NK cells are introduced into the subject. Some embodiments of this aspect include collecting blood from the subject. In these embodiments, the introducing is a reintroducing of the cells that were collected and genetically modified before reintroduction. The entire process, in illustrative embodiments, is a shorter process than prior art methods, as for other aspects herein. For example, the entire process can be completed in less than 48 hours, less than 24 hours, or less than 12 hours. The entire process in other embodiments, can be completed in 2, 4, 6, or 8 hours on the low end of the range, and 12, 24, 36, or 48 hours on the high end of the range.

[371] Accordingly, in some embodiments of the methods and compositions provided herein, the *in vivo* control element is a molecular chaperone. As compared to other embodiments herein with other *in vivo* control elements, such as riboswitches that typically bind a compound to affect expression of a lymphoproliferative element or other component of a first or second engineered signaling polypeptide herein, the molecular chaperones are compounds that are the *in vivo* control elements and as such, directly affect activity of, typically by binding to, a lymphoproliferative element or other component of a first or second engineered signaling polypeptide herein. In illustrative examples of such embodiments of methods herein that include the administration of molecular chaperones, a lymphoproliferative element, membrane-bound cytokine, and/or CAR component, can be a less active or inactive lymphoproliferative element, membrane-bound cytokine, and/or CAR component, that is bound by the molecular chaperone to increase its activity. Thus, the target bound by a molecular chaperone is typically a target polypeptide. In some embodiments, as indicated the polypeptide can be a first and/or a second engineered signaling polypeptide, or a polypeptide component thereof, whose activity is affected by binding to the molecular

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chaperone, which in illustrative embodiments is a small molecule molecular chaperone. In some embodiments, the polypeptide can include a lymphoproliferative element whose activity is regulated, in illustrative embodiments, up-regulated by a molecular chaperone, preferably a small molecule molecular chaperone. The molecular chaperone in the methods provided herein can be a compound that binds to the mutant lymphoproliferative element and/or inactive CAR component, thus rendering them active.

[372] In other embodiments, a lymphoproliferative element or other signaling domain has been mutated to permit transit to the plasma membrane only in the presence of a small molecular synthetic chaperone. In other embodiments, the chaperone promotes stability of the lymphoproliferative element or other signaling domain or protein and half-life as a potentiator.

[373] It will be understood that aspects and embodiments of the present invention include many of the same steps and compositions provided in detail herein. Accordingly, it will be understood that the teachings throughout this specification that relate to these common elements apply to aspects and embodiments that utilize a molecular chaperone as the *in vivo* control element, which typically binds a lymphoproliferative element or other target molecule directly, in addition to, or instead of other *in vivo* control elements provided herein, such as riboswitches, which typically utilize a molecule, such as a drug, that binds the riboswitch.

[374] In some embodiments, the molecular chaperone is a compound that can regulate sub-cellular localization of a target, for example, the proper folding and transit of a target protein, such as a lymphoproliferative element and/or a component of a CAR, from the endoplasmic reticulum to the plasma membrane or its half-life on the surface. In other embodiments, the molecular chaperone can promote the functional conformation of a dysfunctional target, thus acting as a potentiator. Examples of molecules that act as chaperones or potentiators to naturally mutated proteins include lumacaftor and ivacaftor. These proteins act upon the mutant CFTR chloride channel variants such as G551D or F508del. Ivacaftor potentiates the activity of the G551D or F508del mutated ion channel, whereas lumacaftor promotes stabilization of mutant chloride channels and subsequent potentiation by ivacaftor. Such chaperone dependent proteins can be generated from naturally functional proteins and screening for functional activity only in the presence of the molecular chaperones. Thus, such proteins are only active when the chaperone is present. Examples of such molecules which can be screened for specific chaperone activity include small molecule antivirals or anti-infectives that show no activity to normal human proteins. Accordingly, in one embodiment, the molecular chaperone used in methods herein is a small molecule antiviral or anti-infective compound that shows no activity to normal human proteins.

[375] In some embodiments, genetically modified lymphocytes can be exposed and/or a subject can be administered the molecular chaperone. In some embodiments, the compound is administered to the subject before, during, and/or after PBLs are isolated from the blood and before T cells and/or NK cells

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are contacted with a recombinant retrovirus. The recombinant retrovirus in such embodiments includes a less active or inactive lymphoproliferative element and/or CAR component that binds to, and is regulated by, the molecular chaperone compound.

[376] For any of the embodiments provided herein for modifying and expanding lymphocytes, which can be part of methods of adoptive cell therapy, the compound can be administered to the subject for between 5, 10, 15, 30, and 60 minutes on the low end of the range, and 1.5, 2, 3, 4, 5, 6, 8, 12, or 24 hours on the high end of the range, before PBLs are isolated from the blood or before T cells and/or NK cells are contacted with a recombinant retrovirus. In some embodiments, the compound is administered to the subject for between 1.5, 2, 3, 4, 5, 6, 8, 12, or 24 hours on the low end of the range, ½, 1, 2, 3, 4, 5, 6, 7, 10, 14, 21, or 28 days on the high end of the range, after PBLs are isolated from the blood or after T cells and/or NK cells are contacted with a recombinant retrovirus in methods provided herein. In some embodiments, the compound is administered to the subject for at least 1.5, 2, 3, 4, 5, 6, 8, 12, or 24 hours, or at least 2, 3, 4, 5, 6, 7, 10, 14, 21, or 28 days after PBLs are isolated from the blood or after T cells and/or NK cells are contacted with a recombinant retrovirus in methods provided herein. In some embodiments, the compound is administered to the subject for at least 1, 2, 3, 4, 5, 7, 10, 14, 21, 28, 30, 60, 90, or 120 days or 5, 6, 9, 12, 24, 36, 48, 60, 72, 84, 96, 120 months or indefinitely after the PBLs have been reinfused into the subject. In any of the embodiments disclosed herein, the compound can be administered before and/or during the reinfusion of the PBLs and/or after the PBLs have been reinfused.

[377] For any of the embodiments herein, molecular chaperones are not in the *in vivo* control elements that are bound by compounds that regulate and/or activate them. Molecular chaperones are compounds, preferably small molecule compounds, that are the *in vivo* control elements and regulate the activity of lymphoproliferative elements and/or functional components of CARs.

PACKAGING CELL LINES/METHODS OF MAKING RECOMBINANT RETROVIRUSES

[378] In one aspect, provided herein is a retroviral packaging system including: a mammalian cell including: a) a first transactivator expressed from a constitutive promoter and capable of binding a first ligand and a first inducible promoter for affecting expression of a nucleic acid sequence operably linked thereto in the presence versus absence of the first ligand; b) a second transactivator capable of binding a second ligand and a second inducible promoter, and affecting expression of a nucleic acid sequence operably linked thereto in the presence versus absence of a second ligand; and c) a packagable RNA genome for a retroviral particle, wherein the first transactivator regulates expression of the second transactivator, and wherein the second transactivator regulates expression of retroviral polypeptides involved in viral packaging, such as, for example, a gag polypeptide, a pol polypeptide, and/or a pseudotyping element, and optionally other polypeptides that will become incorporated in or on the

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recombinant retrovirus and are believed to be toxic to packaging cell lines, such as, for example, HEK-293. In certain aspects, the second transactivator itself is cytotoxic to packaging cell lines. Pseudotyping elements are typically capable of binding to a cell membrane of a target cell and facilitating fusion thereto, as discussed in detail herein. Thus, not to be limited by theory, the system provides the ability to accumulate certain polypeptides/proteins that do not inhibit, or do not substantially inhibit, or are not believed to inhibit proliferation or survival of the mammalian cells, for example, non-toxic proteins, while culturing a population of the mammalian cells for days or indefinitely, and controlling induction of polypeptides that are desired for retroviral product but that are inhibitory or can be inhibitory or have been reported to be inhibitory to the survival and/or proliferation of the mammalian cell, for example toxic polypeptides, until a later time closer to the time of when retroviruses will be produced and harvested. The packagable RNA genome is typically encoded by a polynucleotide operably linked to a promoter, sometimes referred to herein as a third promoter for convenience, wherein said third promoter is typically inducible by either the first transactivator or the second transactivator. In illustrative embodiments, the packagable RNA genome is encoded by a polynucleotide operably linked to a third promoter, wherein said third promoter is inducible by the second transactivator. As such, the packagable RNA genome can be produced at the later time point, closer to when the retrovirus will be harvested.

[379] A skilled artisan will appreciate many different transactivators, ligands, and inducible promoters can be used in the retroviral packaging system. Such inducible promoters can be isolated and derived from many organisms, e.g., eukaryotes and prokaryotes. Modification of inducible promoters derived from a first organism for use in a second organism, e.g., a first prokaryote and a second a eukaryote, a first eukaryote and a second a prokaryote, etc., is well known in the art. Such inducible promoters, and systems based on such inducible promoters but also including additional control proteins, include, but are not limited to, alcohol regulated promoters (e.g., alcohol dehydrogenase I (alcA) gene promoter, promoters responsive to alcohol transactivator proteins (AlcR), etc.), tetracycline regulated promoters, (e.g., promoter systems including TetActivators, TetON, TetOFF, etc.), steroid regulated promoters (e.g., rat glucocorticoid receptor promoter systems, human estrogen receptor promoter systems, retinoid promoter systems, thyroid promoter systems, ecdysone promoter systems, mifepristone promoter systems, etc.), metal regulated promoters (e.g., metallothionein promoter systems, etc.), pathogenesis-related regulated promoters (e.g., salicylic acid regulated promoters, ethylene regulated promoters, benzothiadiazole regulated promoters, etc.), temperature regulated promoters (e.g., heat shock inducible promoters (e.g., HSP-70, HSP-90, soybean heat shock promoter, etc.), light regulated promoters, synthetic inducible promoters, and the like. In some embodiments, a mifepristone-regulated system can be used. In some embodiments, a mifepristone-inducible system with an autoregulatory feedback loop can be used. In some embodiments, a GAL4 regulatory fusion protein is expressed from one construct that also

contains the transposon terminal repeats and lox and FRT sites. In some embodiments, the GAL4 regulatory fusion protein controls expression of a reverse tet transactivator (rtTA) and BiTRE. In some embodiments, another construct with lox and FRT sites contains a GAL4 upstream activating sequences (UAS) and an E1b TATA box promoter driving a reporter like mCherry. In some embodiments, a GAL4 regulatory fusion protein binds to GAL4 upstream activating sequences (UAS) in both the promoter controlling expression of the GAL4 regulatory fusion protein and the promoter controlling expression of a target polynucleotide. In some embodiments, mifepristone, doxycycline, and puromycin will be used for induction and selection of packaging cell line.

[380] In some embodiments, either or both transactivators can be split into two or more polypeptides. In some embodiments, the two or more polypeptides can include a DNA binding domain and an activation domain capable of stimulating transcription on separate polypeptides. This "activation domain" is not to be confused with an "activation element," such as a polypeptide that binds CD3, which is capable of activating a T cell and/or NK cell, and typically does activate such T cell and/or NK cell when contacted with it, as discussed in detail herein. The separate polypeptides can further include fusions with polypeptides capable of dimerization through the addition of a ligand. In some embodiments, the activation domain can be the p65 activation domain or a functional fragment thereof. In illustrative embodiments of the packaging systems herein, the DNA binding domain can be the DNA binding domain from ZFHD1 or a functional fragment thereof. In some embodiments, one polypeptide can be a fusion with FKBP, or functional mutants and/or fragments thereof, or multiple FKBPs and another polypeptide can be a fusion with the FRB domain of mTOR, or functional mutants and/or fragments thereof, and the ligand can be rapamycin or a functional rapalog. In some embodiments, the FRB contains the mutations K2095P, T2098L, and/or W2101F. In some embodiments, the separate polypeptides can be FKBP, or functional fragments thereof, and CalcineurinA, or functional fragments thereof, and the dimerizing agent can be FK506. In some embodiments, the separate polypeptides can be FKBP, or functional fragments thereof, and CyP-Fas, or functional fragments thereof, and the dimerizing agent can be FKCsA. In some embodiments, the separate polypeptides can be GAI, or functional fragments thereof, and GID1, or functional fragments thereof, and the dimerizing agent can be gibberellin. In some embodiments, the separate polypeptides can be Snap-tag and HaloTag, or functional fragments thereof, and the dimerizing agent can be HaXS. In some embodiments, the separate polypeptides can include the same polypeptide. For example, the DNA binding domain and activation domain can be expressed as fusion proteins with FKBP or GyrB and the dimerizing agent can be FK1012 or coumermycin, respectively. In some embodiments, the inducible promoter can be the DNA sequence where the DNA binding domain typically binds. In some embodiments, the inducible promoter can vary from the DNA sequence where the DNA binding domain typically binds. In some embodiments, either transactivator can be an rtTA, the ligand

can be tetracycline or doxycycline, and the inducible promoter can be a TRE. In illustrative embodiments, the first transactivator is the p65 activation domain fused to FRB and the ZFHD1 DNA binding domain fused to three FKBP polypeptides and the first ligand is rapamycin. In further illustrative embodiments, the second transactivator can be an rtTA, the second ligand can be tetracycline or doxycycline, and the inducible promoter can be a TRE.

[381] In some embodiments, the first transactivator can regulate expression of an element to control the nuclear export of transcripts containing a consensus sequence, such as an HIV Rev and the consensus sequence can be the Rev response element. In illustrative embodiments, the target cell is a T cell.

[382] In some embodiments, the pseudotyping element is a retroviral envelope polypeptide. The pseudotyping element typically includes a binding polypeptide and a fusogenic polypeptide for binding to and facilitating membrane fusion of the target cell and viral membranes, as discussed in more detail herein. In some embodiments, the pseudotyping element is the feline endogenous virus (RD114) envelope protein, the oncoretroviral amphotropic envelope protein, the oncoretroviral ecotropic envelope protein, and/or vesicular stomatitis virus (VSV-G) envelope protein. In illustrative embodiments, the pseudotyping element includes a binding polypeptide and a fusogenic polypeptide derived from different proteins, as discussed in further detail herein. For example, in an illustrative embodiment, especially where the target cell is a T cell and/or NK cell, the binding polypeptide is a hemagglutinin (H) polypeptide of a Measles Virus (such as the Edmonston strain of the Measles Virus), or a cytoplasmic domain deletion variant thereof, and the fusogenic polypeptide other is a fusion (F) polypeptide of a Measles Virus (such as the Edmonston strain of the Measles Virus), or a cytoplasmic domain deletion variant thereof. In some embodiments, the fusogenic polypeptide can include multiple elements expressed as one polypeptide. In some embodiments, the binding polypeptide and the fusogenic polypeptide can be translated from the same transcript but from separate ribosome binding sites, or the polypeptide is cleaved after translation using a peptide cleavage signal or a ribosomal skip sequence, as disclosed elsewhere herein, to generate the binding polypeptide and the fusogenic polypeptide. In some embodiments, where the binding polypeptide is a Measles Virus H polypeptide, or a cytoplasmic domain deletion thereof, and the fusogenic polypeptide is a Measles Virus F polypeptide, or a cytoplasmic domain deletion thereof, translation of the F and H polypeptides from separate ribosome binding sites results in a higher amount of the F polypeptide as compared to the H polypeptide. In some embodiments, the ratio of the F polypeptides (or cytoplasmic domain deletions thereof) to H polypeptides (or cytoplasmic domain deletions thereof) is at least 2:1, at least 3:1, at least 4:1, at least 5:1, at least 6:1, at least 7:1, or at least 8:1.

[383] In some embodiments, the first transactivator can regulate the expression of an activation element capable of binding to and activating a target cell, such as a T cell. Any of the activation elements

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disclosed herein can be expressed. For example, in these embodiments, the activation element can include: a.) a membrane-bound polypeptide capable of binding to and activating CD3: and/or b.) a membrane-bound polypeptide capable of binding to and activating CD28. In some embodiments, the membrane-bound polypeptide capable of binding to and activating CD28 is CD80, CD86, or functional fragments thereof, such as the extracellular domain of CD80.

[384] In some embodiments, the second transactivator can regulate the expression of an RNA that encodes one or more target polypeptides, including as a non-limiting example, any of the engineered signaling polypeptides disclosed herein. It should be noted that it is envisioned that the retroviral packaging system aspect, and the method of making a recombinant retrovirus aspect, are not limited to making recombinant retroviruses for transduction of T cell and/or NK cells, but rather for any cell type that can be transduced by recombinant retroviruses. The RNA, in certain illustrative embodiments, includes in opposite orientation (e.g., encoding on the opposite strand and in the opposite orientation), retroviral components such as gag and pol. For example, the RNA can include from 5′ to 3′: a 5′ long terminal repeat, or active truncated fragment thereof; a nucleic acid sequence encoding a retroviral cis-acting RNA packaging element; a nucleic acid sequence encoding a first and optionally second target polypeptide, such as, but not limited to, an engineered signaling polypeptide(s), which can be driven off a promoter, which in some embodiments is called a "fourth" promoter for convenience only; a promoter that is active in a target cell; and a 3′ long terminal repeat, or active truncated fragment thereof. In some embodiments, the RNA can include a central polypurine tract (cPPT)/central termination sequence (CTS) element. In some embodiments, the retroviral cis-acting RNA packaging element can be HIV Psi. In some embodiments, the retroviral cis-acting RNA packaging element can be the Rev Response Element. The engineered signaling polypeptide in illustrative embodiments, is one or more of the engineered signaling polypeptides disclosed herein.

[385] It will be understood that promoter number, such as a first, second, third, fourth, etc. promoter is for convenience only. A promoter that is called a "fourth" promoter should not be taken to imply that there are any additional promoters, such as first, second or third promoters, unless such other promoters are explicitly recited.

[386] In some embodiments, the engineered signaling polypeptide can include a first lymphoproliferative element. Suitable lymphoproliferative elements are disclosed in other sections herein. As a non-limiting example, the lymphoproliferative element can be expressed as a fusion with a recognition domain, such as an eTag, as disclosed herein. In some embodiments, the packagable RNA genome can further include a nucleic acid sequence encoding a second engineered polypeptide including a chimeric antigen receptor, encoding any CAR embodiment provided herein. For example, the second engineered polypeptide can include a first antigen-specific targeting region, a first transmembrane

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domain, and a first intracellular activating domain. Examples of antigen-specific targeting regions, transmembrane domains, and intracellular activating domains are disclosed elsewhere herein. In some embodiments where the target cell is a T cell, the promoter that is active in a target cell is active in a T cell, as disclosed elsewhere herein.

[387] In some embodiments, the packagable RNA genome can further include a riboswitch, as discussed in other sections herein. In some embodiments, the nucleic acid sequence encoding the engineered signaling polypeptide can be in reverse orientation. In further embodiments, the packagable RNA genome can further include a riboswitch and, optionally, the riboswitch can be in reverse orientation. In any of the embodiments disclosed herein, a polynucleotide including any of the elements can include a primer binding site. In illustrative embodiments, transcription blockers or polyA sequences can be placed near genes to prevent or reduce unregulated transcription. In any of the embodiments disclosed herein, a nucleic acid sequence encoding Vpx can be on the second or an optional third transcriptional unit, or on an additional transcriptional unit that is operably linked to the first inducible promoter.

[388] In another aspect, provided herein is a method for making a recombinant retrovirus, including: culturing a population of packaging cells to accumulate a first transactivator, wherein the packaging cells include the first transactivator expressed from a constitutive promoter, wherein the first transactivator is capable of binding a first ligand and a first inducible promoter for affecting expression of a nucleic acid sequence operably linked thereto in the presence versus absence of the first ligand, and wherein expression of a second transactivator is regulated by the first transactivator; incubating the population of packaging cells including accumulated first transactivator in the presence of the first ligand to accumulate the second transactivator, wherein the second transactivator is capable of binding a second ligand and a second inducible promoter for affecting expression of a nucleic acid sequence operably linked thereto in the presence versus absence of the second ligand; and incubating the population of packaging cells including accumulated second transactivator in the presence of the second ligand thereby inducing expression of retroviral polypeptides involved in viral packaging, such as, for example, a gag polypeptide, a pol polypeptide, and/or a pseudotyping element, and optionally other polypeptides that are believed to inhibit mammalian cell proliferation or survival that will become incorporated in or on the recombinant retrovirus, thereby making the recombinant retrovirus. In illustrative embodiments, a packagable RNA genome is encoded by a polynucleotide operably linked to a promoter, sometimes referred to for convenience as a "third" promoterwherein said third promoter is either constitutively active or inducible by either the first transactivator or, in illustrative embodiments, the second transactivator, thereby making the recombinant retrovirus. The pseudotyping elements are typically capable of binding to a cell membrane of a target cell and facilitating fusion of the target cell membrane to the recombinant retrovirus

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membrane. The pseudotyping elements can be any envelope proteins known in the art. In some embodiments, the envelope protein can be vesicular stomatitis virus (VSV-G) envelope protein, feline endogenous virus (RD114) envelope protein, oncoretroviral amphotropic envelope protein, and/or oncoretroviral ecotropic envelope protein. A skilled artisan will appreciate many different transactivators, ligands, and inducible promoters can be used in the method for making a recombinant retrovirus. Suitable transactivators, ligands, and inducible promoters are disclosed elsewhere herein, including above. A skilled artisan will further appreciate that the teachings hereinabove related to a retroviral packaging system aspect provided herein, apply to method of making recombinant retrovirus aspects as well, and the reverse.

[389] In some embodiments, the first transactivator can regulate expression of an element to control the nuclear export of transcripts containing a consensus sequence, such as an HIV Rev and the consensus sequence can be the Rev Response Element (RRE). In illustrative embodiments, the target cell is typically a T cell. In some embodiments, the HIV RREs and the polynucleotide region encoding HIV Rev can be replaced with HIV-2 RREs and a polynucleotide region encoding the HIV-2 Rev, respectively. In some embodiments, the HIV RREs and the polynucleotide region encoding HIV Rev can be replaced with SIV RREs and a polynucleotide region encoding the SIV Rev, respectively. In some embodiments, the HIV RREs and the polynucleotide region encoding HIV Rev can be replaced with RemREs and a polynucleotide region encoding a betaretrovirus Rem, respectively. In some embodiments, the HIV RREs and the polynucleotide region encoding HIV Rev can be replaced with a deltaretrovirus RexRRE and a polynucleotide region encoding a deltaretrovirus Rex, respectively. In some embodiments, a Rev-like protein is not required and the RREs can be replaced with cis-acting RNA elements, such as the constitutive transport element (CTE).

[390] In some embodiments, the pseudotyping element is a viral envelope protein. The pseudotyping element typically includes a binding polypeptide and a fusogenic polypeptide for binding to and facilitating membrane fusion of viral and target cell membranes. In some embodiments, the pseudotyping element can be the feline endogenous virus (RD114) envelope protein, the oncoretroviral amphotropic envelope protein, the oncoretroviral ecotropic envelope protein, and/or vesicular stomatitis virus (VSV-G) envelope protein. In illustrative embodiments, the pseudotyping element includes a binding polypeptide and a fusogenic polypeptide derived from different proteins, as discussed in further detail herein. For example, in an illustrative embodiment, especially where the target cell is a T cell and/or NK cell, the binding polypeptide can be a cytoplasmic domain deletion variant of a Measles Virus H polypeptide and the fusogenic polypeptide can be the cytoplasmic domain deletion variant of a Measles Virus F polypeptide. In some embodiments, the fusogenic polypeptide can include multiple elements expressed as one polypeptide. In some embodiments, the binding polypeptide and fusogenic polypeptide can be

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translated from the same transcript and translated from separate ribosome binding sites, or the polypeptide can be cleaved after translation using a peptide cleavage signal or a ribosomal skip sequence, as disclosed elsewhere herein, to generate the binding polypeptide and the fusogenic polypeptide. In some embodiments, the translation of the binding polypeptide and fusogenic polypeptide from separate ribosome binding sites results in a higher amount of the fusogenic polypeptide as compared to the binding polypeptide. In some embodiments, the ratio of the fusogenic polypeptide to the binding polypeptide is at least 2:1, at least 3:1, at least 4:1, at least 5:1, at least 6:1, at least 7:1, or at least 8:1.

[391] In some embodiments, the first transactivator can regulate the expression of an activation element capable of binding to and activating a target cell, such as a T cell. In these embodiments, the activation element can include: a.) aa membrane-bound polypeptide capable of binding to and activating CD3: and/or b.) a membrane-bound polypeptide capable of binding to and activating CD28. In some embodiments, the membrane-bound polypeptide capable of binding to and activating CD28 is CD80, CD86, or functional fragments thereof. In some embodiments, the recombinant retrovirus can include the activation element on a retroviral membrane and the retroviral RNA within a nucleocapsid, thereby making a recombinant retrovirus.

[392] In some embodiments, the second transactivator can regulate the expression of an RNA including from 5′ to 3′: a 5′ long terminal repeat, or active truncated fragment thereof; a nucleic acid sequence encoding a retroviral cis-acting RNA packaging element; a nucleic acid sequence encoding a first target polypeptide and optional second target polypeptide, as non-limiting example, one or two engineered signaling polypeptides; a promoter that is active in a target cell; and a 3′ long terminal repeat, or active truncated fragment thereof. In some embodiments, the RNA can include a cPPT/CTS element. In some embodiments, the RNA can include a primer binding site. In some embodiments, the retroviral cis-acting RNA packaging element can be HIV Psi. In some embodiments, the retroviral cis-acting RNA packaging element can be the Rev Response Element. In any of the embodiments disclosed herein, retroviral components on the RNA, including RRE and Psi, can be located in any position, as a skilled artisan will understand. The engineered signaling polypeptide in illustrative embodiments, is one or more of the engineered signaling polypeptides disclosed herein.

[393] In some embodiments, the engineered signaling polypeptide can include a first lymphoproliferative element. Suitable lymphoproliferative elements are disclosed in other sections herein. In some illustrative embodiments, the lymphoproliferative element is an IL-7 receptor mutant fused to a recognition domain, such as an eTag. In some embodiments, the packagable RNA genome can further include a nucleic acid sequence encoding a second engineered polypeptide including a chimeric antigen receptor, encoding any CAR embodiment provided herein. For example, the second engineered polypeptide can include a first antigen-specific targeting region, a first transmembrane domain, and a first

intracellular activating domain. Examples of antigen-specific targeting regions, transmembrane domains, and intracellular activating domains are disclosed elsewhere herein. In some embodiments where the target cell is a T cell, the promoter that is active in a target cell is active in a T cell, as disclosed elsewhere herein.

[394] In some embodiments, the packagable RNA genome can further include a riboswitch, as discussed in other sections herein. In some embodiments, the nucleic acid sequence encoding the engineered signaling polypeptide can be in reverse orientation. In further embodiments, the packagable RNA genome can further include a riboswitch and, optionally, the riboswitch can be in reverse orientation. In any of the embodiments disclosed herein, a polynucleotide including any of the elements can include a primer binding site. In illustrative embodiments, transcription blockers or polyA sequences can be placed near genes to prevent or reduce unregulated transcription. In any of the embodiments disclosed herein, a nucleic acid sequence encoding Vpx can be on the second or an optional third transcriptional unit, or on an additional transcriptional unit that is operably linked to the first inducible promoter.

[395] In some embodiments of the packaging system or methods for making retrovirus aspects, the encoded RNA can include an intron, which can be transcribed, for example, from the same promoter for expressing the target polypeptide(s). Such intron can encode 1, 2, 3, or 4 miRNAs, in certain illustrative embodiments. In these and other embodiments of the packaging system or methods for making retrovirus aspects, the packagable RNA genome is 11,000 KB or less and in some instances 10,000 KB or less in size.

[396] In some embodiments, the first transactivator can affect the expression of one or more polypeptides that are non-toxic. In some embodiments, the second transactivator can affect the expression of one or more polypeptides that are toxic. For example, the first transactivator can induce expression of the retroviral proteins Rev and Vpx in addition to polypeptides that will be transported to the cell membrane of the packaging cell and the second transactivator can induce expression of the retroviral proteins GAG, POL, MV(Ed)-FΔ30, and either MV(Ed)-HΔ18 or MV(Ed)-HΔ24 and expression of the lentiviral genome. In some embodiments, the first transactivator can affect the expression of one or more polypeptides that are toxic and/or the second transactivator can affect the expression of one or more polypeptides that are non-toxic.

[397] In another aspect, provided herein is a mammalian packaging cell, including: a.) a first transcriptional unit in the genome of the mammalian packaging cell, including a nucleic acid sequence encoding a first transactivator, wherein said first transcriptional unit is operably linked to a constitutive promoter and wherein said transactivator is capable of binding a first inducible promoter and affecting expression of a nucleic acid sequence operably linked thereto in the presence versus absence of a first

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ligand, and wherein said first transactivator is capable of binding said first ligand; b.) a second and optional third transcriptional unit in the genome of the mammalian packaging cell, including a nucleic acid sequence encoding a retroviral REV protein and a nucleic acid sequence encoding a second transactivator capable of binding a second inducible promoter and affecting expression of a nucleic acid sequence operably linked thereto in the presence versus absence of a second ligand, wherein the second transactivator is capable of binding the second ligand, and wherein the second and optional third transcriptional units are operably linked to the first inducible promoter; c.) a fourth and optional fifth transcriptional unit in the genome of the mammalian packaging cell, including a nucleic acid sequence encoding a retroviral gag polypeptide and a retroviral pol polypeptide, and a binding polypeptide and a fusogenic polypeptide that are capable of binding to and facilitating fusion of a target cell membrane and the retroviral membrane, wherein the fourth and optional fifth transcriptional unit are operably linked to the second inducible promoter; and d) a sixth transcriptional unit in the genome of the mammalian packaging cell, including , from 5′ to 3′, a 5′ LTR, or active truncated fragment thereof, a nucleic acid sequence encoding a retroviral cis-acting RNA packaging element, a cPPT/CTS element, a reverse complement of a nucleic acid sequence encoding an engineered signaling polypeptide, an intron, a promoter that is active in a target cell, and a 3′ LTR, or active truncated fragment thereof, wherein the sixth transcriptional unit is operably linked to the second inducible promoter.

[398] In another aspect, provided herein is a method for making a recombinant retrovirus, including: 1.) culturing a population of packaging cells to accumulate a first transactivator, wherein the packaging cells include: a.) a first transcriptional unit in the genome of the mammalian packaging cell, including a nucleic acid sequence encoding a first transactivator, wherein said first transcriptional unit is operably linked to a constitutive promoter and wherein said transactivator is capable of binding a first inducible promoter and affecting expression of a nucleic acid sequence operably linked thereto in the presence versus absence of a first ligand, and wherein said first transactivator is capable of binding said first ligand; b.) a second and optional third transcriptional unit in the genome of the mammalian packaging cell, including a nucleic acid sequence encoding a retroviral REV protein and a nucleic acid sequence encoding a second transactivator capable of binding a second inducible promoter and affecting expression of a nucleic acid sequence operably linked thereto in the presence versus absence of a second ligand, wherein the second transactivator is capable of binding the second ligand, and wherein the second and optional third transcriptional units are operably linked to the first inducible promoter; c.) a fourth and optional fifth transcriptional unit in the genome of the mammalian packaging cell, including a nucleic acid sequence encoding a retroviral gag polypeptide and a retroviral pol polypeptide, and a binding polypeptide and a fusogenic polypeptide that are capable of binding to and facilitating fusion of the retroviral membrane with a target cell membrane, wherein the fourth and optional fifth transcriptional unit are operably linked

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to the second inducible promoter; and d.) a sixth transcriptional unit in the genome of the mammalian packaging cell, including from 5′ to 3′, a 5′ LTR, or active truncated fragment thereof, a primer binding site (PBS), a nucleic acid sequence encoding a retroviral cis-acting RNA packaging element, a cPPT/CTS element, a reverse complement of a nucleic acid sequence encoding an engineered signaling polypeptide, an intron, a target cell promoter that is active in a target cell, a 3′ LTR, or active truncated fragment thereof, wherein the fifth transcriptional unit is operably linked to the second inducible promoter; and 2.) incubating the population of packaging cells including the first transactivator in the presence of the first ligand to accumulate the second transactivator and the retroviral REV protein; and 3.) incubating the population of packaging cells including the second transactivator and the retroviral REV protein in the presence of the second ligand thereby inducing expression of the retroviral gag polypeptide, the retroviral pol polypeptide, the binding polypeptide, the fusogenic polypeptide, and a retroviral RNA including from 5′ to 3′, a 5′ LTR, or active fragment thereof, the PBS, the retroviral cis-acting RNA packaging element, the reverse complement of the nucleic acid sequence encoding the engineered signaling polypeptide, the target cell promoter, and a 3′ LTR, or active truncated fragment thereof, wherein recombinant retroviruses are formed and release from the packaging cells, and wherein the recombinant retroviruses include the binding polypeptide and/or the fusogenic polypeptide on a retroviral membrane and the retroviral RNA within a nucleocapsid, thereby making recombinant retroviruses.

[399] In one aspect provided herein, the retroviral packaging system can include a mammalian cell including: 1.) a first transactivator expressed from a constitutive promoter and capable of binding a first ligand and a first inducible promoter for affecting expression of a nucleic acid sequence operably linked thereto in the presence versus absence of the first ligand; 2.) a second transactivator capable of binding a second ligand and a second inducible promoter and affecting expression of a nucleic acid sequence operably linked thereto in the presence versus absence of a second ligand; and 3.) a packagable RNA genome for a retroviral particle, wherein the first transactivator regulates expression of the second transactivator, HIV REV, an IL7 GPI DAF, and an activation element, and wherein the second transactivator regulates expression of a gag polypeptide, a pol polypeptide, a retroviral cis-acting RNA packaging element, and one or more envelope polypeptides. In illustrative embodiments, the first transactivator can be an FRB domain fused to a p65 activation domain and one or more FKBP domains fused to a ZFHD1 DNA binding domain, the first ligand can be rapamycin, and the first inducible promoter can be one or more ZFHD1 binding sites. In illustrative embodiments, the second transactivator can be an rtTA protein, the second ligand can be tetracycline or doxycycline, and the second inducible promoter can be a TRE promoter or a bi-directional TRE promoter. In illustrative embodiments, the retroviral cis-acting RNA packaging element can be HIV Psi. In illustrative embodiments, the one or more envelope proteins include the cytoplasmic domain deletion variants of F and H polypeptides of a

Measles Virus. In illustrative embodiments, transcription blockers or polyA sequences can be placed near genes to prevent or reduce unregulated transcription. In some embodiments, a rapamycin-doxycycline inducible lentiviral genome with riboswitch can be used (SEQ ID NO:83). In some embodiments, a rapamycin-doxycycline inducible GAG POL ENV can be used (SEQ ID NO:84). In some embodiments, a rapamycin-inducible TET activator can be used (SEQ ID NO:85). In some embodiments, a rapamycin inducer inducible REV srcVpx can be used (SEQ ID NO:86).

[400] Some aspects of the present disclosure include or are cells, in illustrative examples, mammalian cells, that are used as packaging cells to make retroviruses, such as lentiviruses, for transduction of T cells and/or NK cells. Any of a wide variety of cells can be selected for *in vitro* production of a virus, such as a redirected retrovirus, according to the invention. Eukaryotic cells are typically used, particularly mammalian cells including human, simian, canine, feline, equine and rodent cells. In illustrative examples, the cells are human cells. In further illustrative embodiments, the cells reproduce indefinitely, and are therefore immortal. Examples of cells that can be advantageously used in the present invention include NIH 3T3 cells, COS cells, Madin-Darby canine kidney cells, human embryonic 293T cells and any cells derived from such cells, such as gpnlslacZ φNX cells, which are derived from 293T cells. Highly transfectable cells, such as human embryonic kidney 293T cells, can be used. By "highly transfectable" it is meant that at least about 50%, more preferably at least about 70% and most preferably at least about 80% of the cells can express the genes of the introduced DNA.

[401] Suitable mammalian cells include primary cells and immortalized cell lines. Suitable mammalian cell lines include human cell lines, non-human primate cell lines, rodent (e.g., mouse, rat) cell lines, and the like. Suitable mammalian cell lines include, but are not limited to, HeLa cells (e.g., American Type Culture Collection (ATCC) No. CCL-2), CHO cells (e.g., ATCC Nos. CRL9618, CCL61, CRL9096), 293 cells (e.g., ATCC No. CRL-1573), Vero cells, NIH 3T3 cells (e.g., ATCC No. CRL-1658), Huh-7 cells, BHK cells (e.g., ATCC No. CCLlO), PC12 cells (ATCC No. CRL1721), COS cells, COS-7 cells (ATCC No. CRL1651), RATl cells, mouse L cells (ATCC No. CCLI.3), human embryonic kidney (HEK) cells (ATCC No. CRL1573), HLHepG2 cells, Hut-78, Jurkat, HL-60, NK cell lines (e.g., NKL, NK92, and YTS), and the like.

[402] In any of the embodiments disclosed herein, the methods of making a recombinant retrovirus can include growing a mammalian packaging cells to 50%, 60%, 70%, 80%, 90% or 95% confluence or confluence or to 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 95% peak cell density or peak cell density and then splitting or diluting the cells. In some embodiments, a stirred tank reactor can be used to grow the cells. In some embodiments, the cells can be split at least about 1:2, 1:3, 1:4, 1:5, 1:6, 1:7, 1:8, 1:9, 1:10, 1:12, 1:15, or 1:20 using methods a skilled artisan will understand. In some embodiments, the cells can be diluted to 25%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 95% peak cell density. In some

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embodiments, after splitting or diluting the cells the cells can be grown for 1, 2, 3, 4, 5, 6, 7, 8, 10, or 16 hours or 1, 2, 3, 4, 5, 6, or 7 days before adding the first ligand. In some embodiments, the cells are grown in the presence of the first ligand for 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 21, or 28 days in the presence of the first ligand, which in illustrative embodiments can be rapamycin or a rapalog. In some embodiments, the second ligand can be added and the cells can be grown for at least 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 21, or 28 days which in illustrative embodiments can be tetracycline or doxycyline. Conditions for culturing will depend on the cells and ligands used and the methods are known in the art. A specific example of conditions for culturing and inducing HEK293S cells is shown in Example 8.

[403] As disclosed herein, recombinant retroviruses are a common tool for gene delivery (Miller, Nature (1992) 357:455-460). The ability of recombinant retroviruses to deliver an unrearranged nucleic acid sequence into a broad range of rodent, primate and human somatic cells makes recombinant retroviruses well suited for transferring genes to a cell. In some embodiments, the recombinant retrovirus can be derived from the Alpharetrovirus genus, the Betaretrovirus genus, the Gammaretrovirus genus, the Deltaretrovirus genus, the Epsilonretrovirus genus, the Lentivirus genus, or the Spumavirus genus. There are many retroviruses suitable for use in the methods disclosed herein. For example, murine leukemia virus (MLV), human immunodeficiency virus (HIV), equine infectious anaemia virus (EIAV), mouse mammary tumor virus (MMTV), Rous sarcoma virus (RSV), Fujinami sarcoma virus (FuSV), Moloney murine leukemia virus (Mo-MLV), FBR murine osteosarcoma virus (FBR MSV), Moloney murine sarcoma virus (Mo-MSV), Abelson murine leukemia virus (A-MLV), Avian myelocytomatosis virus-29 (MC29), and Avian erythroblastosis virus (AEV) can be used. A detailed list of retroviruses may be found in Coffin et al ("Retroviruses" 1997 Cold Spring Harbor Laboratory Press Eds: J M Coffin, S M Hughes, H E Varmus pp 758-763). Details on the genomic structure of some retroviruses may be found in the art. By way of example, details on HIV may be found from the NCBI Genbank (i.e. Genome Accession No. AF033819).

[404] In illustrative embodiments, the recombinant retrovirus can be derived from the Lentivirus genus. In some embodiments, the recombinant retrovirus can be derived from HIV, SIV, or FIV. In further illustrative embodiments, the recombinant retrovirus can be derived from the human immunodeficiency virus (HIV) in the Lentivirus genus. Lentiviruses are complex retroviruses which, in addition to the common retroviral genes gag, pol and env, contain other genes with regulatory or structural function. The higher complexity enables the lentivirus to modulate the life cycle thereof, as in the course of latent infection. A typical lentivirus is the human immunodeficiency virus (HIV), the etiologic agent of AIDS. *In vivo*, HIV can infect terminally differentiated cells that rarely divide, such as lymphocytes and macrophages.

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[405] In illustrative embodiments, recombinant retroviruses provided herein contain Vpx polypeptide. Vpx polypeptide can be expressed in a packaging cell line, after integration of a Vpx coding nucleic acid in its genome, for example as a cell membrane bound protein that gets incorporated into a retrovirus membrane (Durand et al., *J. Virol.* (2013) 87: 234-242). A retroviral membrane bound Vpx can be constructed with a processing sequence for a viral protease such that free Vpx is released once incorporated in a viral particle. Such an example of a Vpx fusion with this functionality is Src-Flag-Vpx, which includes a membrane-targeting domain (MGSSKSKPKDP) (SEQ ID NO:227) of the first 11 amino acids of c-Src followed by a viral protease cleavage domain KARVLAEA (SEQ ID NO:228) followed by Flag-tagged Vpx.

[406] Not to be limited by theory, Vpx polypeptide aids in transduction of resting cells by stimulating the efficiency of the process of reverse transcription by degrading the restriction factor SAMHD1. Accordingly, it is believed that in the methods provided herein where Vpx is present in a recombinant retrovirus used to transduce T cells and/or NK cells, Vpx is released into the cytoplasm of a resting T cell or a resting NK cell upon transduction of the cell by a recombinant retrovirus that contains Vpx. Vpx then degrades SAMHD1, which causes an increase in free dNTPs, which in turn, stimulates reverse transcription of the retroviral genome.

RETROVIRAL GENOME SIZE

[407] In the methods and compositions provided herein, the recombinant retroviral genomes, in non-limiting illustrative examples, lentiviral genomes, have a limitation to the number of polynucleotides that can be packaged into the viral particle. In some embodiments provided herein, the polypeptides encoded by the polynucleotide encoding region can be truncations or other deletions that retain a functional activity such that the polynucleotide encoding region is encoded by less nucleotides than the polynucleotide encoding region for the wild-type polypeptide. In some embodiments, the polypeptides encoded by the polynucleotide encoding region can be fusion polypeptides that can be expressed from one promoter. In some embodiments, the fusion polypeptide can have a cleavage signal to generate two or more functional polypeptides from one fusion polypeptide and one promoter. Furthermore, some functions that are not required after initial *ex vivo* transduction are not included in the retroviral genome, but rather are present on the surface of the virus or retrovirus via the packaging cell membrane. These various strategies are used herein to maximize the functional elements that packaged within the retrovirus.

[408] In some embodiments, the recombinant retroviral genome to be packaged can be between 1,000, 2,000, 3,000, 4,000, 5,000, 6,000, 7,000, and 8,000 nucleotides on the low end of the range and 2,000, 3,000, 4,000, 5,000, 6,000, 7,000, 8,000, 9,000, 10,000, and 11,000 nucleotides on the high end of the range. The retroviral genome to be packaged includes one or more polynucleotide regions encoding a first

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and second engineering signaling polypeptide as disclosed in detail herein. In some embodiments, the recombinant retroviral genome to be packaged can be less than 5,000, 6,000, 7,000, 8,000, 9,000, 10,000, or 11,000 nucleotides. Functions discussed elsewhere herein that can be packaged include required retroviral sequences for retroviral assembly and packaging, such as a retroviral rev, gag, and pol coding regions, as well as a 5′ LTR and a 3′ LTR, or an active truncated fragment thereof, a nucleic acid sequence encoding a retroviral cis-acting RNA packaging element, and a cPPT/CTS element. Furthermore, in illustrative embodiments a recombinant virus or retrovirus herein can include any one or more or all of the following, in some embodiments in reverse orientation of these retroviral functional regions: one or more polynucleotide regions encoding a first and second engineering signaling polypeptide, at least one of which includes a lymphoproliferative element and can further include an ASTR; a second engineered signaling polypeptide that can include a chimeric antigen receptor;an *in vivo* control element, such as a riboswitch, which typically regulates expression of the first and/or the second engineering signaling polypeptide; a recognition domain, an intron, a promoter that is active in a target cell, such as a T cell, a 2A cleavage signal and/or an IRES.

RECOMBINANT RETROVIRUSES

[409] Recombinant retroviruses are disclosed in methods and compositions provided herein, for example, to transduce T cells and/or NK cells to make genetically modified T cells and/or NK cells. The recombinant retroviruses are themselves aspects of the present invention. In some embodiments, the recombinant retroviruses are replication incompetent, meaning that a retrovirus cannot replicate once it leaves the packaging cell. In some embodiments, the recombinant retroviruses can be adenoviruses, adeno-associated viruses, herpesviruses, cytomegaloviruses, poxviruses, avipox viruses, influenza viruses, vesicular stomatitis virus (VSV), or Sindbis virus. A skilled artisan will appreciate how to modify the methods disclosed herein for use with different retroviruses. For example, in some embodiments, the HIV RREs and the polynucleotide region encoding HIV Rev can be replaced with N-terminal RGG box RNA binding motifs and a polynucleotide region encoding ICP27. In some embodiments, the polynucleotide region encoding HIV Rev can be replaced with one or more polynucleotide regions encoding adenovirus E1B 55-kDa and E4 Orf6.

[410] Accordingly, provided herein in some embodiments, is a recombinant retrovirus that includes (i) a pseudotyping element capable of binding to a T cell and/or NK cell and facilitating membrane fusion of the recombinant retrovirus thereto; (ii) a polynucleotide having one or more transcriptional units operatively linked to a promoter active in T cells and/or NK cells, wherein the one or more transcriptional units encode a first engineered signaling polypeptide having a chimeric antigen receptor that includes an antigen-specific targeting region, a transmembrane domain, and an intracellular activating domain, and a

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second engineered signaling polypeptide that includes a lymphoproliferative element; wherein expression of the first engineered signaling polypeptide and/or the second engineered signaling polypeptide are regulated by an *in vivo* control element; and (iii) an activation element on its surface, wherein the activation element is capable of binding to a T cell and/or NK cell and is not encoded by a polynucleotide in the recombinant retrovirus. In some embodiments, the active in T cells and/or NK cells is not active in the packaging cell line. In any of the embodiments disclosed herein, either of the first and second engineered signaling polypeptides can have a chimeric antigen receptor and the other engineered signaling polypeptide can have a lymphoproliferative element.

GENETICALLY MODIFIED T CELLS AND NK CELLS

[411] In embodiments of the methods and compositions herein, genetically modified lymphocytes are produced, which themselves are a separate aspect of the invention. In some embodiments, genetically modified lymphocytes are lymphocytes such as T cells and/or NK cells that have been genetically modified to express a first engineered signaling polypeptide comprising a lymphoproliferative element and/or a second engineered signaling polypeptide comprising a chimeric antigen receptor, which includes an antigen-specific targeting region (ASTR), a transmembrane domain, and an intracellular activating domain.

[412] In the methods and compositions disclosed herein, expression of one or both of the engineered signaling polypeptides is typically regulated by an *in vivo* control element, and in some embodiments, the *in vivo* control element is a polynucleotide comprising a riboswitch. In certain embodiments, the riboswitch is capable of binding a nucleoside analog and when the nucleoside analog is present, one or both of the engineered signaling polypeptides are expressed.

[413] The genetically modified lymphocytes disclosed herein can also have polypeptides expressed on their surface, such as one or more polypeptides that function as an activation element, one or more polypeptides that function as a pseudotyping element, and/or one or more fusion polypeptides that include a cytokine. In some embodiments, the genetically modified lymphocytes have an activation element on their surface. The activation element can have a membrane-bound polypeptide capable of binding to CD3; and/or a membrane-bound polypeptide capable of binding to CD28. In some embodiments, the activation element is anti-CD3 scFvFc fused to a heterologous GPI anchor attachment sequence and/or CD80 fused to a heterologous GPI anchor attachment sequence. In some embodiments, the genetically modified lymphocytes have a pseudotyping element on their surface. In some embodiments, the genetically modified lymphocytes have a fusion polypeptide on their surface in which the fusion polypeptide is a cytokine covalently attached to DAF. In some embodiments, the cytokine is IL-7 or IL-15. In illustrative embodiments, the cytokine is IL-7. In some embodiments, the cytokine is without its

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signal sequence. In illustrative embodiments, the cytokine is inserted into DAF behind its signal sequence.

NUCLEIC ACIDS

[414] The present disclosure provides nucleic acid encoding polypeptides of the present disclosure. A nucleic acid will in some embodiments be DNA, including, e.g., a recombinant expression vector. A nucleic acid will in some embodiments be RNA, e.g., *in vitro* synthesized RNA.

[415] In some cases, a nucleic acid provides for production of a polypeptide of the present disclosure, e.g., in a mammalian cell. In other cases, a subject nucleic acid provides for amplification of the nucleic acid encoding a polypeptide of the present disclosure.

[416] A nucleotide sequence encoding a polypeptide of the present disclosure can be operably linked to a transcriptional control element, e.g., a promoter, and enhancer, etc.

[417] Suitable promoter and enhancer elements are known in the art. For expression in a bacterial cell, suitable promoters include, but are not limited to, lacI, lacZ, T3, T7, gpt, lambda P and trc. For expression in a eukaryotic cell, suitable promoters include, but are not limited to, light and/or heavy chain immunoglobulin gene promoter and enhancer elements; cytomegalovirus immediate early promoter; herpes simplex virus thymidine kinase promoter; early and late SV40 promoters; promoter present in long terminal repeats from a retrovirus; mouse metallothionein-I promoter; and various art-known tissue specific promoters.

[418] Suitable reversible promoters, including reversible inducible promoters are known in the art. Such reversible promoters may be isolated and derived from many organisms, e.g., eukaryotes and prokaryotes. Modification of reversible promoters derived from a first organism for use in a second organism, e.g., a first prokaryote and a second a eukaryote, a first eukaryote and a second a prokaryote, etc., is well known in the art. Such reversible promoters, and systems based on such reversible promoters but also comprising additional control proteins, include, but are not limited to, alcohol regulated promoters (e.g., alcohol dehydrogenase I (alcA) gene promoter, promoters responsive to alcohol transactivator proteins (AlcR), etc.), tetracycline regulated promoters, (e.g., promoter systems including TetActivators, TetON, TetOFF, etc.), steroid regulated promoters (e.g., rat glucocorticoid receptor promoter systems, human estrogen receptor promoter systems, retinoid promoter systems, thyroid promoter systems, ecdysone promoter systems, mifepristone promoter systems, etc.), metal regulated promoters (e.g., metallothionein promoter systems, etc.), pathogenesis-related regulated promoters (e.g., salicylic acid regulated promoters, ethylene regulated promoters, benzothiadiazole regulated promoters, etc.), temperature regulated promoters (e.g., heat shock inducible promoters (e.g., HSP-70, HSP-90, soybean heat shock promoter, etc.), light regulated promoters, synthetic inducible promoters, and the like.

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[419] In some instances, the locus or construct or trans gene containing the suitable promoter is irreversibly switched through the induction of an inducible system. Suitable systems for induction of an irreversible switch are well known in the art, e.g., induction of an irreversible switch may make use of a Cre-lox-mediated recombination (see, e.g., Fuhrmann-Benzakein, et al., *PNAS* (2000) 28:e99, the disclosure of which is incorporated herein by reference). Any suitable combination of recombinase, endonuclease, ligase, recombination sites, etc. known to the art may be used in generating an irreversibly switchable promoter. Methods, mechanisms, and requirements for performing site-specific recombination, described elsewhere herein, find use in generating irreversibly switched promoters and are well known in the art, see, e.g., Grindley et al. (2006) *Annual Review of Biochemistry,* 567-605 and Tropp (2012) Molecular Biology (Jones & Bartlett Publishers, Sudbury, MA), the disclosures of which are incorporated herein by reference.

[420] In some cases, the promoter is a CD8 cell-specific promoter, a CD4 cell-specific promoter, a neutrophil-specific promoter, or an NK-specific promoter. For example, a CD4 gene promoter can be used; see, e.g., Salmon et al. (1993) *Proc. Natl. Acad. Sci. USA* 90:7739; and Marodon et al. (2003) *Blood* 101:3416. As another example, a CD8 gene promoter can be used. NK cell-specific expression can be achieved by use of an *Neri (p46)* promoter; see, e.g., Eckelhart et al. (2011) *Blood* 117:1565.

[421] In some embodiments, e.g., for expression in a yeast cell, a suitable promoter is a constitutive promoter such as an ADH1 promoter, a PGK1 promoter, an ENO promoter, a PYK1 promoter and the like; or a regulatable promoter such as a GAL1 promoter, a GAL10 promoter, an ADH2 promoter, a PH05 promoter, a CUP1 promoter, a GAL7 promoter, a MET25 promoter, a MET3 promoter, a CYC1 promoter, a HIS3 promoter, an ADH1 promoter, a PGK promoter, a GAPDH promoter, an ADC1 promoter, a TRP1 promoter, a URA3 promoter, a LEU2 promoter, an ENO promoter, a TP1 promoter, and AOX1 (e.g., for use in *Pichia).* Selection of the appropriate vector and promoter is well within the level of ordinary skill in the art.

[422] Suitable promoters for use in prokaryotic host cells include, but are not limited to, a bacteriophage T7 RNA polymerase promoter; a trp promoter; a lac operon promoter; a hybrid promoter, e.g., a lac/tac hybrid promoter, a tac/trc hybrid promoter, a trp/lac promoter, a T7/lac promoter; a trc promoter; a tac promoter, and the like; an araBAD promoter; *in vivo* regulated promoters, such as an *ssaG* promoter or a related promoter *(see,* e.g., U.S. Patent Publication No. 20040131637), a *pagC* promoter (Pulkkinen and Miller, *J. Bacterial.,* 1991: 173(1): 86-93; Alpuche-Aranda et al., PNAS, 1992; 89(21): 10079-83), a *nirB* promoter (Harborne et al. (1992) *Mal. Micro.* 6:2805-2813), and the like *(see,* e.g., Dunstan et al. (1999) *Infect. Immun.* 67:5133-5141; McKelvie et al. (2004) *Vaccine* 22:3243-3255; and Chatfield et al. (1992) *Biotechnol.* 10:888-892); a sigma70 promoter, e.g., a consensus sigma70 promoter (see, e.g., GenBank Accession Nos. AX798980, AX798961, and AX798183); a stationary phase

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promoter, e.g., a *dps* promoter, an *spv* promoter, and the like; a promoter derived from the pathogenicity island SPI-2 *(see,* e.g., W096/17951); an actA promoter *(see,* e.g., Shetron-Rama et al. (2002) *Infect. Immun.* 70:1087-1096); an rpsM promoter *(see,* e.g., Valdivia and Falkow (1996). *Mal. Microbial.* 22:367); a tet promoter *(see,* e.g., Hillen,W. and Wissmann,A. (1989) In Saenger,W. and Heinemann,U. (eds), *Topics in Molecular and Structural Biology, Protein-Nucleic Acid Interaction.* Macmillan, London, UK, Vol. 10, pp. 143-162); an SP6 promoter *(see,* e.g., Melton et al. (1984) *Nucl. Acids Res.* 12:7035); and the like. Suitable strong promoters for use in prokaryotes such as *Escherichia coli* include, but are not limited to Trc, Tac, T5, T7, andPLambda Non-limiting examples of operators for use in bacterial host cells include a lactose promoter operator (Laci repressor protein changes conformation when contacted with lactose, thereby preventing the Laci repressor protein from binding to the operator), a tryptophan promoter operator (when complexed with tryptophan, TrpR repressor protein has a conformation that binds the operator; in the absence of tryptophan, the TrpR repressor protein has a conformation that does not bind to the operator), and a tac promoter operator (see, for example, deBoer et al. (1983) Proc. Natl. Acad. Sci. U.S.A. 80:21-25).

[423] A nucleotide sequence encoding a polypeptide of the disclosure can be present in an expression vector and/or a cloning vector. Nucleotide sequences encoding two separate polypeptides can be cloned in the same or separate vectors. An expression vector can include a selectable marker, an origin of replication, and other features that provide for replication and/or maintenance of the vector. Suitable expression vectors include, e.g., plasmids, viral vectors, and the like.

[424] Large numbers of suitable vectors and promoters are known to those of skill in the art; many are commercially available for generating a subject recombinant constructs. The following bacterial vectors are provided by way of example: pBs, phagescript, PsiXl74, pBluescript SK, pBs KS, pNH8a, pNH16a, pNH18a, pNH46a (Stratagene, La Jolla, CA, USA); pTrc99A, pKK223-3, pKK233-3, pDR540, and pRIT5 (Pharmacia, Uppsala, Sweden). The following eukaryotic vectors are provided by way of example: pWLneo, pSV2cat, pOG44, PXRl, pSG (Stratagene) pSVK3, pBPV, pMSG, and pSVL (Pharmacia).

[425] Expression vectors generally have convenient restriction sites located near the promoter sequence to provide for the insertion of nucleic acid sequences encoding heterologous proteins. A selectable marker operative in the expression host may be present. Suitable expression vectors include, but are not limited to, viral vectors (e.g. viral vectors based on vaccinia virus; poliovirus; adenovirus (see, e.g., Li et al., Invest Opthalmol Vis Sci 35:2543 2549, 1994; Borras et al., Gene Ther 6:515 524, 1999; Li and Davidson, PNAS 92:7700 7704, 1995; Sakamoto et al., H Gene Ther 5:1088 1097, 1999; WO 94/12649, WO 93/03769; WO 93/19191; WO 94/28938; WO 95/11984 and WO 95/00655); adeno-associated virus (see, e.g., Ali et al., Hum Gene Ther 9:81 86, 1998, Flannery et al., PNAS 94:6916 6921, 1997; Bennett et al., Invest Opthalmol Vis Sci 38:2857 2863, 1997; Jomary et al., Gene Ther 4:683 690, 1997, Rolling et

al., Hum Gene Ther 10:641 648, 1999; Ali et al., Hum Mol Genet 5:591 594, 1996; Srivastava in WO 93/09239, Samulski et al., J. Vir. (1989) 63:3822-3828; Mendelson et al., Virol. (1988) 166:154-165; and Flotte et al., PNAS (1993) 90: 10613-10617); SV40; herpes simplex virus; gamma retrovirus; human immunodeficiency virus (see, e.g., Miyoshi et al., PNAS 94:10319 23, 1997; Takahashi et al., J Virol 73:7812 7816, 1999); a retroviral vector (e.g., Murine Leukemia Virus, spleen necrosis virus, and vectors derived from retroviruses such as Rous Sarcoma Virus, Harvey Sarcoma Virus, avian leukosis virus, human immunodeficiency virus, myeloproliferative sarcoma virus, and mammary tumor virus); and the like.

[426] As noted above, in some embodiments, a nucleic acid encoding a polypeptide of the present disclosure will in some embodiments be RNA, e.g., *in vitro* synthesized RNA. Methods for *in vitro* synthesis of RNA are known in the art; any known method can be used to synthesize RNA including a nucleotide sequence encoding a polypeptide of the present disclosure. Methods for introducing RNA into a host cell are known in the art. See, e.g., Zhao et al. (2010) Cancer Res. 15:9053. Introducing RNA including a nucleotide sequence encoding a polypeptide of the present disclosure into a host cell can be carried out *in vitro* or *ex vivo* or *in vivo*. For example, a host cell (e.g., an NK cell, a cytotoxic T lymphocyte, etc.) can be electroporated *in vitro* or *ex vivo* with RNA comprising a nucleotide sequence encoding a polypeptide of the present disclosure.

CELLS

[427] The present disclosure provides mammalian cell lines that produce recombinant retroviruses that genetically modify target mammalian cells and the target mammalian cells themselves.

[428] Suitable mammalian cells include primary cells and immortalized cell lines. Suitable mammalian cell lines include human cell lines, non-human primate cell lines, rodent (e.g., mouse, rat) cell lines, and the like. Suitable mammalian cell lines include, but are not limited to, HeLa cells (e.g., American Type Culture Collection (ATCC) No. CCL-2), CHO cells (e.g., ATCC Nos. CRL9618, CCL61, CRL9096), 293 cells (e.g., ATCC No. CRL-1573), Vero cells, NIH 3T3 cells (e.g., ATCC No. CRL-1658), Huh-7 cells, BHK cells (e.g., ATCC No. CCLlO), PC12 cells (ATCC No. CRL1721), COS cells, COS-7 cells (ATCC No. CRL1651), RATl cells, mouse L cells (ATCC No. CCLI.3), human embryonic kidney (HEK) cells (ATCC No. CRL1573), HLHepG2 cells, Hut-78, Jurkat, HL-60, NK cell lines (e.g., NKL, NK92, and YTS), and the like.

[429] In some instances, the cell is not an immortalized cell line, but is instead a cell (e.g., a primary cell) obtained from an individual or an *ex vivo* cell. For example, in some cases, the cell is an immune cell obtained from an individual. As another example, the cell is a stem cell or progenitor cell obtained from an individual.

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METHODS OF ACTIVATING AN IMMUNE CELL

[430] The present disclosure provides methods of activating an immune cell *in vitro, in vivo,* or *ex vivo.* The methods generally involve contacting an immune cell *(in vitro, in vivo,* or *ex vivo)* with one or more target antigens, where the immune cell has been genetically modified to produce a microenvironment restricted CAR of the present disclosure. In the presence of the one or more target antigens, the microenvironment restricted CAR activates the immune cell, thereby producing an activated immune cell. Immune cells include, e.g., a cytotoxic T lymphocyte, an NK cell, a $CD4^+$ T cell, a T regulatory (Treg) cell, a γδ T cell, an NK-T cell, neutrophils, etc.

[431] Contacting the genetically modified immune cell (e.g., a T lymphocyte, an NK cell) with one or more target antigens can increase production of a cytokine by the immune cell by at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 40%, at least about 50%, at least about 75%, at least about 2-fold, at least about 2.5-fold, at least about 5-fold, at least about 10-fold, or more than 10-fold, compared with the amount of cytokine produced by the immune cell in the absence of the one or more target antigens. Cytokines whose production can be increased include, but are not limited to, IL-2 and IFN-γ.

[432] Contacting a genetically modified cytotoxic cell (e.g., cytotoxic T lymphocyte) with AAR can increase cytotoxic activity of the cytotoxic cell by at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 40%, at least about 50%, at least about 75%, at least about 2-fold, at least about 2.5-fold, at least about 5-fold, at least about 10-fold, or more than 10-fold, compared to the cytotoxic activity of the cytotoxic cell in the absence of the one or more target antigens.

[433] Contacting a genetically modified cytotoxic cell (e.g., cytotoxic T lymphocyte) with one or more target antigens can increase cytotoxic activity of the cytotoxic cell by at least about 10%, at least about 15%, at least about 20%, at least about 25%, at least about 30%, at least about 40%, at least about 50%, at least about 75%, at least about 2-fold, at least about 2.5-fold, at least about 5-fold, at least about 10-fold, or more than 10-fold, compared to the cytotoxic activity of the cytotoxic cell in the absence of the one or more target antigens.

[434] In other embodiments, e.g., depending on the host immune cell, contacting a genetically modified host cell with an antigen can increase or decrease cell proliferation, cell survival, cell death, and the like.

METHODS FOR MAKING A MICROENVIRONMENT RESTRICTED ANTIGEN-SPECIFIC TARGETING REGION

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[435] In some embodiments, antigen binding domains (also referred to herein as "antigen-specific target regions" or "ASTRs") of CARs constitutively bind their cognate antigens. In other embodiments, the ASTRs can be microenvironment restricted, preferentially or only binding their cognate antigen under certain aberrant conditions, such as those that exist in the tumor microenvironment, as disclosed in more detail herein. Microenvironment restricted ASTRs that bind preferentially or exclusively under aberrant conditions of a tumor microenvironment, can provide a reduction in on-target off-tumor effects as binding to the antigen in normal physiological conditions is reduced, in some situations to levels below detection by immunoassays. In certain aspects, CARs provided herein include a microenvironment restricted ASTR that specifically binds to a target protein, wherein the ASTR is an scFv fragment that includes a heavy chain variable region and a light chain variable region.

[436] Certain illustrative embodiments of the aspects disclosed herein, for example the methods, cells, cells lines, retroviruses, polynucleotides, or vectors disclosed herein, include CARs that include microenvironment restricted antigen-specific targeting regions.

[437] Accordingly, in one aspect, provided herein is a chimeric antigen receptor for binding a target antigen, that includes:

a) a microenvironment restricted antigen-specific targeting region that exhibits an increase in binding to the target antigen in an aberrant condition compared to a normal physiological environment, wherein the antigen-specific targeting region binds to the target;

b) a transmembrane domain; and

c) an intracellular activating domain.

[438] In another aspect, provided herein is a chimeric antigen receptor for binding a target antigen, that includes:

a) at least one microenvironment restricted antigen specific targeting region selected by panning a polypeptide library and having an increase in activity in a target antigen binding assay at an aberrant condition compared to a normal physiological condition;

b) a transmembrane domain; and

c). an intracellular activating domain.

[439] In some embodiments of any aspect disclosed herein, any of the chimeric antigen receptors can be microenvironment restricted such that they exhibit an increase in binding activity at an aberrant condition compared to a normal physiological condition. In some illustrative embodiments of any aspect disclosed herein, the microenvironment restricted ASTR is identified from an initial polypeptide library without mutating/evolving members of the library before screening/evolving and/or without mutating during or

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between optional repeated rounds of screening. Exemplary transmembrane domains and intracellular activating domains can be any of those disclosed herein for CARs.

[440] In one aspect, provided herein is a method for selecting a microenvironment restricted ASTR, comprising panning a polypeptide display library by:

a. subjecting polypeptides of the polypeptide display library to a target antigen binding assay under a normal physiological condition and a target antigen binding assay under an aberrant condition; and

b. selecting a polypeptide which exhibits an increase in target antigen binding activity at the aberrant condition compared to the physiological condition, thereby selecting the microenvironment restricted antigen specific targeting region.

[441] In another aspect, provided herein is a method for isolating a microenvironment restricted ASTR, that includes panning a polypeptide library by:

contacting the polypeptide library under aberrant conditions with a target antigen bound to a solid support, wherein clones expressing polypeptides that bind the target antigen remain bound to the solid support through the target antigen;

incubating the solid supports with bound polypeptides under physiological conditions; and

collecting clones that elute from the solid support under the physiological conditions, thereby isolating the microenvironment restricted antigen-specific targeting region.

[442] In some illustrative embodiments of any aspect disclosed herein, the microenvironment restricted antigen-specific targeting region is identified from an initial polypeptide library screen without mutating/evolving members of the library before screening and/or without mutating/evolving during or between optional repeated rounds of screening or panning.

[443] Normal physiological conditions can include those of temperature, pH, osmotic pressure, osmolality, oxidative stress, and electrolyte concentration that would be considered within a normal range at the site of administration, or at the tissue or organ at the site of action, to a subject. An aberrant condition is that which deviates from the normally acceptable range for that condition. In one aspect, a microenvironment restricted antigen-specific targeting region (i.e. polypeptide) is virtually inactive at normal conditions but is active at other than normal conditions at a level that is equal or better than at normal conditions. For example, in one aspect, the microenvironment restricted antigen-specific targeting region is virtually inactive at body temperature, but is active at lower temperatures. In another aspect, the microenvironment restricted antigen-specific targeting region is reversibly or irreversibly inactivated at the normal conditions. In a further aspect, the microenvironment restricted antigen-specific targeting region is a therapeutic protein. In another aspect, the microenvironment restricted antigen-specific targeting region is used as a drug, or therapeutic agent. In yet another aspect, the microenvironment

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restricted antigen-specific targeting region is more or less active in highly oxygenated blood, such as, for example, after passage through the lung or in the lower pH environments found in the kidney.

[444] In some embodiments, a single round of selection is performed to obtain the microenvironment restricted antigen-specific targeting region. In certain embodiments, the screening or panning method is repeated after identifying free polypeptides that bound antigen under aberrant conditions and did not bind under physiological conditions, or cells expressing a test polypeptide that had these properties, or phage coated with a test polypeptide that has such properties in an initial or previous round. In some methods, phage that are collected are used to infect cells, which can be infected with helper phage as well, in order to amplify the collected phage. In other methods where polypeptides on the surface of cells are tested, collected cells can be grown to "amplify" the polypeptides expressed by the cells by amplifying polynucleotides in the cells that encode the polypeptides. In some embodiments, the amplifying is done by growing cells that express the identified polypeptides without performing a process to mutate the polynucleotides encoding the identified polypeptides between rounds. Thus, polypeptides that were collected in a previous round are enriched by amplifying cells that contain polynucleotides encoding these collected polypeptides.

[445] The panning or screening method can be performed a single time, or repeated for 1 to 1000 times. In illustrative embodiments, the panning is repeated 1 to 20 times or 2 to 10 times or 2 to 5 times.

[446] In other methods, microenvironment restricted ASTRs against an antigen of interest (i.e. target antigen) are performed using one or more rounds of mutation/evolution between rounds of panning. In one method, a wild-type protein is identified for example by generating a polypeptide or protein library and screening the polypeptide or protein library for a polypeptide or protein with a desired binding affinity to a target antigen. In some embodiments where the wild-type proteins are antibodies, the wild-type antibodies can be discovered by generating and screening polyclonal or monoclonal antibody libraries, including phage display antibody libraries, for example phage display humanized antibody libraries.

[447] Evolved ASTRs can be generated by subjecting the wild-type protein, or a nucleic acid sequence encoding the wild-type protein, to a process of mutagenesis to produce a population of mutant polypeptides that can be screened to identify a mutant ASTR with an increased activity (e.g. enhanced binding affinity to the target antigen) in a tumor environment and/or in an *in vitro* tumor surrogate assay condition, compared to a normal physiological environment. Examples of such methods are provided in WO2016033331 ("CONDITIONALLY ACTIVE CHIMERIC ANTIGEN RECEPTORS FOR MODIFIED T CELLS") or U.S. Patent No. 8,709,755, both herein incorporated by reference in their entirety. This method of generating a microenvironment restricted antibody is hereby incorporated by reference in its entirety herein.

[448] In other embodiments, microenvironment restricted antigen-specific polypeptides (i.e. targeting regions, e.g. antibodies) can be identified by screening an initial polypeptide library under aberrant versus physiological conditions and identifying a test polypeptide from the initial polypeptide library, that binds preferentially or exclusively under aberrant vs. physiological conditions. In some examples, the identified and isolated microenvironment restricted antigen-specific polypeptides (i.e. targeting regions, e.g. antibodies) identified from an initial polypeptide library in an initial polypeptide library screen, bind their cognate antigen preferentially or exclusively under aberrant vs. physiological conditions. In such instances, no rounds of mutating/evolving are performed. Accordingly, the method in illustrative embodiments is performed without mutating polynucleotides encoding the isolated microenvironment restricted antigen-specific targeting region between rounds of screening (e.g. rounds of panning), or performed for only a single binding assay under aberrant versus physiological conditions to isolate and identify the microenvironment restricted antigen-specific polypeptide (i.e. targeting region, e.g. antibody). The method can be performed by culturing, high fidelity amplifying, and/or diluting polynucleotides encoding antigen-specific targeting regions, or host organisms including the same, between rounds of screening and/or panning, without any mutating/evolving. Furthermore, the method can be performed without repeating the screening and/or panning and can be performed without mutating/evolving a polynucleotide encoding the isolated microenvironment restricted antigen-specific targeting region, after the microenvironment restricted antigen-specific polypeptide (i.e. target region, e.g. antibody) is isolated.

[449] Assays for use in the methods provided herein to detect binding of a polypeptide to a cognate binding partner include cell based assays, and in particular assays performed using cell surface display systems, such as mammalian cell surface display systems. In an exemplary method, nucleic acids encoding a polypeptide or a library of variant polypeptides, including a library of modified polypeptides, can be introduced into a vector suitable for expression in cells, such as mammalian cells. Cells are then transfected with the vector, and the polypeptide(s) is/are expressed by the cells. The library of cells containing surface-expressed polypeptides can be contacted with a solution containing a soluble or surface-bound cognate binding partner. Binding activity can be detected using any assay that can detect the binding to the surface of the cells. Activity also can be assessed by assessing a functional activity of the polypeptide or polypeptide. Any cell based assay known to the skilled artisan is contemplated for use in the methods provided herein, including cell proliferation assays, cell death assays, flow cytometry, cell separation techniques, fluorescence activated cell sorting (FACS), phase microscopy, fluorescence microscopy, receptor binding assays, cell signaling assays, immunocytochemistry and reporter gene assays. In some examples, the assays are fluorescence activated cell sorting (FACS) assays.

[450] Polypeptides or proteins can be expressed by mammalian cells as secreted, soluble molecules, cell surface molecules, or intracellular antibodies. In an exemplary method, cells can be transfected with a

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library of proteins under conditions whereby most or all of the cells display a member of the protein library anchored on the cell surface. Optionally, an expression system can be used in which most of mammalian cell transfectants have only one plasmid integrated in their genome. Therefore, most (i.e., at least about 70% or about 80% or about 90%) of the transfectants express one or more molecules of one polypeptide. This can be verified, for example, by isolating and culturing individual transfectants; and amplifying and sequencing the expressed sequences to determine whether they have a single sequence.

[451] In some examples of the methods provided herein, the polypeptides are antibodies displayed on the surface of mammalian cells. Any antibody described herein can be expressed on the surface of mammalian cells, including full length, bivalent, functional antibodies, such as IgG antibodies. The antibody can be a fragment, for example, Fab fragments or scFv fragments. Antibodies can include an Fc region, such as an scFv-Fc or a full length antibody, which comprises two heavy and two light chains. The skilled artisan can select a suitable antibody fragment. For example, an ScFv-Fcs and full length antibodies made in mammalian cells can have several advantages over scFv's or Fab fragments.

[452] Solid supports that can be used in the binding assays provided herein include any carrier that is capable of being affixed with a binding partner of a polypeptide such as a ligand, receptor or antigen. Typically, to facilitate high throughput screening a cognate binding partner is affixed to the solid support. Examples of carriers for use as solid supports in the methods provided herein include, but are not limited to, glass, polystyrene, polypropylene, polyethylene, dextran, nylon, amyloses, natural and modified celluloses, polyacrylamides, agaroses and magnetic solid supports, such as solid supports that include magnetite. The solid support can be one or more beads or particles, microspheres, a surface of a tube or plate, a filter membrane, and other solid supports known in the art. Exemplary solid support systems include, but are not limited to, a flat surface constructed, for example, of glass, silicon, metal, nylon, cellulose, plastic or a composite, including multiwell plates or membranes; or can be in the form of a bead such as a silica gel, a controlled pore glass, a magnetic or cellulose bead. Further, such methods can be adapted for use in suspension or in the form of a column.In some embodiments, the microenvironment restricted antigen-specific polypeptide (i.e. target region, e.g. antibody) is identified and isolated by biopanning a phage display or yeast surface display (Colby et al., "Engineering Antibody Affinity by Yeast Surface Display," *Meth. Enzym.* 388, 26 (2004)) antibody (e.g. humanized antibody) library with an immobilized target antigen. For example, either a naïve humanized antibody library or a synthetic humanized antibody library can be panned using the phage display or yeast surface display methods herein. In some embodiments, an initial phage display process, phage clones can be transferred to a mammalian vector and used to a mammalian cell surface screening method (See e.g., Yoon et al., *BMC Biotechnology* 12:62; 1472-6750 (2012)). An exemplary method for performing phage display to isolate a microenvironment restricted antigen-specific target region is provided in Example 2.

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[453] A microenvironment restricted ASTR identified using methods provided herein, can be an antibody, an antigen, a ligand, a receptor binding domain of a ligand, a receptor, a ligand binding domain of a receptor, or an affibody. In embodiments where the microenvironment restricted ASTR is an antibody, it can be a full-length antibody, a single-chain antibody, an Fab fragment, an Fab' fragment, an (Fab')2 fragment, an Fv fragment, and a divalent single-chain antibody or a diabody. wherein the antigen-specific targeting region comprises a heavy chain and a light chain from an antibody. In some embodiments, the microenvironment restricted ASTR is a single-chain variable fragment. Such single-chain variable fragment can have heavy and light chains separated by a linker, wherein the linker is between 6 and 100 amino acids in length. In some embodiments the heavy chain is positioned N-terminal to the light chain on the chimeric antigen receptor. In other embodiments, the light chain is positioned N-terminal to the heavy chain. The microenvironment restricted ASTR can be a bispecific ASTR.

[454] Microenvironment restricted ASTRs identified using methods provided herein are typically polypeptides and more specifically polypeptide antibodies, and in illustrative embodiments, single chain antibodies. These polypeptides can bind to their cognate antigens with higher or lower affinity under aberrant conditions vs. normal conditions, but in illustrative embodiments, bind with higher affinity under aberrant conditions than normal conditions. In some embodiments, these polypeptides can bind to their cognate antigen with a 10%, 20%, 25%, 50%, 75%, 90%, 95% or 99% greater affinity under aberrant conditions than physiological (i.e. normal) conditions. In some embodiments, the ASTRs identifying using methods provided herein do not bind to their cognate antigens under normal physiological conditions to any detectable level above background levels obtained using negative controls, such as negative control antibodies.

[455] The nucleotide sequence encoding a microenvironment restricted ASTR isolated by the method provided herein, can be determined by sequencing nucleotides of the collected cell expressing the microenvironment restricted antigen-specific targeting. This nucleotide sequence information can then be used to make a microenvironment restricted biologic chimeric antigen receptor (MRB-CAR) by generating a polynucleotide that encodes a polypeptide comprising the microenvironment restricted antigen-specific targeting region, a transmembrane domain, and an intracellular activating domain. Microenvironment restricted antigen-specific targeting regions can be cloned into a CAR construct expression system, which can be used to generate recombinant lentiviruses that include the CAR in their genome, and then the recombinant lentiviruses can be used to transduce T cells for testing for CAR-mediated tumor antigen expressing target cell killing in a tumor-selective environment compared to physiologic conditions.

CONDITIONS FOR CONDITIONAL ACTIVITY

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[456] In the methods provided herein, the activity of one or more polypeptides, such as, for example, single chain antibodies, is screened or tested under two different sets of conditions that simulate a condition or conditions in two different physiologic environments such as, for example, a diseased microenvironment and the normal physiologic condition of a non-diseased microenvironment. Typically, the conditions are conditions that can be simulated or replicated *in vitro*. A set of conditions can include one or more conditions to simulate a microenvironment associated with a disease. Disease can alter intracellular and extracellular homeostasis. For example, the diseased microenvironment can simulate one or more conditions in a tumor microenvironment or a cancer microenvironment. Typically, the difference or differences in activity under the two sets of conditions can result in the conditional activity of the molecule. Thus, a molecule that exhibits greater activity under the first set of conditions (e.g. simulating conditions in a tumor microenvironment) compared to the second set of conditions (e.g. simulating conditions in a normal or non-diseased environment) is identified as a candidate molecule that is microenvironment restricted.

[457] The two sets of conditions can be selected to vary by one or more parameters that differ in two physiologic environments, such as described herein or known to one of skill in the art, including but not limited to chemical conditions, biological conditions, or physical conditions. Parameters that can be varied between the two sets of conditions can include one or more conditions selected from among pressure, temperature, pH, ionic strength, osmotic pressure, osmolality, oxidative stress, turbidity, exposure to light (including UV, infrared or visible light), concentration of one or more solutes, such as electrolytes, concentration of glucose, concentration of hyaluronan, concentration of lactic acid or lactate, concentration of albumin, levels of adenosine, levels of R-2-hydroxyglutarate, concentration of pyruvate, concentration of oxygen, and/or presence of oxidants, reductants, or co-factors. By varying the electrolyte and buffer systems in the calibration solutions, physiological conditions such as pH, buffer capacity, ionic environment, temperature, glucose concentration, and ionic strength can be adjusted to those of the biological environment to be simulated. The set of conditions that simulate a normal physiologic environment can be selected to be different from the set of conditions that simulate a diseased microenvironment, such as a tumor microenvironment, by one or more conditions described herein.

[458] For example, as discussed below, various parameters of the tumor microenvironment differ compared to a non-tumor microenvironment, including, but not limited to, oxygen concentration, pressure, presence of co-factors, pH, hyaluronan concentration, lactate concentration, albumin concentration, levels of adenosine, levels of R-2-hydroxyglutarate, and pyruvate concentration. Any of these parameters can be replicated *in vitro* to simulate one or more conditions that exist in a tumor or cancer environment compared to conditions that exist in a non-tumor or a normal environment. The normal physiologic conditions that can be simulated include environments found in healthy or

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nondiseased tissue at any location of the body such as the GI tract, the skin, the vasculature, the blood, and extracellular matrix. Typically, in the assays herein, physiologic conditions can be simulated *in vitro* by the choice of buffer that is used to assess the activity of the protein. For example, any one or more conditions of a diseased microenvironment (such as a tumor microenvironment) and a non-diseased environment can be simulated by differences in the assay buffer used to assess activity in the assay. Hence, in the methods herein to identify a microenvironment restricted polypeptide, a component or components or characteristic or characteristics of an assay buffer are altered or made to be different in a first assay to test activity under a first condition and in a second assay to test activity under a second condition. For example, as discussed herein, various parameters of the tumor microenvironment are different compared to a non-tumor environment including, but not limited to, oxygen, pressure, presence of co-factors, pH, hyaluronan concentration (such as increased or decreased hyaluronan concentration), lactate concentration (such as increased or decreased lactate concentration), albumin concentration (such as increased or decreased albumin concentration), levels of adenosine (such as increased or decreased adenosine levels), levels of R-2-hydroxyglutarate (such as increased or decreased R-2-hydroxyglutarate levels) and pyruvate concentration (including increased or decreased pyruvate concentration). More specifically, conditions in a tumor microenvironment can include lower pH, higher concentrations of hyaluronan, higher concentrations of lactate and pyruvate, higher concentrations of albumin, increased levels of adenosine, increased levels of R-2-hydroxyglutarate, hypoxia, lower concentration of glucose, and slightly higher temperature in comparison with non-tumor microenvironment. For example, a microenvironment restricted ASTR is virtually inactive at normal body temperature, but is active at a higher temperature in a tumor microenvironment. In yet another aspect, the microenvironment restricted antibody is less active in normal oxygenated blood, but more active under a less oxygenated environment that exists in a tumor. In yet another aspect, the microenvironment restricted antibody is less active in normal physiological pH 7.2-7.8, but more active under an acidic pH 5.8-7.0, or 6.0-6.8 that exists in a tumor microenvironment. For example, the microenvironment restricted antibody is more active at a pH of 6.7 than at pH 7.4. There are other conditions in the tumor microenvironment known to a person skilled in the field that may also be used as the condition in the present invention under which the conditionally active ASTRs have different binding affinities. *In vitro* assay conditions that mimic these *in vivo* tumor conditions are referred to herein as *in vitro* tumor surrogate assay conditions.

[459] Any one or more of these conditions can be simulated *in vitro* by choice of the particular assay buffer. The composition of the assay buffer that simulates a diseased microenvironment can be selected to be identical to the composition of the assay buffer that simulate a normal environment, with the exception of one or more conditions known or described herein that is altered in the diseased microenvironment. Further, in screening or identifying the activity of one or more polypeptides under two different sets of

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conditions, generally the only conditions that are varied in the assay relate to the buffer conditions simulating the *in vivo* microenvironment. The other conditions of the assay, such as time, temperature and incubation conditions, can be the same for both sets of conditions. Typically, the same base buffer is used in the set of conditions that simulate a diseased microenvironment and conditions that simulate a normal microenvironment, but the design of the buffer composition can be made to differ in one or more parameters such as pH, oxygen, pressure, presence of co-factors, pH, hyaluronan concentration (such as increased or decreased hyaluronan concentration), lactate concentration (such as increased or decreased lactate concentration), albumin concentration (such as increased or decreased hyaluronan concentration) and/or pyruvate concentration (including increased or decreased pyruvate concentration). In the conditions that simulate a diseased microenvironment and the conditions that simulate a normal microenvironment, any base buffer known to one of skill in the art that can be used

METHODS OF GENERATING A MICROENVIRONMENT RESTRICTED CELL

[460] The present disclosure provides a method of generating a microenvironment restricted cell. The method generally involves genetically modifying a mammalian cell with an expression vector (e.g. a plasmid or a retrovirus), or an RNA (e.g., *in vitro* transcribed RNA), including nucleotide sequences encoding microenvironment restricted CARs of the present disclosure. The genetically modified cell is microenvironment restricted in the presence of one or more target antigens. The genetic modification can be carried out *in vivo, in vitro,* or *ex vivo.* The cell can be an immune cell (e.g., a T lymphocyte, a T-helper cell, or an NK cell), a stem cell, a progenitor cell, etc.

[461] In some cases, the genetic modification is carried out *ex vivo.* For example, a T lymphocyte, a stem cell, a T-helper cell, or an NK cell is obtained from an individual; and the cell obtained from the individual is genetically modified to express a CAR of the present disclosure. The genetically modified cell is microenvironment restrictablein the presence of one or more target antigens. In some cases, the genetically modified cell is activated *ex vivo.* In other cases, the genetically modified cell is introduced into an individual (e.g., the individual from whom the cell was obtained); and the genetically modified cell is activated *in vivo.* For example, where the one or more target antigens are present on the surface of a cell in the individual, there is no need to administer the antigen. The genetically modified cell comes into contact with the antigen present on the surface of a cell in the individual and the genetically modified cell is activated. For example, where the genetically modified cell is a T lymphocyte, the genetically modified cell can exhibit cytotoxicity toward a cell that expresses the one or more target antigens on its surface to which the CAR binds.

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METHODS OF TRANSIENT REDUCTION OF TUMOR MICROENVIRONMENT SENSTITIVE CAR-T TARGET BINDING

[462] Provided herein is a method of transient reduction of tumor microenvironment sensitive CAR-T target binding through pharmacologic modification of vascular and tissue pH. Microenvironmentally controlled scFvs in CAR-T cells provide an additional level of protection against on-target off tumor toxicity, requiring tumor local environmental conditions to enable T cell engagement. While attractive for some monoclonal antibody therapies, adoptive cellular therapy may create local environments that are transiently permissive for their CAR-T targets. For example, CAR-T cells activated in local tissues may further reduce local pH, depending on cytoplasmic domains present in the CAR construct. In other instances, cytokine release syndrome and other morbidity associated with adoptive cellular therapy may result in loss of bicarbonate buffering capacity of blood, leading to lactic acidosis. It is established that adoptive cellular therapies administered by intravenous infusion result in temporary pulmonary entrapment. For some cellular therapies, infusion rate requires constant monitoring of dissolved oxygen (Fischer et al. *Stem Cells Dev.* 2009 Jun; 18(5): 683–691). The extent of pulmonary entrapment is dependent upon cell size, activation state, cell dose and infusion rate. Cruz et al (*Cytotherapy.* 2010 Oct; 12(6): 743–749) report the adverse findings from over 300 T cell infusions, that low doses and slow infusion may reduce pulmonary entrapment. However, with certain high potency CAR-T cells, targets present even in low levels on lung endothelium, such as Her2 (Morgan et al. *Mol Ther.* 2010 Apr; 18(4): 843–851), can result in immediate toxicity that cannot be controlled, and results in rapid patient deterioration due to the initial high CAR-T cellular concentration in the lung following infusion and the presence of the T cell target in these tissues. In other cases, the presence of T cell targets in other off target tissues such as bile duct may create on target off tumor toxicities that cannot be controlled (Lamers *Mol Ther.* 2013 Apr;21(4):904-12) and result in severe organ toxicity before other agents such as steroids or cell elimination epitopes can be utilized. While venous and arterial plasma have strong buffering capacity against acidosis, conditions of respiratory acidosis, shock, metabolic acidosis and ischemic acidosis can occur in patients with cancer treated with adoptive cellular therapy.

[463] In some embodiments, methods for transiently increasing vascular pH at certain conditions to reduce affinity of microenvironmentally controlled scFvs for their antigens are provided. A 0.4U shift in blood pH can reduce affinity of certain scFvs by greater than 10-fold. In some embodiments, therapeutic pH control can be achieved via IV or oral administration routes. In some embodiments, inactivation of binding affinity can be achieved with bicarbonate. In other embodiments, Tris-hydroxylmethyl aminomethane (also known as tromethamine, trometamol, and THAM) and Carbicarb™ (and equimolar hypertonic solution of sodium bicarbonate and sodium carbonate) are utilized to increase blood pH in a sufficient amount to alleviate on target off tumor toxicities. In still other embodiments, small molecule

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proton pump inhibitors can also be utilized to increase blood pH and/or tissue pH in a sufficient amount to alleviate on target off tumor toxicities. Proton pump inhibitors include, but are not limited to, esomeprazole (Nexium), esomeprazole and naproxen (Vimovo), lansoprazole (Prevacid), omeprazole (Prilosec and Zegerid), and rabeprazole (Aciphex). Administration of proton pump inhibitors can be used effectively over longer time periods to modulate the binding affinity of the antigen biding domain to its cognate antigen for days, weeks, months, or years. In other embodiments, the affinity of the antigen binding domain for its cognate antigen can also be modulated by altering the blood pH and/or tissue pH by controlling the transcription, translation, membrane expression, and stability of transporters and pumps. Examples of such transporters and pumps to modulate pH include, but are not limited to, proton pumps, members of the sodium proton exchange family (NHE), bicarbonate transporter family (BCT), and monocarboxylate transporter family. In certain embodiments, bicarbonate, THAM, or CaricarbTM may be administered prior to or concurrent with infusion of patients CAR-T cells expressing pH controlled scFvs. Such treatment will alleviate the immediate cytoxicity that is otherwise associated with the temporary pulmonary entrapment of CAR-T cell infusions.

FURTHER EMBODIMENTS

[464] In certain embodiments, methods provided herein for the present disclosure include inhibiting expression of one or more endogenous genes expressed in T cell and/or NK cells. Methods provided herein illustrate the ability to make recombinant retroviruses that express miRNA or shRNA, for example, that can be used for such methods. In fact, the methods provided herein illustrate that such miRNA or shRNA can be encoded within introns, including for example, an Ef1a intron. This takes advantage of the present teachings of methods to maximize the functional elements that can be included in a packagable retroviral genome to overcome shortcomings of prior teachings and maximize the effectiveness of such recombinant retroviruses in adoptive T cell therapy.

[465] In some embodiments, 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 miRNAs, in illustrative embodiments between 2 and 5, for example 4 miRNAs, that bind nucleic acids encoding one or more of the following target endogenous T cell expressed genes, can be included in the recombinant retroviral genome and delivered to T cells and/or NK cells using methods provided herein. In fact, as provided herein 1, 2, 3, or 4 miRNAs can be delivered in a single intron such as the EF1a intron. The target endogenous genes expressed on T cells can include the following, with a non-limiting expected benefit of such inactivation in parenthesis: PD-1 (prevent inactivation); CTLA4 (prevent inactivation); TCRa (safety - prevent autoimmunity); TCRb (safety - prevent autoimmunity); CD3Z (safety – prevent autoimmunity); SOCS (prevent inactivation); SMAD2 (prevent inactivation); miR-155 (promote activation); IFN gamma (reduce CRS); cCBL (prolong

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signaling); TRAIL2 (prevent death); PP2A (prolong signaling); ABCG1 (increase cholesterol microdomain content by limiting clearance of cholesterol).

[466] In certain embodiments, miRNA against target genes with similar expected utilities can be combined. In other embodiments, miRNA against target genes with complementary utilities can be combined. In some embodiments, the combinations can include CD3Z, PD1, SOCS1, and/or IFN gamma.

TREATMENT METHODS

[467] The present disclosure provides various treatment methods using a CAR. A CAR of the present disclosure, when present in a T lymphocyte or an NK cell, can mediate cytotoxicity toward a target cell. A CAR of the present disclosure binds to an antigen present on a target cell, thereby mediating killing of a target cell by a T lymphocyte or an NK cell genetically modified to produce the CAR. The ASTR of the CAR binds to an antigen present on the surface of a target cell.

[468] The present disclosure provides methods of killing, or inhibiting the growth of, a target cell, the method involving contacting a cytotoxic immune effector cell (e.g., a cytotoxic T cell, or an NK cell) that is genetically modified to produce a subject CAR, such that the T lymphocyte or NK cell recognizes an antigen present on the surface of a target cell, and mediates killing of the target cell.

[469] The present disclosure provides a method of treating a disease or disorder in an individual having the disease or disorder, the method including: a. introducing an expression vector including a polynucleotide sequence encoding a CAR into peripheral blood cells obtained from the subject to produce a genetically engineered cytotoxic cell; and b. administering the genetically engineered cytotoxic cell to the subject.

SUBJECTS SUITABLE FOR TREATMENT

[470] A variety of subjects are suitable for treatment with the methods and compositions presented herein. Suitable subjects include any individual, e.g., a human or non-human animal who has a disease or disorder, who has been diagnosed with a disease or disorder, who is at risk for developing a disease or disorder, who has had a disease or disorder and is at risk for recurrence of the disease or disorder, who has been treated with an agent for the disease or disorder and failed to respond to such treatment, or who has been treated with an agent for the disease or disorder but relapsed after initial response to such treatment.

[471] Subjects suitable for treatment with an immunomodulatory method include individuals who have an autoimmune disorder; individuals who are organ or tissue transplant recipients; and the like; individuals who are immunocompromised; and individuals who are infected with a pathogen.

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[472] The following non-limiting examples are provided purely by way of illustration of exemplary embodiments, and in no way limit the scope and spirit of the present disclosure. Furthermore, it is to be understood that any inventions disclosed or claimed herein encompass all variations, combinations, and permutations of any one or more features described herein. Any one or more features may be explicitly excluded from the claims even if the specific exclusion is not set forth explicitly herein. It should also be understood that disclosure of a reagent for use in a method is intended to be synonymous with (and provide support for) that method involving the use of that reagent, according either to the specific methods disclosed herein, or other methods known in the art unless one of ordinary skill in the art would understand otherwise. In addition, where the specification and/or claims disclose a method, any one or more of the reagents disclosed herein may be used in the method, unless one of ordinary skill in the art would understand otherwise.

EXAMPLES

Example 1. Generation of riboswitches that respond specifically to nucleoside analogue antiviral drugs.

[473] This example provides a method to screen libraries based on natural structural riboswitches that bind guanosine and deoxyguanosine. These riboswitches were used as scaffolds to develop biased libraries for the selection of aptamers that bind specifically to a ligand nucleoside analogue. Previously, isothermal titration calorimetry has been used to show these natural riboswitches bind to their native ligands. Additional tests showed a deoxyguanosine switch also interacted weakly with the nucleoside analogues acyclovir and penciclovir, leading to the re-design of this sequence into a new library. The single-stranded regions of the riboswitch were targeted for mutation and variant sequences that specifically respond to acyclovir or penciclovir were selected for.

Materials

[474] Selection components guanine, guanosine, deoxyguanosine, acyclovir, and penciclovir were ordered from Sigma-Aldrich (St. Louis, MO). Acyclovir was the initial target while penciclovir was a special interest analyte used in latter rounds and guanine, guanosine, and deoxyguanosine were used as counter-targets. Graphene oxide (GrO), to be used as the partitioning medium, was purchased from Angstron Materials (Dayton, OH). HEPES (pH 7.3) and $MgCl_2$ were purchased from Amersco LLC. (Solon, OH). KCl was purchased from Teknova (Hollister, CA). Selection buffer was prepared at 5X (1X as 50 mM HEPES, 100 mM KCl, 20 mM $MgCl_2$, pH 7.3). Targets, counter-targets, and oligos were reconstituted in nuclease-free water for preliminary analysis and aptamer screening. Aliquots were prepared for all targets and stored at -20 °C to maximize shelf life.

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Generation of the aptamer library

[475] The initial aptamer library template was synthesized by IBA GmbH (Gottingen, Germany) as the reverse complement of the sequences in FIG. 14. In FIG. 14, the nucleotides in boxes are single-stranded in the known sequences, with "mutations" introduced during synthesis to allow for better binding to analogues of the original targets. For nucleotides within the boxes outlined with solid lines, substitution mutations were allowed; for nucleotides within the boxes outlined with dashed lines, substitution mutations as well as insertions or deletions were allowed. Primers were synthesized by IDT (Coralville, IA) as single-stranded DNA. T7 primer (SEQ ID NO:240) was combined with library template sequences for primer extension with Titanium Taq DNA polymerase (Clontech; Mountain View, CA). Primer-extended material was transcribed using the Ampliscribe T7 High Yield Transcription Kit (Epicentre; Madison, WI) and then purified on 10% denaturing polyacrylamide gel electrophoresis (PAGE) with 8 M urea before use in selection. During selection, the library was reverse-transcribed using SuperScript IV Reverse Transcriptase (Invitrogen; Carlsbad, CA) using reverse primer (SEQ ID NO:241) and amplified using Titanium Taq DNA polymerase (Clontech; Mountain View, CA). The aptamer with SEQ ID NO:248 had a J2-3 loop variation of -3 to -1 and a diversity of ~2.25×10^{10}. The aptamer with SEQ ID NO:250 had a J2-3 loop variation of 0 (native) to +5 and a diversity of ~9.38×10^{14}. The two oligonucleotides (SEQ ID NOs:249 and 250) were mixed at a ratio of 1:4160 to produce equimolar diversity in the combined library pool, with a total diversity of ~9.38×10^{13}.

Library screening

[476] Library screening was conducted using a graphene oxide-Systematic Evolution of Ligands by EXponential enrichment (GO-SELEX) approach (FIG. 15) (Park *et al.*, 2012), taking advantage of the π-π interaction that grants graphene oxide a high affinity for single-stranded nucleic acids (Zeng *et al.*, 2015). The goal was to select sequences that did not interact with the 1X selection buffer or with the counter-targets (guanine, guanosine, and deoxyguanosine) but did bind to the positive target acyclovir.

[477] For each round, a given amount of library was first refolded in 1X selection buffer (5-minute denaturing at 90 °C, 5 minutes at 4 °C, then room temperature). The counter-targets were then added to refolded libraries and incubated for 30 minutes at 37 °C. The exceptions to this were rounds 1 and 2, where the counter-targets were only briefly (< 1 minute) included to help load the library onto the GrO. After allowing the library to interact with the counter-targets and buffer components, unbound library was loaded onto GrO (mass equal to 100 times the mass of the library at the start of the round) over the course of a 10-minute incubation at 37 °C. The solution was then centrifuged at 7,000 x *g* to sediment the GrO. The supernatant, which contained sequences bound to the counter-targets and/or to the buffer, was

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removed. The sediment was then washed twice with 200 μL 1X selection buffer, centrifuging at 7,000 x *g* and removing the supernatant after each wash. A positive target-containing solution was then added and allowed to elute library from the GrO under the conditions indicated in Table 1 for up to 60 minutes at 37 °C, essentially allowing the target to compete with graphene oxide for library binding. Sequences that bound more strongly to the target would desorb from graphene oxide and remain bound to the target at the end of the incubation. A final centrifugation step separated the released material, located in the supernatant, from the non-responsive library that remained bound to the graphene oxide.

[478] After positive selection, the recovered RNA purified using 10% denaturing PAGE with 8 M Urea, was then quantified using a spectrophotometer reading (Table 1), reverse-transcribed with SuperScript IV, and amplified using PCR with Titanium Taq DNA polymerase. Amplification products were transcribed into RNA for the next round of selection.

[479] Three tiers of stringency were implemented over the course of selection (Table 1). The first two rounds of selection did not include screening against counter-targets to maximize library loading onto GrO. Additionally, a large excess of acyclovir was used in positive incubations to maximize library recovery, thus the low-stringency designation. Counter-target incubations were introduced after library recovery was achieved, as middle-stringency conditions. The ratio of acyclovir to library was also reduced during these three rounds to increase library competition for binding to target. Once greater than 10% recovery was achieved, the final rounds of high-stringency selection were implemented. Counter-targets/library ratio remained high and positive target/library ratio was brought to 1:1 while positive incubation time was reduced, to select for faster binding sequences. Once library recovery was shown to remain over 10% after more than two rounds of the high-stringency conditions, parallel assessments were conducted.

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Generation (Stringency)	Library:X-Targets (30-min inc.)	Library:(+) Target	(+) Incubation Time (min)	Recovery (%)
G0/R1 (low)	1:1000*	1:1000	60	0.43
G1/R2 (low)	1:1000*	1:1000	60	2.00
G2/R3 (middle)	1:1000	1:500	60	3.60
G3/R4 (middle)	1:1000	1:100	60	8.73
G4/R5 (middle)	1:1000	1:10	60	10.20
G5/R6 (high)	1:1000	1:1	60	12.00
G6/R7 (high)	1:1000	1:1	60	8.60
G7/R8 (high)	1:1000	1:1	60	9.72
G8/R9 (high)	1:1000	1:1	30	20.08
G9/R10 (high)	1:1000	1:1	30	10.62
G10(-)† (parallel 1)	-	-	30	3.74
G10(X)† (parallel 1)	1:40	-	30	3.60
G10(+)† (parallel 1)	-	1:4	30	14.14
G10(P)† (parallel 1)	-	1:4	30	5.46
G11(-)‡ (parallel 2)	-	-	30	4.60
G11(X)‡ (parallel 2)	1:40	-	30	5.26
G11(+)† (parallel 2)	-	1:2	30	9.34
G11(P)‡ (parallel 2)	-	1:4	30	6.32

Table 1. Selection and Assessment Conditions. Conditions used for each round of selection or incubation, with recovery as the ratio between recovered sample and input library for each round. Library enrichment was monitored over the course of selection. *Counter-targets used for loading, not extended incubation. †Pre-loading incubation conducted with pooled counter-targets. ‡Pre-loading incubation conducted with positive target acyclovir. This was done to minimize the recovery of cross-reactive species. The following abbreviations are used in this table: "X-Targets" are counter-targets; "(+) Target" is acyclovir or penciclovir; "(+) Incubation Time (min)" is the time the "Library:(+) Target" solution was incubated on the GrO. G0 is Generation 0 and so on; R1 is Round 1 and so on. For the parallel assessment (parallel 1 and parallel 2) the incubations were performed with: (-) 1X selection buffer only, (X) counter-targets in 1X selection buffer, (+) acyclovir in 1X selection buffer, and (P) penciclovir in 1X selection buffer.

[480] For the two parallel assessments, library to be assessed was divided into four equal amounts for preparation and refolding as above (FIG. 16). For each condition, 50 pmoles of library were combined with 1X selection buffer, refolded (90 °C for 5 minutes, 4 °C for 5 minutes), and then incubated with 200

μL of 10 μM combined counter-targets in 1X selection buffer for 30 minutes at 37 °C. These samples were then loaded onto an amount of graphene oxide equal to 100 times the mass of library in the sample and incubated for 10 minutes at 37 °C and then washed twice with 200 μL of 1X selection buffer as before. The loaded graphene oxide samples were then incubated in parallel with 200 μL of the appropriate assessment condition (1X selection buffer only, 10 μM pooled counter-targets, 1 μM penciclovir, 1 μM acyclovir for the first parallel assessment, or 0.5 μM acyclovir for the second parallel assessment; in Table 1 these conditions are shown as: (-); (X); (P); (+); and (+), respectively) in 1X selection buffer for 30 minutes at 37 °C. A final centrifugation step separated desorbed responsive library from non-responsive graphene oxide-bound library. The responsive libraries were quantified using spectrophotometric reading (Table 1), verified using 10% denaturing PAGE with 8 M urea, and prepared for a second parallel assessment. This follow-up assessment continued to use counter-targets for the positive sample's pre-loading incubation, but utilized positive target acyclovir for each other samples' pre-incubation. This was done to minimize representation of cross-reactive sequences in a given sample (i.e. responsive to counter-targets in the positive sample, responsive to acyclovir in the negative, counter-targets, or penciclovir samples). Material recovered from the second parallel assessment was quantified using spectrophotometric reading (Table 1), verified using 10% denaturing PAGE with 8 M urea, and prepared for sequencing by reverse transcription and PCR to generate double-stranded DNA.

Sequencing

[481] The initial library was subjected to over 10 rounds of GrO-based selection and parallel assessment (Table 1). The GO-SELEX process is designed to enrich for sequences over multiple rounds of selection that bind to the given targets of interest and remove sequences that bind to the non-target compounds or buffer components. As a result, the populations to be sequenced are expected to contain multiple copies of potential aptamer candidates.

[482] The Illumina MiSeq system (San Diego, CA) was implemented to sequence the aptamer libraries after parallel assessment using a single-end read technique. Deep sequencing and subsequent data analysis reduces the large number of screening rounds traditional SELEX requires, which may introduce error and bias due to the screening process (Schütze *et al.*, 2011). Five samples were sequenced: the final generation library that responded to acyclovir, the final generation library that responded to the counter-targets, the final generation library that responded to 1X selection buffer (negative condition), the penultimate generation library that responded to acyclovir, and the final generation library that responded to the additional target of interest, penciclovir. From these sets of data, sequence families were constructed at 95% homology (sequence similarity considering mutations, deletions, and insertion) for aptamer candidate identification. There were 1,711,535 raw sequences (124,600 unique sequences) from

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the library that responded to acyclovir and 2,074,832 raw sequences (110,149 unique sequences) from the library that responded to penciclovir.

Aptamer candidate selection

[483] Sequence family construction focused primarily on sequence similarity. This means that a sequence's frequency in the positive target population was factored in, but greater emphasis was placed on the degree of variation between similar sequences, with 95% homology being the minimum requirement (100% match over the entire sequence is not necessary to join a family, up to 2 bases can be mismatched, inserted, or deleted). One would therefore expect families with the greatest number of members to rank highly as aptamer candidates. After families are constructed, consideration can be given to the relative presence of a family in a given population – families that occur frequently in the negative and counter-target populations are considered weaker candidates, as they demonstrate a degree on non-specific interaction in binding to buffer or counter-target components. Additionally, families that demonstrate a high rate of enrichment (i.e. large ratio between the final positive population and penultimate positive population) improve their candidacy, as enrichment rate has been linked to the binding affinity of a candidate relative to the rest of the population (Levay et al., 2015; Wang et al., 2014). Under these conditions, several candidate families appeared to be strong candidates for binding acyclovir (Table 2) and penciclovir.

Candidate Family-Sequence Number	SEQ ID NO:	Sequence	Length	% Identity	
				Consensus	Wildtype
582-1	108	ACAGCTTAGCGTAATGGCTACTGACG CCGTCCAAACCTATTTACAGACT	49	100	80.77
582-2	109	ACAGCTTAGGATAATGGCTACTGACG CCGTCCAAACCTATTTACAGACT	49	95.92	80.77
582-3	110	ACAGCTTAGCATAATGGCTACTGACG CCGTCCAAACCTATTCACAGACT	49	95.92	80.77
582-4	111	ACAGCTTAGCATAATGGCTACTGACG CCGTCCAAACCTATTGACAGACT	49	95.92	80.77
582-5	112	ACAGCATAGCATAATGGCTACTGAC GCCGTCCAAACCTATTTACAGACT	49	95.92	82.69
582-6	113	ACAGCTTAGCATAATGGCTACTGACG CCGTCCAAACCTATGTACAGACT	49	95.92	80.77
582-7	114	ACAGCTAGCGTAATGGCTACTGACGC CGTCCAAACCTATTTACAGACT	48	97.96	80.77

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582-8	115	ACAGCTTAGCATTATGGCTACTGACG CCGTCCAAACCTATTTACAGACT	49	95.92	80.77
582-9	116	ACAGTTAGCATAATGGCTACTGACGC CGTCCAAACCTATTTACAGACT	48	95.92	82.69
582-10	117	ACAGCTTAGCATAATGGCTACTGACG CGGTCCAAACCTATTTACAGACT	49	95.92	80.77
582-11	118	ACAGCTTAGCTTAATGGCTACTGACG CCGTCCAAACCTATTTACAGACT	49	97.96	80.77
582-12	119	ACAGCTTAGCATAATGGCTACTGACG CCGTCCAAACCCATTTACAGACT	49	95.92	80.77
582-13	120	ACAGCTTAGCATAATGGCTACTGACG CCGTCCAAACCAATTTACAGACT	49	95.92	80.77
582-14	121	ACAGCTTAGCATAATGGATACTGACG CCGTCCAAACCTATTTACAGACT	49	95.92	80.77
582-15	122	ACAGCTTAGCATTGTGGCTACTGACG CCGTCCAAACCTATTTACAGACT	49	93.88	78.85
582-16	123	ACAGGTTAGCATAATGGCTACCGAC GCCGTCCAAACCTATTTACAGACT	49	93.88	82.69
582-17	124	ACAGCTTAGCGTAATGGCTACTGACG CCGCCCAAACCTATTTACAGACT	49	97.96	82.69
582-18	125	ACAGCTTAGCATAATGGCTACTGACG CCGTCCAAAACTATTTCCAGACT	49	93.88	80.77
582-19	126	ACAGCCTAGCATAAGGGCTACTGAC GCCGTCCAAACCTATTTACAGACT	49	93.88	82.69
582-20	127	ACAGCTTAGCATAATGGCTACTGAGG CCGTCCAAACCTATTTACAGACT	49	95.92	80.77
582-21	128	ACAGCTTACCTTAATGGCTACTGACG CCGTCCAAACCTATTTACAGACT	49	95.92	78.85
582-22	129	ACAGCTTAGCATAATGGCTACCGACG CTGTCCAAACCTATTTACAGACT	49	93.88	78.85
582-23	130	ACAGCTTAGCGTAATGGCTACTGGCG CCGTCCAAACCTATTTACAGACT	49	97.96	78.85
582-24	131	ACAGCTTAGCATACTGGCTACTGACG CCGCCCAAACCTATTTACAGACT	49	93.88	82.69
582-25	132	ACAGCTTAGCATAATGGCTACTGACG CCGTCCTAACCTATTTACAGACT	49	95.92	80.77
582-26	133	ACAGGTTAGCATAATGCCTACTGACG CCGTCCAAACCTATTTACAGACT	49	93.88	82.69
582-27	134	ACAGCTTAGCATAATTGCTACTGACG CCGTTCAAACCTATTTACAGACT	49	93.88	82.69
582-28	135	ACAGCTTAGCATAAAGGCTACTGAC GCCGTCCAAACCTATTTACAGACT	49	95.92	80.77

582-29	136	ACAGCTTAGCGTAATGGCTACTGACG CCGTCTAAACCTATTTCCAGACT	49	95.92	80.77
582-30	137	ACAGGTTAGCATAATGGCTACTGACG CCGTCCAAACCTATTTAGAGACT	49	93.88	86.54
582-31	138	ACAGGGTAGCGTAATGGCTACTGAC GCCGTCCAAACCTATTTACAGACT	49	95.92	84.62
582-32	139	ACAGCGTAGCATAATGGCTACTGAC GCCGTTCAAACCTATTTACAGACT	49	93.88	86.54
582-33	140	ACAGCTTAGCATAATGGCTACTGACG CCGTCCAAACTCATTTACAGACT	49	93.88	78.85
582-34	141	ACAGCGTAGCATAGTGGCTACTGAC GCCGTCCAAACCTATTTACAGACT	49	93.88	82.69
582-35	142	ACAGCTTAGTGTAATGGCTACTGACG CTGTCCAAACCTATTTACAGACT	49	95.92	76.92
582-36	143	ACAGCTTAGCATAATGGCTACTGACG GCGTTCAAACCTATTTACAGACT	49	93.88	82.69
582-37	144	ACAGGTTAGCATAATGGCTACTGACG CCGTCCAAACCTATTTATAGACT	49	93.88	84.62
582-38	145	ACAGCTTAGCATAATGGCTACTGACG CCGTCCAAACCTATTGTCGACT	48	91.84	80.77
582-39	146	ACAGCTTAGCATAATGGCTACTGACG CCGTCCAAACCTATTTACGACT	48	95.92	80.77
582 Consensus Sequence	222	ACAGNNTASBDTWVDKSMTACYGRS GSBGYYYWAAMYHATKBHBNGACT Where the N at position 5 can be C, G, or no nucleotide, the N at position 6 can be A, C, G, T, or no nucleotide, and the N at position 45 can be A or no nucleotide.	49	-	-
769-1	147	ACAGGTCAGCATAATGTGCTAGTGCG CCTTCAAACCTATTTAGAGACT	48	100	82.69
769-2	148	ACAGGTCAGCATAATGTGCTAGTGCG CCCTCAAACCTATTTAGAGACT	48	97.92	80.77
769-3	149	ACAGGTTAGCATAATGTGCTATTGCG CCTTCAAACCTATTTAGAGACT	48	95.83	84.62
769-4	150	ACAGGTCAGCATAATGTGCTAGTGCG CATTCAAACCTATTTAGAGACT	48	97.92	80.77
769-5	151	ACAGGTTAGCATAATGTGCTAGTGCG CCTTCAAACCTATTTTGAGACT	48	95.83	84.62
769-6	152	ACAGGTTATCATAATGTGCTAGTGCG CCTTCAAACCTATTTAGAGACT	48	95.83	82.69
769-7	153	ACAGGTTAGCATGATGTGCTAGTGCG CCTTCAAACCTATTTAGAGACT	48	95.83	82.69

769-8	154	ACAGGTTAGCATAATGGGCTAGTGC GCCTTCAAACCTATTTAGAGACT	48	95.83	86.54
769-9	155	ACAGGTCAGCAAAATGTGCAAGTGC GCCTTCAAACCTATTTAGAGACT	48	95.83	78.85
769-10	156	ACAGGTCAGCATAATGTGCTAGTGCG CCTTCAAACCTATCTGGAGACT	48	95.83	82.69
769-11	157	ACAGCTTAGCATAATGTGCTAGTGCG CCTTCAAACCTATTTAGAGACT	48	95.83	82.69
769-12	158	ACAGGTCAGCATAATGTGCTAGTGCG CCTTCAAACCTATTTACAGACT	48	97.92	80.77
769-13	159	ACAGGTCAGCATAATGTGCTAGTGCG CCTTCAAACATATTTAGAGACT	48	97.92	80.77
769-14	160	ACAGGGTAGCATAATGTGCTAGTGC GCCTTCAAACCTATTTAGAGACT	48	95.83	86.54
769-15	161	ACAGGTTAGCATAATGTGCTAGTGCG CCCTCAAACCTATTTAGAGACT	48	95.83	82.69
769-16	162	ACAGGTTAGCATAATGTGCCAGTGCG CCTTCAAACCTATTTAGAGACT	48	95.83	82.69
769-17	163	ACAGGTCAGCATAATGGGCTAGTGC GCCTTCAAACCTATTTAGAGACT	48	97.92	84.62
769 Consensus Sequence	223	ACAGSKYAKCAWRATGKGCHAKTGC GCMYTCAAACMTATYTDSAGACT	48	-	-
795-1	164	ACAGCGAAGCATAATGGCTACTGAC GCCCTCAAACCCTATTTGCAGACT	49	100	83.02
795-2	165	ACAGCGAAGCATAATGGCTACTGAC GCCCTCAAACCCTATTTACAGACT	49	97.96	81.13
795-3	166	ACAGCGAAGCATAATGGCTTCTGAC GCCCTCAAACCCTATTTGCAGACT	49	97.96	81.13
795-4	167	ACAGCCAAGCATACTGGCTACTGAC GCCCTCAAACCCTATTTGCAGACT	49	95.92	79.25
795-5	168	ACAGCGAAGCATAATGGCTACTGAC GCCCGCAAACCCTATTTGCAGACT	49	97.96	81.13
795-6	169	ACAGCGAAGCATAATGGCTACTGAC GGCCTCAAACCCTATTTGCAGACT	49	97.96	80.77
795-7	170	ACAGCGAGGCATAATGGCTACTGAC GCCCTCAAACCCTATTTGCAGACT	49	97.96	81.13
795-8	171	ACAGCGAAGCATAATGGCTACTGAC GCCTTCAAACCCTATTTGCAGACT	49	97.96	84.91
795-9	172	ACAGCGAAGCATAATGGCTACAGAC GCCCTCAAAACCTATTTGCAGACT	49	95.92	80.77
795-10	173	ACAGCGAAGCATAATGGCTACTGAC GCCCTCAAACCCTATTTGAGACT	48	97.96	83.02

795-11	174	ACAGCGAAGCATAATGGCTACTGAC GCCCTCAAACCCTATTGTCGACT	48	93.88	76.92
795-12	175	ACAGCCAAGCATAATGGCTACTGAC GCCCTCAAACCCTATTTGCAGACT	49	97.96	81.13
795-13	176	ACAGCGAAGCATAATGGCTACTGAC GCCCTCAAACCCTATTTGGCGACT	49	95.92	83.02
795-14	177	ACAGCGAAGCATAATGTCTACTGAC GCCCTCAAACCCTATTTGCAGACT	49	97.96	81.13
795-15	178	ACAGCGAAGCATAATGGCTACTGAC GCCGTCAAACCCTATTTGTAGACT	49	95.92	83.02
795-16	179	ACAGCGAAGCATAATGGCTACTGAC GCCCTCAAACCTTATTTGCAGACT	49	97.96	83.02
795-17	180	ACAGGTAGCATAATGGCTACTGACG CCCTCAAACCCTATTTGCAGACT	48	95.92	84.91
795-18	181	ACAGCGAAGCATAATGGCTACTGAC GCCCTCAAACCCTATTTCTAGACT	49	95.92	81.13
795-19	182	ACAGCGAAGCATAATGGCTACTGAC GCCCTCAAACCCTATTTGTAGACT	49	97.96	83.02
795 Consensus Sequence	224	ACAGNSWRGCATAMTGKCTWCWGA CGSCBKCAAAMCYTANTTVNMGACT Where the N at position 5 can be C or no nucleotide, the N at position 40 can be T or no nucleotide, and the N at position 44 can be C, G, T, or no nucleotide	49	-	-
935-1	183	ACAGGGTAGCATAATGGGCTACTTG ACGCCTTCACCTATTTGTAGACT	48	100	86.79
935-2	184	ACAGGGTAGCATAATGGGCTACTTG ACGCCTTCACCTATTTGAGACT	47	97.92	86.79
935-3	185	ACAGGGTAGCATAATGGGCTACTTTA CGCCTTCACCTATTTGTAGACT	48	97.92	84.62
935-4	186	ACAGGGTAGCATAATGGGCTACTTG ACGCCTTCACCTATTTCTAGACT	48	97.92	84.91
935-5	187	ACAGGGTAGCATAATGGGCTACTTG ACGCCTTCACCTATTTGGAGACT	48	97.92	88.68
935-6	188	ACAGGGTAGCATAGTGGGCTACTTG ACGCCTTCACCTATTTGTAGACT	48	97.92	84.91
935-7	189	ACAGGGTAGCATGATGGGCTACTTG ACGCCTTCACCTATTTGTAGACT	48	97.92	84.91
935-8	190	ACAGGGTAGCATAATGGGCTACTTG ACGCCTTCACCTATTAGTAGACT	48	97.92	84.91
935-9	191	ACAGGGTAGCATAATGGGCTATTTGA CGCCTTCACCTATTTGTAGACT	48	97.92	84.91

935-10	192	ACAGGGTAGCATAATGGGCTACTTGC CGCCTTCACCTATTTGTAGACT	48	97.92	86.54
935-11	193	ACAGTGTAGCATAATTGGCTACTTGA CGCCTTCACCTATTTGTAGACT	48	95.83	83.02
935-12	194	ACAGGGTAGCATAATGGGCTACTTG ACGCTTTCACCTTTTTGTAGACT	48	95.83	83.02
935-13	195	ACAGGGTAGCATAAGGGGCTACTTG ACGCCTTCACCTATTTGTAGACT	48	97.92	84.91
935-14	196	ACAGGGTAGCATAATGGACTACTTG ACGCCTCCACCTATTTGTAGACT	48	95.83	81.13
935-15	197	ACAGGGTAGCATAATGGGCTACTTGT CGCCTTCACCTATTTGTAGACT	48	97.92	84.62
935 Consensus Sequence	225	ACAGKGTCGCATRRKKGRCTAYTTKH CGCYTYCACCTWTTWSNAGACT Where the N at position 43 can be G, T, or no nucleotide.	48	-	-
946-1	198	ACAGCGTAGCATAATGGGCTGCAGA CGCCGTCAAACCTATTTGCAGACT	49	100	84.62
946-2	199	ACAGCGTAGCATAATGGGCTGCAGA CGCAGTCAAACCTATTTGCAGACT	49	97.96	82.69
946-3	200	ACATGTAGCATAATGGGCTACTGACG CCGTCAAACCTATTTGCAGACT	48	91.84	86.54
946-4	201	ACAGCGTAGCATAGTGGGCTGCAGA CGCCGTCAAACCTATTTGCAGACT	49	97.96	82.69
946-5	202	ACAGTGTAGCATAATGGGCTGCAGA CGCCTTCAAACCTATTTGGAGACT	49	93.88	88.46
946-6	203	ACAGTGTAGCATAATGGGCTGCTGAC GCCGTCAAACCTATTTGAAGACT	49	93.88	86.54
946-7	204	ACAGCGTAGCATAATGGGCTACAGG CGCCGTCAAACCTATTTGCAGACT	49	95.92	84.62
946-8	205	ACAGCGTAGCATAATGGGCTACTGG CGCCGTCAAACCTATTTGCAGACT	49	93.88	86.54
946-9	206	ACAGCGTAGCATAATGGGCTGCAGA CGCCGTCAAACCTATTTGAGACT	48	97.96	84.62
946-10	207	ACAGGTAGCATAATGGGCTGCAGAC GCCGTCAAACCTATTTGCAGACT	48	97.96	84.62
946-11	208	ACAGGTAGCATAATGGGCTGCTGAC GCCGTCAAACCTATTTACAGACT	48	93.88	84.62
946-12	209	ACAGCGTAGCATATTGGGCTGCAGA CGCCGTCAAACCTATTTGCAGACT	49	97.96	82.69
946-13	210	ACAGCGTAGCATAATGGGCTGCAGA CGCCTTCAAACCTATTTGGAGACT	49	95.92	88.46

946-14	211	ACAGTGTAGCATAATGGGCTGCAGA CGCCGTCAAACCTATTTGAGACT	48	95.92	84.62
946-15	212	ACAGCGTAGCATAATGGGCTGCTGA CGCCGTCAAACCTATTTGGAGACT	49	95.92	88.46
946-16	213	ACAGCGTAGCATAATGGGCTGCAGA CGCCGTCAAACCTATTTACAGACT	49	97.96	82.69
946-17	214	ACAGCGTAGCATAATGGGCTGCTGA CGCCGTCAAACCTATTTGCAGACT	49	97.96	86.54
946-18	215	ACAGGGTAGCATAATGGGCTGCAGA CGCCGTCAAACCTATTTGGAGACT	49	95.92	88.46
946-19	216	ACAGCGTAGCATAATGGGCTACAGA CGCCGTCAAACCTATTTGCAGACT	49	97.96	86.54
946-20	217	ACAGCGTCGCATAATGGGCTGCAGA CGCCGTCAAATCTATTTGCAGACT	49	95.92	80.77
946-21	218	ACAGCGTAGCATAATGGGCTTCAGA CGCCGTCAAACCTATTTGCAGACT	49	97.96	84.62
946-22	219	ACATGTAGCATAATGGGCTGCAGAC GCCGTCAAACCTATTTGGAGACT	48	93.88	84.62
946 Consensus Sequence	226	ACANNGTMGCATADTGGGCTDCWGR CGCMKTCAAAYCTATTTRNAGACT Where the N at position 4 can be G or no nucleotide, the N at position 5 can be C, G, T, or no nucleotide, and the N at position 44 can be A, C, G, or no nucleotide.	49	-	-
961-1	220	ACACCGTAGCATAATGGGCTACTGCC GCCGTCGACCTTTTGGAGACT	47	100%	82.69
996-1	221	ACAGGGTAGCATAATGGCTTAGGAC GCCTTCAAACCTATCAAGACT	46	100%	76.92

Table 2. DNA sequences corresponding to the non-stem regions of the acyclovir binding RNA riboswitches. Seven families were identified in the screen: 582, 769, 795, 935, 946, 961, and 996 with between 1 and 39 sequences in each family. The percent identity for each sequence in the family was compared to the most prevalent sequence within each family (582-1, 769-1, 795-1, 935-1, 946-1, 961-1, and 996-1). The percent identity for each sequence in the family was also compared to the wild-type sequence.

[484] Positive target acyclovir produced seven strong candidates (SEQ ID NOs:87-93; RNA sequences including stem regions) corresponding to 582-1 (SEQ ID NO:108), 769-1 (SEQ ID NO:147), 795-1 (SEQ ID NO:164), 935-1 (SEQ ID NO:183), 946-1 (SEQ ID NO:198), 961-1 (SEQ ID NO:220), and 996-1 (SEQ ID NO:221), each designated F1A (FIG. 17). These sequences were the most prevalent sequences

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in each family (the DNA sequences of all the members of each family are: 582 (SEQ ID NOs:108-146); 769 (SEQ ID NOs:147-163); 795 (SEQ ID NOs:164-182); 935 (SEQ ID NOs:183-197); 946 (SEQ ID NOs:198-219); 961 (SEQ ID NO:220); and 996 (SEQ ID NO:221)). The consensus sequences show all possible substitutions or gaps at each nucleotide position for each family (SEQ ID NOs:222-226). As the goal was to identify aptamers from a library based on RNA that is known to bind to deoxyguanosine, strong candidates needed to have minimal presence in the counter-targets population. Candidates F1A-795, F1A-935, and F1A-946 met this criterion very well, as they were not detected in the counter-target population. F1A-996 and F1A-961 are considered the next best candidates in this regard, although they do show up to a small degree in the counter-targets population. In addition, candidates should appear minimally in the negative population, as those sequences desorbed from GrO without the influence of acyclovir and could represent false positives. F1A-935 and F1A-946 performed ideally under this criterion as well, as they were not found in the negative population. Candidate F1A-769 was minimally detected in the negative population, with candidates F1A-961, F1A-795 and F1A-996 performing less well. Enrichment rate was the final condition to be considered, with F1A-935, F1A-946, and F1A-769 performing adequately. Candidate F1A-582 was included because it exhibited the greatest enrichment rate, although it did not perform well under the other criteria. The remaining candidates did not perform well relative to these four, but exhibited acceptable characteristics.

[485] Additional target penciclovir produced seven strong candidates (SEQ ID NOs:94-100), each designated F1P (FIG. 18). As before, the goal was to identify aptamers from a library based on RNA that is known to bind to deoxyguanosine, diverging from libraries enriched for binding to acyclovir (acyclovir) after Round 10. Strong candidates needed to have minimal presence in both the acyclovir and the counter-targets populations to minimize cross-reactivity. Candidate F1P-923 met the first criterion, candidate F1P-710 met the second criterion, and candidate F1P-584 met both criteria to a degree. Candidate F1P-584 also demonstrated moderate favorability for penciclovir over the negative condition, as well as moderate enrichment relative to the previous generation's response to acyclovir. The remaining candidates demonstrated either minimal favoring of penciclovir over acyclovir or minimal favoring of penciclovir over counter-targets (F1P-837 and F1P-932; F1P-991 and F1P-718; respectively). These four candidates demonstrated some favorability for penciclovir over the negative condition which minimizes the chance of a false positive, although this criterion is not as significant if a candidate does not demonstrate selectivity for penciclovir over its analogues. Enrichment rate was the final condition to be considered, with F1P-923, F1P-932, and F1P-584 performing adequately.

[486] Qualitative PAGE assessment of selected aptamers was performed. Individually synthesized and transcribed aptamers were subjected to selection on Graphene Oxide (GrO) under physiological Mg++

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(0.5 mM) and elution with either acyclovir (+) or counter-targets (x). The specifically eluted aptamer fractions for each sample were subjected to PAGE for analysis.

[487] 100 pmoles of each aptamer candidate (per trial/lane) was resuspended in 1X modified selection buffer (50 mM HEPES, 100 mM KCl, 0.5 mM $MgCl_2$, pH 7.3) and refolded (90 °C for 5 min, then 4 °C for 5 min), then incubated at 37 °C for 30 minutes with 200 pmoles (each) of pooled counter-targets or target. Final library concentration was 0.5 μM, target/counter-targets concentration was 1 μM (incubation volume was 200 μl).

[488] After target/counter-target incubation, 250 μg of GrO (Angstron Materials (Dayton, OH)) was added to adsorb unbound candidate (10-minute incubation at 37 °C).

[489] Samples were centrifuged for 5 minutes at 7,000 x g. Supernatant was recovered, denatured using 2X Formamide with 40 mM EDTA, and run on 10% denaturing PAGE with 8 M urea (supplier: American Bioanalytical; catalog #'s AB13021-01000. AB13022-01000). Running buffer was 1X TBE (supplier: Amresco/VWR; catalog # 0658-20L, diluted using DI water). DNA ladder was 20/100 DNA ladder (IDT). Gels stained with Gel Star (Lonza, 50535) and imaged on a blue light transilluminator.

[490] Candidates F1A-769, F1A-795, F1A-946, and F1A-996 appear to exhibit selective positive response in this qualitative PAGE assessment (good elution of the Aptamer from GrO with Acyclovir target and relatively lower or minimal elution with counter-targets).

Conclusion

[491] Strong candidates for acyclovir were identified after twelve rounds of iterative screening and parallel assessment; reasonable candidates for penciclovir were identified after two rounds of screening and parallel assessment.

Example 2: Isolation of conditional scFv's.

[492] Potential splice site liabilities are removed and tumor antigen specific scFv's are synthesized by overlapping oligo synthesis and cloned into the CAR shuttle construct containing the acyclovir responsive element and the primate CD3ζ promoter. As an initial prototype, anti-ECD of EPCAM or ERBB2scFv with a CD8-alpha signal peptide, stalk, and transmembrane domain is utilized. Solid tumor microenvironment restricted CAR products are generated either using methods as described in US Patent No. 8,709,755 and PCT Publication No. WO/2016/033331A1 or by direct selection from human phage libraries under permissive and non-permissive conditions. Briefly, a human V_H x V_L library from Creative Biolabs (Shirley, NY) is panned in the following tumor permissive conditions: 100 μg/ml hyaluronan, 100 kDa fraction (Lifecore Biomedical, Chaska, MN), 20 mg/ml recombinant HSA (Cyagen, Santa Clara, CA), 200 ng/ml recombinant human VEGF in 25 mM sodium bicarbonate buffer, 2 μM adenosine, 10

mM sodium lactate pH 6.7, following clearance with streptavidin magnetic beads (ThermoFisher, Carlsbad, CA) bound to biotinylated human IgG. Binding to biotinylated-target receptor ECD of EPCAM and ERBB2 conjugated beads at 37 °C is performed under permissive conditions followed by serial washes in permissive conditions. Phage are released with physiologic conditions (1 µg/ml hyaluronan, 20 mg/ml HSA, 25 mM bicarbonate, 1 mM sodium lactate pH 7.2) followed by elution of tight variants with acid elution and rapid neutralization with 1 M Tris. Phage are expanded and genomic DNA is split for deep sequence analysis of V_H x V_L chains using long read sequencing (PacBio, Menlo Park, CA). Panning can be repeated for enrichment. V_H x V_L sequences showing preferential amplification of reads during the phage culturing process over enrichment to target are excluded for further analysis. Phage with selective binding to the target that are enriched under tumor permissive conditions but released under physiologic conditions are chosen for further characterization by cloning into the CAR construct expression system, generation of lentivirus, and transduction into T cells for testing CAR-mediated tumor antigen expressing target cell killing in a tumor-selective environment compared to physiologic conditions.

Example 3: Generation of MRB-CARs Using Microenvironment restricted scFv's.

[493] Microenvironment restricted ASTRs were obtained that were made by subjecting V_H and V_L sequences with low selectivity for the tumor microenvironment to evolution as described in application WO/2016/033331A1. Chimeric antigen receptors (CARs) for binding either of two tumor antigens, Axl or Ror2, with increased activity at the reduced pH of a tumor environment compared to normal tissue (such microenvironment restricted biologics is sometimes referred to herein as (MRB-CARs) were made by incorporating the heavy chains and light chains of the microenvironment restricted single-chain antibodies into lentiviral expression vectors along with other CAR domains to generate MRB-CARs. These CARs included various combinations of modules from amino to carboxy terminus, which included a CD8 signal peptide (P1) (SEQ ID NO:74); microenvironment restricted anti-Ror2 and Anti-Axl V_H and V_L combinations; a stalk and transmembrane domain from CD8 (SEQ ID NO:75) or CD28 (SEQ ID NO:76) (P5); a co-stimulatory domain from CD 137 (SEQ ID NO:1) or ICΔ (SEQ ID NO:3) (P6); an activation domain from CD3Z (SEQ ID NO:13) (P7); a 2A-1 ribosomal skip sequence (SEQ ID NO:77) (P8); and an exemplary eTAG (SEQ ID NO:78) (P9).

[494] Pan T cells were transduced with the recombinant lentiviral particles expressing the candidate CARs and the percent transfected cells was determined by determining the percent of cells expressing the eTag using FACS. Pan T cells were successfully transduced with the recombinant lentiviral particles encoding the candidate CARs and displayed conditional activity in these transduced T cell assays at pH 6.7 vs. pH 7.4.

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[495] The cytotoxic activity of the candidate CARs against target cells expressing either Axl or Ror2 was analyzed at a pH of 7.4 (physiological pH) or a pH of 6.7 (pH surrogate tumor assay condition). Many of the candidate CARs were more effective at lysing target cells at a pH of 6.7 than a pH of 7.4.

Example 4. Construction of ligand-inducible riboswitches.

[496] Deoxyguanosine riboswitch aptamer and guanine riboswitch aptamers (Pikovskaya, 2014; Kim, 2007) or other purine riboswitch aptamers are synthesized as oligonucleotides. In one example, the deoxyguanosine IA riboswitch from *Mesoplasma florum* (underlined and in bold in FIG. 6; FIG. 7) is selected for evolution to generate an acyclovir-responsive riboswitch. In another example, the guanine xpt riboswitch from *Bacillus subtilis* (underlined and in bold in FIG. 10; FIG. 11) is selected for evolution to generate an acyclovir-responsive riboswitch. For each of these two examples, a random RNA library is generated with alternate nucleotides at targeted sequence positions in the P2, P3, J1-2, and J2-3 segments (FIGs. 7 and 11). Each segment allows for 3 alternate nucleic acids at each targeted sequence position, or alternatively base deletion and insertion of 4 nucleotides in the +1 site at each targeted sequence position for saturation mutagenesis as indicated in FIGs. 8A-8B and 9 (*M. florum* IA) and FIGs. 12A-12B and 13 (*B. subtilis* xpt). Primer extension and reagent preparation is followed by RNA transcription. The resultant RNA library is negatively selected on graphene oxide in the presence of guanine, guanosine, and deoxyguanosine followed by positive selection with acyclovir or penciclovir. During the negative and positive selection processes, human cell physiologic magnesium levels (0.5 mM to 1.2 mM) are used and the temperature is kept at 37 °C. Recovered aptamers are reverse transcribed and PCR amplified followed by transcription and subsequent screening for at least 8 successive rounds of selection. In a parallel approach, aptamers are screened with an additional negative screen at 40 °C. Resultant positive pools are examined by NextGen sequencing and analysis. Individual aptamers are synthesized and examined for affinity by isothermal calorimetry at 35-40 °C in human cell physiologic magnesium levels. Following selection for positive acyclovir and penciclovir specific aptamers, aptamers are integrated with ribozyme hammerhead and pistol ribozymes. Positive acyclovir selective aptamers are combined with pistol ribozymes to identify acyclovir regulated ribozymes. (Harris KA RNA. 2015 Nov;21(11):1852-8. doi: 10.1261/rna). Variants are subjected to gel shift based PAGE purification in the presence of acyclovir and absence of penciclovir. Additionally, the acycloguanosine selective riboswitch is placed immediately 3′ in a loop to a splice acceptor upstream of the CAR/IL-7 construct. In the absence of acyclovir, the splice site position is bound in the riboswitch complex but in the presence of acyclovir becomes accessible, generating a functional CAR transcript.

Example 5. Construction of *in vivo* propagation domains.

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[497] A series of constitutively active IL7 receptor (IL7R) transmembrane mutants from T cell lymphoblastic leukemias (243 InsPPCL (SEQ ID NO:82); 246 InsKCH (SEQ ID NO:101); 241 InsFSCGP (SEQ ID NO:102); 244 InsCHL (SEQ ID NO:103); and 244 InsPPVCSVT (SEQ ID NO:104); all from Shochat et al 2011, J. Exp. Med. Vol. 208 No. 5 901-908) are synthesized by overlapping oligo nucleotide synthesis (DNA2.0, Newark, California).The synthesized constitutively active IL7R transmembrane mutants are inserted into a constitutively expressing lentiviral vector backbone immediately behind a 2A ribosomal skip sequence followed by an anti-CD19 CD3ζ expression cassette, which includes a CD8A stalk (SEQ ID NO:79) and a leader peptide (SEQ ID NO:74). HEK293 packaging cells are transfected with the IL7R transmembrane mutant lentiviral vectors and lentiviral packaging constructs, grown, and viral supernatants are harvested using methods known in the art. CD3/CD28-stimulated T cells are transduced with the viral supernatants and grown in IL2 deficient AIM V, CTS OpTmizer T Cell Expansion SFM, or X-VIVO 15 media for 4 weeks, supplemented weekly with frozen PBMCs from the same donor. The resulting expanded transduced T cells expressing IL7R variants are cloned by FACS sorting and the sequences of the IL7R constructs are identified by sequencing RT-PCR products. The 243 InsPPCL variant (PPCL) (SEQ ID NO:82) is selected for further evolution to generate a conditionally active CAR.

Example 6. Screening of accessory components for CAR-T activation and propagation.

[498] A series of protein-encoding domains (ABCG1, SOCS1, SMAD2, TGFBR2, cCBL, and PD1) and miRNA sequences are constructed for incorporation into a synthetic intron on the reverse strand of a CD3-promoter driven CAR cassette. Each construct containing the CD3-promoter driven CAR cassette and a protein-encoding domain or miRNA sequence includes a unique bar code for deep sequencing and is assembled using Gibson assembly followed by transformation and library expansion in *E. coli*. Viral stocks are produced and used to transduce CD3/CD28-stimulated T cells in AIM V, CTS OpTmizer T Cell Expansion SFM, or X-VIVO 15 media without IL2 and allowed to grow for 4 weeks in culture with serial sampling of DNA for amplification and deep sequencing for code identification. The library is also subject to PACBio full length sequencing to determine library diversity and to decode the bar code components. The miRNA sequences and protein-encoding domains are tested for synergistic activation of CAR CD3ζ domains.

Example 7. Engineering a retroviral packaging and transducing system to target resting T cells for selective T cell integration and expression from PBMCs.

[499] Although producing high-titer lentiviral vectors by transient transfection is possible, this method carries the risk of generating replication competent retroviruses (RCRs) and is not scalable for clinical

applications. Herein, a stable retroviral packaging cell line is generated by the simultaneous introduction of multiple constructs encoding inducible promoters and their regulators into HEK293 suspension-adapted cells (HEK293S) to stably produce the viral components, CAR genes, and their regulatory components. Two distinct inducible systems can be used to temporally control the expression of genes. One system is based on rapamycin- or rapalog-induced dimerization of two transcription factors. One transcription factor consists of three copies of the FKPB protein fused to a ZFHD1 DNA binding domain and the other transcription factor consists of a FRB protein fused to a p65 activation domain. Rapamycin or a rapalog dimerizes the transcription factors to form ZFHD1/p65 AD and can activate gene transcription at 12xZFHD1 binding sites.

[500] A series of vectors as shown in FIGs. 3A-3E are generated with flanking transposon sequences for integration into the HEK293S genome. Once integrated into the genome of a cell, these sequences function as regulatory components and lox and/or FRT sites for subsequent integration using Cre and/or flp recombinases, herein referred to as landing pads. The initial 5 constructs contain polynucleotide sequences encoding puromycin resistance, GFP, RFP, and an extracellular MYC tag that is targeted to the cell membrane through an N-terminal PLss (bovine prolactin signal peptide) and anchored to the cell membrane through a platelet-derived growth factor receptor (PDGFR) C-terminal transmembrane anchoring domain. The initial 5 constructs can also include constitutive minimal CMV and minimal IL-2 promoters, a rapamycin-regulated ZFHD1-based promoter, a tetracycline-responsive element (TRE) promoter, or a bidirectional TRE (BiTRE) promoter. The construct in FIG. 3A contains a polynucleotide sequence encoding FRB domain fused to the NFκB p65 activator domain (p65 AD) and ZFHD1 DNA binding domain fused to three FKBP repeats that is constitutively expressed. The construct in FIG. 3A also includes HIV1 REV and HSV VP65 domain SrcFlagVpx under the rapamycin-inducible ZFHD1/p65 AD promoter. The construct in FIG. 3B includes a polynucleotide encoding an rtTA sequence under the control of the ZFHD1/p65 AD promoter. The construct in FIG. 3C includes a polynucleotide encoding a puromycin resistance gene flanked by loxP sites and the extracellular MYC tag flanked by lox2272 sites. Both of these selectable markers are under the control of a BiTRE promoter, which is flanked by FRT sites. The construct in FIG. 3D includes a polynucleotide encoding GFP flanked by loxP sites that is under the control of a TRE promoter. The construct in FIG. 3D also includes a single FRT site between the TRE promoter and the 5′ loxP site of GFP. The construct in FIG. 3E includes a polynucleotide encoding RFP flanked by loxP sites that is under the control of the ZFHD1/p65 AD promoter. The construct in FIG. 3E also includes a single FRT site between the ZFHD1/p65 AD promoter and the 5′ loxP site of RFP. The constructs in FIGs. 3C-3E function as landing pads for other polynucleotide sequences to insert into the genome of the packaging cell line. The polynucleotide sequences to be inserted can be flanked by lox sites and inserted into the genome using Cre recombinase and the loxP sites. This results in insertion and simultaneous removal of

the genomic regions encoding puromycin resistance, the extracellular MYC tag, GFP, and RFP. Alternatively, the polynucleotide sequences can be flanked by FRT sites and inserted into the genome using flp recombinase and the FRT sites followed by removal of the polynucleotide sequences encoding puromycin resistance, the extracellular MYC tag, GFP, and RFP using Cre recombinase.

[501] To generate the packaging cell line with landing pads integrated into the genome, HEK293S cells are co-transfected with equimolar concentrations of the 5 plasmids (FIGs. 3A-3E) plus 5 μg of *in vitro*-transcribed piggybac transposase mRNA or 5 μg of a plasmid with a promoter for expressing piggybac transposase in the presence of PEI at a ratio of 2:1 or 3:1 PEI to DNA (w/w) or 2-5 μg piggybac transposase protein using a cationic peptide mixture. The transfected cells are selected with puromycin in the presence of 100 nm rapamycin and 1ug/mL doxycycline for 2-5 days followed by fluorescence-activated cell sorting to collect cells expressing GFP and RFP. The sorted cells are grown 5 days in the absence of puromycin, rapamycin, and doxycycline and cells expressing GFP and RFP are removed also myc positive cells are removed with myc beads. Individual clones from negatively sorted cells are then screened for induction of GFP and RFP by rapamycin and doxycycline and single cell cloned. The DNA from clones is harvested and sequenced for integration analysis. Clones positive for strong inducible expression of GFP and RFP in the presence of rapamycin and doxycycline with limited background expression in the absence of rapamycin and doxycycline are expanded and banked.

[502] The HEK293S cells with the constructs from FIGs. 3A-3E integrated into the genome are then transfected with a construct containing a tricistronic polynucleotide encoding a DAF signal sequence/anti-CD3 scFvFc (UCHT1)/CD14 GPI anchor attachment site (SEQ ID NO:252), a DAF signal sequence/CD80 extra-cellular domain capable of binding CD28/CD16B GPI anchor attachment site (SEQ ID NO:253), and a DAF signal sequence/IL-7 /DAF (SEQ ID NO:107) and transposon sequences flanking the polynucleotide region for integration into the HEK293S genome (FIG. 4A). After transfection, cells are expanded for 2 days in the absence of rapamycin and doxycycline and colonies that are constitutively red are selected. Positive colonies are then transiently transfected with a construct for expressing Cre recombinase to remove remaining genomic DNA, and the RFP encoding region. Another construct (FIG. 4B) containing a polynucleotide with a BiTRE promoter and a polynucleotide region encoding the gag and pol polypeptides in one direction and a polynucleotide region encoding the measles virus F and H proteins in the other direction is transfected at the same time. The Cre recombinase integrates the construct into the genome to generate the integrated sequence shown in FIG. 4B. Resultant colonies are evaluated for protein expression in the presence of doxycycline and rapamycin and analyzed by deep sequencing for genomic integration. The remaining TRE responsive GFP site is retained for the lentiviral genome insertion.

Example 8. Generation of lentivirus vector and retroviral packaging.

[503] The retroviral packaging stable cell line generated in Example 7 is transfected with a construct (FIG. 4C) for expressing Flp recombinase and a construct containing a polynucleotide sequence encoding a CAR and the lymphoproliferative element IL7Rα-insPPCL under the control of a CD3Z promoter that is not active in HEK293S cells, wherein the CAR and IL7Rα-insPPCL are separated by a polynucleotide sequence encoding a T2A ribosomal skip sequence and the IL7Rα-insPPCL has an acyclovir riboswitch controlled ribozyme. The CAR-containing construct further includes cPPT/CTS and RRE sequences and a polynucleotide sequence encoding HIV-1 Psi. The entire polynucleotide sequence on the CAR-containing construct to be integrated into the genome is flanked by FRT sites. Successful integration of the CAR-containing construct causes constitutive expression of GFP that is consequently removed by transient transfection with a construct for expressing Cre recombinase. The HEK293S line is grown in serum free media. Following growth to peak cell density in a stirred tank reactor, the cells are diluted to 70% peak cell density and treated with 100 nM rapamycin for 2 days to induce expression of early genes REV, Vpx, and aCD3 scFv CD16B GPI, aCD28 scFv CD16B GPI, and IL-7 SD GPI DAF followed by the addition of 1 ug/ mL doxycycline in the media to induce expression of structural elements like Gag Pol, MV(Ed)-FΔ30, MV(Ed)-HΔ18, and lentiviral genome including the therapeutic target. Levels of virus production are examined by qPCR of the packaging sequence and p24 ELISA. Virus is harvested by depth filtration of cells, and concentration/diafiltration using a TFF cartridge followed by flash freezing for vialing.

Example 9. Peripheral blood mononuclear cell (PBMC) isolation, transduction, and expansion.

[504] The following example illustrates the use of a closed system for *ex vivo* processing of PBMCs before *in vivo* expansion. As an example, 30 to 200 ml of human blood is drawn from a subject with Acid Citrate Dextrose Solution (ACD) as an anticoagulant into a blood collection bag. Alternatively, blood is drawn into Vacutainer tubes, a syringe, or an equivalent and is transferred to an empty blood collection or IV bag. The whole blood is processed using a Neat Cell kit (Cat # CS-900.2, Omniamed) on a Sepax 2 cell processing system (BioSafe) according to the manufacturers' instructions. The peripheral blood mononuclear cells (PBMCs) are collected either into a culture bag, or alternatively a syringe. An aliquot is taken aseptically for cell counting to determine the number of viable cells. The PBMCs are transferred to a G-Rex100MCS Gas Permeable Cell Culture System device (Wilson Wolf) at a final concentration of 0.1 – 1.0 x 10^6 viable cells/ml in X-VIVO 15 (Cat # 08-879H, Lonza) or CTS OpTmizer Cell Expansion SFM (Cat # A1048501, Thermo Fisher Scientific) media with 10-300 IU/ml IL-2 (Cat # 202-IL-010, R&D Systems) in up to 200 ml final volume. In addition to IL-2, CTS Immune Cell SR (Cat # A2596101, Thermo Fisher Scientific) can be added to the media. The closed G-Rex Gas Permeable Cell Culture System device can be pre-coated with Retronectin (Cat # CH-296, Takara), or a similar fibronectin-derived equivalent, according to the manufacturer's instructions.

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[505] The PBMCs isolated from peripheral blood are loaded onto a PALL PBMC filter, washed once through the filter with 10 ml of AIM V (Thermo Fisher Scientific) or X-VIVO 15 media followed by perfusion with 10-60 ml of lentivirus stock (as prepared in Example 8) at 37 °C at 5 ml/hr. The PBMCs are then washed again with AIM V, CTS OpTmizer T Cell Expansion SFM, or X-VIVO 15 media containing recombinant human DNase (Pulmozyme, Genentech) followed by a wash with DNase-free Lactated Ringers (Cat # L7500, Braun). The PBMCs are then reverse perfused through the filter into a syringe. The cells (target levels of cells are 5×10^5 to 1×10^6 cells/kg) are then reinfused into the subject through intravenous infusion.

[506] Depending upon the riboswitch contained within the retroviral genome, the subject is given the respective nucleoside analogue antiviral drug or nucleoside analogue antiviral prodrug (acyclovir, valaciclovir, penciclovir, or famciclovir). Subjects can be given any therapeutically effective dose, such as 500 mg of the nucleoside analogue antiviral drug or prodrug orally three times/day. Treatment with the nucleoside analogue antiviral drug or prodrug preferably begins before reinfusion, such as 2 hours before, and can also begin at the time of reinfusion or at some time after reinfusion. The treatment can continue for at least 1, 2, 3, 4, 5, 7, 10, 14, 21, 28, 30, 60, 90, 120 days or 5, 6, 9, 12, 24, 36, or 48 months or longer. The treatment can include administration of the nucleoside analogue antiviral drug or prodrug once, twice, three, or four times daily. After reinfusion and treatment is begun, the number of infected cells is determined through blood counts on days 2, 5, 7, 11, 13, 18, 28, and 56 post-reinfusion using qPCR to quantitate the amount of viral genome. A subject experiencing fever or cytokine release syndrome may have the dose or frequency of the nucleoside analogue antiviral drug or prodrug reduced or halted. If the infected T cells fail to amplify 10,000-100,000 fold by day 18, the dose or frequency of the nucleoside analogue antiviral drug or prodrug may be increased. The clinical response of the subject can be measured through FDG PET imaging and serial CT scan. Oral dosing of the nucleoside analogue antiviral drug or prodrug can be reduced or halted following prolonged remission or in the event of excessive T cell propagation beyond 30% of total peripheral T cell counts.

Example 10. Therapeutic intervention to raise vascular or tissue pH.

[507] To reduce the binding of an antigen binding domain to its cognate antigen, $NaHCO_3$ is administered as an IV bolus or by IV infusion. The standard dosage is 1 mg/kg of body weight as the initial dose followed by 0.5 mg/kg every 10 minutes. A 50-milliliter bolus of $NaHCO_3$ will raise the serum pH approximately 0.1 of a pH unit. If the pH is 7.0, it requires four 50 mEq ampules of $NaHCO_3$ to correct the pH to 7.40

Example 11. Testing activity of IL-7 receptor lymphoproliferative/survival elements in PBMCs

[508] To test IL-7Rα variants for their ability to mediate antigen-independent survival of T cells, thirty milliliters of human blood were drawn with acid citrate dextrose (ACD) as an anticoagulant into Vacutainer tubes. The whole blood was processed using density gradient centrifugation with Ficoll-Pacque™ (General Electric) following manufacturer's instruction, to obtain peripheral blood mononuclear cells (PBMCs). Aliquots of the PBMCs were transferred aseptically to wells of a 12 well tissue culture plate, along with X-Vivo™ 15 media (Lonza) to a final concentration of 0.5 million viable cells / mL in a final volume of 1mL. Recombinant human interleukin-2 (IL-2) (Novoprotein) was also added to a concentration of 100IU/ml in some samples. Activating anti-CD3 Ab (OKT3, Novoprotein) was added at a concentration of 50ng/ml, to activate the PBMC for viral transduction. The plates were incubated overnight in a standard humidified tissue culture incubator at 37 degrees C and 5% Carbon Dioxide. After overnight incubation, lentivirus particle preparations containing the desired test constructs (FIG. 19A) were added to individual wells at a multiplicity of infection (MOI) of 5. The plate was incubated overnight in a standard humidified tissue culture incubator at 37 degrees C and 5% Carbon Dioxide. Following the overnight incubation, the contents of each of the wells of the 12 well plate were collected and centrifuged to obtain a pellet. The samples were washed once with D-PBS + 2% Human Serum Albumin (HSA), resuspended in X-Vivo15™ media, and transferred to wells of G-Rex® 6-well gas permeable cell culture devices (Wilson Wolf). Additional X-Vivo™ 15 media was added to bring the final volume of each well to 30ml. Matching control samples for each of the constructs were transferred to wells of G-Rex® 6-well gas permeable cell culture devices (Wilson Wolf) and additional media was added to bring the final volume to 30ml with 100IU/ml IL-2 for some control samples. The G-Rex® device was incubated in a standard humidified tissue culture incubator at 37 degrees C and 5% Carbon Dioxide for 7 days. Fresh IL-2 was added to the control samples containing IL-2 during the culture every 2-3days. Matched test samples without IL-2 were not supplemented. Samples were removed for tracking cell numbers and viability during expansion (Countess, Thermo Fisher) at day 7.

[509] FIG. 19A provides a schematic of the IL7Rα constructs that were tested. These constructs were inserted into a recombinant lentiviral genome. The recombinant retroviruses were used to transduce PBMCs. FIG. 19A shows a schematic of wild-type IL7Rα (SEQ ID NO:229), which consists of a signal sequence (SS), an extracellular domain (ECD), a transmembrane (TM), and an intracellular domain (ICD). "1" indicates the site of a fibronectin type III domain; "2" indicates the site of a WSXWS motif; "3" indicates a Box 1 site, "4" indicates the site of a protein kinase C (PKC) phosphorylation site, and "5" indicates a Box 2 site.

[510] Variant "A" is the IL-7Rα with an InsPPCL at position 243 (Shochat et al 2011, *J. Exp. Med.* Vol. 208 No. 5 901-908) but without the S185C mutation, expressed on a transcript with a GFP polypeptide, a

GSG linker, and a P2A ribosomal skip sequence fused to its N-terminus. Variant "B" is the IL-7Rα InsPPCL with a GFP polypeptide, a GSG linker, and a P2A ribosomal skip sequence fused to its N-terminus as well as a Myc Tag between the signal sequence and the extracellular domain. Variant "C" is similar to variant "B" except its intracellular domain is truncated at position 292. Variant "D" is similar to variant "A" except its intracellular domain is truncated at position 292. Variant "E" is the IL-7Rα InsPPCL variant truncated at its N terminus such that the signal sequence and most of the extracellular domain (residues 1-228) are not present; variant "E" also has a GFP polypeptide, a GSG linker, a P2A ribosomal skip sequence, and an eTag fused to the N terminus, in that order from the amino terminus. Numbering of the amino acid residues is based on IL7Rα (NCBI GI No. 002176.2). T cells containing each of the variants were tested for viability in the presence or absence of IL-2 using Trypan Blue exclusion.

[511] As shown in FIG. 19B, PBMCs require IL-2 for survival *in vitro*. As illustrated in FIG. 19B, untransfected PBMCs have about 80% viability in the presence of IL-2 and 0% viability in the absence of IL-2. PBMCs having the full-length versions of IL-7Rα InsPPCL (IL-7Rα variants A and B in FIG. 19A) had over 20% viability in the absence of IL-2, indicating that expression of the constitutively active IL-7Rα InsPPCL receptor has survival activity in these cells. Furthermore, T cells expressing the IL-7Rα InsPPCL variants with a truncated intracellular domain (ICD) (IL-7Rα variants C and D in FIG. 19A) had increased viability compared to the wild-type IL-7 receptor. Finally, the N-terminal IL-7 receptor mutant (IL-7Rα variant E in FIG. 19A) as shown in FIG. 19B had survival activity in these cells. Accordingly, this example illustrates that IL-7 receptor has survival activity when expressed in PBMCs.

Example 12. Transduction efficiency of freshly isolated unstimulated human T cells by MeVpp

[512] Lentiviruses were produced by transient transfection of 293T cells (Lenti-X 293T, Clontech) with the lentiviral expression vectors. The cells were adapted to suspension culture by serial growth in Freestyle 293 Expression Medium (ThermoFisher Scientific). The cells in suspension were seeded at 1×10^6 cells/mL (30mL) in a 125 mL Erlenmeyer flask, and immediately transfected using PEI (Polysciences) dissolved in weak acid.

[513] Plasmid DNA was diluted in 1.5 ml Optimem media for 30 mL of cells. For the VSV-G pseudo-particles, the total DNA (1 μg/mL of culture volume) was a mixture of 4 plasmids with the following molar ratios: 2x genomic plasmid, 1x Rev-containing plasmid, 1x VSVg-containing plasmid, and 1x Gagpol-containing plasmid. For the MV(Ed)-FΔ30/HΔ18 pseudo-particles, the total DNA (1 μg/mL of culture volume) was a mixture of 5 plasmids with the following molar ratios: 2x genomic plasmid, 1x Rev-containing plasmid, (2/3, two thirds) x MV(Ed)-FΔ30-containing plasmid, (1/3, one third) x MV(Ed)-HΔ18-containing plasmid, and 1x Gagpol-containing plasmid. For the MV(Ed)-FΔ30/HΔ24

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pseudo-particles, the total DNA (1 µg/mL of culture volume) was a mixture of 5 plasmids with the following molar ratios: 2x genomic plasmid, 1x Rev-containing plasmid, (2/3, two third) x MV(Ed)-FΔ30-containing plasmid, (1/3, one third)x MV(Ed)-HΔ24-containing plasmid, and 1x Gagpol-containing plasmid. Separately, the PEI was diluted in 1.5 ml Optimem to 2 µg/mL (culture volume, 2:1 ratio to DNA). After a 5-minute room temperature incubation, the two solutions were mixed together thoroughly, and incubated at room temperature for 20 more minutes. The final volume (3 ml) was added to the cells. The cells were then incubated at 37 °C for 48 hours with rotation at 120 rpm and with 5-8% CO_2.

[514] After 48 hours, the supernatants were harvested by centrifugation at 1,000g for 10 minutes. The supernatants were decanted to a fresh tube and ¼ of the supernatants volume in PEG solution (PEG-IT, System Biosciences) was added. The lentiviral pseudotypes were precipitated by incubation overnight at 4 °C followed by centrifugation at 1,500 g for 20 minutes at 4 °C. The supernatant was removed, and the virus was resuspended in 1:100 volume of PBS. Viruses were titered by serial dilution and GFP expression on Raji cells, which express both CD46 and SLAM, 48 hours post-transduction, by flow cytometry.

[515] Enriched peripheral blood T cells were first isolated from a fresh buffy coat of blood collected and distributed by the San Diego Blood Bank, CA. Briefly, SepMate™ (Stemcell™)-based gradient density separation of PBMCs on Ficoll-Paque PLUS® (GE Healthcare Life Sciences) was performed per manufacturers' instructions. Untouched T cells were then further enriched by negative selection from the freshly isolated total PBMCs, using the untouched T cells Dynabeads® kit (Invitrogen) and manufacturer's instructions. After isolation, 2.6E5 of enriched and freshly isolated and unstimulated peripheral blood T lymphocytes were transduced, in duplicate, with the different vectors, at various multiplicities of infection (MOI). The transductions were conducted for 14h, at 37°C, in 100 uL RPMI-2%HIFCS final, in a 96 wells plate format. After incubation with the vectors for 14h, the cells were washed three times with PBS-2%HIFCS, and finally incubated at a cell density of 0.5E6/mL in RPMI-10%HIFCS at 37°C until day 3.

[516] Three days post-transduction with the VSV-Gpp or MeVpp, 1E5 cells were collected and analyzed by flow cytometry for expression of GFP in the CD3+ cell population. FIG. 20 shows a plot of transduction efficiency against MOI for negatively selected and unstimulated T cells. Symbols are staggered for improved clarity. Unstimulated T cells were more efficiently transduced using MV(Ed)-FΔ30/ MV(Ed)-HΔ18 pseudo-particles (~70-80%) as compared to VSV-Gpp (~0-5%) at an MOI of 1. Unstimulated T cells were also more efficiently transduced using MV(Ed)-FΔ30/ MV(Ed)-HΔ24 pseudotyping particles at an MOI of 5 (~70-80%) as compared to VSV-Gpp at an MOI of 6 (~5-10%). Transduction efficiency of freshly isolated unstimulated human T cells by pseudotyped lentivectors is

much higher when truncated MeV-envelope polypeptides are used for pseudotyping than when VSV-G is used.

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Example 13. Demonstrating functionality of miRNAs inserted into the EF-1alpha promoter intron

[517] Four separate gBlocks® Gene Fragments were designed, each containing the miR-155 framework. For each gBlock®, a unique miRNA targeting the CD3zeta mRNA transcript was used to replace the miR-155 target sequence. Each gBlock® contained a 40bp overlap sequence designed to facilitate assembly of all four gBlocks® as a single chain into the EF-1alpha promoter intron. The gBlocks® were assembled using a commercial kit for performing Gibson® assembly ultra (NEBuilder, New England Biolabs, Inc.).

[518] The EF-1alpha promoter and intron A (SEQ ID NO:255) was part of a transgene expression cassette driving expression of GFP and eTag contained in a lentivirus vector backbone (the lentivirus vector backbone with the GFP and exemplary eTag recognized by cetuximab is referred to herein as F02). The nucleotide positions of each gBlock® and its respective components in SEQ ID NO:255 are denoted in Table 3. Proper assembly of four miRNA into the lentivirus vector backbone was confirmed by comprehensive sequencing of the EF-1alpha promoter.

Feature	Nucleotide positions in SEQ ID NO:255
gBlock® 1	927-1138
EF1alpha overlap	927-966
miR155 framework – 5' arm	967-994
siRNA1	995-1015
miR terminal loop	1016-1034
siRNA1	1035-1042
siRNA1	1043-1053
miR155 framework – 3' arm	1054-1098
gBlock® 2	1099-1310
40bp 50% GC Linker 1	1099-1138
miR155 framework – 5' arm	1139-1166
siRNA2	1167-1187
miR terminal loop	1188-1206
siRNA2	1207-1214
siRNA2	1215-1225
miR155 framework – 3' arm	1226-1270

gBlock® 3	1271-1482
40bp 50% GC Linker 2	1271-1310
miR155 framework – 5' arm	1311-1338
siRNA3	1339-1359
miR terminal loop	1360-1378
siRNA3	1379-1386
siRNA3	1387-1397
miR155 framework – 3' arm	1398-1442
gBlock® 4	1443-1654
40bp 50% GC Linker 4	1443-1482
miR155 framework – 5' arm	1483-1510
siRNA4	1511-1531
miR terminal loop	1532-1550
siRNA4	1551-1558
siRNA4	1559-1569
miR155 framework – 3' arm	1570-1614
EF-1alpha overlap	1615-1654

Table 3. Nucleotide positions of features in SEQ ID NO:255

[519] Lentiviruses containing the four miRNAs directed against CD3zeta were produced by transient co-transfection of four plasmids into suspension HEK293 cells: the plasmid containing the four miRNAs targeting the CD3zeta mRNA transcript, a plasmid containing VSV-G, a plasmid containing REV, and a plasmid containing GAG-POL. Viral supernatant was harvested after 48 hours and PEG-precipitated for 24 hours. Supernatants were centrifuged, and pelleted virus was re-suspended in complete PBMC growth media without IL-2. Viral titers were calculated by 48 hour transduction of Jurkat cells.

[520] For transduction, PBMCs were thawed on Day 0 and incubated for 24 hours with 100U/mL of hrIL-2. On Day 1, PBMCs were activated via CD3/CD28 conjugated beads. On Day 2, activated PBMCs were transduced with the lentivirus containing the miRNAs at an MOI of 10. Cells were expanded until Day 11, with fresh hrIL-2 added every two days. On days 7, 9, and 11, 1 million cells were harvested for FACS analysis.

[521] Cells were stained for CD3 Epsilon surface expression, using PE conjugated OKT-3 antibody (Biolegend). Expression levels were determined by the mean fluorescence intensity (MF) of PE in the

GFP positive population (*transduced cells*). Expression levels of transduced cells were compared between wild-type (F02) virus and F02 virus containing the CD3z miRNAs. FIG. 21 shows that the miRNAs targeting CD3zeta that are in the EF-1alpha promoter intron are able to knockdown expression of the CD3 complex.

[522] The disclosed embodiments, examples and experiments are not intended to limit the scope of the disclosure or to represent that the experiments below are all or the only experiments performed. Efforts have been made to ensure accuracy with respect to numbers used (e.g., amounts, temperature, etc.) but some experimental errors and deviations should be accounted for. It should be understood that variations in the methods as described may be made without changing the fundamental aspects that the experiments are meant to illustrate.

[523] Those skilled in the art can devise many modifications and other embodiments within the scope and spirit of the present disclosure. Indeed, variations in the materials, methods, drawings, experiments, examples, and embodiments described may be made by skilled artisans without changing the fundamental aspects of the present disclosure. Any of the disclosed embodiments can be used in combination with any other disclosed embodiment.

[524] In some instances, some concepts have been described with reference to specific embodiments. However, one of ordinary skill in the art appreciates that various modifications and changes can be made without departing from the scope of the invention as set forth in the claims below. Accordingly, the specification and figures are to be regarded in an illustrative rather than a restrictive sense, and all such modifications are intended to be included within the scope of invention.

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CLAIMS

1. An isolated polynucleotide, comprising:

a) an aptamer RNA comprising an aptamer domain capable of binding graphene oxide in the presence of guanine, guanosine, and 2′-deoxyguanosine, and capable of eluting from graphene oxide by binding acyclovir or penciclovir;

b) a reverse complement of the aptamer RNA;

c) a coding DNA encoding the aptamer RNA; or

d) a reverse complement DNA encoding the reverse complement of the aptamer RNA, and

wherein the aptamer domain comprises a non-stem region encoded by a DNA that shares at least 80% sequence identity to any one of SEQ ID NOs: 108, 147, 164, 183, 198, 220, or 221, or a reverse complement thereof.

2. The isolated polynucleotide according to claim 1, wherein the aptamer domain comprises a non-stem region encoded by a DNA that shares at least 90% sequence identity to any one of SEQ ID NOs: 108, 147, 164, 183, 198, 220, or 221, or a reverse complement thereof.

3. An isolated polynucleotide according to claim 1 or claim 2, wherein the isolated polynucleotide comprises the reverse complement of the aptamer RNA, and wherein the isolated polynucleotide further comprises the reverse complement of a target polynucleotide encoding a target polypeptide operably linked to a promoter.

4. The isolated polynucleotide of claim 3, wherein the isolated polynucleotide further comprises the reverse complement of a function switching domain.