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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
07 September 2018 (07.09.2018)

WIPO | PCT

(10) International Publication Number
WO 2018/160993 A1

(51) International Patent Classification:
A61P 37/02 (2006.01) *C07K 14/55* (2006.01)
C07K 14/54 (2006.01) *A61K 39/39* (2006.01)

(21) International Application Number:
PCT/US2018/020704

(22) International Filing Date:
02 March 2018 (02.03.2018)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

62/466,601	03 March 2017 (03.03.2017)	US
62/484,063	11 April 2017 (11.04.2017)	US
62/542,402	08 August 2017 (08.08.2017)	US

(71) Applicant: **OBSIDIAN THERAPEUTICS, INC.** [US/US]; 1030 Massachusetts Avenue, Fourth Floor, Cambridge, MA 02138 (US).

(72) Inventors: **SURI, Vipin**; 3 Coolidge Road, Belmont, MA 02478 (US). **ELPEK, Kutlu Goksu**; 5 Florence Terrace, Arlington, MA 02476 (US). **OLS, Michelle, Lynn**; 65 Oak Avenue, Northborough, MA 01532 (US). **DOLINSKI, Brian**; 1030 Massachusetts Avenue, Fourth Floor, Cambridge, MA 02138 (US). **KOSMIDER, Nicole**; 1030 Massachusetts Avenue, Fourth Floor, Cambridge, MA 02138 (US). **HELLER, Scott, Francis**; 559 Plain St., Stoughton, MA 02072 (US). **KULKARNI, Abhishek**; 360 Tappan Street #3, Brookline, MA 02445 (US). **INNISS, Mara, Christine**; 11 Highland Ave. Unit 2, Beverly, MA 01915 (US). **LI, Dan Jun**; 1030 Massachusetts Avenue, Fourth Floor, Cambridge, MA 02138 (US).

(74) Agent: **WARD, Donna, T.** et al.; DT Ward, P. C., 142A Main Street, Groton, MA 01450 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

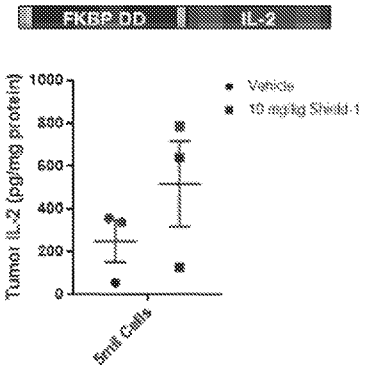
(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:
— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: COMPOSITIONS AND METHODS FOR IMMUNOTHERAPY

(57) Abstract: The present invention provides biocircuit systems, effector modules and compositions for cancer immunotherapy. Methods for inducing anti-cancer immune responses in a subject are also provided.

Figure 19



WO 2018/160993 A1

COMPOSITIONS AND METHODS FOR IMMUNOTHERAPY

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to the US Provisional Patent Application No. 62/466,601, filed on March 3, 2017 entitled Compositions and Methods for Immunotherapy, US Provisional Patent Application No. 62/484,063, filed on April 11, 2017 entitled Compositions and Methods for Immunotherapy, and US Provisional Patent Application 62/542,402, filed on August 8, 2017 entitled Compositions and Methods for Immunotherapy, the contents of each of which are herein incorporated by reference in their entirety.

SEQUENCE LISTING

[0002] The present application is being filed along with a Sequence Listing in electronic format. The Sequence Listing is provided as a file entitled 2095_1207PCT_SL.txt, created on March 2, 2018, which is 1,566,189 bytes in size. The information in the electronic format of the sequence listing is incorporated herein by reference in its entirety.

FIELD OF THE INVENTION

[0003] The present invention relates to compositions and methods for immunotherapy. Provided in the present invention include polypeptides of biocircuit systems, effector modules, stimulus response elements (SREs) and immunotherapeutic agents, polynucleotides encoding the same, vectors and cells containing the polypeptides and/or polynucleotides for use in cancer immunotherapy. In one embodiment, the compositions comprise destabilizing domains (DDs) which tune protein stability.

BACKGROUND OF THE INVENTION

[0004] Cancer immunotherapy aims to eradicate cancer cells by rejuvenating the tumoricidal functions of tumor-reactive immune cells, predominantly T cells. Strategies of cancer immunotherapy including the recent development of checkpoint blockade, adoptive cell transfer (ACT) and cancer vaccines which can increase the anti-tumor immune effector cells have produced remarkable results in several tumors.

[0005] The impact of host anti-tumor immunity and cancer immunotherapy is impeded by three major hurdles: 1) low number of tumor antigen-specific T cells due to clonal deletion; 2) poor activation of innate immune cells and accumulation of tolerogenic antigen-presenting cells in the tumor microenvironment; and 3) formation of an immunosuppressive tumor microenvironment. Particularly, in solid tumors the therapeutic efficacy of immunotherapeutic regimens remains unsatisfactory due to lack of an effective an anti-tumor response in the immunosuppressive tumor microenvironment. Tumor cells often induce immune tolerance or

suppression and such tolerance is acquired because even truly foreign tumor antigens will become tolerated. Such tolerance is also active and dominant because cancer vaccines and adoptive transfer of pre-activated immune effector cells (e.g., T cells), are subject to suppression by inhibitory factors in the tumor microenvironment (TME).

[0006] In addition, administration of engineered T cells could result in on/off target toxicities as well as a cytokine release syndrome (reviewed by Tey *Clin. Transl. Immunol.*, 2014, 3: e17 10.1038).

[0007] Development of a tunable switch that can turn on or off the transgenic immunotherapeutic agent expression is needed in case of adverse events. For example, adoptive cell therapies may have a very long and an indefinite half-life. Since toxicity can be progressive, a safety switch is desired to eliminate the infused cells. Systems and methods that can tune the transgenic protein level and expression window with high flexibility can enhance therapeutic benefit, and reduce potential side effects.

[0008] To develop regulatable therapeutic agents for disease therapy, in particular cancer immunotherapy, the present invention provides biocircuit systems to control the expression of immunotherapeutic agents. The biocircuit system comprises a stimulus and at least one effector module that responds to the stimulus. The effector module may include a stimulus response element (SRE) that binds and is responsive to a stimulus and an immunotherapeutic agent operably linked to the SRE. In one example, a SRE is a destabilizing domain (DD) which is destabilized in the absence of its specific ligand and can be stabilized by binding to its specific ligand.

SUMMARY OF THE INVENTION

[0009] The present invention provides compositions and methods for immunotherapy. The compositions relate to tunable systems and agents that induce anti-cancer immune responses in a cell or in a subject. The tunable system and agent may be a biocircuit system comprising at least one effector module that is responsive to at least one stimulus. The biocircuit system may be, but is not limited to, a destabilizing domain (DD) biocircuit system, a dimerization biocircuit system, a receptor biocircuit system, and a cell biocircuit system. These systems are further taught in co-owned U.S. Provisional Patent Application No. 62/320,864 filed April 11, 2016, 62/466,596 filed March 3, 2017 and the International Publication WO2017/180587 (the contents each of which are herein incorporated by reference in their entirety).

[0010] In some embodiments, the composition for inducing an immune response may comprise a first effector module. In some embodiments, the effector module may comprise a first

stimulus response element (SRE) operably linked to at least one payload. In one aspect, the payload may be an immunotherapeutic agent.

[0011] In some embodiments, the immunotherapeutic agent may be selected from, but is not limited to a cytokine, a safety switch, a regulatory switch, a chimeric antigen receptor and combinations thereof.

[0012] In one aspect, the first SRE of the composition may be responsive to or interact with at least one stimulus.

[0013] In some embodiments, the first SRE may comprise a destabilizing domain (DD). The DD may be derived from a parent protein or from a mutant protein having one, two, there, or more amino acid mutations compared to the parent protein. In some embodiments, the parent protein may be selected from, but is not limited to, human protein FKBP, comprising the amino acid sequence of SEQ. ID NO. 3; human DHFR (hDHFR), comprising the amino acid sequence of SEQ. ID NO. 2; E. Coli DHFR, comprising the amino acid sequence of SEQ. ID NO. 1; PDE5, comprising the amino acid sequence of SEQ. ID NO. 4; PPAR, gamma comprising the amino acid sequence of SEQ. ID NO. 5; CA2, comprising the amino acid sequence of SEQ. ID NO. 6; or NQO2, comprising the amino acid sequence of SEQ. ID NO. 7.

[0014] In one aspect, the parent protein is hDHFR and the DD comprises a mutant protein having at least one mutation selected from M1del,V2A, C7R, I8V, V9A, A10T, A10V, Q13R, N14S, G16S, I17N, I17V, K19E, N20D, G21T, G21E, D22S, L23S, P24S, L28P, N30D, N30H, N30S, E31G, E31D, F32M, R33G, R33S, F35L, Q36R, Q36S, Q36K, Q36F, R37G, M38V, M38T, T40A, V44A, K47R, N49S, N49D, M53T, G54R, K56E, K56R, T57A, F59S, I61T, K64R, N65A, N65S, N65D, N65F, L68S, K69E, K69R, R71G, I72T, I72A, I72V, N73G, L74N, V75F, R78G, L80P, K81R, E82G, H88Y, F89L, R92G, S93G, S93R, L94A, D96G, A97T, L98S, K99G, K99R, L100P, E102G, Q103R, P104S, E105G, A107T, A107V, N108D, K109E, K109R, V110A, D111N, M112T, M112V, V113A, W114R, I115V, V116I, G117D, V121A, Y122C, Y122D, Y122I, K123R, K123E, A125F, M126I, N127R, N127S, N127Y, H128R, H128Y, H131R, L132P, K133E, L134P, F135P, F135L, F135S, F135V, V136M, T137R, R138G, R138I, I139T, I139V, M140I, M140V, Q141R, D142G, F143S, F143L, E144G, D146G, T147A, F148S, F148L, F149L, P150L, E151G, I152V, D153A, D153G, E155G, K156R, Y157R, Y157C, K158E, K158R, L159P, L160P, E162G, Y163C, V166A, S168C, D169G, V170A, Q171R, E172G, E173G, E173A, K174R, I176A, I176F, I176T, K177E, K177R, Y178C, Y178H, F180L, E181G, V182A, Y183C, Y183H, E184R, E184G, K185R, K185del, K185E, N186S, N186D, D187G, and D187N.

[0015] In one aspect, the stimulus of the SRE may be Trimethoprim or Methotrexate.

[0016] In some embodiments, the immunotherapeutic agent may be a cytokine. In one aspect, the cytokine may be an interleukin, an interferon, a tumor necrosis factor, a transforming growth factor B, a CC chemokine, a CXC chemokine, a CX3C chemokine or a growth factor. In some embodiments, the cytokine is an interleukin. In some embodiments, the interleukin is selected from a group consisting of IL1, IL1-alpha, IL1-beta, IL1-delta, IL1-epsilon, IL1-eta, IL1-zeta, IL-RA, IL2, IL3, IL4, IL5, IL6, IL7, IL8, IL9, IL10, IL10C, IL10D, IL11a, IL11b, IL13, IL14, IL16, IL17, IL-17A, IL17B, IL17C, IL17E, IL17F, IL18, IL19, IL20, IL20L, IL21, IL22, IL23, IL23A, IL24, IL25, IL26, IL27, IL28, IL29, IL30, IL31, IL32, IL33, IL34, IL36α, IL36β, IL36γ, IL36RN, IL37, IL37a, IL37b, IL37c, Il37d, IL37e, and IL38.

[0017] In one aspect, the interleukin may be IL2, comprising the amino acid sequence of SEQ ID NO. 51.

[0018] In one aspect, the immunotherapeutic agent may be a safety switch. In some embodiments, the safety switch may be selected from a Caspase 9, an inducible FAS (iFAS), an inducible caspase 9 (icasp9), a CD20/anti-CD20 antibody pair, a protein tag/anti-tag antibody, and a compact suicide gene (RQR8). In one aspect, the safety switch may be Caspase 9 comprising the amino acid sequence of SEQ ID NO. 65.

[0019] In one aspect, the immunotherapeutic agent may encode a regulatory switch. In some embodiments, the regulatory switch may be selected from a FOXP3, a Nr4a, a FOXO, and a NF-κB. In one aspect, the regulatory switch may be a FOXP3, comprising the amino acid sequence of SEQ ID NO. 103-106.

[0020] In one aspect, the immunotherapeutic agent may be a chimeric antigen receptor (CAR). In some embodiments, the CAR may be selected from a GD2 CAR, a Her2 CAR, a BCMA CAR, a CD33 CAR, an ALK CAR, a CD22 CAR, and a CD276 CAR. The CARs described herein may comprise an extracellular moiety, a transmembrane domain, an intracellular signaling domain, and optionally, one or more co-stimulatory domains.

[0021] In one aspect, the CAR may be selected from, but is not limited, to a standard CAR, a split CAR, an off-switch CAR, an on-switch CAR, a first-generation CAR, a second-generation CAR, a third-generation CAR, or a fourth-generation CAR.

[0022] In some embodiments, the extracellular target moiety of the CAR may be selected from, but is not limited to an Ig NAR, a Fab fragment, a Fab' fragment, a F(ab)'2 fragment, a F(ab)'3 fragment, an Fv, a single chain variable fragment (scFv), a bis-scFv, a (scFv)2, a minibody, a diabody, a triabody, a tetrabody, an intrabody, a disulfide stabilized Fv protein (dsFv), a unibody, a nanobody, and an antigen binding region derived from an antibody that may

specifically bind to any of a protein of interest, a ligand, a receptor, a receptor fragment or a peptide aptamer.

[0023] In some embodiments, the extracellular target moiety may be selected from an ALK target moiety, comprising the amino acid sequence of SEQ ID NO. 242- 257 and 422-429, a CD22 target moiety, comprising the amino acid sequence of SEQ ID NO.258-262 and 430-432, a CD276 target moiety, comprising the amino acid sequence of SEQ ID NO. 263-270 and 433-436, a GD2 target moiety, comprising the amino acid sequence of SEQ ID NO.271-349 and 437-465, a CD33 target moiety, comprising the amino acid sequence of SEQ ID NO. 350-357, a BCMA target moiety, comprising the amino acid sequence of SEQ ID NO. 358-365, and a Her2 target moiety, comprising the amino acid sequence of SEQ ID NO. 366-421 and 466-473.

[0024] In some embodiments, the intracellular signaling domain of the CAR may be derived from T cell receptor CD3zeta or a cell surface molecule selected from the group consisting of FcR gamma, FcR beta, CD3 gamma, CD3 delta, CD3 epsilon, CD5, CD22, CD79a, CD79b, and CD66d.

[0025] In some embodiments, the CAR may comprise a co-stimulatory domain. The costimulatory domain may be selected from the group consisting of 2B4, HVEM, ICOS, LAG3, DAP10, DAP12, CD27, CD28, 4-1BB (CD137), OX40 (CD134), CD30, CD40, ICOS (CD278), glucocorticoid-induced tumor necrosis factor receptor (GITR), lymphocyte function-associated antigen- 1 (LFA-1), CD2, CD7, LIGHT, NKG2C, and B7-H3.

[0026] In one embodiment, the transmembrane domain of the CAR may be derived from a transmembrane domain. In one aspect, the transmembrane domain may comprise the amino acid sequence selected from, but not limited to SEQ ID NO. 527-624.

[0027] In some embodiments, the CAR of the effector module may further comprise a hinge region near the transmembrane domain. In one aspect, the hinge region may comprise an amino acid sequence selected from the group consisting of any of SEQ ID NOs. 628-694.

[0028] In one aspect, the first effector module may comprise an IL2-DD, comprising the amino acid sequence of any of SEQ ID NOs. 52-54.

[0029] In one aspect, the first effector module may comprise a Caspase 9-DD, comprising the amino acid sequence of any of SEQ ID NOs. 72-80.

[0030] In one aspect, the first effector module may comprise a FOXP3-DD, comprising the amino acid sequence of any of SEQ ID NOs. 107-116.

[0031] In one aspect, the first effector module may comprise a BCMA CAR-DD, comprising the amino acid sequence of any of SEQ ID NOs. 775-777.

[0032] In one aspect, the first effector module may comprise a HER2-DD, comprising the amino acid sequence of any of SEQ ID NO. 906.

[0033] The present invention, also provides polynucleotides encoding the compositions of the invention.

[0034] In one aspect, the polynucleotides may be a DNA or RNA molecule. In one aspect, the polynucleotides may comprise spatiotemporally selected codons. In some embodiments, the polynucleotides may be an RNA molecule. In one aspect, the RNA molecule may be a messenger molecule. In some embodiments, the RNA molecule may be chemically modified. In some embodiments, the polynucleotides may comprise spatiotemporally selected codons.

[0035] In some embodiments, the polynucleotides may further comprise, at least one additional feature selected from, but not limited to, a promoter, a linker, a signal peptide, a tag, a cleavage site and a targeting peptide.

[0036] The present invention also provides vectors comprising polynucleotides described herein. In one aspect, the vector may be a viral vector. In some embodiments, the viral vector may be a retroviral vector, a lentiviral vector, a gamma retroviral vector, a recombinant AAV vector, an adeno viral vector, and an oncolytic viral vector.

[0037] The present invention also provides immune cells for adoptive cell transfer (ACT) which may express the compositions of the invention, the polynucleotides described herein. In one aspect, the immune cells may be infected or transfected with the vectors described herein. The immune cells for ACT may be selected from, but not limited to a CD8+ T cell, a CD4+ T cell, a helper T cell, a natural killer (NK) cell, a NKT cell, a cytotoxic T lymphocyte (CTL), a tumor infiltrating lymphocyte (TIL), a memory T cell, a regulatory T (Treg) cell, a cytokine-induced killer (CIK) cell, a dendritic cell, a human embryonic stem cell, a mesenchymal stem cell, a hematopoietic stem cell, or a mixture thereof.

[0038] In one aspect, the immune cell may comprise a destabilizing domain DD, wherein the DD is derived from human protein FKBP comprising the amino acid sequence of SEQ ID NO. 3, DHFR comprising the amino acid sequence of SEQ ID NO. 1-2, PDE5 comprising the amino acid sequence of SEQ ID NO. 4, PPAR gamma comprising the amino acid sequence of SEQ ID NO. 5, CA2 comprising the amino acid sequence of SEQ ID NO. 6 and NQO2 comprising the amino acid sequence of SEQ ID NO. 7.

[0039] In one aspect, the DD may be derived from a parent protein and the parent protein is hDHFR and the DD comprises a mutant protein having at least one mutation selected from M1del, V2A, C7R, I8V, V9A, A10T, A10V, Q13R, N14S, G16S, I17N, I17V, K19E, N20D, G21T, G21E, D22S, L23S, P24S, L28P, N30D, N30H, N30S, E31G, E31D, F32M, R33G,

R33S, F35L, Q36R, Q36S, Q36K, Q36F, R37G, M38V, M38T, T40A, V44A, K47R, N49S, N49D, M53T, G54R, K56E, K56R, T57A, F59S, I61T, K64R, N65A, N65S, N65D, N65F, L68S, K69E, K69R, R71G, I72T, I72A, I72V, N73G, L74N, V75F, R78G, L80P, K81R, E82G, H88Y, F89L, R92G, S93G, S93R, L94A, D96G, A97T, L98S, K99G, K99R, L100P, E102G, Q103R, P104S, E105G, A107T, A107V, N108D, K109E, K109R, V110A, D111N, M112T, M112V, V113A, W114R, I115V, V116I, G117D, V121A, Y122C, Y122D, Y122I, K123R, K123E, A125F, M126I, N127R, N127S, N127Y, H128R, H128Y, H131R, L132P, K133E, L134P, F135P, F135L, F135S, F135V, V136M, T137R, R138G, R138I, I139T, I139V, M140I, M140V, Q141R, D142G, F143S, F143L, E144G, D146G, T147A, F148S, F148L, F149L, P150L, E151G, I152V, D153A, D153G, E155G, K156R, Y157R, Y157C, K158E, K158R, L159P, L160P, E162G, Y163C, V166A, S168C, D169G, V170A, Q171R, E172G, E173G, E173A, K174R, I176A, I176F, I176T, K177E, K177R, Y178C, Y178H, F180L, E181G, V182A, Y183C, Y183H, E184R, E184G, K185R, K185del, K185E, N186S, N186D, D187G, and D187N.

[0040] In some embodiments, the immune cells may be autologous, allogeneic, syngeneic, or xenogeneic in relation to a particular individual subject.

[0041] The present invention provides methods for reducing a tumor volume or burden in a subject comprising contacting the subject with the immune cells of the invention. Also provided herein, is a method for inducing an anti-tumor immune response in a subject, comprising administering the immune cells of the system to the subject.

[0042] Also provided herein, is a method for inducing an immune response in a subject, administering the compositions of the invention, the polynucleotides of the invention, and/or the immune cells of the invention to the subject.

[0043] The present invention also provides methods for preventing or reversing T cell exhaustion in a subject in need thereof. Such methods may comprise administering to the subject, a therapeutically effective amount of compositions described herein, the polynucleotides of the invention, the vectors of the invention, or the immune cells described herein. Such methods may comprise an SRE that responds to a stimulus and tunes the expression and/or function of the immunotherapeutic agent, thereby preventing or reversing T cell exhaustion.

[0044] In some aspects, the immunotherapeutic agent is a chimeric antigen receptor. In some embodiments, the chimeric antigen receptor may be a GD2 CAR, a BCMA CAR, a CD33 CAR, a Her2 CAR, an ALK CAR, a CD22 CAR, or a CD276 CAR.

[0045] Also provided herein, is a method for detecting cancer in a mammal, comprising the steps of (a) contacting a sample comprising one or more cells from the mammal with the

compositions, the polynucleotides, the vector or the immune cells of the invention, and (b) detecting the complex, wherein the detection of the complex may be indicative of the presence of cancer in the mammal.

[0046] In some embodiments, the effector module comprises a stimulus response element (SRE) and at least one payload comprising a protein of interest (POI).

[0047] In some embodiments, the SRE may be a destabilizing domain (DD). In some examples, the DD is a mutant domain derived from a protein such as FKBP (FK506 binding protein), E. coli DHFR (Dihydrofolate reductase) (ecDHFR), human DHFR (hDHFR), or any protein of interest. In this context, the biocircuit system is a DD biocircuit system.

[0048] The payload may be any immunotherapeutic agent used for cancer immunotherapy such as a cytokine such as IL2, a safety switch such as Caspase 9, a regulatory switch encoding FOXP3, a chimeric antigen receptor such as BCMA CAR, CD33 CAR, GD2 CAR, Her2 CAR, ALK CAR, CD22 CAR, CD276 CAR or any agent that can induce an immune response. The SRE and payload may be operably linked through one or more linkers and the positions of components may vary within the effector module.

[0049] In some embodiments, the effector module may further comprise of one or more additional features such as linker sequences (with specific sequences and lengths), cleavage sites, regulatory elements (that regulate expression of the protein of interest such as microRNA targeting sites), signal sequences that lead the effector module to a specific cellular or subcellular location, penetrating sequences, or tags and biomarkers for tracking the effector module.

[0050] The invention provides isolated biocircuit polypeptides, effector modules, stimulus response elements (SREs) and payloads, as well as polynucleotides encoding any of the foregoing; vectors comprising polynucleotides of the invention; and cells expressing polypeptides, polynucleotides and vectors of the invention. The polypeptides, polynucleotides, viral vectors and cells are useful for inducing anti-tumor immune responses in a subject.

[0051] In some embodiments, the vector of the invention is a viral vector. The viral vector may include, but is not limited to a retroviral vector, an adenoviral vector, an adeno-associated viral vector, or a lentiviral vector.

[0052] In some embodiments, the vector of the invention may be a non-viral vector, such as a nanoparticles and liposomes.

[0053] The present invention also provides immune cells engineered to include one or more polypeptides, polynucleotides, or vectors of the present invention. The cells may be immune effector cells, including T cells such as cytotoxic T cells, helper T cells, memory T cells, regulatory T cells, natural killer (NK) cells, NK T cells, cytokine-induced killer (CIK) cells,

cytotoxic T lymphocytes (CTLs), and tumor infiltrating lymphocytes (TILs). The engineered cell may be used for adoptive cell transfer for treating a disease (e.g., a cancer).

[0054] The present invention also provides methods for inducing immune responses in a subject using the compositions of the invention. Also provided are methods for reducing a tumor burden in a subject using the compositions of the invention and methods for preventing or reversing T cell exhaustion.

BRIEF DESCRIPTION OF THE DRAWINGS

[0055] Figure 1 shows an overview diagram of a biocircuit system of the invention. The biocircuit comprises a stimulus and at least one effector module responsive to a stimulus, where the response to the stimulus produces a signal or outcome. The effector module comprises at least one stimulus response element (SRE) and one payload.

[0056] Figure 2 shows representative effector modules carrying one payload. The signal sequence (SS), SRE and payload may be located or positioned in various arrangements without (A to F) or with (G to Z, and AA to DD) a cleavage site. An optional linker may be inserted between each component of the effector module.

[0057] Figure 3 shows representative effector modules carrying two payloads without a cleavage site. The two payloads may be either directly linked to each other or separated.

[0058] Figure 4 shows representative effector modules carrying two payloads with a cleavage site. In one embodiment, an SS is positioned at the N-terminus of the construct, while other components: SRE, two payloads and the cleavage site may be located at different positions (A to L). In another embodiment, the cleavage site is positioned at the N-terminus of the construct (M to X). An optional linker may be inserted between each component of the effector module.

[0059] Figure 5 shows effector modules of the invention carrying two payloads, where an SRE is positioned at the N-terminus of the construct (A to L), while SS, two payloads and the cleavage site can be in any configuration. An optional linker may be inserted between each component of the effector module.

[0060] Figure 6 shows effector modules of the invention carrying two payloads, where either the two payloads (A to F) or one of the two payloads (G to X) is positioned at the N-terminus of the construct (A to L), while SS, SRE and the cleavage site can be in any configuration. An optional linker may be inserted between each component of the effector module.

[0061] Figure 7 depicts representative configurations of the stimulus and effector module within a biocircuit system. A trans-membrane effector module is activated either by a free stimulus (Figure 7A) or a membrane bound stimulus (Figure 7B) which binds to SRE. The

response to the stimulus causes the cleavage of the intracellular signal/payload, which activates down-stream effector/payload.

[0062] Figure 8 depicts a dual stimulus-dual presenter biocircuit system, where two bound stimuli (A and B) from two different presenters (e.g., different cells) bind to two different effector modules in a single receiver (e.g., another single cell) simultaneously and create a dual-signal to downstream payloads.

[0063] Figure 9 depicts a dual stimulus-single presenter biocircuit system, where two bound stimuli (A and B) from the same presenter (e.g., a single cell) bind to two different effector modules in another single cell simultaneously and create a dual-signal.

[0064] Figure 10 depicts a single-stimulus-bridged receiver biocircuit system. In this configuration, a bound stimulus (A) binds to an effector module in the bridge cell and creates a signal to activate a payload which is a stimulus (B) for another effector module in the final receiver (e.g., another cell).

[0065] Figure 11 depicts a single stimulus-single receiver biocircuit system, wherein the single receiver contains the two effector modules which are sequentially activated by a single stimulus.

[0066] Figure 12 depicts a biocircuit system which requires a dual activation. In this embodiment, one stimulus must bind the transmembrane effector module first to prime the receiver cell being activated by the other stimulus. The receiver only activates when it senses both stimuli (B).

[0067] Figure 13 is a line graph depicting the effect of Shield-1 on DD-IL2 levels.

[0068] Figure 14 denotes the frequency of IFNgamma positive T cells.

[0069] Figure 15A depicts IFN gamma production in T cells. Figure 15B depicts T cell expansion with IL15/IL15Ra treatment. Figure 15C is a dot plot depicting percentage human cells after *in vivo* cell transfer. Figure 15D is scatter plot depicting CD4+/CD8+ T cells.

[0070] Figure 16A is a western blot depicting luciferase levels in DD-luciferase expressing cells. Figure 16B depicts luciferase activity.

[0071] Figure 17A and Figure 17B are western blot depicting DD regulated expression of FOXP3.

[0072] Figure 18 is a bar graph representing the effect of promoters on transgene expression.

[0073] Figure 19 represents Shield-1 regulation of DD-IL2 secretion from HCT116 cells *in vivo*.

[0074] Figure 20 depicts the viability of T cells cultured with different ratios of CD3/CD8 beads.

[0075] Figure 21 represents the percentage BCMA CAR positive T cells with ligand treatment.

DETAILED DESCRIPTION OF THE INVENTION

[0076] The details of one or more embodiments of the invention are set forth in the accompanying description below. Although any materials and methods similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred materials and methods are now described. Other features, objects and advantages of the invention will be apparent from the description. In the description, the singular forms also include the plural unless the context clearly dictates otherwise. 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. In the case of conflict, the present description will control.

I. INTRODUCTION

[0077] Cancer immunotherapy aims` at the induction or restoration of the reactivity of the immune system towards cancer. Significant advances in immunotherapy research have led to the development of various strategies which may broadly be classified into active immunotherapy and passive immunotherapy. In general, these strategies may be utilized to directly kill cancer cells or to counter the immunosuppressive tumor microenvironment. Active immunotherapy aims at induction of an endogenous, long-lasting tumor-antigen specific immune response. The response can further be enhanced by non-specific stimulation of immune response modifiers such as cytokines. In contrast, passive immunotherapy includes approaches where immune effector molecules such as tumor-antigen specific cytotoxic T cells or antibodies are administered to the host. This approach is short lived and requires multiple applications.

[0078] Despite significant advances, the efficacy of current immunotherapy strategies is limited by associated toxicities. These are often related to the narrow therapeutic window associated with immunotherapy, which in part, emerges from the need to push therapy dose to the edge of potentially fatal toxicity to get a clinically meaningful treatment effect. Further, dose expands *in vivo* since adoptively transferred immune cells continue to proliferate within the patient, often unpredictably.

[0079] A major risk involved in immunotherapy is the on-target but off tumor side effects resulting from T-cell activation in response to normal tissue expression of the tumor associated antigen (TAA). Clinical trials utilizing T cells expressing T-cell receptor against specific TAA reported skin rash, colitis and hearing loss in response to immunotherapy.

[0080] Immunotherapy may also produce on target, on-tumor toxicities that emerge when tumor cells are killed in response to the immunotherapy. The adverse effects include tumor lysis syndrome, cytokine release syndrome and the related macrophage activation syndrome.

Importantly, these adverse effects may occur during the destruction of tumors, and thus even a successful on-tumor immunotherapy might result in toxicity. Approaches to regulatably control immunotherapy are thus highly desirable since they have the potential to reduce toxicity and maximize efficacy.

[0081] The present invention provides systems, compositions, immunotherapeutic agents and methods for cancer immunotherapy. These compositions provide tunable regulation of gene expression and function in immunotherapy. The present invention also provides biocircuit systems, effector modules, stimulus response elements (SREs) and payloads, as well as polynucleotides encoding any of the foregoing. In one aspect, the systems, compositions, immunotherapeutic agents and other components of the invention can be controlled by a separately added stimulus, which provides a significant flexibility to regulate cancer immunotherapy. Further, the systems, compositions and the methods of the present invention may also be combined with therapeutic agents such as chemotherapeutic agents, small molecules, gene therapy, and antibodies.

[0082] The tunable nature of the systems and compositions of the invention has the potential to improve the potency and duration of the efficacy of immunotherapies. Reversibly silencing the biological activity of adoptively transferred cells using compositions of the present invention allows maximizing the potential of cell therapy without irretrievably killing and terminating the therapy.

[0083] The present invention provides methods for fine tuning of immunotherapy after administration to patients. This in turn improves the safety and efficacy of immunotherapy and increases the subject population that may benefit from immunotherapy.

II. COMPOSITIONS OF THE INVENTION

[0084] According to the present invention, biocircuit systems are provided which comprise, at their core, at least one effector module system. Such effector module systems comprise at least one effector module having associated, or integral therewith, one or more stimulus response elements (SREs). The overall architecture of a biocircuit system of the invention is illustrated in Figure 1. In general, a stimulus response element (SRE) may be operably linked to a payload construct which could be any protein of interest (POI) (e.g., an immunotherapeutic agent), to form an effector module. The SRE, when activated by a particular stimulus, e.g., a small molecule, can produce a signal or outcome, to regulate transcription and/or protein levels of the linked payload either up or down by perpetuating a stabilizing signal or destabilizing signal, or any other types of regulation. A much-detailed description of a biocircuit system are taught in co-owned U.S. Provisional Patent Application No. 62/320,864 filed April 11, 2016, 62/466,596

filed March 3, 2017 and the International Publication WO2017/180587 (the contents each of which are herein incorporated by reference in their entirety). In accordance with the present invention, biocircuit systems, effector modules, SREs and components that tune expression levels and activities of any agents used for immunotherapy are provided.

[0085] As used herein, a "biocircuit" or "biocircuit system" is defined as a circuit within or useful in biologic systems comprising a stimulus and at least one effector module responsive to a stimulus, where the response to the stimulus produces at least one signal or outcome within, between, as an indicator of, or on a biologic system. Biologic systems are generally understood to be any cell, tissue, organ, organ system or organism, whether animal, plant, fungi, bacterial, or viral. It is also understood that biocircuits may be artificial circuits which employ the stimuli or effector modules taught by the present invention and effect signals or outcomes in acellular environments such as with diagnostic, reporter systems, devices, assays or kits. The artificial circuits may be associated with one or more electronic, magnetic, or radioactive components or parts.

[0086] In accordance with the present invention, a biocircuit system may be a destabilizing domain (DD) biocircuit system, a dimerization biocircuit system, a receptor biocircuit system, and a cell biocircuit system. Any of these systems may act as a signal to any other of these biocircuit systems.

Effector modules and SREs for immunotherapy

[0087] In accordance with the present invention, biocircuit systems, effector modules, SREs, and components that tune expression levels and activities of any agents used for immunotherapy are provided. As non-limiting examples, an immunotherapeutic agent may be an antibody and fragments and variants thereof, a cancer specific T cell receptor (TCR) and variants thereof, an anti-tumor specific chimeric antigen receptor (CAR), a chimeric switch receptor, an inhibitor of a co-inhibitory receptor or ligand, an agonist of a co-stimulatory receptor and ligand, a cytokine, chemokine, a cytokine receptor, a chemokine receptor, a soluble growth factor, a metabolic factor, a suicide gene, a homing receptor, or any agent that induces an immune response in a cell and a subject.

[0088] As stated, the biocircuits of the invention include at least one effector module as a component of an effector module system. As used herein, an "effector module" is a single or multi-component construct or complex comprising at least (a) one or more stimulus response elements (i.e. proteins of interest (POIs). As used herein a "stimulus response element (SRE)" is a component of an effector module which is joined, attached, linked to or associated with one or more payloads of the effector module and in some instances, is responsible for the responsive

nature of the effector module to one or more stimuli. As used herein, the "responsive" nature of an SRE to a stimulus may be characterized by a covalent or non-covalent interaction, a direct or indirect association or a structural or chemical reaction to the stimulus. Further, the response of any SRE to a stimulus may be a matter of degree or kind. The response may be a partial response. The response may be a reversible response. The response may ultimately lead to a regulated signal or output. Such output signal may be of a relative nature to the stimulus, e.g., producing a modulatory effect of between 1% and 100% or a factored increase or decrease such as 2-fold, 3-fold, 4-fold, 5-fold, 10-fold or more.

[0089] In some embodiments, the present invention provides methods for modulating protein expression, function or level. In some aspects, the modulation of protein expression, function or level refers to modulation of expression, function or level by at least about 20%, such as by at least about 30%, 40%, 50%, 60%, 70%, 80%, 85%, 90%, 95% and 100%, or at least 20-30%, 20-40%, 20-50%, 20-60%, 20-70%, 20-80%, 20-90%, 20-95%, 20-100%, 30-40%, 30-50%, 30-60%, 30-70%, 30-80%, 30-90%, 30-95%, 30-100%, 40-50%, 40-60%, 40-70%, 40-80%, 40-90%, 40-95%, 40-100%, 50-60%, 50-70%, 50-80%, 50-90%, 50-95%, 50-100%, 60-70%, 60-80%, 60-90%, 60-95%, 60-100%, 70-80%, 70-90%, 70-95%, 70-100%, 80-90%, 80-95%, 80-100%, 90-95%, 90-100% or 95-100%.

[0090] In some embodiments, the present invention provides methods for modulating protein, expression, function or level by measuring the stabilization ratio and destabilization ratio. As used herein, the stabilization ratio may be defined as the ratio of expression, function or level of a protein of interest in response to the stimulus to the expression, function or level of the protein of interest in the absence of the stimulus specific to the SRE. In some aspects, the stabilization ratio is at least 1, such as by at least 1-10, 1-20, 1 -30, 1-40, 1-50, 1- 60, 1-70, 1-80, 1- 90, 1-100, 20-30, 20-40, 20-50, 20-60, 20-70, 20-80, 20-90, 20-95, 20-100, 30-40, 30-50, 30-60, 30-70, 30-80, 30-90, 30-95, 30-100, 40-50, 40-60, 40-70, 40-80, 40-90, 40-95, 40-100, 50-60, 50-70, 50-80, 50-90, 50-95, 50-100, 60-70, 60-80, 60-90, 60-95, 60-100, 70-80, 70-90, 70-95, 70-100, 80-90, 80-95, 80-100, 90-95, 90-100 or 95-100. As used herein, the destabilization ratio may be defined as the ratio of expression, function or level of a protein of interest in the absence of the stimulus specific to the effector module to the expression, function or level of the protein of interest, that is expressed constitutively and in the absence of the stimulus specific to the SRE. As used herein "constitutively" refers to the expression, function or level of a protein of interest that is not linked to an SRE, and is therefore expressed both in the presence and absence of the stimulus. In some aspects, the destabilization ratio is at least 0, such as by at least 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9, or at least, 0-0.1, 0-0.2, 0 -0.3, 0-0.4, 0-0.5, 0-0.6, 0-0.7, 0-0.8, 0-0.9,

0.1-0.2, 0.1 -0.3, 0.1-0.4, 0.1-0.5, 0.1-0.6, 0.1-0.7, 0.1-0.8, 0.1-0.9, 0.2 -0.3, 0.2-0.4, 0.2-0.5, 0.2-0.6, 0.2-0.7, 0.2-0.8, 0.2-0.9, 0.3-0.4, 0.3-0.5, 0.3-0.6, 0.3-0.7, 0.3-0.8, 0.3-0.9, 0.4-0.5, 0.4-0.6, 0.4-0.7, 0.4-0.8, 0.4-0.9, 0.5-0.6, 0.5-0.7, 0.5-0.8, 0.5-0.9, 0.6-0.7, 0.6-0.8, 0.6-0.9,0.7-0.8, 0.7-0.9 or 0.8-0.9.

[0091] The SRE of the effector module may be selected from, but is not limited to, a peptide, peptide complex, peptide-protein complex, protein, fusion protein, protein complex, protein-protein complex. The SRE may comprise one or more regions derived from any natural or mutated protein, or antibody. In this aspect, the SRE is an element, when responding to a stimulus, can tune intracellular localization, intramolecular activation, and/or degradation of payloads.

[0092] In some embodiments, effector modules of the present invention may comprise additional features that facilitate the expression and regulation of the effector module, such as one or more signal sequences (SSs), one or more cleavage and/or processing sites, one or more targeting and/or penetrating peptides, one or more tags, and/or one or more linkers. Additionally, effector modules of the present invention may further comprise other regulatory moieties such as inducible promoters, enhancer sequences, microRNA sites, and/or microRNA targeting sites. Each aspect or tuned modality may bring to the effector module or biocircuit a differentially tuned feature. For example, an SRE may represent a destabilizing domain, while mutations in the protein payload may alter its cleavage sites or dimerization properties or half-life and the inclusion of one or more microRNA or microRNA binding site may impart cellular detargeting or trafficking features. Consequently, the present invention embraces biocircuits which are multifactorial in their tenability. Such biocircuits may be engineered to contain one, two, three, four or more tuned features.

[0093] In some embodiments, effector modules of the present invention may include one or more degrons to tune expression. As used herein, a "degron" refers to a minimal sequence within a protein that is sufficient for the recognition and the degradation by the proteolytic system. An important property of degrons is that they are transferrable, that is, appending a degron to a sequence confers degradation upon the sequence. In some embodiments, the degron may be appended to the destabilizing domains, the payload or both. Incorporation of the degron within the effector module of the invention, confers additional protein instability to the effector module and may be used to minimize basal expression. In some embodiments, the degron may be an N-degron, a phospho degron, a heat inducible degron, a photosensitive degron, an oxygen dependent degron. As a non-limiting example, the degron may be an Ornithine decarboxylase degron as described by Takeuchi et al. (Takeuchi J et al. (2008). Biochem J. 2008 Mar

1;410(2):401-7; the contents of which are incorporated by reference in their entirety). Other examples of degrons useful in the present invention include degrons described in International patent publication Nos. WO2017004022, WO2016210343, and WO2011062962; the contents of each of which are incorporated by reference in their entirety.

[0094] As shown in Figure 2, representative effector module embodiments comprising one payload, i.e. one immunotherapeutic agent are illustrated. Each components of the effector module may be located or positioned in various arrangements without (A to F) or with (G to Z, and AA to DD) a cleavage site. An optional linker may be inserted between each component of the effector module.

[0095] Figures 3 to 6 illustrate representative effector module embodiments comprising two payloads, i.e. two immunotherapeutic agents. In some aspects, more than two immunotherapeutic agents (payloads) may be included in the effector module under the regulation of the same SRE (e.g., the same DD). The two or more agents may be either directly linked to each other or separated (Figure 3). The SRE may be positioned at the N-terminus of the construct, or the C-terminus of the construct, or in the internal location.

[0096] In some aspects, the two or more immunotherapeutic agents may be the same type such as two antibodies, or different types such as a CAR construct and a cytokine IL12. Biocircuits and components utilizing such effector molecules are given in Figures 7-12.

[0097] In some embodiments, biocircuits of the invention may be modified to reduce their immunogenicity. Immunogenicity is the result of a complex series of responses to a substance that is perceived as foreign and may include the production of neutralizing and non-neutralizing antibodies, formation of immune complexes, complement activation, mast cell activation, inflammation, hypersensitivity responses, and anaphylaxis. Several factors can contribute to protein immunogenicity, including, but not limited to protein sequence, route and frequency of administration and patient population. In a preferred embodiment, compositions of the invention may be engineered to reduce the immunogenicity of the compositions of the invention. In some embodiments, modifications to reduce immunogenicity may include modifications that reduce binding of the processed peptides derived from the parent sequence to major histocompatibility complex (MHC) proteins. For example, amino acid modifications may be engineered such that the minimum number of immune epitopes are available to bind with high affinity to any prevalent MHC alleles. Several methods of identifying MHC binding epitopes of known protein sequences are known in the art and may be useful in the present invention. Such methods are disclosed in US Patent Publication No. US 20020119492, US20040230380, and US 20060148009; the contents of each of which are incorporated by reference in their entirety.

[0098] Epitope identification and subsequent sequence modification may be applied to reduce immunogenicity. The identification of immunogenic epitopes may be achieved using physical or computational methods. Physical methods of epitope identification may include, for example, mass spectrometry and tissue culture/cellular techniques. Computational approaches use antigen processing, loading and display information, structural and/or proteomic data to identify non-self-peptides produced by antigen processing with good MHC groove binding characteristics. One or more mutations may be introduced into the biocircuits of the invention directing the expression of the protein, to maintain its functionality while simultaneously rendering the identified epitope less or non-immunogenic.

[0099] Protein modifications may also be employed to interfere with antigen processing and peptide loading e.g. glycosylation and PEGylation. Compositions of the invention may also be engineered to include non-classical amino acid side chains to design less immunogenic compositions. Any of the methods discussed in International Patent Publication No. WO2005051975 for reducing immunogenicity may be useful in the present invention (the contents of which are incorporated by reference in their entirety).

[00100] In one embodiment, patients may also be stratified according to the immunogenic peptides presented by their immune cells and may be utilized as a parameter to determine suitable patient cohorts that may therapeutically benefit for the compositions of the invention.

[00101] In some embodiments, reduced immunogenicity may be achieved by limiting immunoproteasome processing. The proteasome is an important cellular protease that is found in two forms: the constitutive proteasome, which is expressed in all cell types and which contains active e.g. catalytic subunits and the immunoproteasome that is expressed in cell of the hematopoietic lineage, and which contains different active subunits termed low molecular weight proteins (LMP) namely LMP-2, LMP- 7 and LMP-10. Immunoproteasomes exhibit altered peptidase activities and cleavage site preferences that result in more efficient liberation of many MHC class I epitopes. A well described function of the immunoproteasome is to generate peptides with hydrophobic C terminus that can be processed to fit in the groove of MHC class I molecules. Deol P et al. have shown that immunoproteasomes may lead to a frequent cleavage of specific peptide bonds and thereby to a faster appearance of a certain peptide on the surface of the antigen presenting cells; and enhanced peptide quantities (Deol P et al. (2007) *J Immunol* 178 (12) 7557-7562; the contents of which are incorporated herein reference in its entirety). This study indicates that reduced immunoproteasome processing may be accompanied by reduced immunogenicity. In some embodiments, immunogenicity of the compositions of the invention may be reduced by modifying the sequence encoding the compositions of the invention to

prevent immunoproteasome processing. Biocircuits of the present invention may also be combined with immunoproteasome-selective inhibitors to achieve the same effects. Examples of inhibitors useful in the present invention include UK-101 (B1i selective compound), IPSI-001, ONX 0914 (PR-957), and PR-924 (IPSI).

[00102] Another embodiment of the invention provides a method of detecting the presence of cancer in a mammal, comprising: (a) contacting a sample comprising one or more cells from the mammal with any of the CARs, nucleic acids, recombinant expression vectors, host cells, population of cells, or pharmaceutical compositions of the invention, thereby forming a complex, (b) and detecting the complex, wherein detection of the complex is indicative of the presence of cancer in the mammal. The sample may be obtained by any suitable method, e.g., biopsy or necropsy. A biopsy is the removal of tissue and/or cells from an individual. Such removal may be to collect tissue and/or cells from the individual in order to perform experimentation on the removed tissue and/or cells. This experimentation may also be used to determine if the individual has and/or is suffering from cancer.

[00103] With respect to an embodiment of the inventive method of detecting the presence of cancer in a mammal, the sample comprising cells of the mammal can be a sample comprising whole cells, lysates thereof, or a fraction of the whole cell lysates, e.g., a nuclear or cytoplasmic fraction, a whole protein traction, or a nucleic acid fraction. If the sample comprises whole cells, the cells can be any cells of the mammal, e.g., the cells of any organ or tissue, including tumor cells. The contacting can take place in vitro or in vivo with respect, to the mammal. Preferably, the contacting is in vitro. Also, detection of the complex can occur through any number of ways known in the art. For instance, the inventive CARs, nucleic acids, recombinant expression vectors, host cells, populations of cells, or pharmaceutical compositions described herein can be labeled with a detectable label such as, for instance, a radioisotope, a fluorophore (e.g., fluorescein isothiocyanate (FITC), phycoerythrin (PE)), an enzyme (e.g., alkaline phosphatase, horseradish peroxidase), and element particles (e.g., gold particles). Methods of testing the compositions of the invention for the ability to recognize target cells and for antigen specificity are known in the art. For instance, Clay et al, J. Immunol, 163: 507-51 3 (1999), teaches methods of measuring the release of cytokines (e.g., interferon-γ, granulocyte/monocyte colony stimulating factor (GM-CSF), tumor necrosis factor a (TNF-a) or interleukin 2 (IL2)). In addition, anti-CD276 material function can be evaluated by measurement of cellular cytotoxicity, as described in Zhao et al., J. Immunol, 1 74: 44) 5-4423 (2005).

[00104] In some embodiments, the stimulus of the present invention maybe ultrasound stimulation. In some embodiments, the SREs of the present invention may derived from

mechanosensitive proteins. In one embodiment, the SRE of the present invention may be the mechanically sensitive ion channel, Piezo1.

[00105] Expression of the payload of interest in such instances is tuned by providing focused ultrasound stimulation. In other embodiments, the SREs of the present invention may be derived from calcium biosensors, and the stimulus of the present invention may calcium. The calcium may be generated by the ultrasound induced mechanical stimulation of mechanosensitive ion channels. The ultrasound activation of the ion channel causes a calcium influx thereby generating the stimulus. In one embodiment, the mechanosensitive ion channel is Piezo 1. Mechanosensors may be advantageous to use since they provide spatial control to a specific location in the body.

1. Destabilizing domains (DDs)

[00106] In some embodiments, biocircuit systems, effector modules, and compositions of the present invention relate to post-translational regulation of protein (payload) function anti-tumor immune responses of immunotherapeutic agents. In one embodiment, the SRE is a stabilizing/destabilizing domain (DD). The presence, absence or an amount of a small molecule ligand that binds to or interacts with the DD, can, upon such binding or interaction modulate the stability of the payload(s) and consequently the function of the payload. Depending on the degree of binding and/or interaction the altered function of the payload may vary, hence providing a "tuning" of the payload function.

[00107] In some embodiments, destabilizing domains described herein or known in the art may be used as SREs in the biocircuit systems of the present invention in association with any of the immunotherapeutic agents (payloads) taught herein. Destabilizing domains (DDs) are small protein domains that can be appended to a target protein of interest. DDs render the attached protein of interest unstable in the absence of a DD-binding ligand such that the protein is rapidly degraded by the ubiquitin-proteasome system of the cell (Stankunas, K., et al., Mol. Cell, 2003, 12: 1615–1624; Banaszynski, *et al.*, Cell; 2006, 126(5): 995–1004; reviewed in Banaszynski, L.A., and Wandless, T.J. *Chem. Biol.*; 2006, 13:11–21 and Rakhit R et al., *Chem Biol.* 2014; 21(9):1238-1252). However, when a specific small molecule ligand binds its intended DD as a ligand binding partner, the instability is reversed and protein function is restored. The conditional nature of DD stability allows a rapid and non-perturbing switch from stable protein to unstable substrate for degradation. Moreover, its dependency on the concentration of its ligand further provides tunable control of degradation rates.

[00108] In some embodiments, the desired characteristics of the DDs may include, but are not limited to, low protein levels in the absence of a ligand of the DD (i.e. low basal stability), large

dynamic range, robust and predictable dose-response behavior, and rapid kinetics of degradation. DDs that bind to a desired ligand but not endogenous molecules may be preferred.

[00109] Several protein domains with destabilizing properties and their paired small molecules have been identified and used to control protein expression, including FKBP/shield-1 system (Egeler et al., *J Biol. Chem.* 2011, 286(36): 32328-31336; the contents of which are incorporated herein by reference in their entirety), ecDHFR and its ligand trimethoprim (TMP); estrogen receptor domains which can be regulated by several estrogen receptor antagonists (Miyazaki et al., *J Am Chem. Soc.*, 2012, 134(9): 3942-3945; the contents of which are incorporated by reference herein in their entirety); and fluorescent destabilizing domain (FDD) derived from bilirubin-inducible fluorescent protein, UnaG and its cognate ligand bilirubin (BR) (Navarro et al., *ACS Chem Biol.*, 2016, June 6; the contents of which are incorporated herein by reference in their entirety).

[00110] Known DDs also include those described in U.S. Pat. NO. 8,173,792 and U.S. Pat. NO. 8,530,636, the contents of which are each incorporated herein by reference in their entirety.

[00111] In some embodiments, the DDs of the present invention may be derived from some known sequences that have been approved to be capable of post-translational regulation of proteins. For example, Xiong et al., have demonstrated that the non-catalytic N-terminal domain (54-residues) of ACS7 (1-aminocyclopropane-1-carboxylate synthase) in *Arabidopsis*, when fused to the β-glucuronidase (GUS) reporter, can significantly decrease the accumulation of the GUS fusion protein (Xiong et al., *J. Exp. Bot.*, 2014, 65(15): 4397-4408). Xiong et al. further demonstrated that both exogenous 1-aminocyclopropane-1-carboxylic acid (ACC) treatment and salt can rescue the levels of accumulation of the ACS N-terminal and GUS fusion protein. The ACS N-terminus mediates the regulation of ACS7 stability through the ubiquitin–26S proteasome pathway.

[00112] Another non-limiting example is the stability control region (SCR, residues 97-118) of Tropomyosin (Tm), which controls protein stability. A destabilizing mutation L110A, and a stabilizing mutation A109L dramatically affect Tropomyosin protein dynamics (Kirwan and Hodges, *J. Biol. Chem.*, 2014, 289: 4356-4366). Such sequences can be screened for ligands that bind them and regulate their stability. The identified sequence and ligand pairs may be used as components of the present invention.

[00113] In some embodiments, the DDs of the present invention may be developed from known proteins. Regions or portions or domains of wild type proteins may be utilized as SREs/DDs in whole or in part. They may be combined or rearranged to create new peptides, proteins, regions

or domains of which any may be used as SREs/DDs or the starting point for the design of further SREs and/or DDs.

[00114] Ligands such as small molecules that are well known to bind candidate proteins can be tested for their regulation in protein responses. The small molecules may be clinically approved to be safe and have appropriate pharmaceutical kinetics and distribution. In some embodiments, the stimulus is a ligand of a destabilizing domain (DD), for example, a small molecule that binds a destabilizing domain and stabilizes the POI fused to the destabilizing domain. In some embodiments, ligands, DDs and SREs of the present invention, include without limitation, any of those taught in Tables 2-4 of copending commonly owned U.S. Provisional Application NOs. 62/320,864, filed on 4/11/2016, or in US Provisional Application No. 62/466,596 filed March 3, 2017 and the International Publication WO2017/180587, the contents of each which are incorporated herein by reference in their entirety. Some examples of the proteins that may be used to develop DDs and their ligands are listed in Table 1.

Table 1: Proteins and their binding ligands

Protein	Protein Sequence	Protein SEQ ID NOS.	Ligands
E. coli Dihydrofolate reductase (ecDHFR) (Uniprot ID: P0ABQ4)	MISLIAALAVDRVIGMENAMPWNLPADLAWFKRNT LNKPVIMGRHTWESIGRPLPGRKNIILSSQPGTDDRVT WVKSVDEAIAACGDVPEIMVIGGGRVYEQFLPKAQK LYLTHIDAEVEGDTHFPDYEPDDWESVFSEFHDADA QNSHSYCFEILERR	1	Methotrexate (MTX) Trimethoprim (TMP)
Human Dihydrofolate reductase (hDHFR) (Uniprot ID: P00374)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQ RMTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRIN LVLSRELKEPPQGAHFLSRSLDDALKLTEQPELANKV DMVWIVGGSSVYKEAMNHPGHLKLFVTRIMQDFES DTFFPEIDLEKYKLLPEYPGVLSDVQEEKGIKYKFEV YEKND	2	Methotrexate (MTX) Trimethoprim (TMP)
FK506 binding protein (FKBP) (Uniprot ID: P62942)	GVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKF DSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQR AKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLE	3	Shield-1
Phosphodiesterase 5 (PDE5), ligand binding domain (Uniprot ID: Uniprot ID O76074)	MEETRELQSLAAAVVPSAQTLKITDFSFSDFELSDLET ALCTIRMFTDLNLVQNFQMKHEVLCRWILSVKKNYR KNVAYHNWRHAFNTAQCMFAALKAGKIQNKLTDL EILALLIAALSHDLDHRGVNNSYIQRSEHPLAQLYCH SIMEHHHFDQCLMILNSPGNQILSGLSIEEYKTTLKIIK QAILATDLALYIKRRGEFFELIRKNQFNLEDPHQKELF LAMLMTACDLSAITKPWPIQQRIAELVATEFFDQGDR ERKELNIEPTDLMNREKKNKIPSMQVGFIDAICLQLY EALTHVSEDCFPLLDGCRKNRQKWQALAEQQ	4	Sildenafil; Vardenafil; Tadalafil
PPAR gamma (PPARg), ligand binding domain (Uniprot ID: P37231; amino acids 317-505)	SVEAVQEITEYAKSIPGFVNLDLNDQVTLLKYGVHEII YTMLASLMNKDGVLISEGQGFMTREFLKSLRKPFGD FMEPKFEFAVKFNALELDDSDLAIFIAVIILSGDRPGL LNVKPIEDIQDNLLQALELQLKLNHPESSQLFAKLLQ KMTDLRQIVTEHVQLLQVIKKTETDMSLHPLLQEIYK DLY	5	Posiglitazone Pioglitazone

Carbonic anhydrase II (CA2) (Uniprot ID: P00918)	MSHHWGYGKHNGPEHWHKDFPIAKGERQSPVDIDT HTAKYDPSLKPLSVSYDQATSLRILNNGHAFNVEFD DSQDKAVLKGGPLDGTYRLIQFHFHWGSLDGQGSEH TVDKKKYAAELHLVHWNTKYGDFGKAVQQPDGLA VLGIFLKVGSAKPGLQKVVDVLDSIKTKGKSADFTNF DPRGLLPESLDYWTYPGSLTTPPLLECVTWIVLKEPIS VSSEQVLKFRKLNFNGEGEPEELMVDNWRPAQPLKN RQIKASFK	6	Celecoxib Acetazolamide
NRH: Quinone oxidoreductase 2 (NQO2) (Uniprot ID: P16083)	MAGKKVLIVYAHQEPKSFNGSLKNVAVDELSRQGC TVTVSDLYAMNLEPRATDKDITGTLSNPEVFNYGVE THEAYKQRSLASDITDEQKKVREADLVIFQFPLYWFS VPAILKGWMDRVLCQGFAFDIPGFYDSGLLQGKLAL LSVTTGGTAEMYTKTGVNGDSRYFLWPLQHGTLHF CGFKVLAPQISFAPEIASEEERKGMVAAWSQRLQTIW KEEPIPCTAHWHFGQ	7	Imatinib Melatonin
Dipeptidyl peptidases (DPPIV) (Uniprot ID: P27487)	MKTPWKVLLGLLGAAALVTIITVPVVLLNKGTDDAT ADSRKTYTLTDYLKNTYRLKLYSLRWISDHEYLYKQ ENNILVFNAEYGNSSVFLENSTFDEFGHSINDYSISPD GQFILLEYNYVKQWRHSYTASYDIYDLNKRQLITEER IPNNTQWVTWSPVGHKLAYVWNNDIYVKIEPNLPSY RITWTGKEDIIYNGITDWVYEEEVFSAYSALWWSPN GTFLAYAQFNDTEVPLIEYSFYSDESLQYPKTVRVPY PKAGAVNPTVKFFVVNTDSLSSVTNATSIQITAPASM LIGDHYLCDVTWATQERISLQWLRRIQNYSVMDICD YDESSGRWNCLVARQHIEMSTTGWVGRFRPSEPHFT LDGNSFYKIISNEEGYRHICYFQIDKKDCTFITKGTWE VIGIEALTSDYLYYISNEYKGMPGGRNLYKIQLSDYT KVTCLSCELNPERCQYYSVSFSKEAKYYQLRCSGPG LPLYTLHSSVNDKGLRVLEDNSALDKMLQNVQMPS KKLDFIILNETKFWYQMILPPHFDKSKKYPLLLDVYA GPCSQKADTVFRLNWATYLASTENIIVASFDGRGSG YQGDKIMHAINRRLGTFEVEDQIEAARQFSKMGFVD NKRIAIWGWSYGGYVTSMVLGSGSGVFKCGIAVAPV SRWEYYDSVYTERYMGLPTPEDNLDHYRNSTVMSR AENFKQVEYLLIHGTADDNVHFQQSAQISKALVDVG VDFQAMWYTDEDHGIASSTAHQHIYTHMSHFIKQCF SLP	224	Sitagliptin, Saxagliptin, Denagliptin

[00115] In some embodiments, DDs of the invention may be FKBP DD or ecDHFR DDs such as those listed in Table 2. The position of the mutated amino acid listed in Table 2 is relative to the ecDHFR (Uniprot ID: P0ABQ4) of SEQ ID NO. 1 for ecDHFR DDs and relative to FKBP (Uniprot ID: P62942) of SEQ ID NO. 3 for FKBP DDs.

Table 2: ecDHFR DDs and FKBP DDs

DD	Sequence	SEQ ID NO.
ecDHFR (R12Y, Y100I)	MISLIAALAVDYVIGMENAMPWNLPADLAWFKRNTLNKP VIMGRHTWESIGRPLPGRKNIILSSQPGTDDRVTWVKSVDE AIAACGDVPEIMVIGGGRVIEQFLPKAQKLYLTHIDAEVEG DTHFPDYEPDDWESVFSEFHDADAQNSHSYCFEILERR	8
ecDHFR (Amino acid 2-159 of WT) (R12Y, Y100I)	ISLIAALAVDYVIGMENAMPWNLPADLAWFKRNTLNKPVI MGRHTWESIGRPLPGRKNIILSSQPGTDDRVTWVKSVDEAI AACGDVPEIMVIGGGRVIEQFLPKAQKLYLTHIDAEVEGDT HFPDYEPDDWESVFSEFHDADAQNSHSYCFEILERR	9

ecDHFR (Amino acid 2-159 of WT) (R12H, E129K)	ISLIAALAVDHVIGMENAMPWNLPADLAWFKRNTLNKPVI MGRHTWESIGRPLPGRKNIILSSQPGTDDRVTWVKSVDEAI AACGDVPEIMVIGGGRVYEQFLPKAQKLYLTHIDAEVEGD THFPDYKPDDWESVFSEFHDADAQNSHSYCFEILERR	10
FKBP (F36V, L106P)	GVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKVDSSR DRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAKLTISPD YAYGATGHPGIIPPHATLVFDVELLKPE	11
FKBP (E31G, F36V, R71G, K105E)	GVQVETISPGDGRTFPKRGQTCVVHYTGMLGDGKKVDSSR DRNKPFKFMLGKQEVIRGWEEGVAQMSVGQGAKLTISPD YAYGATGHPGIIPPHATLVFDVELLELE	12

[00116] Inventors of the present invention have tested and identified several candidate human proteins that may be used to develop destabilizing domains. As show in Table 2, these candidates include human DHFR (hDHFR), PDE5 (phosphodiesterase 5), PPAR gamma (peroxisome proliferator-activated receptor gamma), CA2 (Carbonic anhydrase II) and NQO2 (NRH: Quinone oxidoreductase 2). Candidate destabilizing domain sequence identified from protein domains of these proteins (as a template) may be mutated to generate libraries of mutants based on the template candidate domain sequence. Mutagenesis strategies used to generate DD libraries may include site-directed mutagenesis e.g. by using structure guided information; or random mutagenesis e.g. using error-prone PCR, or a combination of both. In some embodiments, destabilizing domains identified using random mutagenesis may be used to identify structural properties of the candidate DDs that may be required for destabilization, which may then be used to further generate libraries of mutations using site directed mutagenesis.

[00117] In some embodiments, novel DDs derived from E.coli DHFR (ecDHFR) may comprise amino acids 2-159 of the wild type ecDHFR sequence. This may be referred to as an M1del mutation.

[00118] In some embodiments, novel DDs derived from ecDHFR may comprise amino acids 2-159 of the wild type ecDHFR sequence (also referred to as an M1del mutation), and may include one, two, three, four, five or more mutations including, but not limited to, M1del, R12Y, R12H, Y100I, and E129K.

[00119] In some embodiments, novel DDs derived from FKBP may comprise amino acids 2-107 of the wild type FKBP sequence. This may be referred to as an M1del mutation.

[00120] In some embodiments, novel DDs derived from FKBP may comprise amino acids 2-107 of the wild type FBKP sequence (also referred to as an M1del mutation), and may include one, two, three, four, five or more mutations including, but not limited to, M1del, E31G, F36V, R71G, K105E, and L106P.

[00121] In some embodiments, DD mutant libraries may be screened for mutations with altered, preferably higher binding affinity to the ligand, as compared to the wild type protein. DD

libraries may also be screened using two or more ligands and DD mutations that are stabilized by some ligands but not others may be preferentially selected. DD mutations that bind preferentially to the ligand compared to a naturally occurring protein may also be selected. Such methods may be used to optimize ligand selection and ligand binding affinity of the DD. Additionally, such approaches can be used to minimize deleterious effects caused by off-target ligand binding.

[00122] In some embodiments, suitable DDs may be identified by screening mutant libraries using barcodes. Such methods may be used to detect, identify and quantify individual mutant clones within the heterogeneous mutant library. Each DD mutant within the library may have distinct barcode sequences (with respect to each other). In other instances, the polynucleotides can also have different barcode sequences with respect to 2,3,4,5,6,7,8,9,10 or more nucleic acid bases. Each DD mutant within the library may also comprise a plurality of barcode sequences. When used in plurality may be used such that each barcode is unique to any other barcode. Alternatively, each barcode used may not be unique, but the combination of barcodes used may create a unique sequence that can be individually tracked. The barcode sequence may be placed upstream of the SRE, downstream of the SRE, or in some instances may be placed within the SRE. DD mutants may be identified by barcodes using sequencing approaches such as Sanger sequencing, and next generation sequencing, but also by polymerase chain reaction and quantitative polymerase chain reaction. In some embodiments, polymerase chain reaction primers that amplify a different size product for each barcode may be used to identify each barcode on an agarose gel. In other instances, each barcode may have a unique quantitative polymerase chain reaction probe sequence that enables targeted amplification of each barcode.

[00123] In some embodiments, DDs of the invention may be derived from human dihydrofolate reductase (hDHFR). hDHFR is a small (18 kDa) enzyme that catalyzes the reduction of dihydrofolate and plays a vital role in variety of anabolic pathway. Dihydrofolate reductase (DHFR) is an essential enzyme that converts 7,8-dihydrofolate (DHF) to 5,6,7,8, tetrahydrofolate (THF) in the presence of nicotinamide adenine dihydrogen phosphate (NADPH). Anti-folate drugs such as methotrexate (MTX), a structural analogue of folic acid, which bind to DHFR more strongly than the natural substrate DHF, interferes with folate metabolism, mainly by inhibition of dihydrofolate reductase, resulting in the suppression of purine and pyrimidine precursor synthesis. Other inhibitors of hDHFR such as folate, TQD, Trimethoprim (TMP), epigallocatechin gallate (EGCG) and ECG (epicatechin gallate) can also bind to hDHFR mutants and regulates its stability.

[00124] In one aspect of the invention, the DHFR DDs of the invention may include mutations such as, but not limited to V2A, C7R, I8V, V9A, A10T, A10V, Q13R, N14S, G16S, I17N, I17V,

K19E, N20D, G21T, G21E, D22S, L23S, P24S, L28P, N30D, N30H, N30S, E31G, E31D, F32M, R33G, R33S, F35L, Q36R, Q36S, Q36K, Q36F, R37G, M38V, M38T, T40A, V44A, K47R, N49S, N49D, M53T, G54R, K56E, K56R, T57A, F59S, I61T, K64R, N65A, N65S, N65D, N65F, L68S, K69E, K69R, R71G, I72T, I72A, I72V, N73G, L74N, V75F, R78G, L80P, K81R, E82G, H88Y, F89L, R92G, S93G, S93R, L94A, D96G, A97T, L98S, K99G, K99R, L100P, E102G, Q103R, P104S, E105G, A107T, A107V, N108D, K109E, K109R, V110A, D111N, M112T, M112V, V113A, W114R, I115V, V116I , G117D, V121A, Y122C, Y122D, Y122I, K123R, K123E, A125F, M126I, N127R, N127S, N127Y, H128R, H128Y, H131R, L132P, K133E, L134P, F135P, F135L, F135S, F135V, V136M, T137R, R138G, R138I, I139T, I139V, M140I, M140V, Q141R, D142G, F143S, F143L, E144G, D146G, T147A, F148S, F148L, F149L, P150L, E151G, I152V, D153A, D153G, E155G, K156R, Y157R, Y157C, K158E, K158R, L159P, L160P, E162G, Y163C, V166A, S168C, D169G, V170A, Q171R, E172G, E173G, E173A, K174R, I176A, I176F, I176T, K177E, K177R, Y178C, Y178H, F180L, E181G, V182A, Y183C, Y183H, E184R, E184G, K185R, K185del, K185E, N186S, N186D, D187G, and D187N.

[00125] In one embodiment, the stimulus is a small molecule that binds to a SRE in order to post-translationally regulate protein levels. In one aspect, DHFR ligands: trimethoprim (TMP) and methotrexate (MTX) are used to stabilize hDHFR mutants. The hDHFR based destabilizing domains are listed in Table 3. The position of the mutated amino acid listed in Table 3 is relative to the human DHFR (Uniprot ID: P00374) of SEQ ID NO. 2 for human DHFR. In Table 3, the mutations are underlined and in bold. In Table 3, "del" means that the mutation is the deletion of the amino acid at that position relative to the wild type sequence.

Table 3: Human DHFR mutants and novel destabilizing domains

Mutants	Amino acid Sequence	SEQ ID NO
hDHFR (I17V)	MVGSLNCIVAVSQNMGVGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVW IVGGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFPEID LEKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	13
hDHFR (F59S)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWSSIPEKNRPLKGRINLVL SRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVW IVGGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFPEID LEKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	14
hDHFR (N65D)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKDRPLKGRINLVL SRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVW IVGGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFPEID LEKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	15

hDHFR (K81R)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELREPPQGAHFLSRSLDDALKLTEQPELANKVDMVW IVGGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFPEID LEKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	16
hDHFR (A107V)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAHFLSRSLDDALKLTEQPELVNKVDMVW IVGGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFPEID LEKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	17
hDHFR (Y122I)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVW IVGGSSVIKEAMNHPGHLKLFVTRIMQDFESDTFFPEIDL EKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	18
hDHFR (N127Y)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVW IVGGSSVYKEAMYHPGHLKLFVTRIMQDFESDTFFPEID LEKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	19
hDHFR (M140I)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVW IVGGSSVYKEAMNHPGHLKLFVTRIIQDFESDTFFPEIDL EKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	20
hDHFR (K185E)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVW IVGGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFPEID LEKYKLLPEYPGVLSDVQEEKGIKYKFEVYEEND	21
hDHFR (N186D)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVW IVGGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFPEID LEKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKDD	22
hDHFR (C7R, Y163C)	MVGSLNRIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVW IVGGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFPEID LEKYKLLPECPGVLSDVQEEKGIKYKFEVYEKND	23
hDHFR (A10V, H88Y)	MVGSLNCIVVVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAYFLSRSLDDALKLTEQPELANKVDMVW IVGGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFPEID LEKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	24
hDHFR (Q36K, Y122I)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFKR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVW IVGGSSVIKEAMNHPGHLKLFVTRIMQDFESDTFFPEIDL EKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	25
hDHFR (M53T, R138I)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVITGKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVW IVGGSSVYKEAMNHPGHLKLFVTIIMQDFESDTFFPEIDL EKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	26
hDHFR (T57A, I72A)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKAWFSIPEKNRPLKGRANLV LSRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMV WIVGGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFPEI DLEKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	27

hDHFR (E63G, I176F)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPGKNRPLKGRINLVL SRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVW IVGGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFPEID LEKYKLLPEYPGVLSDVQEEKGFKYKFEVYEKND	28
hDHFR (G21T, Y122I)	MVGSLNCIVAVSQNMGIGKNTDLPWPPLRNEFRYFQRM TTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVLS RELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWI VGGSSVIKEAMNHPGHLKLFVTRIMQDFESDTFFPEIDL EKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	29
hDHFR (L74N, Y122I)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINNVL SRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVW IVGGSSVIKEAMNHPGHLKLFVTRIMQDFESDTFFPEIDL EKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	30
hDHFR (V75F, Y122I)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLFL SRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVW IVGGSSVIKEAMNHPGHLKLFVTRIMQDFESDTFFPEIDL EKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	31
hDHFR (L94A, T147A)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAHFLSRSADDALKLTEQPELANKVDMVW IVGGSSVYKEAMNHPGHLKLFVTRIMQDFESDAFFPEID LEKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	32
DHFR (V121A, Y22I)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVW IVGGSSAIKEAMNHPGHLKLFVTRIMQDFESDTFFPEIDL EKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	33
hDHFR (Y122I, A125F)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVW IVGGSSVIKEFMNHPGHLKLFVTRIMQDFESDTFFPEIDL EKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	34
hDHFR (H131R, E144G)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVW IVGGSSVYKEAMNHPGRLKLFVTRIMQDFGSDTFFPEID LEKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	35
hDHFR (T137R, F143L)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVW IVGGSSVYKEAMNHPGHLKLFVRRIMQDLESDTFFPEID LEKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	36
hDHFR (Y178H, E18IG)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVW IVGGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFPEID LEKYKLLPEYPGVLSDVQEEKGIKHKFGVYEKND	37
hDHFR (Y183H, K185E)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVW IVGGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFPEID LEKYKLLPEYPGVLSDVQEEKGIKYKFEVHEEND	38
hDHFR (V9A, S93R, P150L)	MVGSLNCIAAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAHFLSRRLDDALKLTEQPELANKVDMVW IVGGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFLEID LEKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	39

hDHFR (I8V, K133E, Y163C)	MVGSLNCVVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVW IVGGSSVYKEAMNHPGHLELFVTRIMQDFESDTFFPEID LEKYKLLPECPGVLSDVQEEKGIKYKFEVYEKND	40
hDHFR (L23S, V121A, Y157C)	MVGSLNCIVAVSQNMGIGKNGDSPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVW IVGGSSAYKEAMNHPGHLKLFVTRIMQDFESDTFFPEID LEKCKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	41
hDHFR (K19E, F89L, E181G)	MVGSLNCIVAVSQNMGIGENGDLPWPPLRNEFRYFQRM TTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVLS RELKEPPQGAHLLSRSLDDALKLTEQPELANKVDMVWI VGGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFPEIDL EKYKLLPEYPGVLSDVQEEKGIKYKFGVYEKND	42
hDHFR (Q36F, N65F, Y122I)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFFRM TTTSSVEGKQNLVIMGKKTWFSIPEKFRPLKGRINLVLS RELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWI VGGSSVIKEAMNHPGHLKLFVTRIMQDFESDTFFPEIDL EKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	43
hDHFR (G54R, M140V, S168C)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMRKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVW IVGGSSVYKEAMNHPGHLKLFVTRIVQDFESDTFFPEIDL EKYKLLPEYPGVLCDVQEEKGIKYKFEVYEKND	44
hDHFR (V110A, V136M, K177R)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAHFLSRSLDDALKLTEQPELANKADMVW IVGGSSVYKEAMNHPGHLKLFMTRIMQDFESDTFFPEID LEKYKLLPEYPGVLSDVQEEKGIRYKFEVYEKND	45
hDHFR (Amino acid 2-187 of WT; Q36F, Y122I, A125F)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFFRMT TTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVLSR ELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWIV GGSSVIKEFMNHPGHLKLFVTRIMQDFESDTFFPEIDLEK YKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	46
hDHFR (N49D, F59S, D153G)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQDLVIMGKKTWSSIPEKNRPLKGRINLVL SRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVW IVGGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFPEIG LEKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	47
hDHFR (G21E, I72V, I176T)	MVGSLNCIVAVSQNMGIGKNEDLPWPPLRNEFRYFQRM TTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRVNLVLS RELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWI VGGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFPEIDL EKYKLLPEYPGVLSDVQEEKGTKYKFEVYEKND	48
hDHFR (L100P, E102G, Q103R, P104S, E105G, N108D, V113A, W114R, Y122C, M126I, N127R, H128Y, L132P, F135P, I139T, F148S, F149L, I152V, D153A, D169G, V170A, I176A, K177R, V182A, K185R, N186S)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAHFLSRSLDDALKPTGRSGLADKVDMAR IVGGSSVCKEAIRYPGHPKLPVTRTMQDFESDTSLPEVA LEKYKLLPEYPGVLSGAQEEKGARYKFEAYERSD	225
hDHFR (V2A, R33G, Q36R, L100P, K185R)	MAGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFGYFRR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAHFLSRSLDDALKPTEQPELANKVDMVW IVGGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFPEID LEKYKLLPEYPGVLSDVQEEKGIKYKFEVYERND	226
hDHFR (G16S, I17V, F89L, D96G, K123E, M140V, D146G, K156R)	MVGSLNCIVAVSQNMSVGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAHLLSRSLDGALKLTEQPELANKVDMVW	227

	IVGGSSVYEEAMNHPGHLKLFVTRIVQDFESGTFFPEIDL ERYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	
hDHFR (F35L, R37G, N65A, L68S, K69E, R71G, L80P, K99G, G117D, L132P, I139V, M140I, D142G, D146G, E173G, D187G)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYLQG MTTTSSVEGKQNLVIMGKKTWFSIPEKARPSEGGINLVL SREPKEPPQGAHFLSRSLDDALGLTEQPELANKVDMVW IVDGSSVYKEAMNHPGHPKLFVTRVIQGFESGTFFPEIDL EKYKLLPEYPGVLSDVQEGKGIKYKFEVYEKNG	228
hDHFR (I17N, L98S, K99R, M112T, E151G, E162G, E172G)	MVGSLNCIVAVSQNMGNGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAHFLSRSLDDASRLTEQPELANKVDTVWI VGGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFPGIDL EKYKLLPGYPGVLSDVQGEKGIKYKFEVYEKND	229
hDHFR (R138G, D142G, F143S, K156R, K158E, E162G, V166A, K177E, Y178C, K185E, N186S)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVW IVGGSSVYKEAMNHPGHLKLFVTGIMQGSESDTFFPEID LERYELLPGYPGALSDVQEEKGIECKFEVYEESD	230
hDHFR (K81R, K99R, L100P, E102G, N108D, K123R, H128R, D142G, F180L, K185E)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELREPPQGAHFLSRSLDDALRPTGQPELADKVDMVW IVGGSSVYREAMNRPGHLKLFVTRIMQGFESDTFFPEID LEKYKLLPEYPGVLSDVQEEKGIKYKLEVYEEND	231
hDHFR (N14S, P24S, F35L, M53T, K56E, R92G, S93G, N127S, H128Y, F135L, F143S, L159P, L160P, E173A, F180L)	MVGSLNCIVAVSQSMGIGKNGDLSWPPLRNEFRYLQRM TTTSSVEGKQNLVITGKETWFSIPEKNRPLKGRINLVLSR ELKEPPQGAHFLSGGLDDALKLTEQPELANKVDMVWIV GGSSVYKEAMSYPGHLKLLVTRIMQDSESDTFFPEIDLE KYKPPPEYPGVLSDVQEAKGIKYKLEVYEKND	232
hDHFR (V2A, I17V, N30D, E31G, Q36R, F59S, K69E, I72T, H88Y, F89L, N108D, K109E, V110A, I115V, Y122D, L132P, F135S, M140V, E144G, T147A, Y157C, V170A, K174R, N186S)	MAGSLNCIVAVSQNMGVGKNGDLPWPPLRDGFRYFRR MTTTSSVEGKQNLVIMGKKTWSSIPEKNRPLEGRTNLV LSRELKEPPQGAYLLSRSLDDALKLTEQPELADEAGMV WVVGGSSVDKEAMNHPGHPKLSVTRIVQDFGSDAFFPE IDLEKCKLLPEYPGVLSDAQEERGIKYKFEVYEKSD	233
hDHFR (L28P, N30H, M38V, V44A, L68S, N73G, R78G, A97T, K99R, A107T, K109R, D111N, L134P, F135V, T147A, I152V, K158R, E172G, V182A, E184R)	MVGSLNCIVAVSQNMGIGKNGDLPWPPPRHEFRYFQRV TTTSSAEGKQNLVIMGKKTWFSIPEKNRPSKGRIGLVLS GELKEPPQGAHFLSRSLDDTLRLTEQPELTNRVNMVWI VGGSSVYKEAMNHPGHLRPVVTRIMQDFESDAFFPEVD LEKYRLLPEYPGVLSDVQGEKGIKYKFEAYRKND	234
hDHFR (A10T, Q13R, N14S, N20D, P24S, N30S, M38T, T40A, K47R, N49S, K56R, I61T, K64R, K69R, I72A, R78G, E82G, F89L, D96G, N108D, M112V, W114R, Y122D, K123E, I139V, Q141R, D142G, F148L, E151G, E155G, Y157R, Q171R, Y183C, E184G, K185del, D187N)	MVGSLNCIVTVSRSMGIGKDGDLSWPPLRSEFRYFQRTT ATSSVEGRQSLVIMGKRTWFSTPERNRPLRGRANLVLS GELKGPPQGAHLLSRSLDGALKLTEQPELADKVDVVRI VGGSSVDEEAMNHPGHLKLFVTRVMRGFESDTLFPGID LGKRKLLPEYPGVLSDVREEKGIKYKLEVCGNN	235
hDHFR (Amino acid 2-187 of WT; I17V, Y122I)	VGSLNCIVAVSQNMGVGKNGDLPWPPLRNEFRYFQRM TTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVLS RELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWI VGGSSVIKEAMNHPGHLKLFVTRIMQDFESDTFFPEIDL EKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	236
hDHFR (Amino acid 2-187 of WT; Y122I, M140I)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQRMT TTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVLSR ELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWIV GGSSVIKEAMNHPGHLKLFVTRIIQDFESDTFFPEIDLEK YKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	237
hDHFR (Amino acid 2-187 of WT; N127Y, Y122I)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQRMT TTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVLSR ELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWIV	238

	GGSSVIKEAMYHPGHLKLFVTRIMQDFESDTFFPEIDLE KYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	
hDHFR (Amino acid 2-187 of WT; Y122I, H131R, E144G)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQRMT TTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVLSR ELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWIV GGSSVIKEAMNHPGRLKLFVTRIMQDFGSDTFFPEIDLE KYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	239
hDHFR (Amino acid 2-187 of WT; D22S, F32M, R33S, Q36S, N65S)	VGSLNCIVAVSQNMGIGKNGSLPWPPLRNEMSYFSRMT TTSSVEGKQNLVIMGKKTWFSIPEKSRPLKGRINLVLSR ELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWIV GGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFPEIDLE KYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	240
hDHFR (Amino acid 2-187 of WT; E31D, F32M, V116I)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNDMRYFQRM TTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVLS RELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWII GGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFPEIDLE KYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	241
hDHFR (Amino acid 2-187 of WT; E162G, I176F)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQRMT TTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVLSR ELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWIV GGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFPEIDLE KYKLLPGYPGVLSDVQEEKGFKYKFEVYEKND	854
hDHFR (Amino acid 2-187 of WT; K185E)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQRMT TTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVLSR ELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWIV GGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFPEIDLE KYKLLPEYPGVLSDVQEEKGIKYKFEVYEEND	855
hDHFR (Amino acid 2-187 of WT; Y122I, A125F)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQRMT TTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVLSR ELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWIV GGSSVIKEFMNHPGHLKLFVTRIMQDFESDTFFPEIDLEK YKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	856
hDHFR (Amino acid 2-187 of WT; Q36F, N65F, Y122I)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFFRMT TTSSVEGKQNLVIMGKKTWFSIPEKFRPLKGRINLVLSR ELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWIV GGSSVIKEAMNHPGHLKLFVTRIMQDFESDTFFPEIDLE KYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	857
hDHFR (Amino acid 2-187 of WT; N127Y)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQRMT TTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVLSR ELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWIV GGSSVYKEAMYHPGHLKLFVTRIMQDFESDTFFPEIDLE KYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	858
hDHFR (Amino acid 2-187 of WT; H131R, E144G)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQRMT TTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVLSR ELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWIV GGSSVYKEAMNHPGRLKLFVTRIMQDFGSDTFFPEIDLE KYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	859
hDHFR (Amino acid 2-187 of WT; I17V)	VGSLNCIVAVSQNMGVGKNGDLPWPPLRNEFRYFQRM TTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVLS RELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWI VGGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFPEIDL EKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	860
hDHFR (Amino acid 2-187 of WT; Y122I)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQRMT TTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVLSR ELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWIV GGSSVIKEAMNHPGHLKLFVTRIMQDFESDTFFPEIDLE KYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	861
hDHFR (E162G, I176F)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVW	862

	IVGGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFPEID LEKYKLLPGYPGVLSDVQEEKGFKYKFEVYEKND	
hDHFR (Amino acid 2-187 of WT; Q36K, Y122I)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFKRMT TTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVLSR ELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWIV GGSSVIKEAMNHPGHLKLFVTRIMQDFESDTFFPEIDLE KYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	863

[00126] In some embodiments, DD mutations that do not inhibit ligand binding may be preferentially selected. In some embodiments, ligand binding may be improved by mutation of residues in DHFR. Amino acid positions selected for mutation include aspartic acid at position 22 of SEQ ID NO. 2, glutamic acid at position 31 of SEQ ID NO. 2; phenyl alanine at position 32 of SEQ ID NO. 2; arginine at position 33 of SEQ ID NO. 2; glutamine at position 36 of SEQ ID NO. 2; asparagine at position 65 of SEQ ID NO. 2; and valine at position 115 of SEQ ID NO. 2. In some embodiments, one or more of the following mutations may be utilized in the DDs of the present invention to improve TMP binding, including but not limited to, D22S, E31D, F32M, R33S, Q36S, N65S, and V116I. The position of the mutated amino acids is relative to the wildtype human DHFR (Uniprot ID: P00374) of SEQ ID NO. 2.

[00127] In some embodiments, novel DDs derived from human DHFR may include one, two, three, four, five or more mutations including, but not limited to, M1del, V2A, C7R, I8V, V9A, A10T, A10V, Q13R, N14S, G16S, I17N, I17V, K19E, N20D, G21T, G21E, D22S, L23S, P24S, L28P, N30D, N30H, N30S, E31G, E31D, F32M, R33G, R33S, F35L, Q36R, Q36S, Q36K, Q36F, R37G, M38V, M38T, T40A, V44A, K47R, N49S, N49D, M53T, G54R, K56E, K56R, T57A, F59S, I61T, K64R, N65A, N65S, N65D, N65F, L68S, K69E, K69R, R71G, I72T, I72A, I72V, N73G, L74N, V75F, R78G, L80P, K81R, E82G, H88Y, F89L, R92G, S93G, S93R, L94A, D96G, A97T, L98S, K99G, K99R, L100P, E102G, Q103R, P104S, E105G, A107T, A107V, N108D, K109E, K109R, V110A, D111N, M112T, M112V, V113A, W114R, I115V, I115L, V116I, G117D, V121A, Y122C, Y122D, Y122I, K123R, K123E, A125F, M126I, N127R, N127S, N127Y, H128R, H128Y, H131R, L132P, K133E, L134P, F135P, F135L, F135S, F135V, V136M, T137R, R138G, R138I, I139T, I139V, M140I, M140V, Q141R, D142G, F143S, F143L, E144G, D146G, T147A, F148S, F148L, F149L, P150L, E151G, I152V, D153A, D153G, E155G, K156R, Y157R, Y157C, K158E, K158R, L159P, L160P, E162G, Y163C, V166A, S168C, D169G, V170A, Q171R, E172G, E173G, E173A, K174R, I176A, I176F, I176T, K177E, K177R, Y178C, Y178H, F180L, E181G, V182A, Y183C, Y183H, E184R, E184G, K185R, K185del, K185E, N186S, N186D, D187G, and D187N.

[00128] In some embodiments, novel DDs derived from human DHFR may comprise amino acids 2-187 of the wild type human DHFR sequence. This may be referred to as an M1del mutation.

[00129] In some embodiments, novel DDs derived from human DHFR may comprise amino acids 2-187 of the wild type human DHFR sequence (also referred to as an M1del mutation), and may include one, two, three, four, five or more mutations including, but not limited to, M1del, V2A, C7R, I8V, V9A, A10T, A10V, Q13R, N14S, G16S, I17N, I17V, K19E, N20D, G21T, G21E, D22S, L23S, P24S, L28P, N30D, N30H, N30S, E31G, E31D, F32M, R33G, R33S, F35L, Q36R, Q36S, Q36K, Q36F, R37G, M38V, M38T, T40A, V44A, K47R, N49S, N49D, M53T, G54R, K56E, K56R, T57A, F59S, I61T, K64R, N65A, N65S, N65D, N65F, L68S, K69E, K69R, R71G, I72T, I72A, I72V, N73G, L74N, V75F, R78G, L80P, K81R, E82G, H88Y, F89L, R92G, S93G, S93R, L94A, D96G, A97T, L98S, K99G, K99R, L100P, E102G, Q103R, P104S, E105G, A107T, A107V, N108D, K109E, K109R, V110A, D111N, M112T, M112V, V113A, W114R, I115V, I115L, V116I, G117D, V121A, Y122C, Y122D, Y122I, K123R, K123E, A125F, M126I, N127R, N127S, N127Y, H128R, H128Y, H131R, L132P, K133E, L134P, F135P, F135L, F135S, F135V, V136M, T137R, R138G, R138I, I139T, I139V, M140I, M140V, Q141R, D142G, F143S, F143L, E144G, D146G, T147A, F148S, F148L, F149L, P150L, E151G, I152V, D153A, D153G, E155G, K156R, Y157R, Y157C, K158E, K158R, L159P, L160P, E162G, Y163C, V166A, S168C, D169G, V170A, Q171R, E172G, E173G, E173A, K174R, I176A, I176F, I176T, K177E, K177R, Y178C, Y178H, F180L, E181G, V182A, Y183C, Y183H, E184R, E184G, K185R, K185del, K185E, N186S, N186D, D187G, and D187N.

2. Stimulus

[00130] Biocircuits of the invention are triggered by one or more stimuli. Stimuli may be selected from a ligand, an externally added or endogenous metabolite, the presence or absence of a defined ligand, pH, temperature, light, ionic strength, radioactivity, cellular location, subject site, microenvironment, the presence or the concentration of one or more metal ions.

[00131] In some embodiments, the stimulus is a ligand. Ligands may be nucleic acid-based, protein-based, lipid based, organic, inorganic or any combination of the foregoing. In some embodiments, the ligand is selected from the group consisting of a protein, peptide, nucleic acid, lipid, lipid derivative, sterol, steroid, metabolite derivative and a small molecule. In some embodiments, the stimulus is a small molecule. In some embodiments, the small molecules are cell permeable. Ligands useful in the present invention include without limitation, any of those taught in Table 2 of copending commonly owned US serial number 62/320,684, filed on 4/11/2016, or in US Provisional Application No. 62/466,596 filed March 3, 2017 and the International Publication WO2017/180587, the contents of each of which are incorporated herein

by reference in their entirety. In some embodiments, the small molecules are FDA-approved, safe and orally administered.

[00132] In some embodiments, the ligand binds to dihydrofolate reductase. In some embodiments, the ligand binds to and inhibits dihydrofolate reductase function and is herein referred to as a dihydrofolate inhibitor.

[00133] In some embodiments, the ligand may be a selective inhibitor of human DHFR. Ligands of the invention may also be selective inhibitors of dihydrofolate reductases of bacteria and parasitic organisms such as *Pneumocystis spp.*, *Toxoplasma spp.*, *Trypanosoma spp.*, *Mycobacterium spp.*, and *Streptococcus spp.* Ligands specific to other DHFR may be modified to improve binding to human dihydrofolate reductase.

[00134] Examples of dihydrofolate inhibitors include, but are not limited to, Trimethoprim (TMP), Methotrexate (MTX), Pralatrexate, Piritrexim Pyrimethamine, Talotrexin, Chloroguanide, Pentamidine, Trimetrexate, aminopterin, C1 898 trihydrochloride, Pemetrexed Disodium, Raltitrexed, Sulfaguanidine, Folotyn, Iclaprim and Diaveridine. Other examples of DHFR inhibitors include BAL0030543, BAL0030544 and BAL0030545, developed by Basillea Pharmaceuticals; as well as WR 99210, and P218. Any of the inhibitors described by Zhang Q et al. (2015) Int J Antimicrob Agents. 2015 Aug; 46(2): 174–182 (the contents of which are incorporated herein by reference in their entirety). Some inhibitors contain bulky benzyl groups that dramatically diminish binding to human DHFR. In some embodiments, the inhibitors may be designed without bulky benzyl groups to improve TMP binding.

[00135] In some embodiments, ligands of the present invention may be polyglutamate or non polyglutamylatable. Like naturally occurring folates, polyglutamatable folates also contain a glutamic acid residue and therefore undergo intracellular polyglutamylation. In contrast, non-polyglutamatable antifolates are devoid of a glutamate residue and thus are not available for polyglutamylation. In some embodiments, polyglutamylatable ligands may be preferred to increase intracellular retention as they can no longer be exported out of the cell. In other embodiments, non polyglutamylatable ligands may be preferred to decrease intracellular retention.

[00136] In some embodiments, ligands of the present invention may include dihydrofolic acid or any of its derivatives that may bind to human DHFR. In some embodiments, the ligands of the present invention, may be 2,4, diaminohetrocyclic compounds. In some embodiments, the 4-oxo group in dihydrofolate may be modified to generate DHFR inhibitors. In one example, the 4 -oxo group may be replaced by 4-amino group. Various diamino heterocycles, including pteridines, quinazolines, pyridopyrimidines, pyrimidines, and triazines, may also be used as scaffolds to

develop DHFR inhibitors and may be used in the present invention. The crystal structure of DHFR in complex with known DHFR inhibitors may be utilized in the rational design of better DHFR ligands. The ligands used herein include a 2,4-diaminopyrimidine ring with a propargyl group linked to an optionally substituted aryl or heteroaryl ring (as described in US Patent No. US 8,426,432; the contents of which are incorporated herein by reference in their entirety).

[00137] In some embodiments, ligands include TMP- derived ligands containing portions of the ligand known to mediate binding to DHFR. Ligands may also be modified to reduce off-target binding to other folate metabolism enzymes and increase specific binding to DHFR.

3. Payloads: Immunotherapeutic agents

[00138] In some embodiments, payloads of the present invention may be immunotherapeutic agents that induce immune responses in an organism. The immunotherapeutic agent may be, but is not limited to a cytokine, a safety switch (e.g., a suicide gene), a regulatory switch, a chimeric antigen receptor, or any agent that induces an immune response. In one embodiment, the immunotherapeutic agent induces an anti-cancer immune response in a cell, or in a subject.

[00139] In some embodiments, the payload of the invention may be any of the co-stimulatory molecules and/or intracellular domains described herein. In some embodiments, one or more co-stimulatory molecules, each under the control of different SRE may be used in the present invention. SRE regulated co- stimulatory molecules may also be expressed in conjunction with a first generation CAR, a second generation CAR, a third generation CAR, a fourth generation, or any other CAR design described herein.

Cytokines, chemokines and other soluble factors

[00140] In accordance with the present invention, payloads of the present invention may be cytokines, chemokines, growth factors, and soluble proteins produced by immune cells, cancer cells and other cell types, which act as chemical communicators between cells and tissues within the body. These proteins mediate a wide range of physiological functions, from effects on cell growth, differentiation, migration and survival, to several effector activities. For example, activated T cells produce a variety of cytokines for cytotoxic function to eliminate tumor cells.

[00141] In some embodiments, payloads of the present invention may be cytokines, and fragments, variants, analogs and derivatives thereof, including but not limited to interleukins, tumor necrosis factors (TNFs), interferons (IFNs), TGF beta and chemokines. It is understood in the art that certain gene and/or protein nomenclature for the same gene or protein may be inclusive or exclusive of punctuation such as a dash "-" or symbolic such as Greek letters. Whether these are included or excluded herein, the meaning is not meant to be changed as would be understood by one of skill in the art. For example, IL2, IL2 and IL 2 refer to the same

interleukin. Likewise, TNFalpha, TNFα, TNF-alpha, TNF-α, TNF alpha and TNF α all refer to the same protein. In some embodiments, payloads of the present invention may be cytokines that stimulate immune responses. In other embodiments, payloads of the invention may be antagonists of cytokines that negatively impact anti-cancer immune responses.

[00142] In some embodiments, payloads of the present invention may be cytokine receptors, recombinant receptors, variants, analogs and derivatives thereof; or signal components of cytokines.

[00143] In some embodiments, cytokines of the present invention may be utilized to improve expansion, survival, persistence, and potency of immune cells such as CD8+T_{EM}, natural killer cells and tumor infiltrating lymphocytes (TIL) cells used for immunotherapy. In other embodiments, T cells engineered with two or more DD regulated cytokines are utilized to provide kinetic control of T cell activation and tumor microenvironment remodeling. In one aspect, the present invention provides biocircuits and compositions to minimize toxicity related to cytokine therapy. Despite its success in mitigating tumor burden, systemic cytokine therapy often results in the development of severe dose limiting side effects. Two factors contribute to the observed toxicity (a) Pleiotropism, wherein cytokines affect different cells types and sometimes produce opposing effects on the same cells depending on the context (b) Cytokines have short serum half-life and thus need to be administered at high doses to achieve therapeutic effects, which exacerbates the pleiotropic effects. In one aspect, cytokines of the present invention may be utilized to modulate cytokine expression in the event of adverse effects. In some embodiments, cytokines of the present invention may be designed to have prolonged life span or enhanced specificity to minimize toxicity.

[00144] In some embodiments, the payload of the present invention may be an interleukin (IL) cytokine. Interleukins (ILs) are a class of glycoproteins produced by leukocytes for regulating immune responses. As used herein, the term "interleukin (IL)" refers to an interleukin polypeptide from any species or source and includes the full-length protein as well as fragments or portions of the protein. In some aspects, the interleukin payload is selected from IL1, IL1alpha (also called hematopoietin-1), IL1beta (catabolin), IL1 delta, IL1epsilon, IL1eta, IL1 zeta, interleukin-1 family member 1 to 11 (IL1F1 to IL1F11), interleukin-1 homolog 1 to 4 (IL1H1 to IL1H4), IL1 related protein 1 to 3 (IL1RP1 to IL1RP3), IL2, IL3, IL4, IL5, IL6, IL7, IL8, IL9, IL10, IL10C, IL10D, IL11, IL11a, IL11b, IL12, IL13, IL14, IL15, IL16, IL17, IL17A, Il17B, IL17C, IL17E, IL17F, IL18, IL19, IL20, IL20 like (IL20L), Il21, IL22, IL23, IL23A, IL23-p19, IL23-p40, IL24, Il25, IL26, IL27, IL28A, IL28B, IL29, IL30, IL31, IL32, IL33, IL34, IL35, IL36 alpha, IL36 beta, IL36 gamma, IL36RN, IL37, IL37a, IL37b, IL37c, IL37d, IL37e

and IL38. In other aspects, the payload of the present invention may be an interleukin receptor selected from CD121a, CDw121b, IL2Rα/CD25, IL2Rβ/CD122, IL2Rγ/CD132, CDw131, CD124, CD131, CDw125, CD126, CD130, CD127, CDw210, IL8RA, IL11Rα, CD212, CD213α1, CD213α2, IL14R, IL15Rα, CDw217, IL18Rα, IL18Rβ, IL20Rα, and IL20Rβ.

[00145] In one embodiment, the payload of the invention may comprise IL2. In one aspect, the effector module of the invention may be a DD-IL2 fusion polypeptide. The amino acid sequences corresponding to DD-IL2 and its components are listed in the Table 4. The amino acid sequences in Table 4 may comprise a stop codon which is denoted in the table with a "*" at the end of the amino acid sequence.

Table 4: DD-IL2 construct sequences

Description	Amino Acid Sequence	Amino Acid SEQ ID NO	Nucleic Acid SEQ ID NO/ Sequence
IL2 signal sequence	MYRMQLLSCIALSLALVTNS	49	55-56, 117-118
Linker	EFSTEF	50	57
Linker	MH	-	ATGCAC
IL2	APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRM LTFKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNF HLRPRDLISNINVIVLELKGSETTFMCEYADETATIVEFL NRWITFCQSIISTLT	51	58-59
FKBP (F36V, L106P)	GVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKVDS SRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAKLTI SPDYAYGATGHPGIIPPHATLVFDVELLKPE	11	60, 878-882
ecDHFR (Amino acid 2-159 of WT) (R12Y, Y100I)	ISLIAALAVDYVIGMENAMPWNLPADLAWFKRNTLNKP VIMGRHTWESIGRPLPGRKNIILSSQPGTDDRVTWVKSV DEAIAACGDVPEIMVIGGGRVIEQFLPKAQKLYLTHIDA EVEGDTHFPDYEPDDWESVFSEFHDADAQNSHSYCFEIL ERR	9	61, 869-874
OT-IL2-001 (IL2 Signal Sequence – Linker (EFSTEF)-FKBP (F36V, L106P) – Linker (MH) – IL2- stop)	MYRMQLLSCIALSLALVTNSEFSTEFGVQVETISPGDGR TFPKRGQTCVVHYTGMLEDGKKVDSSRDRNKPFKFML GKQEVIRGWEEGVAQMSVGQRAKLTISPDYAYGATGH PGIIPPHATLVFDVELLKPEMHAPTSSSTKKTQLQLEHLL LDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHL QCLEEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLEL KGSETTFMCEYADETATIVEFLNRWITFCQSIISTLT	52	62
OT-IL2-002 (IL2 Signal Sequence - IL2- stop)	MYRMQLLSCIALSLALVTNSAPTSSSTKKTQLQLEHLLL DLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQ CLEEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELK GSETTFMCEYADETATIVEFLNRWITFCQSIISTLT	53	63
OT-IL2-003 (IL2 Signal Sequence - Linker (EFSTEF)-ecDHFR (Amino acid 2-159 of WT) (R12Y, Y100I) – Linker (MH) – IL2- stop)	MYRMQLLSCIALSLALVTNSEFSTEFISLIAALAVDYVIG MENAMPWNLPADLAWFKRNTLNKPVIMGRHTWESIGR PLPGRKNIILSSQPGTDDRVTWVKSVDEAIAACGDVPEI MVIGGGRVIEQFLPKAQKLYLTHIDAEVEGDTHFPDYEP DDWESVFSEFHDADAQNSHSYCFEILERRMHAPTSSSTK KTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYM PKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDL ISNINVIVLELKGSETTFMCEYADETATIVEFLNRWITFC QSIISTLT	54	64

[00146] In some aspects of the invention, an IL2 mutein may be used as a payload. As used herein, the term "mutein" is a construct, molecule or sequence of a mutation, change or alteration in a protein and hence is also known as a mutant, e.g., a protein mutant, mutein. Consequently, an "IL2 mutein" is an IL2 mutant. In some embodiments an IL2 mutein is a variant of wild type IL2 protein, where the wildtype IL2 consists of the amino acid sequence of SEQ ID NO. 51. In some aspects, it refers to an IL2 variant which binds to and activates only cells expressing IL2Rαβγ, but does not significantly bind to or activate cell expressing only IL2Rβγ. In some examples, an IL2 mutein may be an IL2 protein in which residues of IL2 responsible for binding to either IL2Rβ or IL2Rγ are substituted to abolish the interaction of IL2 with IL2Rβ or IL2Rγ. In other examples, an IL2 mutein may be an IL2 protein comprising mutations conferring high affinity for IL2Rα. An IL2 mutein may be an IL2 selective agonist ($IL2_{SA}$) which can preferentially activate the high affinity IL2 receptor (i.e., IL2Rαβγ) which is necessary to selectively activate T cells with respect to NK cells. In some embodiments, the IL2 mutein may be IL2 protein which preferentially binds to the lower affinity IL2Rβγ but with reduced affinity to CD25.

[00147] In some embodiments, IL2 muteins may be used to preferentially expand or stimulate Treg cells. As used herein "preferentially expand or stimulate Treg cells" means the IL2 muteins promote the proliferation, survival, activation and /or function of T regulatory cells.

[00148] Exemplary IL2 muteins may include, but are not limited to, N88R substitution (Shanafelt et al., *Nature Biotech.*, 2000, 18:1197-1202), an IL2 with a V91K substitution (e.g., US Patent publication NO. US20140286898); V91K substitution, C125A substitution, an IL2 with three mutations: V69A, N71R, Q74P; an IL2 mutein with high affinity for IL2Rα (N29S, Y31H, K35R, T37A, K48E, V69A, N71R, Q74P); an IL2 mutein with high affinity for IL2Rα and reduced signaling activity (N29S, Y31H, K35R, T37A, K48E, V69A, N71R, Q74P, N88D), and D20H, D20I, N88G, N88I, N88R, and Q126L substitutions as described in PCT application NO. 1999060128; the contents of each of which are incorporated herein by reference in their entirety. In other aspects, IL2 muteins may include those described in US Patent NOs. 4,518,584; 5,116,943; 5,206,344; 6,955,807; 7,105,653; 7,371,371; 7,803,361; 8,124,066; 8,349,311; 8,759,486; and 9,206,243; PCT patent publication NOs. WO2005086751 and WO2012088446; European Patent N.s.: EP0234599 and EP0200280 and Sim, G.C. et al. (2016) Cancer Immunol Res; 4(11):983-994; the contents of each of which are incorporated herein by reference in their entirety.

[00149] In some aspects, the IL2 mutein may be fused to a polypeptide that extends the serum half-life of the IL2 mutein, such as an IgG Fc fragment. Preferred Fc regions are derived from

human IgG, which includes IgG1, IgG2, IgG3, and IgG4. In other aspects, the payload of the invention may be an IL2 fusion protein comparing a second functional polypeptide. In a non-limiting example, an IL2 fusion protein may comprise an IL2 or IL2 mutein polypeptide fused with a pro-apoptotic Bcl-2 family polypeptide (such as Bad, Bik/Nbk, Bid, Bim/Bod, Hrk, Bak or Bax); such fusion protein may be capable of inhibiting cell survival, inhibiting cell proliferation, or enhancing cell death or apoptosis of a target cell expressing an IL2 receptor. Alternatively, an IL2 or IL2 mutein polypeptide may be fused with an anti-apoptotic Bcl-2 family polypeptide (such as Bcl-$_{XL}$, Bcl-w or Bcl-2). The fusion protein may be capable of enhancing cell survival, enhancing cell proliferation, or inhibiting cell death or apoptosis of a target cell expressing an IL2 receptor. See, e.g., US patent publication NOS. US2016/0229901.

[00150] In addition, the IL2 fusion protein may be a IL2-GMCSF fusion protein which can promote cell-cell interaction; therefore, enhances anti-cancer immune responses (Wen et al., *J. Translational Med.*, 2016, 14: 41).

Safety switch

[00151] In some embodiments, payloads of the present invention may comprise SRE regulated safety switches that can eliminate adoptively transferred cells in the case of severe toxicity, thereby mitigating the adverse effects of T cell therapy. Adoptively transferred T cells in immunotherapy may attack normal cells in response to normal tissue expression of TAA. Even on-tumor target activity of adoptively transferred T cells can result in toxicities such as tumor lysis syndrome, cytokine release syndrome and the related macrophage activation syndrome. Safety switches may be utilized to eliminate inappropriately activated adoptively transferred cells by induction of apoptosis or by immunosurveillance.

[00152] In some embodiments, payloads of the present invention may comprise inducible killer/suicide genes that acts as a safety switch. The killer/suicide gene when introduced into adoptively transferred immune cells, could control their alloreactivity. The killer/suicide gene may be an apoptotic gene (e.g., any Caspase gene) which allows conditional apoptosis of the transduced cells by administration of a non-therapeutic ligand of the SRE (e.g., DD).

[00153] In some embodiments, the payload of the present invention may be Caspase 9. In some instances, Caspase 9 may be modified to have low basal expression and lacking the caspase recruitment domain (CARD) (SEQ ID NOS. 26 and SEQ ID NOS. 28 of US Patent No. US9434935B2; the contents of which are incorporated by reference in their entirety).

[00154] In one embodiment, the payload of the present invention is a suicide gene system, iCasp9/Chemical induced dimerization (CID) system which consists of a polypeptide derived from the Caspase9 gene fused to a drug binding domain derived from the human FK506 protein.

Administration of bioinert, small molecule AP1903(rimiducid), induces cross linking of the drug binding domains and dimerization of the fusion protein and in turn the dimerization of Caspase 9. This results in the activation of downstream effector Caspase 3 and subsequent induction of cellular apoptosis (Straathof et al., *Blood*, 2005, 105: 4247–4254; incorporated herein by reference in its entirety). Preclinical trials using CART including an iCasp9 gene have shown effective elimination of CAR T cells *in vivo* in mouse models and demonstrate the potential efficacy of this approach. (Budde et al, *Plos One*, 2013, 8: e82742.10.1371; Hoyos et al., *Leukemia*, 2010; 24(6):1160-1170). In one embodiment, the payload of the invention may comprise Caspase 9. In one aspect, the effector module of the invention may be a DD-Caspase9 fusion polypeptide. The DD-Caspase 9 may comprise the amino acid sequences provided in Table 5. The amino acid sequences in Table 5 may comprise a stop codon which is denoted in the table with a "*" at the end of the amino acid sequence

Table 5: DD-Caspase 9 constructs

Description/ Construct ID	Amino acid sequence	Amino Acid SEQ ID NO.	Nucleic Acid SEQ ID NO./ Sequence
Caspase 9	DEADRRLLRRCRLRLVEELQVDQLWDALLSRELFRPH MIEDIQRAGSGSRRDQARQLIIDLETRGSQALPLFISCLE DTGQDMLASFLRTNRQAAKLSKPTLENLTPVVLRPEIR KPEVLRPETPRPVDIGSGGFGDVGALESLRGNADLAYI LSMEPCGHCLIINNVNFCRESGLRTRTGSNIDCEKLRRR FSSLHFMVEVKGDLTAKKMVLALLELAQQDHGALDC CVVVILSHGCQASHLQFPGAVYGTDGCPVSVEKIVNIF NGTSCPSLGGKPKLFFIQACGGEQKDHGFEVASTSPED ESPGSNPEPDATPFQEGLRTFDQLDAISSLPTPSDIFVSY STFPGFVSWRDPKSGSWYVETLDDIFEQWAHSEDLQSL LLRVANAVSVKGIYKQMPGCFNFLRKKLFFKTS	65	81-82
Caspase delta CD	GVDGFGDVGALESLRGNADLAYILSMEPCGHCLIINNV NFCRESGLRTRTGSNIDCEKLRRRFSSLHFMVEVKGDL TAKKMVLALLELARQDHGALDCCVVVILSHGCQASHL QFPGAVYGTDGCPVSVEKIVNIFNGTSCPSLGGKPKLFF IQACGGEQKDHGFEVASTSPEDESPGSNPEPDATPFQE GLRTFDQLDAISSLPTPSDIFVSYSTFPGFVSWRDPKSGS WYVETLDDIFEQWAHSEDLQSLLLRVANAVSVKGIYK QMPGCFNFLRKKLFFKTS	66	83
Linker	VDYPYDVPDYALD	67	84
Linker	SGGGS	68	85, 69, 86
Linker	QLIGMLQGLMRDL	908	909
Linker	SG	-	AGCGGC
FKBP (F36V, L106P)	GVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKVD SSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAK LTISPDYAYGATGHPGIIPPHATLVFDVELLKPE	11	60, 878-882
FKBP (F36V)	GVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKVD SSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAK LTISPDYAYGATGHPGIIPPHATLVFDVELLKLE	70	87

FKBP (E31G, F36V, R71G, K105E)	GVQVETISPGDGRTFPKRGQTCVVHYTGMLGDGKKV DSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQGA KLTISPDYAYGATGHPGIIPPHATLVFDVELLELE	12	88, 883-889
ecDHFR (R12Y, Y100I)	MISLIAALAVDYVIGMENAMPWNLPADLAWFKRNTL NKPVIMGRHTWESIGRPLPGRKNIILSSQPGTDDRVTW VKSVDEAIAACGDVPEIMVIGGGRVIEQFLPKAQKLYL THIDAEVEGDTHFPDYEPDDWESVFSEFHDADAQNSH SYCFEILERR	8	89
ecDHFR (Amino acid 2-159 of WT) (R12Y, E129K)	ISLIAALAVDYVIGMENAMPWNLPADLAWFKRNTLNK PVIMGRHTWESIGRPLPGRKNIILSSQPGTDDRVTWVK SVDEAIAACGDVPEIMVIGGGRVYEQFLPKAQKLYLTH IDAEVEGDTHFPDYKPDDWESVFSEFHDADAQNSHSY CFEILERR	71	90
ecDHFR (Amino acid 2-159 of WT) (R12Y, Y100I)	ISLIAALAVDYVIGMENAMPWNLPADLAWFKRNTLNK PVIMGRHTWESIGRPLPGRKNIILSSQPGTDDRVTWVK SVDEAIAACGDVPEIMVIGGGRVIEQFLPKAQKLYLTHI DAEVEGDTHFPDYEPDDWESVFSEFHDADAQNSHSYC FEILERR	9	61, 869-874
hDHFR (Y122I)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLV LSRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDM VWIVGGSSVIKEAMNHPGHLKLFVTRIMQDFESDTFFP EIDLEKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	18	91
hDHFR (V75F, Y122I)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLF LSRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDM VWIVGGSSVIKEAMNHPGHLKLFVTRIMQDFESDTFFP EIDLEKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	31	92
hDHFR (L94A, T147A)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLV LSRELKEPPQGAHFLSRSADDALKLTEQPELANKVDM VWIVGGSSVYKEAMNHPGHLKLFVTRIMQDFESDAFF PEIDLEKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	32	93
OT-CASP9-001 (Met – FKBP (F36V, L106P) – Linker (SGGGS) – Caspase 9 - stop)	MGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKK VDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQR AKLTISPDYAYGATGHPGIIPPHATLVFDVELLKPESGG GSDEADRRLLRRCRLRLVEELQVDQLWDALLSRELFR PHMIEDIQRAGSGSRRDQARQLIIDLETRGSQALPLFISC LEDTGQDMLASFLRTNRQAAKLSKPTLENLTPVVLRPE IRKPEVLRPETPRPVDIGSGGFGDVGALESLRGNADLA YILSMEPCGHCLIINNVNFCRESGLRTRTGSNIDCEKLR RRFSSLHFMVEVKGDLTAKKMVLALLELAQQDHGAL DCCVVVILSHGCQASHLQFPGAVYGTDGCPVSVEKIV NIFNGTSCPSLGGKPKLFFIQACGGEQKDHGFEVASTSP EDESPGSNPEPDATPFQEGLRTFDQLDAISSLPTPSDIFV SYSTFPGFVSWRDPKSGSWYVETLDDIFEQWAHSEDL QSLLLRVANAVSVKGIYKQMPGCFNFLRKKLFFKTS*	72	94
OT-CASP9-002 (ecDHFR (R12Y, Y100I) – Linker (SGGGS) - Caspase 9 - stop)	MISLIAALAVDYVIGMENAMPWNLPADLAWFKRNTL NKPVIMGRHTWESIGRPLPGRKNIILSSQPGTDDRVTW VKSVDEAIAACGDVPEIMVIGGGRVIEQFLPKAQKLYL THIDAEVEGDTHFPDYEPDDWESVFSEFHDADAQNSH SYCFEILERRSGGGSDEADRRLLRRCRLRLVEELQVDQ LWDALLSRELFRPHMIEDIQRAGSGSRRDQARQLIIDLE TRGSQALPLFISCLEDTGQDMLASFLRTNRQAAKLSKP TLENLTPVVLRPEIRKPEVLRPETPRPVDIGSGGFGDVG ALESLRGNADLAYILSMEPCGHCLIINNVNFCRESGLRT RTGSNIDCEKLRRRFSSLHFMVEVKGDLTAKKMVLAL LELAQQDHGALDCCVVVILSHGCQASHLQFPGAVYGT DGCPVSVEKIVNIFNGTSCPSLGGKPKLFFIQACGGEQK DHGFEVASTSPEDESPGSNPEPDATPFQEGLRTFDQLD	73	95

	AISSLPTPSDIFVSYSTFPGFVSWRDPKSGSWYVETLDDI FEQWAHSEDLQSLLLRVANAVSVKGIYKQMPGCFNFL RKKLFFKTS*		
OT-CASP9-003 (hDHFR (Y122I) – Linker (SGGGS) - Caspase 9 - stop)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLV LSRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDM VWIVGGSSVIKEAMNHPGHLKLFVTRIMQDFESDTFFP EIDLEKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKNDS GGGSDEADRRLLRRCRLRLVEELQVDQLWDALLSREL FRPHMIEDIQRAGSGSRRDQARQLIIDLETRGSQALPLFI SCLEDTGQDMLASFLRTNRQAAKLSKPTLENLTPVVLR PEIRKPEVLRPETPRPVDIGSGGFGDVGALESLRGNADL AYILSMEPCGHCLIINNVNFCRESGLRTRTGSNIDCEKL RRRFSSLHFMVEVKGDLTAKKMVLALLELAQQDHGA LDCCVVVILSHGCQASHLQFPGAVYGTDGCPVSVEKIV NIFNGTSCPSLGGKPKLFFIQACGGEQKDHGFEVASTSP EDESPGSNPEPDATPFQEGLRTFDQLDAISSLPTPSDIFV SYSTFPGFVSWRDPKSGSWYVETLDDIFEQWAHSEDL QSLLLRVANAVSVKGIYKQMPGCFNFLRKKLFFKTS*	74	96
OT-CASP9-004 (hDHFR (V75F, Y122I) – Linker (SGGGS) - Caspase 9 - stop)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLF LSRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDM VWIVGGSSVIKEAMNHPGHLKLFVTRIMQDFESDTFFP EIDLEKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKNDS GGGSDEADRRLLRRCRLRLVEELQVDQLWDALLSREL FRPHMIEDIQRAGSGSRRDQARQLIIDLETRGSQALPLFI SCLEDTGQDMLASFLRTNRQAAKLSKPTLENLTPVVLR PEIRKPEVLRPETPRPVDIGSGGFGDVGALESLRGNADL AYILSMEPCGHCLIINNVNFCRESGLRTRTGSNIDCEKL RRRFSSLHFMVEVKGDLTAKKMVLALLELAQQDHGA LDCCVVVILSHGCQASHLQFPGAVYGTDGCPVSVEKIV NIFNGTSCPSLGGKPKLFFIQACGGEQKDHGFEVASTSP EDESPGSNPEPDATPFQEGLRTFDQLDAISSLPTPSDIFV SYSTFPGFVSWRDPKSGSWYVETLDDIFEQWAHSEDL QSLLLRVANAVSVKGIYKQMPGCFNFLRKKLFFKTS*	75	97
OT-CASP9-005 (hDHFR (L94A, T147A) – Linker (SGGGS) - Caspase 9 - stop)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLV LSRELKEPPQGAHFLSRSADDALKLTEQPELANKVDM VWIVGGSSVYKEAMNHPGHLKLFVTRIMQDFESDAFF PEIDLEKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND SGGGSDEADRRLLRRCRLRLVEELQVDQLWDALLSRE LFRPHMIEDIQRAGSGSRRDQARQLIIDLETRGSQALPL FISCLEDTGQDMLASFLRTNRQAAKLSKPTLENLTPVV LRPEIRKPEVLRPETPRPVDIGSGGFGDVGALESLRGNA DLAYILSMEPCGHCLIINNVNFCRESGLRTRTGSNIDCE KLRRRFSSLHFMVEVKGDLTAKKMVLALLELAQQDH GALDCCVVVILSHGCQASHLQFPGAVYGTDGCPVSVE KIVNIFNGTSCPSLGGKPKLFFIQACGGEQKDHGFEVAS TSPEDESPGSNPEPDATPFQEGLRTFDQLDAISSLPTPSD IFVSYSTFPGFVSWRDPKSGSWYVETLDDIFEQWAHSE DLQSLLLRVANAVSVKGIYKQMPGCFNFLRKKLFFKTS *	76	98
OT-CASP9-006 (Met – Leu – Glu - FKBP (F36V) – Linker (SGGGS) - Caspase Delta CD – Linker	MLEGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGK KVDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQ RAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKLESG GGSGVDGFGDVGALESLRGNADLAYILSMEPCGHCLII NNVNFCRESGLRTRTGSNIDCEKLRRRFSSLHFMVEVK GDLTAKKMVLALLELARQDHGALDCCVVVILSHGCQ ASHLQFPGAVYGTDGCPVSVEKIVNIFNGTSCPSLGGK PKLFFIQACGGEQKDHGFEVASTSPEDESPGSNPEPDAT	77	99

(QLIGMLQG LMRDL) - stop)	PFQEGLRTFDQLDAISSLPTPSDIFVSYSTFPGFVSWRDP KSGSWYVETLDDIFEQWAHSEDLQSLLLRVANAVSVK GIYKQMPGCFNFLRKKLFFKTSQLIGMLQGLMRDL*		
OT-CASP9-007 (Met - Caspase 9 – Linker (SG) – FKBP (E31G, F36V, R71G, K105E) - stop)	MDEADRRLLRRCRLRLVEELQVDQLWDALLSRELFRP HMIEDIQRAGSGSRRDQARQLIIDLETRGSQALPLFISCL EDTGQDMLASFLRTNRQAAKLSKPTLENLTPVVLRPEI RKPEVLRPETPRPVDIGSGGFGDVGALESLRGNADLAY ILSMEPCGHCLIINNVNFCRESGLRTRTGSNIDCEKLRR RFSSLHFMVEVKGDLTAKKMVLALLELAQQDHGALD CCVVVILSHGCQASHLQFPGAVYGTDGCPVSVEKIVNI FNGTSCPSLGGKPKLFFIQACGGEQKDHGFEVASTSPE DESPGSNPEPDATPFQEGLRTFDQLDAISSLPTPSDIFVS YSTFPGFVSWRDPKSGSWYVETLDDIFEQWAHSEDLQ SLLLRVANAVSVKGIYKQMPGCFNFLRKKLFFKTSSGG VQVETISPGDGRTFPKRGQTCVVHYTGMLGDGKKVDS SRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQGAKL TISPDYAYGATGHPGIIPPHATLVFDVELLELE*	78	100
OT-CASP9-008 (Met - Caspase 9 – Linker (SG) - ecDHFR (Amino acid 2-159 of WT) (R12Y, Y100I) - stop	MDEADRRLLRRCRLRLVEELQVDQLWDALLSRELFRP HMIEDIQRAGSGSRRDQARQLIIDLETRGSQALPLFISCL EDTGQDMLASFLRTNRQAAKLSKPTLENLTPVVLRPEI RKPEVLRPETPRPVDIGSGGFGDVGALESLRGNADLAY ILSMEPCGHCLIINNVNFCRESGLRTRTGSNIDCEKLRR RFSSLHFMVEVKGDLTAKKMVLALLELAQQDHGALD CCVVVILSHGCQASHLQFPGAVYGTDGCPVSVEKIVNI FNGTSCPSLGGKPKLFFIQACGGEQKDHGFEVASTSPE DESPGSNPEPDATPFQEGLRTFDQLDAISSLPTPSDIFVS YSTFPGFVSWRDPKSGSWYVETLDDIFEQWAHSEDLQ SLLLRVANAVSVKGIYKQMPGCFNFLRKKLFFKTSSGI SLIAALAVDYVIGMENAMPWNLPADLAWFKRNTLNK PVIMGRHTWESIGRPLPGRKNIILSSQPGTDDRVTWVK SVDEAIAACGDVPEIMVIGGGRVIEQFLPKAQKLYLTHI DAEVEGDTHFPDYEPDDWESVFSEFHDADAQNSHSYC FEILERR*	79	101
OT-CASP9-009 (Met - Caspase 9 - ecDHFR (Amino acid 2-159 of WT) (R12Y, E129K) - stop)	MDEADRRLLRRCRLRLVEELQVDQLWDALLSRELFRP HMIEDIQRAGSGSRRDQARQLIIDLETRGSQALPLFISCL EDTGQDMLASFLRTNRQAAKLSKPTLENLTPVVLRPEI RKPEVLRPETPRPVDIGSGGFGDVGALESLRGNADLAY ILSMEPCGHCLIINNVNFCRESGLRTRTGSNIDCEKLRR RFSSLHFMVEVKGDLTAKKMVLALLELAQQDHGALD CCVVVILSHGCQASHLQFPGAVYGTDGCPVSVEKIVNI FNGTSCPSLGGKPKLFFIQACGGEQKDHGFEVASTSPE DESPGSNPEPDATPFQEGLRTFDQLDAISSLPTPSDIFVS YSTFPGFVSWRDPKSGSWYVETLDDIFEQWAHSEDLQ SLLLRVANAVSVKGIYKQMPGCFNFLRKKLFFKTSSGI SLIAALAVDYVIGMENAMPWNLPADLAWFKRNTLNK PVIMGRHTWESIGRPLPGRKNIILSSQPGTDDRVTWVK SVDEAIAACGDVPEIMVIGGGRVYEQFLPKAQKLYLTH IDAEVEGDTHFPDYKPDDWESVFSEFHDADAQNSHSY CFEILERR*	80	102

[00155] In some instances, the iCasp9/CID system has been shown to have a basal rate of dimerization even in the absence of rimiducid, resulting in unintended cell death. Regulating the expression levels of iCasp9/CID is critical for maximizing the efficacy of iCasp9/CID system. Biocircuits of the present invention and/or any of their components may be utilized in regulating or tuning the iCasp9/CID system to optimize its utility. Other examples of proteins used in

dimerization-induced apoptosis paradigm may include, but are not limited to Fas receptor, the death effector domain of Fas-associated protein, FADD, Caspase 1, Caspase 3, Caspase 7 and Caspase 8. (Belshaw P.J. et al, *Chem Biol.*, 996,3:731-738; MacCorkle R.A. et al, *Proc Natl Acad Sci,* 1998, 95:3655-3660; Spencer, D.M. et al., *Curr Biol.*1996; 6:839-847; the contents of each of which are incorporated herein by reference in their entirety).

[00156] In some embodiments, the safety switch of the present invention may comprise a metabolic enzyme, such as herpes simplex virus thymidine kinase (HSV-TK) and cytosine deaminase (CD). HSV-TK phosphorylates nucleoside analogs, including acyclovir and ganciclovir (GCV) to generate triphosphate form of nucleosides. When incorporated into DNA, it leads to chain termination and cell death. Unlike the mammalian thymidine kinase, HSV-TK is characterized by 1000-fold higher affinity to nucleoside analogs such as GCV, making it suitable for use as a suicide gene in mammalian cells. Cytosine deaminase (CD) can converts 5-fluorocytosine (5-FC) into the cytotoxic 5-fluorouracil (5-FU) (Tiraby et al., *FEMS* Lett., 1998, 167: 41–49).

[00157] In some embodiments, the safety switch of the present invention may comprise a CYP4B1 mutant (as suicide gene), which may be co-expressed in a CAR engineered T cells (Roellecker et al., *Gen Ther.*, 2016, May 19, doi: 10.1038/gt.2016.38.).

[00158] In some embodiments, the payload of the present invention may comprise a fusion construct that can induce cell death, for example, a polypeptide with the formula of St-R1-S1-Q-S2-R2, wherein the St is a stalk sequence, R1/2 and Q are different epitopes; and S1/2 are optional spacer sequences (See International patent publication NOS. WO2013153391; the content of which are incorporated herein by reference in their entirety).

[00159] In some embodiments, safety switch may be mediated by therapeutic antibodies which specifically bind to an antigen that is expressed in the plasma membrane of adoptively transferred cells. The antigen-antibody interaction allows cell removal after administration of a specific monoclonal antibody against the antigen. As non-limiting examples, payloads of the present invention may comprise the antigen and antibody pair used to mediate safety switch such as CD20 and anti-CD20 antibody (Griffioen et al., *Haematologica*, 2009, 94:1316–1320), a protein tag and anti-tag antibody (Kieback et al., *Natl. Acad. Sci. U.S.A.*, 2008, 105: 623–628), a compact suicide gene (RQR8) combining epitopes from CD34 (as a marker moiety) and CD20 (as a suicide moiety) which enables CD34 selection, cell tracking, as well as cell deletion after anti-CD20 monoclonal antibody administration (Philip et al., *Blood*, 2014, 124: 1277–1287); truncated human EGFR polypeptide and anti-EGFR monoclonal antibody (Wang et al., *Blood*, 2011, 118:1255–1263); and a compact polypeptide safety switch having a structural formula as

discussed in U.S Patent Application Publication NOS. US20150093401; the contents of each of which are incorporated herein by reference in their entirety.

Regulatory switch

[00160] The utility of adoptive cell therapy (ACT) has been limited by the high incidence of graft versus host disease (GVHD). GVHD occurs when adoptively transferred T cells elicit an immune response resulting in host tissue damage. Recognition of host antigens by the graft cells triggers a proinflammatory cytokine storm cascade that signifies acute GVHD. GVHD is characterized as an imbalance between the effector and the regulatory arms of the immune system. In some embodiments, the payloads of the present invention may be used as regulatory switches. As used herein "regulatory switch" refers proteins, which when expressed in target cells increase tolerance to the graft by enhancing the regulatory arm of the immune system.

[00161] In one embodiment, regulatory switches may include payloads that preferentially promote the expansion of regulatory T (Treg cells). Tregs are a distinct population of cells that are positively selected on high affinity ligands in the thymus and play an important role in the tolerance to self-antigens. In addition, T regs have also been shown to play a role in peripheral tolerance to foreign antigens. Since Tregs promote immune tolerance, expansion of Tregs with the compositions of the invention may be desirable to limit GVHD.

[00162] In some embodiments, the regulatory switch may include, but is not limited to T regs activation factors such NFκB, FOXO, nuclear receptor Nr4a, Retinoic acid receptor alpha, NFAT, AP-1 and SMAD. Such factors can result in the expression of Fork headbox P3 (FOXP3) in T cells resulting in the activation of the regulatory T cell program and the expansion of T cells.

[00163] In one embodiment, the regulatory switch may be FOXP3, a transcriptional regulator in T cells. A function of FOXP3 is to suppress the function of NFAT, which leads to the suppression of expression of many genes including IL2 and effector T-cell cytokines. FOXP3 acts also as a transcription activator for genes such as CD2S, Cytotoxic T-Lymphocyte Antigen Cytotoxic T-Lymphocyte Antigen 4 (CTLA4), glucocorticoid-induced TNF receptor family gene (GITR) and folate receptor 4. FOXP3 also inhibits the differentiation of IL17 producing helper T-cells (Th17) by antagonizing RORC (RAR related orphan receptor C). Isoforms of FOXP3 lacking exon2 (FOXP3 delta 2), or exon 7 (FOXP3 delta 7) may also be used as regulatory switches. In one aspect, the effector module of the invention may be a DD-FOXP3 fusion polypeptide. The DD-FOXP3 may comprise the amino acid sequences provided in Table 6. The amino acid sequences in Table 6 may comprise a stop codon which is denoted in the table with a "*" at the end of the amino acid sequence.

Table 6: DD-FOXP3 constructs

Construct/ Description	Amino Acid sequence	Amino Acid SEQ ID NO.	Nucleic Acid SEQ ID NO.
Linker	SGGGS	68	85, 69, 86
Linker	SG	-	AGCGGC
FKBP (F36V, L106P)	GVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKVD SSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRAK LTISPDYAYGATGHPGIIPPHATLVFDVELLKPE	11	60, 878-882
FKBP (E31G, F36V, R71G, K105E)	GVQVETISPGDGRTFPKRGQTCVVHYTGMLGDGKKV DSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQGA KLTISPDYAYGATGHPGIIPPHATLVFDVELLELE	12	88, 883-889
ecDHFR (R12Y, Y100I)	MISLIAALAVDYVIGMENAMPWNLPADLAWFKRNTL NKPVIMGRHTWESIGRPLPGRKNIILSSQPGTDDRVTW VKSVDEAIAACGDVPEIMVIGGGRVIEQFLPKAQKLYL THIDAEVEGDTHFPDYEPDDWESVFSEFHDADAQNSH SYCFEILERR	8	89
ecDHFR (Amino acid 2-159 of WT) (R12Y, Y100I)	ISLIAALAVDYVIGMENAMPWNLPADLAWFKRNTLNK PVIMGRHTWESIGRPLPGRKNIILSSQPGTDDRVTWVK SVDEAIAACGDVPEIMVIGGGRVIEQFLPKAQKLYLTHI DAEVEGDTHFPDYEPDDWESVFSEFHDADAQNSHSYC FEILERR	9	61, 869-874
FOXP3 full length	MPNPRPGKPSAPSLALGPSPGASPSWRAAPKASDLLGA RGPGGTFQGRDLRGGAHASSSSLNPMPPSQLQLPTLPL VMVAPSGARLGPLPHLQALLQDRPHFMHQLSTVDAH ARTPVLQVHPLESPAMISLTPPTTATGVFSLKARPGLPP GINVASLEWVSREPALLCTFPNPSAPRKDSTLSAVPQSS YPLLANGVCKWPGCEKVFEEPEDFLKHCQADHLLDEK GRAQCLLQREMVQSLEQQLVLEKEKLSAMQAHLAGK MALTKASSVASSDKGSCCIVAAGSQGPVVPAWSGPRE APDSLFAVRRHLWGSHGNSTFPEFLHNMDYFKFHNMR PPFTYATLIRWAILEAPEKQRTLNEIYHWFTRMFAFFRN HPATWKNAIRHNLSLHKCFVRVESEKGAVWTVDELEF RKKRSQRPSRCSNPTPGP*	103	911
Amino Acid 2-431 of FOXP3 full length	PNPRPGKPSAPSLALGPSPGASPSWRAAPKASDLLGAR GPGGTFQGRDLRGGAHASSSSLNPMPPSQLQLPTLPLV MVAPSGARLGPLPHLQALLQDRPHFMHQLSTVDAHAR TPVLQVHPLESPAMISLTPPTTATGVFSLKARPGLPPGI NVASLEWVSREPALLCTFPNPSAPRKDSTLSAVPQSSY PLLANGVCKWPGCEKVFEEPEDFLKHCQADHLLDEKG RAQCLLQREMVQSLEQQLVLEKEKLSAMQAHLAGKM ALTKASSVASSDKGSCCIVAAGSQGPVVPAWSGPREAP DSLFAVRRHLWGSHGNSTFPEFLHNMDYFKFHNMRPP FTYATLIRWAILEAPEKQRTLNEIYHWFTRMFAFFRNH PATWKNAIRHNLSLHKCFVRVESEKGAVWTVDELEFR KKRSQRPSRCSNPTPGP	104	912
FOXP3 delta 2	MPNPRPGKPSAPSLALGPSPGASPSWRAAPKASDLLGA RGPGGTFQGRDLRGGAHASSSSLNPMPPSQLQLSTVDA HARTPVLQVHPLESPAMISLTPPTTATGVFSLKARPGLP PGINVASLEWVSREPALLCTFPNPSAPRKDSTLSAVPQS SYPLLANGVCKWPGCEKVFEEPEDFLKHCQADHLLDE KGRAQCLLQREMVQSLEQQLVLEKEKLSAMQAHLAG KMALTKASSVASSDKGSCCIVAAGSQGPVVPAWSGPR EAPDSLFAVRRHLWGSHGNSTFPEFLHNMDYFKFHNM RPPFTYATLIRWAILEAPEKQRTLNEIYHWFTRMFAFFR	105	913

	NHPATWKNAIRHNLSLHKCFVRVESEKGAVWTVDELE FRKKRSQRPSRCSNPTPGP		
FOXP3 delta 2	PNPRPGKPSAPSLALGPSPGASPSWRAAPKASDLLGAR GPGGTFQGRDLRGGAHASSSSLNPMPPSQLQLSTVDA HARTPVLQVHPLESPAMISLTPPTTATGVFSLKARPGLP PGINVASLEWVSREPALLCTFPNPSAPRKDSTLSAVPQS SYPLLANGVCKWPGCEKVFEEPEDFLKHCQADHLLDE KGRAQCLLQREMVQSLEQQLVLEKEKLSAMQAHLAG KMALTKASSVASSDKGSCCIVAAGSQGPVVPAWSGPR EAPDSLFAVRRHLWGSHGNSTFPEFLHNMDYFKFHNM RPPFTYATLIRWAILEAPEKQRTLNEIYHWFTRMFAFFR NHPATWKNAIRHNLSLHKCFVRVESEKGAVWTVDELE FRKKRSQRPSRCSNPTPGP	106	914
OT-FOXP3-001 (FoxP3 - stop)	MPNPRPGKPSAPSLALGPSPGASPSWRAAPKASDLLGA RGPGGTFQGRDLRGGAHASSSSLNPMPPSQLQLPTLPL VMVAPSGARLGPLPHLQALLQDRPHFMHQLSTVDAH ARTPVLQVHPLESPAMISLTPPTTATGVFSLKARPGLPP GINVASLEWVSREPALLCTFPNPSAPRKDSTLSAVPQSS YPLLANGVCKWPGCEKVFEEPEDFLKHCQADHLLDEK GRAQCLLQREMVQSLEQQLVLEKEKLSAMQAHLAGK MALTKASSVASSDKGSCCIVAAGSQGPVVPAWSGPRE APDSLFAVRRHLWGSHGNSTFPEFLHNMDYFKFHNMR PPFTYATLIRWAILEAPEKQRTLNEIYHWFTRMFAFFRN HPATWKNAIRHNLSLHKCFVRVESEKGAVWTVDELEF RKKRSQRPSRCSNPTPGP*	107	193
OT-FOXP3-002 (FoxP3 Delta 2 - stop)	MPNPRPGKPSAPSLALGPSPGASPSWRAAPKASDLLGA RGPGGTFQGRDLRGGAHASSSSLNPMPPSQLQLSTVDA HARTPVLQVHPLESPAMISLTPPTTATGVFSLKARPGLP PGINVASLEWVSREPALLCTFPNPSAPRKDSTLSAVPQS SYPLLANGVCKWPGCEKVFEEPEDFLKHCQADHLLDE KGRAQCLLQREMVQSLEQQLVLEKEKLSAMQAHLAG KMALTKASSVASSDKGSCCIVAAGSQGPVVPAWSGPR EAPDSLFAVRRHLWGSHGNSTFPEFLHNMDYFKFHNM RPPFTYATLIRWAILEAPEKQRTLNEIYHWFTRMFAFFR NHPATWKNAIRHNLSLHKCFVRVESEKGAVWTVDELE FRKKRSQRPSRCSNPTPGP*	108	194
OT-FOXP3-003 (Met - FKBP (F36V, L106P) - Linker (SGGGS) - Amino Acid 2-431 of FOXP3 full length - stop)	MGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKK VDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQR AKLTISPDYAYGATGHPGIIPPHATLVFDVELLKPESGG GSPNPRPGKPSAPSLALGPSPGASPSWRAAPKASDLLG ARGPGGTFQGRDLRGGAHASSSSLNPMPPSQLQLPTLP LVMVAPSGARLGPLPHLQALLQDRPHFMHQLSTVDAH ARTPVLQVHPLESPAMISLTPPTTATGVFSLKARPGLPP GINVASLEWVSREPALLCTFPNPSAPRKDSTLSAVPQSS YPLLANGVCKWPGCEKVFEEPEDFLKHCQADHLLDEK GRAQCLLQREMVQSLEQQLVLEKEKLSAMQAHLAGK MALTKASSVASSDKGSCCIVAAGSQGPVVPAWSGPRE APDSLFAVRRHLWGSHGNSTFPEFLHNMDYFKFHNMR PPFTYATLIRWAILEAPEKQRTLNEIYHWFTRMFAFFRN HPATWKNAIRHNLSLHKCFVRVESEKGAVWTVDELEF RKKRSQRPSRCSNPTPGP*	109	195
OT-FOXP3-004 (ecDHFR (R12Y, Y100I) – Linker (SGGGS) - Amino Acid 2-431 of	MISLIAALAVDYVIGMENAMPWNLPADLAWFKRNTL NKPVIMGRHTWESIGRPLPGRKNIILSSQPGTDDRVTW VKSVDEAIAACGDVPEIMVIGGGRVIEQFLPKAQKLYL THIDAEVEGDTHFPDYEPDDWESVFSEFHDADAQNSH SYCFEILERRSGGGSPNPRPGKPSAPSLALGPSPGASPS WRAAPKASDLLGARGPGGTFQGRDLRGGAHASSSSLN PMPPSQLQLPTLPLVMVAPSGARLGPLPHLQALLQDRP HFMHQLSTVDAHARTPVLQVHPLESPAMISLTPPTTAT GVFSLKARPGLPPGINVASLEWVSREPALLCTFPNPSAP	110	196

FOXP3 full length - stop)	RKDSTLSAVPQSSYPLLANGVCKWPGCEKVFEEPEDFL KHCQADHLLDEKGRAQCLLQREMVQSLEQQLVLEKE KLSAMQAHLAGKMALTKASSVASSDKGSCCIVAAGSQ GPVVPAWSGPREAPDSLFAVRRHLWGSHGNSTFPEFL HNMDYFKFHNMRPPFTYATLIRWAILEAPEKQRTLNEI YHWFTRMFAFFRNHPATWKNAIRHNLSLHKCFVRVES EKGAVWTVDELEFRKKRSQRPSRCSNPTPGP*		
OT-FOXP3-005 (FoxP3 – Linker (SG) - FKBP (E31G, F36V, R71G, K105E) - stop)	MPNPRPGKPSAPSLALGPSPGASPSWRAAPKASDLLGA RGPGGTFQGRDLRGGAHASSSSLNPMPPSQLQLPTLPL VMVAPSGARLGPLPHLQALLQDRPHFMHQLSTVDAH ARTPVLQVHPLESPAMISLTPPTTATGVFSLKARPGLPP GINVASLEWVSREPALLCTFPNPSAPRKDSTLSAVPQSS YPLLANGVCKWPGCEKVFEEPEDFLKHCQADHLLDEK GRAQCLLQREMVQSLEQQLVLEKEKLSAMQAHLAGK MALTKASSVASSDKGSCCIVAAGSQGPVVPAWSGPRE APDSLFAVRRHLWGSHGNSTFPEFLHNMDYFKFHNMR PPFTYATLIRWAILEAPEKQRTLNEIYHWFTRMFAFFRN HPATWKNAIRHNLSLHKCFVRVESEKGAVWTVDELEF RKKRSQRPSRCSNPTPGPSGGVQVETISPGDGRTFPKRG QTCVVHYTGMLGDGKKVDSSRDRNKPFKFMLGKQEV IRGWEEGVAQMSVGQGAKLTISPDYAYGATGHPGIIPP HATLVFDVELLELE*	111	197
OT-FOXP3-006 (FoxP3 – Linker (SG) - ecDHFR (Amino acid 2-159 of WT) (R12Y, Y100I) - stop)	MPNPRPGKPSAPSLALGPSPGASPSWRAAPKASDLLGA RGPGGTFQGRDLRGGAHASSSSLNPMPPSQLQLPTLPL VMVAPSGARLGPLPHLQALLQDRPHFMHQLSTVDAH ARTPVLQVHPLESPAMISLTPPTTATGVFSLKARPGLPP GINVASLEWVSREPALLCTFPNPSAPRKDSTLSAVPQSS YPLLANGVCKWPGCEKVFEEPEDFLKHCQADHLLDEK GRAQCLLQREMVQSLEQQLVLEKEKLSAMQAHLAGK MALTKASSVASSDKGSCCIVAAGSQGPVVPAWSGPRE APDSLFAVRRHLWGSHGNSTFPEFLHNMDYFKFHNMR PPFTYATLIRWAILEAPEKQRTLNEIYHWFTRMFAFFRN HPATWKNAIRHNLSLHKCFVRVESEKGAVWTVDELEF RKKRSQRPSRCSNPTPGPSGISLIAALAVDYVIGMENA MPWNLPADLAWFKRNTLNKPVIMGRHTWESIGRPLPG RKNIILSSQPGTDDRVTWVKSVDEAIAACGDVPEIMVI GGGRVIEQFLPKAQKLYLTHIDAEVEGDTHFPDYEPDD WESVFSEFHDADAQNSHSYCFEILERR*	112	198
OT-FOXP3-007 (Met - FKBP (F36V, L106P) – Linker (SG) - Amino Acid 2-396 of FOXP3 delta 2 - stop)	MGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKK VDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQR AKLTISPDYAYGATGHPGIIPPHATLVFDVELLKPESGP NPRPGKPSAPSLALGPSPGASPSWRAAPKASDLLGARG PGGTFQGRDLRGGAHASSSSLNPMPPSQLQLSTVDAH ARTPVLQVHPLESPAMISLTPPTTATGVFSLKARPGLPP GINVASLEWVSREPALLCTFPNPSAPRKDSTLSAVPQSS YPLLANGVCKWPGCEKVFEEPEDFLKHCQADHLLDEK GRAQCLLQREMVQSLEQQLVLEKEKLSAMQAHLAGK MALTKASSVASSDKGSCCIVAAGSQGPVVPAWSGPRE APDSLFAVRRHLWGSHGNSTFPEFLHNMDYFKFHNMR PPFTYATLIRWAILEAPEKQRTLNEIYHWFTRMFAFFRN HPATWKNAIRHNLSLHKCFVRVESEKGAVWTVDELEF RKKRSQRPSRCSNPTPGP*	113	199
OT-FOXP3-008 (ecDHFR (R12Y, Y100I) – Linker (SG) - Amino Acid 2-396 of	MISLIAALAVDYVIGMENAMPWNLPADLAWFKRNTL NKPVIMGRHTWESIGRPLPGRKNIILSSQPGTDDRVTW VKSVDEAIAACGDVPEIMVIGGGRVIEQFLPKAQKLYL THIDAEVEGDTHFPDYEPDDWESVFSEFHDADAQNSH SYCFEILERRSGPNPRPGKPSAPSLALGPSPGASPSWRA APKASDLLGARGPGGTFQGRDLRGGAHASSSSLNPMP PSQLQLSTVDAHARTPVLQVHPLESPAMISLTPPTTATG VFSLKARPGLPPGINVASLEWVSREPALLCTFPNPSAPR	114	200

FOXP3 delta 2 - stop)	KDSTLSAVPQSSYPLLANGVCKWPGCEKVFEEPEDFLK HCQADHLLDEKGRAQCLLQREMVQSLEQQLVLEKEK LSAMQAHLAGKMALTKASSVASSDKGSCCIVAAGSQG PVVPAWSGPREAPDSLFAVRRHLWGSHGNSTFPEFLH NMDYFKFHNMRPPFTYATLIRWAILEAPEKQRTLNEIY HWFTRMFAFFRNHPATWKNAIRHNLSLHKCFVRVESE KGAVWTVDELEFRKKRSQRPSRCSNPTPGP*		
OT-FOXP3-009 (FoxP3 Delta 2-Linker (SG) - FKBP (E31G, F36V, R71G, K105E) - stop)	MPNPRPGKPSAPSLALGPSPGASPSWRAAPKASDLLGA RGPGGTFQGRDLRGGAHASSSSLNPMPPSQLQLSTVDA HARTPVLQVHPLESPAMISLTPPTTATGVFSLKARPGLP PGINVASLEWVSREPALLCTFPNPSAPRKDSTLSAVPQS SYPLLANGVCKWPGCEKVFEEPEDFLKHCQADHLLDE KGRAQCLLQREMVQSLEQQLVLEKEKLSAMQAHLAG KMALTKASSVASSDKGSCCIVAAGSQGPVVPAWSGPR EAPDSLFAVRRHLWGSHGNSTFPEFLHNMDYFKFHNM RPPFTYATLIRWAILEAPEKQRTLNEIYHWFTRMFAFFR NHPATWKNAIRHNLSLHKCFVRVESEKGAVWTVDELE FRKKRSQRPSRCSNPTPGPSGGVQVETISPGDGRTFPKR GQTCVVHYTGMLGDGKKVDSSRDRNKPFKFMLGKQE VIRGWEEGVAQMSVGQGAKLTISPDYAYGATGHPGIIP PHATLVFDVELLELE*	115	201
OT-FOXP3-010 (FoxP3 Delta 2-Linker (SG) - ecDHFR (Amino acid 2-159 of WT) (R12Y, Y100I) - stop)	MPNPRPGKPSAPSLALGPSPGASPSWRAAPKASDLLGA RGPGGTFQGRDLRGGAHASSSSLNPMPPSQLQLSTVDA HARTPVLQVHPLESPAMISLTPPTTATGVFSLKARPGLP PGINVASLEWVSREPALLCTFPNPSAPRKDSTLSAVPQS SYPLLANGVCKWPGCEKVFEEPEDFLKHCQADHLLDE KGRAQCLLQREMVQSLEQQLVLEKEKLSAMQAHLAG KMALTKASSVASSDKGSCCIVAAGSQGPVVPAWSGPR EAPDSLFAVRRHLWGSHGNSTFPEFLHNMDYFKFHNM RPPFTYATLIRWAILEAPEKQRTLNEIYHWFTRMFAFFR NHPATWKNAIRHNLSLHKCFVRVESEKGAVWTVDELE FRKKRSQRPSRCSNPTPGPSGISLIAALAVDYVIGMENA MPWNLPADLAWFKRNTLNKPVIMGRHTWESIGRPLPG RKNIILSSQPGTDDRVTWVKSVDEAIAACGDVPEIMVI GGGRVIEQFLPKAQKLYLTHIDAEVEGDTHFPDYEPDD WESVFSEFHDADAQNSHSYCFEILERR*	116	202

Antibodies

[00164] In some embodiments, antibodies, fragments and variants thereof are payloads of the present invention.

[00165] In some embodiments, antibodies of the present invention, include without limitation, any of those taught in Table 5 of copending commonly owned U.S. Provisional Patent Application No. 62/320,864, filed on 4/11/2016, or in US Provisional Application No. 62/466,596 filed March 3, 2017 and the International Publication WO2017/180587, the contents of which are incorporated herein by reference in their entirety.

Antibody fragments and variants

[00166] In some embodiments, antibody fragments and variants may comprise antigen binding regions from intact antibodies. Examples of antibody fragments and variants may include, but are not limited to Fab, Fab', F(ab')2, and Fv fragments; diabodies; linear antibodies; single-chain antibody molecules such as single chain variable fragment (scFv); 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. Also produced is a residual "Fc" fragment, whose name reflects its ability to crystallize readily. Pepsin treatment yields an F(ab')2 fragment that has two antigen-binding sites and is still capable of cross-linking with the antigen. Pharmaceutical compositions, biocircuits, biocircuit components, effector modules including their SREs or payloads of the present invention may comprise one or more of these fragments.

[00167] For the purposes herein, an "antibody" may comprise a heavy and light variable domain as well as an Fc region. As used herein, the term "native antibody" usually refers to a heterotetrameric glycoprotein of about 150,000 daltons, composed of two identical light (L) chains and two identical heavy (H) chains. Genes encoding antibody heavy and light chains are known and segments making up each have been well characterized and described (Matsuda et al., *The Journal of Experimental Medicine*. 1998, 188(11): 2151-62 and Li et al., *Blood*, 2004, 103(12): 4602-4609; the content of each of which are herein incorporated by reference in their entirety). Each light chain is linked to a heavy chain by one covalent disulfide bond, while the number of disulfide linkages varies among the heavy chains of different immunoglobulin isotypes. Each heavy and light chain also has regularly spaced intrachain disulfide bridges. Each heavy chain has at one end a variable domain (VH) followed by a number of constant domains. Each light chain has a variable domain at one end (VL) and a constant domain at its other end; the constant domain of the light chain is aligned with the first constant domain of the heavy chain, and the light chain variable domain is aligned with the variable domain of the heavy chain.

[00168] As used herein, the term "variable domain" refers to specific antibody domains found on both the antibody heavy and light chains that differ extensively in sequence among antibodies and are used in the binding and specificity of each particular antibody for its particular antigen. Variable domains comprise hypervariable regions. As used herein, the term "hypervariable region" refers to a region within a variable domain comprising amino acid residues responsible for antigen binding. The amino acids present within the hypervariable regions determine the structure of the complementarity determining regions (CDRs) that become part of the antigen-binding site of the antibody. As used herein, the term "CDR" refers to a region of an antibody comprising a structure that is complimentary to its target antigen or epitope. Other portions of the variable domain, not interacting with the antigen, are referred to as framework (FW) regions. The antigen-binding site (also known as the antigen combining site or paratope) comprises the amino acid residues necessary to interact with a particular antigen. The exact residues making up the antigen-binding site are typically elucidated by co-crystallography with bound antigen,

however computational assessments based on comparisons with other antibodies can also be used (Strohl, W.R. Therapeutic Antibody Engineering. Woodhead Publishing, Philadelphia PA. 2012. Ch. 3, p47-54, the contents of which are herein incorporated by reference in their entirety). Determining residues that make up CDRs may include the use of numbering schemes including, but not limited to, those taught by Kabat (Wu et al., *JEM*, 1970, 132(2):211-250 and Johnson et al., *Nucleic Acids Res.* 2000, 28(1): 214-218, the contents of each of which are herein incorporated by reference in their entirety), Chothia (Chothia and Lesk, J. *Mol. Biol.* 1987, 196, 901, Chothia et al., *Nature*, 1989, 342, 877, and Al-Lazikani et al., *J. Mol. Biol.* 1997, 273(4): 927-948, the contents of each of which are herein incorporated by reference in their entirety), Lefranc (Lefranc et al., *Immunome Res.* 2005, 1:3) and Honegger (Honegger and Pluckthun, *J. Mol. Biol.* 2001, 309(3): 657-70, the contents of which are herein incorporated by reference in their entirety).

[00169] VH and VL domains have three CDRs each. VL CDRs are referred to herein as CDR-L1, CDR-L2 and CDR-L3, in order of occurrence when moving from N- to C- terminus along the variable domain polypeptide. VH CDRs are referred to herein as CDR-H1, CDR-H2 and CDR-H3, in order of occurrence when moving from N- to C- terminus along the variable domain polypeptide. Each of CDRs has favored canonical structures with the exception of the CDR-H3, which comprises amino acid sequences that may be highly variable in sequence and length between antibodies resulting in a variety of three-dimensional structures in antigen-binding domains (Nikoloudis, et al., *PeerJ.* 2014, 2: e456). In some cases, CDR-H3s may be analyzed among a panel of related antibodies to assess antibody diversity. Various methods of determining CDR sequences are known in the art and may be applied to known antibody sequences (Strohl, W.R. Therapeutic Antibody Engineering. Woodhead Publishing, Philadelphia PA. 2012. Ch. 3, p47-54, the contents of which are herein incorporated by reference in their entirety).

[00170] As used herein, the term "Fv" refers to an antibody fragment comprising the minimum fragment on an antibody needed to form a complete antigen-binding site. These regions consist of a dimer of one heavy chain and one light chain variable domain in tight, non-covalent association. Fv fragments can be generated by proteolytic cleavage, but are largely unstable. Recombinant methods are known in the art for generating stable Fv fragments, typically through insertion of a flexible linker between the light chain variable domain and the heavy chain variable domain (to form a single chain Fv (scFv)) or through the introduction of a disulfide bridge between heavy and light chain variable domains (Strohl, W.R. Therapeutic Antibody Engineering. Woodhead Publishing, Philadelphia PA. 2012. Ch. 3, p46-47, the contents of which are herein incorporated by reference in their entirety).

[00171] As used herein, the term "light chain" refers to a component of an antibody from any vertebrate species assigned to one of two clearly distinct types, called kappa and lambda based on amino acid sequences of constant domains. Depending on the amino acid sequence of the constant domain of their heavy chains, antibodies can be assigned to different classes. There are five major classes of intact antibodies: IgA, IgD, IgE, IgG, and IgM, and several of these may be further divided into subclasses (isotypes), e.g., IgG1, IgG2, IgG3, IgG4, IgA, and IgA2.

[00172] As used herein, the term "single chain Fv" or "scFv" refers to a fusion protein of VH and VL antibody domains, wherein these domains are linked together into a single polypeptide chain by a flexible peptide linker. In some embodiments, the Fv polypeptide linker enables the scFv to form the desired structure for antigen binding. In some embodiments, scFvs are utilized in conjunction with phage display, yeast display or other display methods where they may be expressed in association with a surface member (e.g. phage coat protein) and used in the identification of high affinity peptides for a given antigen.

[00173] Using molecular genetics, two scFvs can be engineered in tandem into a single polypeptide, separated by a linker domain, called a "tandem scFv" (tascFv). Construction of a tascFv with genes for two different scFvs yields a "bispecific single-chain variable fragments" (bis-scFvs). Only two tascFvs have been developed clinically by commercial firms; both are bispecific agents in active early phase development by Micromet for oncologic indications, and are described as "Bispecific T-cell Engagers (BiTE)." Blinatumomab is an anti-CD19/anti-CD3 bispecific tascFv that potentiates T-cell responses to B-cell non-Hodgkin lymphoma in Phase 2. MT110 is an anti-EP-CAM/anti-CD3 bispecific tascFv that potentiates T-cell responses to solid tumors in Phase 1. Bispecific, tetravalent "TandAbs" are also being researched by Affimed (Nelson, A. L., MAbs., 2010, Jan-Feb; 2(1):77–83). maxibodies (bivalent scFv fused to the amino terminus of the Fc (CH2-CH3 domains) of IgG may also be included.

[00174] As used herein, the term "bispecific antibody" refers to an antibody capable of binding two different antigens. Such antibodies typically comprise regions from at least two different antibodies. Bispecific antibodies may include any of those described in Riethmuller, G. *Cancer Immunity*. 2012, 12:12-18, Marvin et al., 2005. *Acta Pharmacologica Sinica*. 2005, 26(6): 649-658 and Schaefer et al., *PNAS*. 2011, 108(27):11187-11192, the contents of each of which are herein incorporated by reference in their entirety.

[00175] As used herein, the term "diabody" refers to a small antibody fragment with two antigen-binding sites. Diabodies are functional bispecific single-chain antibodies (bscAb). Diabodies comprise a heavy chain variable domain VH connected to a light chain variable domain VL in the same polypeptide chain. By using a linker that is too short to allow pairing

between the two domains on the same chain, the domains are forced to pair with the complementary domains of another chain and create two antigen-binding sites. Diabodies are described more fully in, for example, EP 404,097; WO 93/11161; and Hollinger et al. (Hollinger, P. et al., "Diabodies": Small bivalent and bispecific antibody fragments. *PNAS*, 1993. 90: 6444-6448); the contents of each of which are incorporated herein by reference in their entirety.

[00176] The term "intrabody" refers to a form of antibody that is not secreted from a cell in which it is produced, but instead targets one or more intracellular proteins. Intrabodies may be used to affect a multitude of cellular processes including, but not limited to intracellular trafficking, transcription, translation, metabolic processes, proliferative signaling and cell division. In some embodiments, methods of the present invention may include intrabody-based therapies. In some such embodiments, variable domain sequences and/or CDR sequences disclosed herein may be incorporated into one or more constructs for intrabody-based therapy.

[00177] As used herein, the term "monoclonal antibody" refers to an antibody obtained from a population of substantially homogeneous cells (or clones), i.e., the individual antibodies comprising the population are identical and/or bind the same epitope, except for possible variants that may arise during production of the monoclonal antibodies, such variants generally being present in minor amounts. In contrast to polyclonal antibody preparations that typically include different antibodies directed against different determinants (epitopes), each monoclonal antibody is directed against a single determinant on the antigen.

[00178] The modifier "monoclonal" indicates the character of the antibody as being obtained from a substantially homogeneous population of antibodies, and is not to be construed as requiring production of the antibody by any particular method. The monoclonal antibodies herein include "chimeric" antibodies (immunoglobulins) in which a portion of the heavy and/or light chain is identical with or homologous to corresponding sequences in antibodies derived from a particular species or belonging to a particular antibody class or subclass, while the remainder of the chain(s) is identical with or homologous to corresponding sequences in antibodies derived from another species or belonging to another antibody class or subclass, as well as fragments of such antibodies.

[00179] As used herein, the term "humanized antibody" refers to a chimeric antibody comprising a minimal portion from one or more non-human (e.g., murine) antibody source(s) with the remainder derived from one or more human immunoglobulin sources. For the most part, humanized antibodies are human immunoglobulins (recipient antibody) in which residues from the hypervariable region from an antibody of the recipient are replaced by residues from the hypervariable region from an antibody of a non-human species (donor antibody) such as mouse,

rat, rabbit or nonhuman primate having the desired specificity, affinity, and/or capacity. In one embodiment, the antibody may be a humanized full-length antibody. As a non-limiting example, the antibody may have been humanized using the methods taught in US Patent Publication NO. US20130303399, the contents of which are herein incorporated by reference in its entirety.

[00180] As used herein, the term "antibody variant" refers to a modified antibody (in relation to a native or starting antibody) or a biomolecule resembling a native or starting antibody in structure and/or function (e.g., an antibody mimetic). Antibody variants may be altered in their amino acid sequence, composition or structure as compared to a native antibody. Antibody variants may include, but are not limited to, antibodies with altered isotypes (e.g., IgA, IgD, IgE, IgG1, IgG2, IgG3, IgG4, or IgM), humanized variants, optimized variants, multispecific antibody variants (e.g., bispecific variants), and antibody fragments.

[00181] In some embodiments, pharmaceutical compositions, biocircuits, biocircuit components, effector modules including their SREs or payloads of the present invention may be antibody mimetics. As used herein, the term "antibody mimetic" refers to any molecule which mimics the function or effect of an antibody and which binds specifically and with high affinity to their molecular targets. In some embodiments, antibody mimetics may be monobodies, designed to incorporate the fibronectin type III domain (Fn3) as a protein scaffold (US 6,673,901; US 6,348,584). In some embodiments, antibody mimetics may be those known in the art including, but are not limited to affibody molecules, affilins, affitins, anticalins, avimers, Centyrins, DARPINSTM, Fynomers and Kunitz and domain peptides. In other embodiments, antibody mimetics may include one or more non-peptide regions.

[00182] In one embodiment, the antibody may comprise a modified Fc region. As a non-limiting example, the modified Fc region may be made by the methods or may be any of the regions described in US Patent Publication NO. US20150065690, the contents of which are herein incorporated by reference in its entirety.

[00183] In some embodiments, payloads of the invention may encode multispecific antibodies that bind more than one epitope. As used herein, the terms "multibody" or "multispecific antibody" refer to an antibody wherein two or more variable regions bind to different epitopes. The epitopes may be on the same or different targets. In one embodiment, the multispecific antibody may be generated and optimized by the methods described in International Patent Publication NO. WO2011109726 and US Patent Publication NO. US20150252119, the contents of which each of which are herein incorporated by reference in their entirety. These antibodies are able to bind to multiple antigens with high specificity and high affinity.

[00184] In certain embodiments, a multi-specific antibody is a "bispecific antibody" which recognizes two different epitopes on the same or different antigens. In one aspect, bispecific antibodies are capable of binding two different antigens. Such antibodies typically comprise antigen-binding regions from at least two different antibodies. For example, a bispecific monoclonal antibody (BsMAb, BsAb) is an artificial protein composed of fragments of two different monoclonal antibodies, thus allowing the BsAb to bind to two different types of antigen. Bispecific antibody frameworks may include any of those described in Riethmuller, G., 2012. Cancer Immunity, 2012, 12:12-18; Marvin et al., Acta Pharmacologica Sinica. 2005, 26(6):649-658; and Schaefer et al., PNAS. 2011, 108(27): 11187-11192, the contents of each of which are herein incorporated by reference in their entirety. New generations of BsMAb, called "trifunctional bispecific" antibodies, have been developed. These consist of two heavy and two light chains, one each from two different antibodies, where the two Fab regions (the arms) are directed against two antigens, and the Fc region (the foot) comprises the two heavy chains and forms the third binding site.

[00185] In some embodiments, payloads may encode antibodies comprising a single antigen-binding domain. These molecules are extremely small, with molecular weights approximately one-tenth of those observed for full-sized mAbs. Further antibodies may include "nanobodies" derived from the antigen-binding variable heavy chain regions (VHHs) of heavy chain antibodies found in camels and llamas, which lack light chains (Nelson, A. L., MAbs.2010. Jan-Feb; 2(1):77–83).

[00186] In some embodiments, the antibody may be "miniaturized". Among the best examples of mAb miniaturization are the small modular immunopharmaceuticals (SMIPs) from Trubion Pharmaceuticals. These molecules, which can be monovalent or bivalent, are recombinant single-chain molecules containing one VL, one VH antigen-binding domain, and one or two constant "effector" domains, all connected by linker domains. Presumably, such a molecule might offer the advantages of increased tissue or tumor penetration claimed by fragments while retaining the immune effector functions conferred by constant domains. At least three "miniaturized" SMIPs have entered clinical development. TRU-015, an anti-CD20 SMIP developed in collaboration with Wyeth, is the most advanced project, having progressed to Phase 2 for rheumatoid arthritis (RA). Earlier attempts in systemic lupus erythrematosus (SLE) and B cell lymphomas were ultimately discontinued. Trubion and Facet Biotechnology are collaborating in the development of TRU-016, an anti-CD37 SMIP, for the treatment of CLL and other lymphoid neoplasias, a project that has reached Phase 2. Wyeth has licensed the anti-CD20 SMIP SBI-087 for the treatment of autoimmune diseases, including RA, SLE and possibly multiple sclerosis, although

these projects remain in the earliest stages of clinical testing. (Nelson, A. L., MAbs, 2010. Jan-Feb; 2(1):77–83).

[00187] On example of miniaturized antibodies is called "unibody" in which the hinge region has been removed from IgG4 molecules. While IgG4 molecules are unstable and can exchange light-heavy chain heterodimers with one another, deletion of the hinge region prevents heavy chain-heavy chain pairing entirely, leaving highly specific monovalent light/heavy heterodimers, while retaining the Fc region to ensure stability and half-life in vivo. This configuration may minimize the risk of immune activation or oncogenic growth, as IgG4 interacts poorly with FcRs and monovalent unibodies fail to promote intracellular signaling complex formation (see, e.g., Nelson, A. L., MAbs, 2010. Jan-Feb; 2(1):77–83).

[00188] In some embodiments, payloads of the invention may encode single-domain antibodies (sdAbs, or nanobodies) which are antibody fragment consisting of a single monomeric variable antibody domain. Like a whole antibody, it is able to bind selectively to a specific antigen. In one aspect, a sdAb may be a "Camel Ig or "camelid VHH". As used herein, the term "camel Ig" refers to the smallest known antigen-binding unit of a heavy chain antibody (Koch-No lte, et al, *FASEB J.*, 2007, 21: 3490- 3498). A "heavy chain antibody" or a "camelid antibody" refers to an antibody that contains two VH domains and no light chains (Riechmann L. et al, *J. Immunol. Methods*, 1999, 231: 25-38; International patent publication NOs. WO1994/04678 and W01994/025591; and U.S. Patent No. 6,005,079). In another aspect, a sdAb may be a "immunoglobulin new antigen receptor" (IgNAR). As used herein, the term "immunoglobulin new antigen receptor" refers to class of antibodies from the shark immune repertoire that consist of homodimers of one variable new antigen receptor (VNAR) domain and five constant new antigen receptor (CNAR) domains. IgNARs represent some of the smallest known immunoglobulin-based protein scaffolds and are highly stable and possess efficient binding characteristics. The inherent stability can be attributed to both (i) the underlying Ig scaffold, which presents a considerable number of charged and hydrophilic surface exposed residues compared to the conventional antibody VH and VL domains found in murine antibodies; and (ii) stabilizing structural features in the complementary determining region (CDR) loops including inter-loop disulphide bridges, and patterns of intra-loop hydrogen bonds.

[00189] In some embodiments, payloads of the invention may encode intrabodies. Intrabodies are a form of antibody that is not secreted from a cell in which it is produced, but instead targets one or more intracellular proteins. Intrabodies are expressed and function intracellularly, and may be used to affect a multitude of cellular processes including, but not limited to intracellular trafficking, transcription, translation, metabolic processes, proliferative signaling and cell

division. In some embodiments, methods described herein include intrabody-based therapies. In some such embodiments, variable domain sequences and/or CDR sequences disclosed herein are incorporated into one or more constructs for intrabody-based therapy. For example, intrabodies may target one or more glycated intracellular proteins or may modulate the interaction between one or more glycated intracellular proteins and an alternative protein.

[00190] The intracellular expression of intrabodies in different compartments of mammalian cells allows blocking or modulation of the function of endogenous molecules (Biocca, et al., *EMBO J.* 1990, 9: 101-108; Colby et al., *Proc. Natl. Acad. Sci. U.S.A.* 2004, 101: 17616-17621). Intrabodies can alter protein folding, protein-protein, protein-DNA, protein-RNA interactions and protein modification. They can induce a phenotypic knockout and work as neutralizing agents by direct binding to the target antigen, by diverting its intracellular trafficking or by inhibiting its association with binding partners. With high specificity and affinity to target antigens, intrabodies have advantages to block certain binding interactions of a particular target molecule, while sparing others.

[00191] Sequences from donor antibodies may be used to develop intrabodies. Intrabodies are often recombinantly expressed as single domain fragments such as isolated VH and VL domains or as a single chain variable fragment (scFv) antibody within the cell. For example, intrabodies are often expressed as a single polypeptide to form a single chain antibody comprising the variable domains of the heavy and light chains joined by a flexible linker polypeptide. Intrabodies typically lack disulfide bonds and are capable of modulating the expression or activity of target genes through their specific binding activity. Single chain intrabodies are often expressed from a recombinant nucleic acid molecule and engineered to be retained intracellularly (e.g., retained in the cytoplasm, endoplasmic reticulum, or periplasm). Intrabodies may be produced using methods known in the art, such as those disclosed and reviewed in: (Marasco et al., *PNAS*, 1993, 90: 7889-7893; Chen et al., *Hum. Gene Ther.* 1994, 5:595-601; Chen et al., 1994, *PNAS*, 91: 5932-5936; Maciejewski et al., 1995, *Nature Med.*, 1: 667-673; Marasco, 1995, *Immunotech*, 1: 1-19; Mhashilkar, et al., 1995, *EMBO J.* 14: 1542-51; Chen et al., 1996, *Hum. Gene Therap.*, 7: 1515-1525; Marasco, *Gene Ther.* 4:11-15, 1997; Rondon and Marasco, 1997, Annu. Rev. Microbiol. 51:257-283; Cohen, et al., 1998, Oncogene 17:2445-56; Proba et al., 1998, J. Mol. Biol. 275:245-253; Cohen et al., 1998, Oncogene 17:2445-2456; Hassanzadeh, et al., 1998, FEBS Lett. 437:81-6; Richardson et al., 1998, Gene Ther. 5:635-44; Ohage and Steipe, 1999, J. Mol. Biol. 291:1119-1128; Ohage et al., 1999, J. Mol. Biol. 291:1129-1134; Wirtz and Steipe, 1999, Protein Sci. 8:2245-2250; Zhu et al., 1999, J. Immunol. Methods 231:207-222; Arafat et al., 2000, Cancer Gene Ther. 7:1250-6; der Maur et al., 2002, J. Biol. Chem.

277:45075-85; Mhashilkar et al., 2002, Gene Ther. 9:307-19; and Wheeler et al., 2003, FASEB J. 17: 1733-5; and references cited therein).

[00192] In some aspects, payloads of the invention may encode biosynthetic antibodies as described in U.S. Patent No. 5,091,513, the contents of which are herein incorporated by reference in their entirety. Such antibody may include one or more sequences of amino acids constituting a region which behaves as a biosynthetic antibody binding site (BABS). The sites comprise 1) non-covalently associated or disulfide bonded synthetic VH and VL dimers, 2) VH-VL or VL-VH single chains wherein the VH and VL are attached by a polypeptide linker, or 3) individuals VH or VL domains. The binding domains comprise linked CDR and FR regions, which may be derived from separate immunoglobulins. The biosynthetic antibodies may also include other polypeptide sequences which function, e.g., as an enzyme, toxin, binding site, or site of attachment to an immobilization media or radioactive atom. Methods are disclosed for producing the biosynthetic antibodies, for designing BABS having any specificity that can be elicited by in vivo generation of antibody, and for producing analogs thereof.

[00193] In some embodiments, payloads may encode antibodies with antibody acceptor frameworks taught in U.S. Patent No. 8,399,625. Such antibody acceptor frameworks may be particularly well suited accepting CDRs from an antibody of interest.

[00194] In one embodiment, the antibody may be a conditionally active biologic protein. An antibody may be used to generate a conditionally active biologic protein which are reversibly or irreversibly inactivated at the wild type normal physiological conditions as well as to such conditionally active biologic proteins and uses of such conditional active biologic proteins are provided. Such methods and conditionally active proteins are taught in, for example, International Publication No. WO2015175375 and WO2016036916 and US Patent Publication No. US20140378660, the contents of each of which are incorporated herein by reference in their entirety.

Antibody preparations

[00195] The preparation of antibodies, whether monoclonal or polyclonal, is known in the art. Techniques for the production of antibodies are well known in the art and described, e.g. in Harlow and Lane "Antibodies, A Laboratory Manual", Cold Spring Harbor Laboratory Press, 1988; Harlow and Lane "Using Antibodies: A Laboratory Manual" Cold Spring Harbor Laboratory Press, 1999 and "Therapeutic Antibody Engineering: Current and Future Advances Driving the Strongest Growth Area in the Pharmaceutical Industry" Woodhead Publishing, 2012.

[00196] The antibodies and fragments and variants thereof as described herein can be produced using recombinant polynucleotides. In one embodiment, the polynucleotides have a modular

design to encode at least one of the antibodies, fragments or variants thereof. As a non-limiting example, the polynucleotide construct may encode any of the following designs: (1) the heavy chain of an antibody, (2) the light chain of an antibody, (3) the heavy and light chain of the antibody, (4) the heavy chain and light chain separated by a linker, (5) the VH1, CH1, CH2, CH3 domains, a linker and the light chain or (6) the VH1, CH1, CH2, CH3 domains, VL region, and the light chain. Any of these designs may also comprise optional linkers between any domain and/or region. The polynucleotides of the present invention may be engineered to produce any standard class of immunoglobulins using an antibody described herein or any of its component parts as a starting molecule.

[00197] Recombinant antibody fragments may also be isolated from phage antibody libraries using techniques well known in the art and described in e.g. Clackson et al., 1991, Nature 352: 624-628; Marks et al., 1991, J. Mol. Biol. 222: 581-597. Recombinant antibody fragments may be derived from large phage antibody libraries generated by recombination in bacteria (Sblattero and Bradbury, 2000, Nature Biotechnology 18:75-80; the contents of which are incorporated herein by reference in its entirety).

Antibodies used for immunotherapy

[00198] In some embodiments, payloads of the present invention may be antibodies, fragments and variants thereof which are specific to tumor specific antigens (TSAs) and tumor associated antigens (TAAs). Antibodies circulate throughout the body until they find and attach to the TSA/TAA. Once attached, they recruit other parts of the immune system, increasing ADCC (antibody dependent cell-mediated cytotoxicity) and ADCP (antibody dependent cell-mediated phagocytosis) to destroy tumor cells. As used herein, the term "tumor specific antigen (TSA)" means an antigenic substance produced in tumor cells, which can trigger an anti-tumor immune response in a host organism. In one embodiment, a TSA may be a tumor neoantigen. The tumor antigen specific antibody mediates complement-dependent cytotoxic response against tumor cells expressing the same antigen.

[00199] In some embodiments, the tumor specific antigens (TSAs), tumor associated antigens (TAAs), pathogen associated antigens, or fragments thereof can be expressed as a peptide or as an intact protein or portion thereof. The intact protein or a portion thereof can be native or mutagenized. Antigens associated with cancers or virus-induced cancers as described herein are well-known in the art. Such a TSA or TAA may be previously associated with a cancer or may be identified by any method known in the art.

[00200] In one embodiment, the antigen may be GD2 ganglioside. In one embodiment, payloads of the present invention may be antibodies, fragments and variants thereof which are

specific to GD2 antigen. Gangliosides expressed on the tumor cell surface can be targets for cancer immunotherapy. GD2 is a disialoganglioside with a molecular formula of C74H134N4032. Gangliosides are acidic glycosphingolipids found on the outer surface of most cell membranes. They are ideal targets for immunotherapy because of the high antigen density, lack of modulation, relative homogeneity in many tumors and the possibility of up regulation by cytokines. Many tumors have abnormal glycolipid composition and structure. GD2 has been found in a wide spectrum of human tumors, including those of neuroectodermal or epithelial origin, virtually all melanomas, and approximately 50% of tumor samples from osteosarcoma and soft tissue sarcoma. Antibodies with high affinity for GD2 include, but not limited to 1B7, 2H12, 1G2, 1E9, 1H3, 2F5, 2F7, 31F9, 31F9V2, 32E2, chl4.18, hul4.18, 3F8, 8B6, 4B5, 1A7, A1G4, GD2 mimotopes, hul4.18K322A, 5F11, 3G6, 14g2a, and 14.18. In one embodiment, the GD antibody is the 14g2a antibody (Mujoo K., et al. (1989) Cancer Res. 1;49(11):2857-61; the contents of which are incorporated herein by reference in its entirety). Any of the GD2 antibodies described in Long A.H. et al. (2015) Nat Med. 21(6):581-90; the contents of which are incorporated by reference in their entirety).

[00201] In one embodiment, the antigen is HER2 antigen. In one embodiment, payloads of the present invention may be antibodies, fragments and variants thereof which are specific to HER2 antigen. HER2 is the oncogene product of human epidermal cell growth factor receptor 2 related oncogenes and is a transmembrane receptor protein having a molecular weight of 185 kDa and having a tyrosine kinase domain. HER2 is a member of the EGFR family consisting of HER1 (EGFR, ERBB1), HER2 (neu, ERBB-2), HER2 (ErbB-3), and Her4 (ErbB-4) and is known to be autophosphorylated at intracellular tyrosine residues by its homodimer formation or heterodimer formation with another EGFR receptor HER1, HER3, HER4 and is activated in this manner. Thereby playing an important role in cell growth, differentiation, and survival in normal cells and tumor cells. In some embodiments, HER2 antibodies useful in the present invention may include 3B5 (from Oncogene Science/BAYER), 2C4 (ATCC HB-12697), 7C2 (ATCC HB-12215), ApoB17F/ocHER2, 8A4 (ATCC PTA-4565), A10A12 (ATCC PTA- 4566), 9G6 , 7H4, AlOE9, A12D6, A6B12, A10E11, B3G4, A5C7, 13A11, 11C11, 13E11, Her2Bi (OKT3 x 9184), Her2Bi (OKT3 x Herc), 7F3 (ATCC HB-12216), huMAb4D5-1, huMAb4D5-2, huMAb4D5-3, huMAb4D5-4, huMAb4D5-6, huMAb4D5-7, 520C9, CB-11 (from Novocastra Laboratories), NCLB12 (from Novocastra Laboratories), humanized 2C4 mutant 560, humanized 2C4 mutant 561, humanized 2C4 mutant 562, humanized 2C4 mutant 568, humanized 2C4 mutant 569, humanized 2C4 mutant 570, humanized 2C4 mutant 571, humanized 2C4 mutant 56869, 3E8, 3H4, Cl 11 (NeoMarkers), HER-81, 452F2, 736G9, 741F8, 758G5, 761B10 , anti-

p185HER2/FcγRIII (CD16) , anti-CD3/anti-p185HER2 , Hu4D5-8 and variants, 4D5-H, CB11 (from Ventana Medical Scientific Instruments), 6E9, 2H11, 5B8, 7D3, HER50, HER66, HER70, scFv C6.5, scFv C6ML3-9 (ML3.9 or C6ML3.9), scFv C6MH3-B1 (B1 or C6MH3.B1), scFv C6-B1D2, (B1D2 or C6MH3-B1D2), ALM, L87, N28 , N12 , MGr6 , 9GG.10 (Neomarkers), MGFc-5 (V379M), MGFc-9 (F243I, V379L), MGFc-10 (K288N, A330S, P396L), MGFc-13 (K334E, T359N, T366S), MGFc-27 (G316D, A378V, D399E), MGFc-37 (K248M), MGFc-39 (E293V Q295E, A327T), MGFc-38 (K392T, P396L), MGFc-41 (H268N, P396L), MGFc-23 (K334E, R292L), MGFc-44, MGFc-45, MDX-210, 17.6.4 , HER2-PY1248, MAb74, FRP5, TAb250, HER-81, PN2A, mAb 191924 (R&D systems), IDMI, scFv23, Ab-3, Ab-5 , 2502A, Rexomun, MAB-1129 (R&D systems), and MM-111. In one embodiment, antibodies with high affinity may be derived from any of the HER2 antibody heavy and light chain variables described in Table 7.

[00202] In one embodiment of the present invention, the antigen is CD33. In one embodiment, payloads of the present invention may be antibodies, fragments and variants thereof which are specific to CD33 antigen. Acute myeloid leukemia (AML) is the second most common acute leukemia in the United States. The commonly applied therapy of leukemic disease includes irradiation and/or chemotherapy. However, very often 65-80% of patients receiving treatment relapse because the cells that survived the chemotherapy are enriched in AML leukemia stem cells (AML-LSCs), and constitute a reservoir of cells capable of re-expanding and causing a relapse. AML-LSCs express a characteristic set of cell surface antigens including among other CD33. CD33 (Sialic acid binding Ig-like lectin 3) or SIGLEC3(UNIPROT ID: P20138) is a transmembrane receptor expressed on cells of myeloid lineage. It is usually considered myeloid specific, but it can also be found on some lymphoid cells. It binds to sialic acid, therefore is a member of the SIGLEC family of lectins. Exemplary antibodies targeting CD33 may include, but are not limited to M195, M2H12, DRB2, My 9-6. In one embodiment, the antibody is derived from My9.6. In some embodiments, antibodies with high affinity may be derived from any of the CD33 antibody heavy and light chain variables described in Table 7.

[00203] In one embodiment, the antigen of the present invention is a BCMA (B-cell maturation antigen), also referred to as the CD269. In one embodiment, payloads of the present invention may be antibodies, fragments and variants thereof which are specific to BCMA antigen. BCMA antigen (UNIPROT ID: Q02223) is encoded by the gene, TNFRS17. BCMA is a member of the TNF receptor super family. It binds to B cell activating factor (BAFF) and a proliferation inducing ligand (APRIL). Among non-malignant cells, BCMA has been reported to be expressed mostly by plasma cells and subsets of mature B cells, but not T cells and NK cells. Therefore,

BCMA represents a suitable therapeutic candidate in the treatment of multiple myeloma. Exemplary antibodies targeting BCMA include, but are not limited to BCMA 50, BCMA30, C11D5.3 and C13F12.1 In one embodiment, the antibody is derived from C11D5.3. In some embodiments, antibodies with high affinity may be derived from any of the BCMA antibody heavy and light chain variables described in Table 7.

[00204] In one embodiment, the antigen of the present invention is a CD276 (also known as B7-H3). In one embodiment, payloads of the present invention may be antibodies, fragment, and variants thereof which are specific to CD276. CD276 is expressed in a variety of human tumors, including pediatric solid tumors and adult carcinomas. Any of the CD276 antibodies taught in International Patent publications WO2017044699 and WO2014160627 (the contents of which are incorporated herein by reference in their entirety), may be useful in the present invention. In some embodiments, antibodies with high affinity may be derived from any of the CD276 antibody heavy and light chain variables described in Table 7.

[00205] In one embodiment, the antigen of the present invention is a ALK protein. The developmentally-regulated cell surface receptor tyrosine kinase, ALK is known to be expressed as a tumor associated antigen as a fusion protein resulting from a chromosomal translocation. Cancer associated ALK was first described as a 2;5 translocation associated with nucleophosphomin (NPM) in anaplastic large cell leukemia. The fusion protein is composed of intracellular component of NPM fused to ALK. In some embodiments, the ALK antigen may be the extracellular portion of the protein. Any of the antibodies, fragment and variants specific to ALK may be useful in the present invention. In one embodiment, the ALK antibodies described in the International Patent Publication, WO2015069922 (the contents of which are incorporated by reference herein in its entirety). In some embodiments, antibodies with high affinity may be derived from any of the ALK antibody heavy and light chain variables described in Table 7.

[00206] In one embodiment, the antigen of the present invention is a CD22 antigen. In one embodiment, payloads of the present invention may be antibodies, fragment, and variants thereof which are specific to CD22. CD22 is a lineage restricted B cell antigen belonging to the immunoglobulin (Ig) superfamily. CD22 is expressed in 60-70% of B cell lymphomas and leukemias (e.g. B-chronic lymphocytic leukemia, hairy cell leukemia, acute lymphocytic leukemia (ALL) and Burkit's lymphoma) and is not present on the cell surface in early stages of B cell development or on to stem cells. In some embodiments, the antibodies, fragments, and variants thereof may be any of those taught in International Patent Publications, WO2016149578, WO2014065961, and WO2013059593A1(the contents of each of which are incorporated by reference in its entirety). In some embodiments, antibodies with high affinity

may be derived from any of the CD22 antibody heavy and light chain variables described in Table 7.

[00207] In some embodiments, the payloads of the present invention may include an antigen binding region comprising variable heavy chain and variable light chains with the amino acid sequences selected from those in Table 7.

Table 7: Variable Heavy and Light Chain Sequences

Target	Description and Clone name	Source	Antibody chain	SEQ ID NO.
ALK	ALK15 VH	SEQ ID NO. 1 in WO2015069922	VH	242
ALK	ALK48 VH	SEQ ID NO. 3 in WO2015069922	VH	243
ALK	ALK53 VH	SEQ ID NO. 5 in WO2015069922	VH	244
ALK	ALK58 VH	SEQ ID NO. 7 in WO2015069922	VH	245
ALK	humanized ALK15 VH	SEQ ID NO. 9 in WO2015069922	VH	246
ALK	humanized ALK48 VH	SEQ ID NO. 11 in WO2015069922	VH	247
ALK	humanized ALK53 VH	SEQ ID NO. 13 in WO2015069922	VH	248
ALK	humanized ALK58 VH	SEQ ID NO. 15 in WO2015069922	VH	249
ALK	ALK15 VL	SEQ ID NO. 2 in WO2015069922	VL	250
ALK	ALK48 VL	SEQ ID NO. 4 in WO2015069922	VL	251
ALK	ALK53 VL	SEQ ID NO. 6 in WO2015069922	VL	252
ALK	ALK58 VL	SEQ ID NO. 8 in WO2015069922	VL	253
ALK	humanized ALK15 VL	SEQ ID NO. 10 in WO2015069922	VL	254
ALK	humanized ALK48 VL	SEQ ID NO. 12 in WO2015069922	VL	255
ALK	humanized ALK53 VL	SEQ ID NO. 14 in WO2015069922	VL	256
ALK	humanized ALK58 VL	SEQ ID NO. 16 in WO2015069922	VL	257
CD22	CD22 VL	SEQ ID NO. 14 in WO2016149578	VL	258
CD22	CD22 (HA22 or BL22) VL	SEQ ID NO. 1 in WO2013059593	VL	259
CD22	CD22 VH	SEQ ID NO. 13 in WO2016149578	VH	260
CD22	CD22 (HA22 or BL22) VH	SEQ ID NO. 3 in WO2013059593	VH	261
CD22	CD22 (HA22 or BL22) VH	SEQ ID NO. 4 in WO2013059593	VH	262
CD276	CD276 VH	SEQ ID NO. 7 in WO2017044699	VH	263
CD276	CD276 VH (CD276.6 (m856))	SEQ ID NO. 7 in WO2014160627	VH	264
CD276	CD276 VH (CD276.1 (m851))	SEQ ID NO. 17 in WO2014160627	VH	265

CD276	CD276 VH (CD276.17 (m8517))	SEQ ID NO. 26 in WO2014160627	VH	266
CD276	CD276 VL	SEQ ID NO. 8 in WO2017044699	VL	267
CD276	CD276 VL (CD276.6 (m856))	SEQ ID NO. 8 in WO2014160627	VL	268
CD276	CD276 VL (CD276.1 (m851))	SEQ ID NO. 18 in WO2014160627	VL	269
CD276	CD276 VL (CD276.17 (m8517))	SEQ ID NO. 27 in WO2014160627	VL	270
GD2	3F8 heavy chain variable	SEQ ID No. 1 in WO2011160119	VH	271
GD2	3F8 light chain variable	SEQ ID No. 2 in WO2011160119	VL	272
GD2	3F8 heavy chain variable	SEQ ID No. 3 in WO2011160119	VH	273
GD2	humanized 3F8 heavy chain variable	SEQ ID No. 4 in WO2011160119	VH	274
GD2	humanized 3F8 light chain variable	SEQ ID No. 5 in WO2011160119	VL	275
GD2	humanized 3F8 heavy chain 2 variable	SEQ ID No. 6 in WO2011160119	VH	276
GD2	humanized 3F8 light chain 2 variable	SEQ ID No. 7 in WO2011160119	VL	277
GD2	humanized 3F8 heavy chain variable	SEQ ID No. 8 in WO2011160119	VH	278
GD2	Human GD2 heavy chain variable	SEQ ID No. 16 in WO2010002822A1	VH	279
GD2	Human GD2 light chain variable	SEQ ID No. 32 in WO2010002822A1	VL	280
GD2	chimeric Ch3F8 heavy chain-gamma 1	Cheung et al., Oncoimmunology, 2012, 1(4): 477-486	VH	281
GD2	chimeric Ch3F8 light chain-kappa	Cheung et al., Oncoimmunology, 2012, 1(4): 477-486	VL	282
GD2	humanized Hu3F8 heavy chain-gamma1	Cheung et al., Oncoimmunology, 2012, 1(4): 477-486	VH	283
GD2	humanized Hu3F8 light chain-kappa	Cheung et al., Oncoimmunology, 2012, 1(4): 477-486	VL	284
GD2	Chimeric Ch3F8 heavy chain-gamma4	Cheung et al., Oncoimmunology, 2012, 1(4): 477-486	VH	285
GD2	humanized Hu3F8 heavy chain-gamma4	Cheung et al., Oncoimmunology, 2012, 1(4): 477-486	VL	286
GD2	GD2 VH	SEQ ID NO. 17 in WO2016134284	VH	287
GD2	GD2 VL	SEQ ID NO. 18 in WO2016134285	VL	288
GD2	Murine KM666 VH (heavy chain variable region) sequence	SEQ ID NO. 9 in WO2015132604	VH	289

GD2	Humanized KM666 VH sequence	SEQ ID NO. 10 in WO2015132604	VH	290
GD2	Murine KM666 VL (light chain variable region) sequence	SEQ ID NO. 11 in WO2015132604	VL	291
GD2	Humanized KM666 VL sequence	SEQ ID NO. 12 in WO2015132604	VL	292
GD2	GD2 VL	SEQ ID NO. 1 in US20040203100	VL	293
GD2	GD2 VH	SEQ ID NO. 2 in US20040203100	VH	294
GD2	Murine KM666 VH (heavy chain variable region) sequence	SEQ ID NO. 9 in US20170066838	VH	295
GD2	Humanized KM666 VH sequence	SEQ ID NO. 10 in US20170066838	VH	296
GD2	Murine KM666 VL (light chain variable region) sequence	SEQ ID NO. 11 in US20170066838	VL	297
GD2	Humanized KM666 VL sequence	SEQ ID NO. 12 in US20170066838	VL	298
GD2	GD2 VL	SEQ ID NO.3 in US20160304620	VL	299
GD2	GD2 VH	SEQ ID NO. 4 in US20160304620	VH	300
GD2	GD2 VH	SEQ ID NO. 2 in US20150353645	VH	301
GD2	GD2 VL	SEQ ID NO. 4 in US20150353645	VL	302
GD2	GD2 VH	SEQ ID NO. 6 in US20150353645	VH	303
GD2	GD2 VL	SEQ ID NO. 8 in US20150353645	VL	304
GD2	GD2 VH	SEQ ID NO. 10 in US20150353645	VH	305
GD2	GD2 VL	SEQ ID NO. 12 in US20150353645	VL	306
GD2	GD2 VH	SEQ ID NO. 14 in US20150353645	VH	307
GD2	GD2 VL	SEQ ID NO. 16 in US20150353645	VL	308
GD2	GD2 VH	SEQ ID NO. 18 in US20150353645	VH	309
GD2	GD2 VL	SEQ ID NO. 20 in US20150353645	VL	310
GD2	GD2 VH	SEQ ID NO. 22 in US20150353645	VH	311
GD2	GD2 VL	SEQ ID NO. 24 in US20150353645	VL	312
GD2	GD2 VH	SEQ ID NO. 26 in US20150353645	VH	313
GD2	GD2 VL	SEQ ID NO. 28 in US20150353645	VL	314
GD2	GD2 VH	SEQ ID NO. 30 in US20150353645	VH	315

GD2	GD2 VL	SEQ ID NO. 32 in US20150353645	VL	316
GD2	GD2 VH	SEQ ID NO. 34 in US20150353645	VH	317
GD2	GD2 VH	SEQ ID NO. 36 in US20150353645	VH	318
GD2	GD2 VL	SEQ ID NO. 38 in US20150353645	VL	319
GD2	GD2 VH	SEQ ID NO. 40 in US20150353645	VH	320
GD2	GD2 VL	SEQ ID NO. 42 in US20150353645	VL	321
GD2	GD2 VL	SEQ ID NO. 3 in US20150139942	VL	322
GD2	GD2 VH	SEQ ID NO. 4 in US20150139942	VH	323
GD2	GD2 VL	SEQ ID NO. 7 in US20150139942	VL	324
GD2	GD2 VH	SEQ ID NO. 8 in US20150139942	VH	325
GD2	GD2 VH	SEQ ID NO. 16 in US20130287691	VH	326
GD2	GD2 VL	SEQ ID NO. 32 in US20130287691	VL	327
GD2	GD2 VH	SEQ ID NO. 40 in US20130287691	VH	328
GD2	GD2 VL	SEQ ID NO. 42 in US20130287691	VL	329
GD2	GD2 VL	SEQ ID NO. 3 in US20140134162	VL	330
GD2	GD2 VH	SEQ ID NO. 4 in US20140134162	VH	331
GD2	GD2 VH	SEQ ID NO. 20 in WO2017055385	VH	332
GD2	GD2 VL	SEQ ID NO. 20 in WO2017055385	VL	333
GD2	GD2 VH	SEQ ID NO. 3 in WO2013189516	VH	334
GD2	GD2 VL	SEQ ID NO. 4 in WO2013189516	VL	335
GD2-0-acetylated	KM8B6 GD2-0-acetylated heavy chain variable	SEQ ID No. 1 in WO2008043777	VH	336
GD2-0-acetylated	KM8B6 GD2-0-acetylated light chain variable	SEQ ID No. 2 in WO2008043777	VL	337
GD2-0-acetylated	O-acetylated-GD2 ganglioside light chain variable region	SEQ ID No. 6 in WO2015067375	VL	338
GD2-0-acetylated	O-acetylated-GD2 ganglioside heavy chain variable region	SEQ ID No. 7 in WO2015067375	VH	339
GD2-0-acetylated	GD2 VL	SEQ ID NO.7 in US20160068608	VL	340

GD2-0-acetylated	GD2 VH	SEQ ID NO.8 in US20160068608	VH	341
GD2-0-acetylated	GD2 VL (8B6)	SEQ ID NO. 7 in WO2014177271A1	VL	342
GD2-0-acetylated	GD2 VH (8B6)	SEQ ID NO. 8 in WO2014177271A1	VH	343
GD2-0-acetylated	GD2 VL	SEQ ID NO. 9 in WO2014177271A1	VL	344
GD2-0-acetylated	GD2 VH	SEQ ID NO. 10 in WO2014177271A1	VH	345
Gangliosides (including GD2)	GMab1-VH	SEQ ID No. 11 in WO2012071216	VH	346
Gangliosides (including GD2)	GMab1-VH	SEQ ID No. 12 in WO2012071216	VL	347
Gangliosides (including GD2)	GMab1-VL	SEQ ID No. 13 in WO2012071216	VH	348
Gangliosides (including GD2)	GMab2-VH	SEQ ID No. 14 in WO2012071216	VL	349
CD33	Anti CD33 VH (Clone M195)	SEQ ID NO. 11 in WO2015150526	VH	350
CD33	Anti CD33 VL (Clone M195)	SEQ ID NO. 12 in WO2015150526	VL	351
CD33	Anti CD33 VH (Clone M2H12)	SEQ ID NO. 13 in WO2015150526	VH	352
CD33	Anti CD33 VL (Clone M2H12)	SEQ ID NO. 14 in WO2015150526	VL	353
CD33	Anti CD33 VH (Clone DRB2)	SEQ ID NO. 15 in WO2015150526	VH	354
CD33	Anti CD33 VL (Clone DRB2)	SEQ ID NO. 16 in WO2015150526	VL	355
CD33	Anti CD33 VH (Clone My9-6)	SEQ ID NO. 17 in WO2015150526	VH	356
CD33	Anti CD33 VL (Clone My9-6)	SEQ ID NO. 18 in WO2015150526	VL	357
BCMA	BCMA VH (Clone BCMA-50)	SEQ ID NO. 11 in WO2015158671	VH	358
BCMA	BCMA VL (Clone BCMA-50)	SEQ ID NO. 12 in WO2015158672	VL	359
BCMA	BCMA VH (Clone BCMA-30)	SEQ ID NO. 13 in WO2015158673	VH	360
BCMA	BCMA VL (Clone BCMA-30)	SEQ ID NO. 14 in WO2015158674	VL	361
BCMA	BCMA VH (Clone C11D5.3)	SEQ ID NO. 15 in WO2015158675	VH	362

BCMA	BCMA VL (Clone C11D5.3)	SEQ ID NO. 16 in WO2015158676	VL	363
BCMA	BCMA VH (Clone C13F12.1)	SEQ ID NO. 17 in WO2015158677	VH	364
BCMA	BCMA VL (Clone C13F12.1)	SEQ ID NO. 18 in WO2015158678	VL	365
Her2	Trastuzumab (Herceptin)	SEQ ID NO. 1 in WO2017093844	VH	366
Her2	Trastuzumab (Herceptin)	SEQ ID NO. 7 in WO2017093844	VL	367
Her2	huMAb4D5-5	SEQ ID NO. 1 in US 8,075,890	VL	368
Her2	huMAb4D5-5	SEQ ID NO. 2 in US 8,075,890	VH	369
Her2	a consensus antibody variable domain	SEQ ID NO. 3 in US 8,075,890	VL	370
Her2	a consensus antibody variable domain	SEQ ID NO. 4 in US 8,075,890	VH	371
Her2	muMAb4D5	SEQ ID NO. 5 in US 8,075,890	VL	372
Her2	muMAb4D5	SEQ ID NO. 6 in US 8,075,890	VH	373
Her2	N29	No SEQ ID in WO1993003741	VH	374
Her2	N29	No SEQ ID in WO1993003741	VL	375
Her2	2C4	SEQ ID NO. 1 in US 7,981,418	VL	376
Her2	2C4	SEQ ID NO. 2 in US 7,981,418	VH	377
Her2	variant 574/Pertuzumab	SEQ ID NO. 3 in US 7,981,418	VL	378
Her2	variant 574/Pertuzumab	SEQ ID NO. 4 in US 7,981,418	VH	379
Her2	human VL consensus (hum. kappa.1, light kappa subgroup 1)	SEQ ID NO. 5 in US 7,981,418	VL	380
Her2	human VH consensus (humIII, heavy subgroup III)	SEQ ID NO. 6 in US 7,981,418	VH	381
Her2	Pertuzumab	SEQ ID NO. 13 in US 7,981,418	VL	382
Her2	Pertuzumab	SEQ ID NO. 14 in US 7,981,418	VH	383
Her2	trastuzumab/humMAb4 D5-8	SEQ ID NO. 15 in US 7,981,418	VL	384
Her2	trastuzumab/humMAb4 D5-8	SEQ ID NO. 16 in US 7,981,418	VH	385
Her2	a variant Pertuzumab light chain sequence	SEQ ID NO. 17 in US 7,981,418	VL	386
Her2	a variant Pertuzumab heavy chain sequence	SEQ ID NO. 18 in US 7,981,418	VH	387

Her2	3. F2 monoclonal antibody	SEQ ID NO. 2 in WO2001009187	VH	388
Her2	3. F2 monoclonal antibody	SEQ ID NO. 4 in WO2001009187	VL	389
Her2	1. D2 monoclonal antibody	SEQ ID NO. 6 in WO2001009187	VH	390
Her2	1. D2 monoclonal antibody	SEQ ID NO. 8 in WO2001009187	VL	391
Her2	2. E8 monoclonal antibody	SEQ ID NO. 10 in WO2001009187	VH	392
Her2	2. E8 monoclonal antibody	SEQ ID NO. 12 in WO2001009187	VL	393
Her2	2C4	SEQ ID NO. 4 in US 7,097,840	VL	394
Her2	variant 574/Pertuzumab	SEQ ID NO. 5 in US 7,097,840	VL	395
Her2	human VL subgroup	SEQ ID NO. 6 in US 7,097,840	VL	396
Her2	4D5	SEQ ID NO. 14 in WO2003068801	VH	397
Her2	Hu4D5-8	SEQ ID NO. 1 in WO2003087131	VL	398
Her2	rhuMAb	SEQ ID NO. 50 in US20040254108	VH	399
Her2	rhuMAb	SEQ ID NO. 52 in US20040254108	VL	400
Her2	her2VHCH - SM5-1 VH; human kappa chain constant (CH)	SEQ ID NO. 54 in US20040254108	VH	401
Her2	her2VLCL - SM5-1 VL; human kappa chain constant (CL)	SEQ ID NO. 56 in US20040254108	VL	402
Her2	her2VH/Fc/FL-- rhuMAb VH; IgG1 Fc; Flt3 ligand extracellular region (hFLex)	SEQ ID NO. 58 in US20040254108	VH	403
Her2	her2VH/Fc/Link/FL – rhuMAb; IgG1 Fc; linker; Flt3 ligand extracellular region (hFLex)	SEQ ID NO. 60 in US20040254108	VH	404
Her2	Herceptin Fab	SEQ ID NO. 9 in US20050260711A1	VL	405
Her2	Herceptin Fab	SEQ ID NO. 10 in US20050260711A1	VH	406
Her2	Pertuzumab with a signal peptide sequence	SEQ ID NO. 17 in US20060018899	VL	407
Her2	Pertuzumab with a signal peptide sequence	SEQ ID NO. 18 in US20060018899	VH	408
Her2	Periplasmic Fab-4D5	SEQ ID NO. 30 in US 7,632,924	VL	409

Her2	Periplasmic Fab-4D5	SEQ ID NO. 31 in US 7,632,924	VH	410
Her2	trastuzumab A88C	SEQ ID NO. 6 in US7521541	VH	411
Her2	trastuzumab A121C	SEQ ID NO. 7 in US7521541	VH	412
Her2	trastuzumab V110C	SEQ ID NO. 8 in US7521541	VL	413
Her2	B1D2	SEQ ID NO. 42 in US7,332,585	VH	414
Her2	B1D2	SEQ ID NO. 47 in US7,332,585	VL	415
Her2	Fab63	SEQ ID NO. 7 in US20100047230	VH	416
Her2	Fab63	SEQ ID NO. 8 in US20100047230	VL	417
Her2	Herceptin	SEQ ID NO. 3 in US20160256561	VH	418
Her2	anti-her2/neu antibody with a signal peptide	SEQ ID NO. 1 in US 9,534,057	VH	419
Her2	anti-her2/neu antibody with a signal peptide	SEQ ID NO. 2 in US 9,534,057	VL	420
anti-Her2/neu - anti-CD3	anti-Her2/neu - anti-CD3 bispecific antibody VH	SEQ ID NO. 3 in WO2014079000A1	VH	421

Chimeric antigen receptors (CARs)

[00208] In some embodiments, payloads of the present invention may be a chimeric antigen receptors (CARs) which when transduced into immune cells (e.g., T cells and NK cells), can re-direct the immune cells against the target (e.g., a tumor cell) which expresses a molecule recognized by the extracellular target moiety of the CAR.

[00209] As used herein, the term "chimeric antigen receptor (CAR)" refers to a synthetic receptor that mimics TCR on the surface of T cells. In general, a CAR is composed of an extracellular targeting domain, a transmembrane domain/region and an intracellular signaling/activation domain. In a standard CAR receptor, the components: the extracellular targeting domain, transmembrane domain and intracellular signaling/activation domain, are linearly constructed as a single fusion protein. The extracellular region comprises a targeting domain/moiety (e.g., a scFv) that recognizes a specific tumor antigen or other tumor cell-surface molecules. The intracellular region may contain a signaling domain of TCR complex (e.g., the signal region of CD3ζ), and/or one or more costimulatory signaling domains, such as those from CD28, 4-1BB (CD137) and OX-40 (CD134). For example, a "first-generation CAR" only has the CD3ζ signaling domain. In an effort to augment T-cell persistence and proliferation, costimulatory intracellular domains are added, giving rise to second generation CARs having a

CD3ζsignal domain plus one costimulatory signaling domain, and third generation CARs having CD3ζ signal domain plus two or more costimulatory signaling domains. A CAR, when expressed by a T cell, endows the T cell with antigen specificity determined by the extracellular targeting moiety of the CAR. Recently, it is also desirable to add one or more elements such as homing and suicide genes to develop a more competent and safer architecture of CAR, so called the fourth-generation CAR.

[00210] In some embodiments, the extracellular targeting domain is joined through the hinge (also called space domain or spacer) and transmembrane regions to an intracellular signaling domain. The hinge connects the extracellular targeting domain to the transmembrane domain which transverses the cell membrane and connects to the intracellular signaling domain. The hinge may need to be varied to optimize the potency of CAR transformed cells toward cancer cells due to the size of the target protein where the targeting moiety binds, and the size and affinity of the targeting domain itself. Upon recognition and binding of the targeting moiety to the target cell, the intracellular signaling domain leads to an activation signal to the CAR T cell, which is further amplified by the "second signal" from one or more intracellular costimulatory domains. The CAR T cell, once activated, can destroy the target cell.

[00211] In some embodiments, the CAR of the present invention may be split into two parts, each part is linked a dimerizing domain, such that an input that triggers the dimerization promotes assembly of the intact functional receptor. Wu and Lim recently reported a split CAR in which the extracellular CD19 binding domain and the intracellular signaling element are separated and linked to the FKBP domain and the FRB* (T2089L mutant of FKBP-rapamycin binding) domain that heterodimerize in the presence of the rapamycin analog AP21967. The split receptor is assembled in the presence of AP21967 and together with the specific antigen binding, activates T cells (Wu et al., *Science*, 2015, 625(6258): aab4077).

[00212] In some embodiments, the CAR of the present invention may be designed as an inducible CAR. Sakemura et al recently reported the incorporation of a Tet-On inducible system to the CD19 CAR construct. The CD19 CAR is activated only in the presence of doxycycline (Dox). Sakemura reported that Tet-CD19CAR T cells in the presence of Dox were equivalently cytotoxic against $CD19^+$ cell lines and had equivalent cytokine production and proliferation upon CD19 stimulation, compared with conventional CD19CAR T cells (Sakemura et al., *Cancer Immuno. Res.*, 2016, Jun 21, Epub ahead of print). In one example, this Tet-CAR may be the payload of the effector module under the control of SREs (e.g., DDs) of the invention. The dual systems provide more flexibility to turn-on and off of the CAR expression in transduced T cells.

[00213] According to the present invention, the payload of the present invention may be a first-generation CAR, or a second-generation CAR, or a third-generation CAR, or a fourth-generation CAR. Representative effector module embodiments comprising CAR constructs are illustrated in Figures 13-18. In some embodiments, the payload of the present invention may be a full CAR construct composed of the extracellular domain, the hinge and transmembrane domain and the intracellular signaling region. In other embodiments, the payload of the present invention may be a component of the full CAR construct including an extracellular targeting moiety, a hinge region, a transmembrane domain, an intracellular signaling domain, one or more co-stimulatory domain, and other additional elements that improve CAR architecture and functionality including but not limited to a leader sequence, a homing element and a safety switch, or the combination of such components.

[00214] CARs regulated by biocircuits and compositions of the present invention are tunable and thereby offer several advantages. The reversible on-off switch mechanism allows management of acute toxicity caused by excessive CAR-T cell expansion. Pulsatile CAR expression using SREs of the present invention may be achieved by cycling ligand level. The ligand conferred regulation of the CAR may be effective in offsetting tumor escape induced by antigen loss, avoiding functional exhaustion caused by tonic signaling due to chronic antigen exposure and improving the persistence of CAR expressing cells *invivo*.

[00215] In some embodiments, biocircuits and compositions of the invention may be utilized to down regulate CAR expression to limit on target on tissue toxicity caused by tumor lysis syndrome. Down regulating the expression of the CARs of the present invention following anti-tumor efficacy may prevent (1) On target off tumor toxicity caused by antigen expression in normal tissue. (2) antigen independent activation *in vivo*.

Extracellular targeting domain/moiety

[00216] In accordance with the invention, the extracellular target moiety of a CAR may be any agent that recognizes and binds to a given target molecule, for example, a neoantigen on tumor cells, with high specificity and affinity. The target moiety may be an antibody and variants thereof that specifically binds to a target molecule on tumor cells, or a peptide aptamer selected from a random sequence pool based on its ability to bind to the target molecule on tumor cells, or a variant or fragment thereof that can bind to the target molecule on tumor cells, or an antigen recognition domain from native T- cell receptor (TCR) (e.g. CD4 extracellular domain to recognize HIV infected cells), or exotic recognition components such as a linked cytokine that leads to recognition of target cells bearing the cytokine receptor, or a natural ligand of a receptor.

[00217] In some embodiments, the targeting domain of a CAR may be a Ig NAR, a Fab fragment, a Fab' fragment, a F(ab)'2 fragment, a F(ab)'3 fragment, Fv, a single chain variable fragment (scFv), a bis-scFv, a (scFv)2, a minibody, a diabody, a triabody, a tetrabody, a disulfide stabilized Fv protein (dsFv), a unitbody, a nanobody, or an antigen binding region derived from an antibody that specifically recognizes a target molecule, for example a tumor specific antigen (TSA). In one embodiment, the targeting moiety is a scFv antibody. The scFv domain, when it is expressed on the surface of a CAR T cell and subsequently binds to a target protein on a cancer cell, is able to maintain the CAR T cell in proximity to the cancer cell and to trigger the activation of the T cell. A scFv can be generated using routine recombinant DNA technology techniques and is discussed in the present invention.

[00218] In some embodiments, natural ligands may be used as the targeting moieties of the CARs of the present invention. Such natural ligands may be capable of binding to the antigens with affinity in the range of the scFvs and can redirect T cells specificity and effector functions to target cells expressing the complementary receptor. In some embodiments, the targeting moiety of the CAR may be neuregulin-1 (NRG1) which is a natural ligand for HER3 and HER4; VEGF which is a natural ligand of VEGFR; IL13 wildtype protein or IL13 mutein e.g. E13Y which binds to IL13Ra2; NKG2D ligand, which is a natural ligand of NKG2D receptor; CD70 which is ligand of CD27; and a proliferation-inducing ligand (APRIL) which is a natural high affinity ligand for BCMA8 and transmembrane activator and CAML interactor (TACI). Any of the ligand based BCMA CARs taught in the US Patent Publication No. US20160362467A1, the contents of which are incorporated by reference in their entirety.

[00219] In one embodiment, the targeting moiety of the CAR may recognize antigen such as, but not limited to a ganglioside, a growth factor receptor, a lectin or any other cell surface antigen. In some embodiments, any of the sequences described in Table 7 or Table 8 may be useful in the present invention.

[00220] In some embodiments, the targeting moiety of a CAR may recognize a tumor specific antigen (TSA), for example a cancer neoantigen that is only expressed by tumor cells because of genetic mutations or alterations in transcription which alter protein coding sequences, therefore creating novel, foreign antigens. The genetic changes result from genetic substitution, insertion, deletion or any other genetic changes of a native cognate protein (i.e. a molecule that is expressed in normal cells

[00221] In some embodiments, the targeting moieties of the present invention may be scFv comprising the amino acid sequences in Table 8.

Table 8: scFv sequences

Target	Description and Clone name	Source	SEQ ID NO.
ALK	ALK15 scFv	SEQ ID NO. 17 in WO2015069922	422
ALK	ALK48 scFv	SEQ ID NO. 18 in WO2015069922	423
ALK	ALK53 scFv	SEQ ID NO. 19 in WO2015069922	424
ALK	ALK58 scFv	SEQ ID NO. 20 in WO2015069922	425
ALK	humanized ALK15 scFv	SEQ ID NO. 21 in WO2015069922	426
ALK	humanized ALK48 scFv	SEQ ID NO. 22 in WO2015069922	427
ALK	humanized ALK53 scFv	SEQ ID NO. 23 in WO2015069922	428
ALK	humanized ALK58 scFv	SEQ ID NO. 24 in WO2015069922	429
CD22	CD22 (m971) scFv	SEQ ID NO. 9 in WO2014065961	430
CD22	CD22 (HA22 or BL22) scFv	SEQ ID NO. 5 in WO2013059593	431
CD22	CD22 (HA22 or BL22) scFv	SEQ ID NO. 6 in WO2013059593	432
CD276	CD276 scFv	SEQ ID NO. 21 in WO2017044699	433
CD276	CD276 scFv (CD276.6)	SEQ ID NO. 10 in WO2014160627	434
CD276	CD276 scFv (CD276.1)	SEQ ID NO. 19 in WO2014160627	435
CD276	CD276 scFv (CD276.17)	SEQ ID NO. 28 in WO2014160627	436
GD2	hu3F8/huOKT3 scFv	SEQ ID No. 23 in WO2011160119	437
GD2	hu3F8/C8.2.5 scFv	SEQ ID No. 24 in WO2011160119	438
Gangliosides including GD2	DMab14-86184 scFv	SEQ ID No. 6 in WO2012071216	439
Gangliosides including GD2	GMab1 scFv	SEQ ID No. 20 in WO2012071216	440
Gangliosides including GD2	GMab2 scFV	SEQ ID No. 21 in WO2012071216	441
Gangliosides including GD2	DMab14 scFV	SEQ ID No. 22 in WO2012071216	442
GD2	GD2 scFv	SEQ ID NO. 19 in WO2016134286	443
GD2	GD2 scFv	SEQ ID NO. 20 in WO2016134287	444
GD2	GD2 scFv	SEQ ID NO. 21 in WO2016134288	445
GD2	Murine KM666 sequence	SEQ ID NO. 7 in WO2015132604	446
GD2	Humanized KM666 sequence	SEQ ID NO. 8 in WO2015132604	447
GD2	GD2 scFv	SEQ ID NO. 11 in US20160032009	448
GD2	GD2 scFv	SEQ ID NO. 12 in US20160032009	449
GD2	GD2 scFv	SEQ ID NO. 13 in US20160032009	450
GD2	GD2 scFv	SEQ ID NO. 14 in US20160032009	451
GD2	GD2 scFv	SEQ ID NO. 15 in US20160032009	452
GD2	GD2 scFv	SEQ ID NO. 16 in US20160032009	453

GD2	GD2 scFv	SEQ ID NO. 17 in US20160032009	454
GD2	GD2 scFv	SEQ ID NO. 18 in US20160032009	455
GD2	GD2 scFv	SEQ ID NO. 19 in US20160032009	456
GD2	GD2 scFv	SEQ ID NO. 20 in US20160032009	457
GD2	GD2 scFv	SEQ ID NO. 21 in US20160032009	458
GD2	GD2 scFv	SEQ ID NO. 22 in US20160032009	459
GD2	GD2 scFv	SEQ ID NO. 23 in US20160032009	460
GD2	GD2 scFv	SEQ ID NO. 24 in US20160032009	461
GD2	GD2 scFv	SEQ ID NO. 25 in US20160032009	462
GD2	Murine KM666 scFv sequence	SEQ ID NO. 7 in US20170066838	463
GD2	Humanized KM666 scFv sequence	SEQ ID NO. 8 in US20170066838	464
GD2	GD2 (clone 1A7) scFv	SEQ ID NO. 1 in US 20050287148A1	465
Her2	F5	SEQ ID NO. 1 in US 9,388,244	466
Her2	C1	SEQ ID NO. 2 in US 9,388,244	467
anti-Her2/neu - anti-CD3	anti-Her2/neu - anti-CD3 bispecific antibody scFv	SEQ ID NO. 1 in WO2014079000A1	468
Her2	F5	SEQ ID NO. 1 in US7,332,580	469
Her2	HER3.B12	SEQ ID NO. 6 in US7,332,580	470
Her2	FL/Fc/HER2Fv - Flt3 ligand extracellular region (hFLex); IgG1 Fc; rhuMAb ScFv	SEQ ID NO. 62 in US20040254108	471
Her2	Periplasmic 6×-His (SEQ ID NO. 933) C terminal scFv-4D5	SEQ ID NO. 26 in US 7,632,924	472
Her2	Periplasmic 6×-His (SEQ ID NO. 933) N terminal scFv-4D5	SEQ ID NO. 28 in US 7,632,924	473

Intracellular signaling domains

[00222] The intracellular domain of a CAR fusion polypeptide, after binding to its target molecule, transmits a signal to the immune effector cell, activating at least one of the normal effector functions of immune effector cells, including cytolytic activity (e.g., cytokine secretion) or helper activity. Therefore, the intracellular domain comprises an "intracellular signaling domain" of a T cell receptor (TCR).

[00223] In some aspects, the entire intracellular signaling domain can be employed. In other aspects, a truncated portion of the intracellular signaling domain may be used in place of the intact chain if it transduces the effector function signal.

[00224] In some embodiments, the intracellular signaling domain of the present invention may contain signaling motifs which are known as immunoreceptor tyrosine-based activation motifs (ITAMs). Examples of ITAM containing cytoplasmic signaling sequences include those derived from TCR CD3zeta, FcR gamma, FcR beta, CD3 gamma, CD3 delta, CD3 epsilon, CD5, CD22, CD79a, CD79b, and CD66d. In one example, the intracellular signaling domain is a CD3 zeta (CD3ζ) signaling domain.

[00225] In some embodiments, the intracellular region of the present invention further comprises one or more costimulatory signaling domains which provide additional signals to the immune effector cells. These costimulatory signaling domains, in combination with the signaling domain can further improve expansion, activation, memory, persistence, and tumor-eradicating efficiency of CAR engineered immune cells (e.g., CAR T cells). In some cases, the costimulatory signaling region contains 1, 2, 3, or 4 cytoplasmic domains of one or more intracellular signaling and /or costimulatory molecules. The costimulatory signaling domain may be the intracellular/cytoplasmic domain of a costimulatory molecule, including but not limited to CD2, CD7, CD27, CD28, 4-1BB (CD137), OX40 (CD134), CD30, CD40, ICOS (CD278), GITR (glucocorticoid-induced tumor necrosis factor receptor), LFA-1 (lymphocyte function-associated antigen- 1), LIGHT, NKG2C, B7-H3. In one example, the costimulatory signaling domain is derived from the cytoplasmic domain of CD28. In another example, the costimulatory signaling domain is derived from the cytoplasmic domain of 4-1BB (CD137). In another example, the co-stimulatory signaling domain may be an intracellular domain of GITR as taught in U.S. Pat. NOS. 9, 175, 308; the contents of which are incorporated herein by reference in its entirety.

[00226] In some embodiments, the intracellular region of the present invention may comprise a functional signaling domain from a protein selected from the group consisting of an MHC class I molecule, a TNF receptor protein, an immunoglobulin-like protein, a cytokine receptor, an integrin, a signaling lymphocytic activation protein (SLAM) such as CD48, CD229, 2B4, CD84, NTB-A, CRACC, BLAME,CD2F-10, SLAMF6, SLAMF7, an activating NK cell receptor, BTLA, a Toll ligand receptor, OX40, CD2, CD7, CD27, CD28, CD30, CD40, CDS, ICAM-1, LFA-1 (CD11a/CD18), 4-1BB (CD137), B7-H3, CDS, ICAM-1, ICOS (CD278), GITR, BAFFR, LIGHT, HVEM (LIGHTR), SLAMF7, NKp80 (KLRF1), NKp44, NKp30, NKp46, CD19, CD4, CD8alpha, CD8beta, IL2R beta, IL2R gamma, IL7R alpha, IL-15Ra, ITGA4, VLA1, CD49a, ITGA4, IA4, CD49D, ITGA6, VLA-6, CD49f, ITGAD, CD11d, ITGAE,

CD103, ITGAL, CD11a, LFA-1, ITGAM, CD11b, ITGAX, CD11c, ITGB1, CD29, ITGB2, CD18, LFA-1, ITGB7, NKG2D, NKG2C, NKD2C SLP76, TNFR2, TRANCE/RANKL, DNAM1 (CD226), SLAMF4 (CD244, 2B4), CD84, CD96 (Tactile), CEACAM1, CRTAM, Ly9 (CD229), CD160 (BY55), PSGL1, CD100 (SEMA4D), CD69, SLAMF6 (NTB-A, Ly108), SLAM (SLAMF1, CD150, IPO-3), BLAME (SLAMF8), SELPLG (CD162), LTBR, LAT, CD270 (HVEM), GADS, SLP-76, PAG/Cbp, CD19a, a ligand that specifically binds with CD83, DAP 10, TRIM, ZAP70, Killer immunoglobulin receptors (KIRs) such as KIR2DL1, KIR2DL2/L3, KIR2DL4, KIR2DL5A, KIR2DL5B, KIR2DS1, KIR2DS2, KIR2DS3, KIR2DS4, KIR2DS5, KIR3DL1/S1, KIR3DL2, KIR3DL3, and KIR2DP1; lectin related NK cell receptors such as Ly49, Ly49A, and Ly49C.

[00227] In some embodiments, the intracellular signaling domain of the present invention may contain signaling domains derived from JAK-STAT. In other embodiments, the intracellular signaling domain of the present invention may contain signaling domains derived from DAP-12 (Death associated protein 12) (Topfer et al., *Immunol.*, 2015, 194: 3201-3212; and Wang et al., Cancer Immunol., 2015, 3: 815-826). DAP-12 is a key signal transduction receptor in NK cells. The activating signals mediated by DAP-12 play important roles in triggering NK cell cytotoxicity responses toward certain tumor cells and virally infected cells. The cytoplasmic domain of DAP12 contains an Immunoreceptor Tyrosine-based Activation Motif (ITAM). Accordingly, a CAR containing a DAP12-derived signaling domain may be used for adoptive transfer of NK cells.

[00228] In some embodiments, T cells engineered with two or more CARs incorporating distinct co-stimulatory domains and regulated by distinct DD may be used to provide kinetic control of downstream signaling.

[00229] In some embodiments, the intracellular domain of the present invention may comprise amino acid sequences of Table 9.

Table 9: Intracellular signaling and co-stimulatory

Domain	Sequence	SEQ ID NO
2B4 co-stimulatory domain	WRRKRKEKQSETSPKEFLTIYEDVKDLKTRRNHEQEQTFPGGGSTIYSMIQ SQSSAPTSQEPAYTLYSLIQPSRKSGSRKRNHSPSFNSTIYEVIGKSQPKAQ NPARLSRKELENFDVYS	474
CD27 co-stimulatory domain	HQRRKYRSNKGESPVEPAEPCRYSCPREEEGSTIPIQEDYRKPEPACSP	475
CD272 (BTLA1) co-stimulatory domain	RRHQGKQNELSDTAGREINLVDAHLKSEQTEASTRQNSQVLLSETGIYDN DPDLCFRMQEGSEVYSNPCLEENKPGIVYASLNHSVIGPNSRLARNVKEAP TEYASICVRS	476

CD272 (BTLA1) co-stimulatory domain	CCLRRHQGKQNELSDTAGREINLVDAHLKSEQTEASTRQNSQVLLSETGI YDNDPDLCFRMQEGSEVYSNPCLEENKPGIVYASLNHSVIGPNSRLARNV KEAPTEYASICVRS	477
CD28 co-stimulatory	FWVLVVVGGVLACYSLLVTVAFIIFWV	478
CD28 co-stimulatory domain	KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL	479
CD28 co-stimulatory domain	FWVRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRS	480
CD28 co-stimulatory domain	RSKRSRGGHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRS	481
CD28 co-stimulatory domain	RSKRSRGGHSDYIVINMTPRRPGPTRKHYQPYAPPRDFAAYRS	482
CD28 co-stimulatory signaling region	MLRLLLALNLFPSIQVTGNKILVKQSPMLVAYDNAVNLSCKYSYNLFSRE FRASLHKGLDSAVEVCVVYGNYSQQLQVYSKTGFNCDGKLGNESVTFYL QNLYVNQTDIYFCKIEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPS KPFWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRP GPTRKHYQPYAPPRDFAAYRS	483
CD30 co-stimulatory domain	RRACRKRIRQKLHLCYPVQTSQPKLELVDSRPRRSSTQLRSGASVTEPVAE ERGLMSQPLMETCHSVGAAYLESLPLQDASPAGGPSSPRDLPEPRVSTEHT NNKIEKIYIMKADTVIVGTVKAELPEGRGLAGPAEPELEEELEADHTPHYP EQETEPPLGSCSDVMLSVEEEGKEDPLPTAASGK	484
GITR co-stimulatory domain	HIWQLRSQCMWPRETQLLLEVPPSTEDARSCQFPEEERGERSAEEKGRLG DLWV	485
HVEM co-stimulatory domain	CVKRRKPRGDVVKVIVSVQRKRQEAEGEATVIEALQAPPDVTTVAVEETI PSFTGRSPNH	486
ICOS co-stimulatory domain	TKKKYSSSVHDPNGEYMFMRAVNTAKKSRLTDVTL	487
ICOS co-stimulatory signaling domain	CWLTKKKYSSSVHDPNGEYMFMRAVNTAKKSRLTDVTL	488
LAG-3 co-stimulatory region	HLWRRQWRPRRFSALEQGIHPPQAQSKIEELEQEPEPEPEPEPEPEPEPEPE QL	489
OX40 co-stimulatory domain	ALYLLRRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKI	490
OX40 co-stimulatory domain	RRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKI	491
4-1BB intracellular domain	KRGRKKLLYIFKQPFMRPVQTIQEEDGCSCRFPEEEEGGCEL	492
4-1BB signaling domain	KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGYEL	493
4-1BB-CD3Zeta intracellular domain	TGTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWA PLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRF PEEEEGGCELRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRR GRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHD GLYQGLSTATKDTYDALHMQALPPR	494
4-1BB-Z endodomain fusion	KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSAD APAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEG LYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALH MQALPPR	495
CD127 intracellular domain	KRIKPIVWPSLPDHKKTLEHLCKKPRKNLNVSFNPESFLDCQIHRVDDIQA RDEVEGFLQDTFPQQLEESEKQRLGGDVQSPNCPSEDVVITPESFGRDSSL TCLAGNVSACDAPILSSSRSLDCRESGKNGPHVYQDLLLSLGTTNSTLPPPF SLQSGILTLNPVAQGQPILTSLGSNQEEAYVTMSSFYQNQ	496
CD137 intracellular domain	RFSVVKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL	497

CD148 intracellular domain	RKKRKDAKNNEVSFSQIKPKKSKLIRVENFEAYFKKQQADSNCGFAEEYE DLKLVGISQPKYAAELAENRGKNRYNNVLPYDISRVKLSVQTHSTDDYIN ANYMPGYHSKKDFIATQGPLPNTLKDFWRMVWEKNVYAIIMLTKCVEQ GRTKCEEYWPSKQAQDYGDITVAMTSEIVLPEWTIRDFTVKNIQTSESHPL RQFHFTSWPDHGVPDTTDLLINFRYLVRDYMKQSPPESPILVHCSAGVGR TGTFIAIDRLIYQIENENTVDVYGIVYDLRMHRPLMVQTEDQYVFLNQCV LDIVRSQKDSKVDLIYQNTTAMTIYENLAPVTTFGKTNGYIA	498
CD27 intracellular domain	QRRKYRSNKGESPVEPAEPCHYSCPREEEGSTIPIQEDYRKPEPACSP	499
CD28 intracellular domain	FAAYRS	500
CD28 signaling chain	FWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPT RKHYQPYAPPRDFAAYRS	501
CD28 signaling domain	RSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRS	502
CD28 signaling domain	SKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRS	503
CD28 signaling domain	IEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPSKPFWVLVVVGGVLA CYSLLVTVAFIIFWRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDF AAYRS	504
CD28, 4-1BB, and/or CD3ζ signaling domain	RSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSRFSVVKRG RKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSADAPA YQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYN ELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQA LPPR	505
CD28/CD3C	AAAIEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPSKPFWVLVVVGG VLACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAP PRDFAAYRSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRG RDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDG LYQGLSTATKDTYDALHMQALPPR	506
CD28-0XZ intracellular domain	RSKRSRLLHSDYNMTPRRPGPTRKHYQPYAPPRDFAAYRSRDQRLPPDAH KPPGGGSFRTPIQEEQADAHSTLAKIRVKFSRSADAPAYQQGQNQLYNEL NLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYS EIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR	507
CD28-4-1BB intracellular domain	MFWVLVVVGGVLACYSLLVTVAFIIFWVKRGRKKLLYIFKQPFMRPVQT TQEEDGCSCRFPEEEEGGCEL	508
CD28-4-1BB intracellular domain	IEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPSKPFWVLVVVGGVLA CYSLLVTVAFIIFWVKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEE EGGCEL	509
CD28-CD3 Zeta intracellular domain	RSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSRVKFSRSA DAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQE GLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDAL HMQALPPR	510
CD28-CD3Zeta intracellular domain	KRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRSRVKFSRSADA PAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGL YNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHM QALPPR	511
CD3 delta chain intracellular signaling domain	MEHSTFLSGLVLATLLSQVSPFKIPIEELEDRVFVNCNTSITWVEGTVGTLL SDITRLDLGKRILDPRGIYRCNGTDIYKDKESTVQVHYRMCQSCVELDPAT VAGIIVTDVIATLLLALGVFCFAGHETGRLSGAADTQALLRNDQVYQPLR DRDDAQYSHLGGNWARNK	512
CD3 delta chain intracellular signaling domain	MEHSTFLSGLVLATLLSQVSPFKIPIEELEDRVFVNCNTSITWVEGTVGTLL SDITRLDLGKRILDPRGIYRCNGTDIYKDKESTVQVHYRTADTQALLRND QVYQPLRDRDDAQYSHLGGNWARNK	513
CD3 delta chain intracellular signaling domain	DQVYQPLRDRDDAQYSHLGGN	514

CD3 delta intracellular domain	MEHSTFLSGLVLATLLSQVSPFKIPIEELEDRVFVNCNTSITWVEGTVGTLL SDITRLDLGKRILDPRGIYRCNGTDIYKDKESTVQVHYRMCQSCVELDPAT VAGIIVTDVIATLLLALGVFCFAGHETGRLSGAADTQALLRNDQVYQPLR DRDDAQYSHLGGNWARNK	515
CD3 delta intracellular domain	MEHSTFLSGLVLATLLSQVSPFKIPIEELEDRVFVNCNTSITWVEGTVGTLL SDITRLDLGKRILDPRGIYRCNGTDIYKDKESTVQVHYRTADTQALLRND QVYQPLRDRDDAQYSHLGGNWARNK	516
CD3 epsilon intracellular domain	MQSGTHWRVLGLCLLSVGVWGQDGNEEMGGITQTPYKVSISGTTVILTCP QYPGSEILWQHNDKNIGGDEDDKNIGSDEDHLSLKEFSELEQSGYYVCYP RGSKPEDANFYLYLRARVCENCMEMDVMSVATIVIVDICITGGLLLLVYY WSKNRKAKAKPVTRGAGAGGRQRGQNKERPPPVPNPDYEPIRKGQRDLY SGLNQRRI	517
CD3 epsilon intracellular domain	NPDYEPIRKGQRDLYSGLNQR	518
CD3 gamma intracellular domain	MEQGKGLAVLILAIILLQGTLAQSIKGNHLVKVYDYQEDGSVLLTCDAEA KNITWFKDGKMIGFLTEDKKKWNLGSNAKDPRGMYQCKGSQNKSKPLQ VYYRMCQNCIELNAATISGFLFAEIVSIFVLAVGVYFIAGQDGVRQSRASD KQTLLPNDQLYQPLKDREDDQYSHLQGNQLRRN	519
CD3 gamma intracellular domain	DQLYQPLKDREDDQYSHLQGN	520
CD3 zeta intracellular domain	MKWKALFTAAILQAQLPITEAQSFGLLDPKLCYLLDGILFIYGVILTALFLR VKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKP QRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTA TKDTYDALHMQALPPR	521
CD3 zeta intracellular domain	NQLYNELNLGRREEYDVLDKR	522
CD3 zeta domain 2 (NM_000734.3)	RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGK PRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTA TKDTYDALHMQALPPR	523
CD3 zeta intracellular domain	DGLYQGLSTATKDTYDALHMQ	524
CD3 zeta intracellular domain	RVKFSRSAEPPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKP RRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTAT KDTYDALHMQALPPR	525
CD3 zeta intracellular domain	RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGK PQRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLST ATKDTYDALHMQALPPR	526
CD3 zeta intracellular domain	RSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMG GKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLS TATKDTYDALHMQALPPR	527
CD3 zeta intracellular domain	RVKFSRSADAPAYQQGEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNEL QKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALP PR	528
CD3 zeta intracellular domain	RVKFSRSADAPAYQQGQNQLYNELNLGRREEVDVLDKRRGRDPEMGGK PRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTA TKDTYDALHMQALPPR	529
CD3 zeta intracellular domain	MIPAVVLLLLLLVEQAAALGEPQLCYILDAILFLVGIVLTLLVCRLKIQVRK AAITSYEKSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRG RDPEMGGKPRRKNPQEGLYNELQKDKMAEAVSEIGMKGERRRGKGHDG LYQGLSTATKDTYDALHMQALPPR	530
CD3 zeta intracellular domain	LRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGG KPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLST ATKDTYDALHMQALPPR	531
CD3 zeta intracellular domain	RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGK PQRRKNPQEGLY	532

CD3 zeta intracellular domain	LRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGG KPQRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLS TATKDTYDALHMQALPPR	533
CD3 zeta intracellular domain	RRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGG KPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLST ATKDTYDALHMQALPPR	534
CD3 zeta intracellular domain	EGLYNELQKDKMAEAYSEIGMK	535
CD3 zeta intracellular domain	RVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGK PRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTA TKDTYDALHMQALPPR	536
CD3 zeta intracellular domain	RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGK PRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTA TKDTYDALHMQALP	537
CD3 zeta intracellular domain	DPKLCYLLDGILFIYGVILTALFLRVKFSRSADAPAYQQGQNQLYNELNLG RREEYDVLDKRRGRDPEMGGKPQRRKNPQEGLYNELQKDKMAEAYSEI GMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR	538
CD3 zeta intracellular domain	MKWKALFTAAILQAQLPITEAQSFGLLDPKLCYLLDGILFIYGVILTALFLR VKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKP RRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTAT KDTYDALHMQALPPR	539
CD40 intracellular domain	RSRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKI	540
CD79A intracellular domain	MPGGPGVLQALPATIFLLFLLSAVYLGPGCQALWMHKVPASLMVSLGED AHFQCPHNSSNNANVTWWRVLHGNYTWPPEFLGPGEDPNGTLIIQNVNK SHGGIYVCRVQEGNESYQQSCGTYLRVRQPPPRPFLDMGEGTKNRIITAEG IILLFCAVVPGTLLLFRKRWQNEKLGLDAGDEYEDENLYEGLNLDDCSMY EDISRGLQGTYQDVGSLNIGDVQLEKP	541
CD79A intracellular domain	MPGGPGVLQALPATIFLLFLLSAVYLGPGCQALWMHKVPASLMVSLGED AHFQCPHNSSNNANVTWWRVLHGNYTWPPEFLGPGEDPNEPPPRPFLDM GEGTKNRIITAEGIILLFCAVVPGTLLLFRKRWQNEKLGLDAGDEYEDENL YEGLNLDDCSMYEDISRGLQGTYQDVGSLNIGDVQLEKP	542
CD79A intracellular domain	ENLYEGLNLDDCSMYEDISRG	543
CD8 intracellular domain	FVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDF ACDIYIWAPLAGTCGVLLLSLVITLYCNHRNR	544
CD8 intracellular domain	FVPVFLPAKPITTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDF ACDIYIWAPLAGTCGVLLLSLVITLYCNHRNR	545
CD8a intracellular domain	PTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDI	546
CTLA4 intracellular domain	AVSLSKMLKKRSPLTTGVFVKMAPTEAECEKQFQPYFIPIN	547
CTLA4 intracellular domain	AVSLSKMLKKRSPLTTGVYMNMTPRRPECEKQFQPYAPPRDFAAYRS	548
DAP10 intracellular domain	RPRRSPAQDGKVYINMPGRG	549
DAP12 intracellular domain	MGGLEPCSRLLLLPLLLAVSGLRPVQAQAQSDCSCSTVSPGVLAGIVMGD LVLTVLIALAVYFLGRLVPRGRGAAEAATRKQRITETESPYQELQGQRSD VYSDLNTQRPYYK	550
DAP12 intracellular domain	MGGLEPCSRLLLLPLLLAVSGLRPVQAQAQSDCSCSTVSPGVLAGIVMGD LVLTVLIALAVYFLGRLVPRGRGAAEATRKQRITETESPYQELQGQRSDV YSDLNTQRPYYK	551

DAP12 intracellular domain	MGGLEPCSRLLLLPLLLAVSDCSCSTVSPGVLAGIVMGDLVLTVLIALAVY FLGRLVPRGRGAAEAATRKQRITETESPYQELQGQRSDVYSDLNTQRPYY K	552
DAP12 intracellular domain	MGGLEPCSRLLLLPLLLAVSDCSCSTVSPGVLAGIVMGDLVLTVLIALAVY FLGRLVPRGRGAAEATRKQRITETESPYQELQGQRSDVYSDLNTQRPYYK	553
DAP12 intracellular domain	MGGLEPCSRLLLLPLLLAVSGLRPVQAQAQSDCSCSTVSPGVLAGIVMGD LVLTVLIALAVYFLGRLVPRGRGAAEAATRKQRITETESPYQELQGQRSD VYSDLNTQRPYYK	554
DAP12 intracellular domain	MGGLEPCSRLLLLPLLLAVSGLRPVQAQAQSDCSCSTVSPGVLAGIVMGD LVLTVLIALAVYFLGRLVPRGRGAAEAATRKQRITETESPYQELQGQRSDV YSDLNTQRPYYK	555
DAP12 intracellular domain	MGGLEPCSRLLLLPLLLAVSDCSCSTVSPGVLAGIVMGDLVLTVLIALAVY FLGRLVPRGRGAAEAATRKQRITETESPYQELQGQRSDVYSDLNTQRPYY K	556
DAP12 intracellular domain	MGGLEPCSRLLLLPLLLAVSDCSCSTVSPGVLAGIVMGDLVLTVLIALAVY FLGRLVPRGRGAAEATRKQRITETESPYQELQGQRSDVYSDLNTQRPYYK	557
DAP12 intracellular domain	ESPYQELQGQRSDVYSDLNTQ	558
GITR intracellular domain	RSQCMWPRETQLLLEVPPSTEDARSCQFPEEERGERSAEEKGRLGDLWV	559
ICOS intracellular domain	TKKKYSSSVHDPNGEFMFMRAVNTAKKSRLTDVTL	560
IL- 15Ra intracellular domain	KSRQTPPLASVEMEAMEALPVTWGTSSRDEDLENCSHHL	561
OX40-CD3 Zeta intracellular domain	RRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKIRVKFSRSADAPAYQ QGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNEL QKDKMAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALP PR	562
ZAP70 intracellular domain	MPDPAAHLPFFYGSISRAEAEEHLKLAGMADGLFLLRQCLRSLGGYVLSL VHDVRFHHFPIERQLNGTYAIAGGKAHCGPAELCEFYSRDPDGLPCNLRK PCNRPSGLEPQPGVFDCLRDAMVRDYVRQTWKLEGEALEQAIISQAPQVE KLIATTAHERMPWYHSSLTREEAERKLYSGAQTDGKFLLRPRKEQGTYAL SLIYGKTVYHYLISQDKAGKYCIPEGTKFDTLWQLVEYLKLKADGLIYCL KEACPNSSASNASGAAAPTLPAHPSTLTHPQRRIDTLNSDGYTPEPARITSP DKPRPMPMDTSVYESPYSDPEELKDKKLFLKRDNLLIADIELGCGNFGSVR QGVYRMRKKQIDVAIKVLKQGTEKADTEEMMREAQIMHQLDNPYIVRLI GVCQAEALMLVMEMAGGGPLHKFLVGKREEIPVSNVAELLHQVSMGMK YLEEKNFVHRDLAARNVLLVNRHYAKISDFGLSKALGADDSYYTARSAG KWPLKWYAPECINFRKFSSRSDVWSYGVTMWEALSYGQKPYKKMKGPE VMAFIEQGKRMECPPECPPELYALMSDCWIYKWEDRPDFLTVEQRMRAC YYSLASKVEGPPGSTQKAEAACA	563
CD28 intracellular domain	MLRLLLALNLFPSIQVTGNKILVKQSPMLVAYDNAVNLSCKYSYNLFSRE FRASLHKGLDSAVEVCVVYGNYSQQLQVYSKTGFNCDGKLGNESVTFYL QNLYVNQTDIYFCKIEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPS KPFWVLVVVGGVLACYSLLVTVAFIIFWVR	564
4-1BB intracellular domain	MGNSCYNIVATLLLVLNFERTRSLQDPCSNCPAGTFCDNNRNQICSPCPPN SFSSAGGQRTCDICRQCKGVFRTRKECSSTSNAECDCTPGFHCLGAGCSM CEQDCKQGQELTKKGCKDCCFGTFNDQKRGICRPWTNCSLDGKSVLVNG TKERDVVCGPSPADLSPGASSVTPPAPAREPGHSPQIISFFLALTSTALLFLL FFLTLRFSVVKRGRKKLLYIFKQPFMRPVQTTQEEDG	565
Fc epsilon Receptor I gamma chain intracellular domain	MIPAVVLLLLLLVEQAAALGEPQLCYILDAILFLYGIVLTLLYCRLKIQVRK AAITSYEKSDGVYTGLSTRNQETYETLKHEKPPQ	566
Fc epsilon Receptor I gamma	DGVYTGLSTRNQETYETLKHE	567

chain intracellular domain		
Fc epsilon Receptor I gamma chain intracellular domain	DPKLCYILDAILFLYGIVLTLLYCRLKIQVRKAAITSYEKSDGVYTGLSTRN QETYETLKHEKPPQ	568
CD28 intracellular domain	SKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRS	569
CD28 signaling domain	IEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPSKPFWVLVVVGGVLA CYSLLVTVAFIIFWVR	570
CD8 signaling domain	FVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDF ACDIYIWAPLAGTCGVLLLSLVITLYCNHRNR	571
intracellular T cell signaling domain comprising CD28 and CD3 zeta	IEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPSKPFWVLVVVGGVLA CYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRD FAAYRSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDP EMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQ GLSTATKDTYDALHMQALPPR	572
Intracellular T cell signaling domain comprising CD28, CD137, and CD3 zeta	FVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDF ACDIYIWAPLAGTCGVLLLSLVITLYCNHRNRSKRSRLLHSDYMNMTPRR PGPTRKHYQPYAPPRDFAAYRSRFSVVKRGRKKLLYIFKQPFMRPVQTTQ EEDGCSCRFPEEEEGGCELRVKFSRSADAPAYQQGQNQLYNELNLGRREE YDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGE RRRGKGHDGLYQGLSTATKDTYDALHMQALPPR	573

Transmembrane domains

[00230] In some embodiments, the CAR of the present invention may comprise a transmembrane domain. As used herein, the term "Transmembrane domain (TM)" refers broadly to an amino acid sequence of about 15 residues in length which spans the plasma membrane. More preferably, a transmembrane domain includes at least 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, 30, 31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41, 42, 43, 44, or 45 amino acid residues and spans the plasma membrane. In some embodiments, the transmembrane domain of the present invention may be derived either from a natural or from a synthetic source. The transmembrane domain of a CAR may be derived from any naturally membrane-bound or transmembrane protein. For example, the transmembrane region may be derived from (i.e. comprise at least the transmembrane region(s) of) the alpha, beta or zeta chain of the T-cell receptor, CD3 epsilon, CD4, CD5, CD8, CD8α, CD9, CD16, CD22, CD33, CD28, CD37, CD45, CD64, CD80, CD86, CD134, CD137, CD152, or CD154.

[00231] Alternatively, the transmembrane domain of the present invention may be synthetic. In some aspects, the synthetic sequence may comprise predominantly hydrophobic residues such as leucine and valine.

[00232] In some embodiments, the transmembrane domain of the present invention may be selected from the group consisting of a CD8α transmembrane domain, a CD4 transmembrane domain, a CD 28 transmembrane domain, a CTLA-4 transmembrane domain, a PD-1 transmembrane domain, and a human IgG_4 Fc region. As non-limiting examples, the

transmembrane domain may be a CTLA-4 transmembrane domain comprising the amino acid sequences of SEQ ID NOs. 1-5 of International Patent Publication NOS. WO2014/100385; and a PD-1 transmembrane domain comprising the amino acid sequences of SEQ ID NOs. 6-8 of International Patent Publication NOS. WO2014100385; the contents of each of which are incorporated herein by reference in their entirety.

[00233] In some embodiments, the CAR of the present invention may comprise an optional hinge region (also called spacer). A hinge sequence is a short sequence of amino acids that facilitates flexibility of the extracellular targeting domain that moves the target binding domain away from the effector cell surface to enable proper cell/cell contact, target binding and effector cell activation (Patel et al., *Gene Therapy*, 1999; 6: 412-419). The hinge sequence may be positioned between the targeting moiety and the transmembrane domain. The hinge sequence can be any suitable sequence derived or obtained from any suitable molecule. The hinge sequence may be derived from all or part of an immunoglobulin (e.g., IgGl, IgG2, IgG3, IgG4) hinge region, i.e., the sequence that falls between the CHI and CH2 domains of an immunoglobulin, e.g., an IgG4 Fc hinge, the extracellular regions of type 1 membrane proteins such as CD8α CD4, CD28 and CD7, which may be a wild type sequence or a derivative. Some hinge regions include an immunoglobulin CH3 domain or both a CH3 domain and a CH2 domain. In certain embodiments, the hinge region may be modified from an IgG1, IgG2, IgG3, or IgG4 that includes one or more amino acid residues, for example, 1, 2, 3, 4 or 5 residues, substituted with an amino acid residue different from that present in an unmodified hinge. Table 10 provides various transmembrane regions that can be used in the CARs described herein.

Table 10: Transmembrane domains

Transmembrane domain	Sequence	SEQ ID NO
CD8 Transmembrane domain	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAV HTRGLDFACDI	574
4-1BB Transmembrane domain	IISFFLALTSTALLFLLFFLTLRFSVVKRGR	575
4-1BB Transmembrane domain	IISFFLALTSTALLFLLFFLTLRFSVV	576
CD134 (OX40) Transmembrane domain	VAAILGLGLVLGLLGPLAILLALYLL	577
CD148 Transmembrane and intracellular domain	AVFGCIFGALVIVTVGGFIFWRKKRKDAKNNEVS FSQIKPKKSKLIRVENFEAYFKKQQADSNCGFAEE YEDLKLVGISQPKYAAELAENRGKNRYNNVLPY DISRVKLSVQTHSTDDYINANYMPGYHSKKDFIA TQGPLPNTLKDFWRMVWEKNVYAIIMLTKCVEQ GRTKCEEYWPSKQAQDYGDITVAMTSEIVLPEVV TIRDFTVKNIQTSESHPLRQFHFTSWPDHGVPDTT	578

	DLLINFRYLVRDYMKQSPPESPILVHCSAGVGRTG TFIAIDRLIYQIENENTVDVYGIVYDLRMHRPLMV QTEDQYVFLNQCVLDIVRSQKDSKVDLIYQNTTA MTIYENLAPVTTFGKTNGYIA	
CD148 Transmembrane domain	AVFGCIFGALVIVTVGGFIFW	579
CD2 Transmembrane domain	KEITNALETWGALGQDINLDIPSFQMSDDIDDIKW EKTSDKKKIAQFRKEKETFKEKDTYKLFKNGTLK IKHLKTDDQDIYKVSIYDTKGKNVLEKIFDLKIQE RVSKPKISWTCINTTLTCEVMNGTDPELNLYQDG KHLKLSQRVITHKWTTSLSAKFKCTAGNKVSKES SVEPVSCPEKGLD	580
CD28 Transmembrane and intracellular domain	IEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPG PSKPFWVLVVVGGVLACYSLLVTVAHIFWVRSK RSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFA AYRS	581
CD28 Transmembrane domain	FWVLVVVGGVLACYSLLVTVAFIIFWV	582
CD28 Transmembrane domain	IEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGP SKPFWVLVVVGGVLACYSLLVTVAFIIFWV	583
CD28 Transmembrane domain	IFWVLVVVGGVLACYSLLVTVAFIIFWVRSKRR	584
CD28 Transmembrane domain	FWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRL LHSDYMNMTPRRPGPTRKHYQP YAPPRDFAAYRS	585
CD28 Transmembrane domain	MFWVLVVVGGVLACYSLLVTVAFIIFWV	586
CD28 Transmembrane domain	FWVLVVVGGVLACYSLLVTVAFHFWV	587
CD28 Transmembrane domain	MFWVLVVVGGVLACYSGGVTVAFIIFWV	588
CD28 Transmembrane domain	WVLVVVGGVLACYSLLVTVAFIIFWV	589
CD28 Transmembrane domain	PFWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSR LLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAY RS	590
CD28 Transmembrane domain and CD28 and CD3 Zeta intracellular domain	FWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRL LHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYR SRVKFSRSADAPAYQQGQNQLYNELNLGRREEY DVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKD KMAEAYSEIGMKGERRRGKGHDGLYQGLSTATK DTYDALHMQALPPR	591
CD28 Transmembrane domain and CD28, OX40, and CD3 Zeta intracellular domain	FWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRL LHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYR SRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLA KIRVKFSRSADAPAYQQGQNQLYNELNLGRREEY DVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKD KMAEAYSEIGMKGERRRGKGHDGLYQGLSTATK DTYDALHMQALPPR	592

CD28 Transmembrane domain and CD3 Zeta intracellular domain	FWVLVVVGGVLACYSLLVTVAFIIFWVRRVKFSR SADAPAYQQGQNQLYNELNLGRREEYDVLDKRR GRDPEMGGKPRRKNPQEGLYNELQKDKMAEAY SEIGMKGERRRGKGHDGLYQGLSTATKDTYDAL HMQALPPR	593
CD28 transmembrane-CD3 zeta signaling domain ("28z")	AAAIEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPL FPGPSKPFWVLVVVGGVLACYSLLVTVAFIIFWV RSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPR DFAAYRSRVKFSRSADAPAYQQGQNQLYNELNL GRREEYDVLDKRRGRDPEMGGKPRRKNPQEGLY NELQKDKMAEAYSEIGMKGERRRGKGHDGLYQ GLSTATKDTYDALHMQALPPR	594
CD3 zeta Transmembrane domain	LCYLLDGILFIYGVILTALFLRV	595
CD3 zeta Transmembrane domain	MKWKALFTAAILQAQLPITEAQSFGLLDPKLCYL LDGILFIYGVILTALFL	596
CD3 zeta Transmembrane domain	LCYLLDGILFIYGVILTALFL	597
CD4 Transmembrane domain	ALIVLGGVAGLLLFIGLGIFFCVRC	598
CD4 Transmembrane domain	MALIVLGGVAGLLLFIGLGIFF	599
CD45 Transmembrane and intracellular domain	ALIAFLAFLIIVTSIALLVVLYKIYDLHKKRSCNLD EQQELVERDDEKQLMNVEPIHADILLETYKRKIA DEGRLFLAEFQSIPRVFSKFPIKEARKPFNQNKNR YVDILPYDYNRVELSEINGDAGSNYINASYIDGFK EPRKYIAAQGPRDETVDDFWRMIWEQKATVIVM VTRCEEGNRNKCAEYWPSMEEGTRAFGDVVVKI NQHKRCPDYIIQKLNIVNKKEKATGREVTHIQFTS WPDHGVPEDPHLLLKLRRRVNAFSNFFSGPIWHC SAGVGRTGTYIGIDAMLEGLEAENKVDVYGYVV KLRRQRCLMVQVEAQYILIHQALVEYNQFGETEV NLSELHPYLHNMKKRDPPSEPSPLEAEFQRLPSYR SWRTQHIGNQEENKSKNRNSNVIPYDYNRVPLKH ELEMSKESEHDSDESSDDDSDSEEPSKYINASFIM SYWKPEVMIAAQGPLKETIGDFWQMIFQRKVKVI VMLTELKHGDQEICAQYWGEGKQTYGDIEVDLK DTDKSSTYTLRVFELRHSKRKDSRTVYQYQYTN WSVEQLPAEPKELISMIQVVKQKLPQKNSSEGNKH HKSTPLLIHCRDGSQQTGIFCALLNLLESAETEEW DIFQVVKALRKARPGMVSTFEQYQFLYDVIASTYP AQNGQVKKNNHQEDKIEFDNEVDKVKQDANCV NPLGAPEKLPEAKEQAEGSEPTSGTEGPEHSVNGP ASPALNQGS	600
CD62L Transmembrane domain	PLFIPVAVMVTAFSGLAFIIWLA	601
CD7 Transmembrane domain	ALPAALAVISFLLGLGLGVACVLA	602
CD8 Transmembrane domain	MALPVTALLLPLALLLHAARP	603
CD8 Transmembrane domain and CD28 signaling domain	AAAFVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLR PEACRPAAGGAVHTRGLDFACDIYIWAPLAGTCG VLLLSLVITLYCNHRNRSKRSRLLHSDYMNMTPR RPGPTRKHYQPYAPPRDFAAYRSRFSVVKRGRKK LLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCE	604

	LRVKFSRSADAPAYQQGQNQLYNELNLGRREEY DVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKD KMAEAYSEIGMKGERRRGKGHDGLYQGLSTATK DTYDALHMQALPPR	
CD8 transmembrane domain-CD137 (4-1BB) signaling domain and CD3 zeta signaling domain ("BBz")	AAATTTPAPRPPTPAPTIASQPLSLRPEACRPAAG GAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITL YCKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRF PEEEEGGCELRVKFSRSADAPAYKQGQNQLYNEL NLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEG LYNELQKDKMAEAYSEIGMKGERRRGKGHDGL YQGLSTATKDTYDALHMQALPPR	605
CD8a Transmembrane domain	FVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEA CRPAAGGAVHTRGLDFACDIYIWAPLAGTCGVLL LSLVITLYCNHRN	606
CD8a Transmembrane domain	IWAPLAGTCGVLLLSLVITLYC	607
CD8a Transmembrane domain	IYIWAPLAGTCGVLLLSLVITLYC	608
CD8a Transmembrane domain	IYIWAPLAGTCGVLLLSLVITLYCR	609
CD8a Transmembrane domain	IYIWAPLAGTCGVLLLSLVITLVCR	610
CD8a Transmembrane domain	IYIWAPLAGTCGVLLLSLVIT	611
CD8a Transmembrane domain	IYIWAPLAGTCGVLLLSLVITLY	612
CD8b Transmembrane domain	LGLLVAGVLVLLVSLGVAIHLCC	613
EpoR Transmembrane domain	APVGLVARLADESGHVVLRWLPPPETPMTSHIRY EVDVSAGNGAGSVQRVEILEGRTECVLSNLRGRT RYTFAVRARMAEPSFGGFWSAWSEPVSLLTPSD	614
FcER1 a- Transmembrane domain	MAPAMESPTLLCVALLFFAPDGVLAVPQKPKVSL NPPWNRIFKGENVTLTCNGNNFFEVSSTKWFHNG SLSEETNSSLNIVNAKFEDSGEYKCQHQQVNESEP VYLEVFSDWLLLQASAEVVMEGQPLFLRCHGWR NWDVYKVIYYKDGEALKYWYENHNISITNATVE DSGTYYCTGKVWQLDYESEPLNITVIKAPREKYW LQFFIPLLVVILFAVDTGLFISTQQQVTFLLKIKRT RKGFRLLNPHPKPNPKNN	615
FceRIa Transmembrane domain	DIFIPLLVVILFAVDTGLFISTQQQVTFLLKIKRTRK GFRLLNPHPKPNPKNNR	616
GITR Transmembrane domain	PLGWLTVVLLAVAACVLLLTSAQLGLHIWQL	617
Her2 Transmembrane domain	SIISAVVGILLVVVLGVVFGILI	618
Her2 Transmembrane domain	CHPECQPQNGSVTCFGPEADQCVACAHYKDPPFC VARCPSGVKPDLSYMPIWKFPDEEGACQPCPINC THSCVDLDDKGCPAEQRASPLTSIISAVVGILLVV VLGVVFGILI	619
ICOS Transmembrane domain	FWLPIGCAAFVVVCILGCILI	620

IgG1Transmembrane domain	EPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTL MIARTPEVTCVVVDVSHEDPEVKFNWYVDGVEV HNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGK EYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLP PSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQ PENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQG NVFSCSVMHEALHNHYTQKSLSLSPGKKD	621
OX40 Transmembrane domain	VAAILGLGLVLGLLGPLAILL	622
Transmembrane domain	IYIWAPLAGTCGVLLLSLVITLYC	623
Transmembrane domain	IYIWAPLAGTCGVLLLSLVITLYC	624
CD28 transmembrane and signaling domains	IEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGP SKPFWVLVVVGGVLACYSLLVTVAFIIFWVRSKR SRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFA AYRS	625
CD28 Transmembrane domain	VMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPS KPFWVLVVVGGVLACYSLLVTVAFIIFWVR	626
CD8 Transmembrane domain	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAV HTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYC	627

[00234] Hinge region sequences useful in the present invention are provided in Table 11.

Table 11: Hinge regions

Hinge Domain	Sequence	SEQ ID NO
Hinge	DKTHT	628
Hinge	CPPC	629
Hinge	CPEPKSCDTPPPCPR	630
Hinge	ELKTPLGDTTHT	631
Hinge	KSCDKTHTCP	632
Hinge	KCCVDCP	633
Hinge	KYGPPCP	634
C233P Hinge	VEPKSPDKTHTCPPCP	635
C233S Hinge	LDPKSSDKTHTCPPCP	636
CD28 Hinge	IEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPSKP	637
CD8a Hinge	GGAVHTRGLDFA	638
CD8a Hinge	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD	639
CD8a Hinge	AKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD	640
CD8a Hinge	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDEPKSP DKTHTCPPCPAPPVAGPSVFLFPPKPKDT	641
CD8a Hinge	PAKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDI Y	642
CD8a Hinge	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYIWA PLAGTCGVLLLSLVITLYC	643
CD8a Hinge	TTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACD	644
CD8a Hinge	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIY	645
Delta5 Hinge	LDKTHTCPPCP	646

EpoR Hinge	APVGLVARLADESGHVVLRWLPPPETPMTSHIRYEVDVSAGNGAGSV QRVEILEGRTECVLSNLRGRTRYTFAVRARMAEPSFGGFWSAWSEPVS LLTPSD	647
FCRIIα Hinge	GLAVSTISSFFPPGYQ	648
Hinge	RWPESPKAQASSVPTAQPQAEGSLAKATTAPATTRNTGRGGEEKKKE KEKEEQEERETKTPECPSHTQPLGVYLLTPAVQDLWLRDKATFTCFVV GSDLKDAHLTWEVAGKVPTGGVEEGLLERHSNGSQSQHSRLTLPRSL WNAGTSVTCTLNHPSLPPQRLMALREPAAQAPVKLSLNLLASSDPPEA ASWLLCEVSGFSPPNILLMWLEDQREVNTSGFAPARPPPQPGSTTFWA WSVLRVPAPPSPQPATYTCVVSHEDSRTLLNASRSLEVSYVTDH	649
Hinge	YVTVSSQDPAEPKSPDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISR TPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYR VVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQV YTLPPSRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP VLDSDGSFFLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPGKKDPK	650
Hinge	KPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFA	651
Hinge	LEPKSCDKTHTCPPCP	652
Hinge	KPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLD	653
Hinge	ELKTPLGDTHTCPRCP	654
Hinge	EPKSCDTPPPCPRCP	655
Hinge	ESKYGPPCPSCP	656
Hinge	ERKCCVECPPCP	657
Hinge (CH2-CH3)	ESKYGPPCPPCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQ EDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWL NGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQ VSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLT VDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGK	658
Hinge (CH3)	ESKYGPPCPPCPGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDI AVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFS CSVMHEALHNHYTQKSLSLSLGK	659
IgD Hinge	RWPESPKAQASSVPTAQPQAEGSLAKATTAPATTRNTGRGGEEKKKE KEKEEQEERETKTPECPSHTQPLGVYLLTPAVQDLWLRDKATFTCFVV GSDLKDAHLTWEVAGKVPTGGVEEGLLERHSNGSQSQHSRLTLPRSL WNAGTSVTCTLNHPSLPPQRLMALREPAAQAPVKLSLNLLASSDPPEA ASWLLCEVSGFSPPNILLMWLEDQREVNTSGFAPARPPPQPGSTTFWA WSVLRVPAPPSPQPATYTCVVSHEDSRTLLNASRSLEVSYVTDH	660
IgD Hinge	RWPESPKAQASSVPTAQPQAEGSLAKATTAPATTRNTGRGGEEKKKE KEKEEQEERETKTPECPSHTQPLGVYLLTPAVQDLWLRDKATFTCFVV GSDLKDAHLTWEVAGKVPTGGVEEGLLERHSNGSQSQHSRLTLPRSL WNAGTSVTCTLHPSLPPQRLMALREPAAQAPVKLSLNLLASSDPPEAA SWLLCEVSGFSPPNILLMWLEDQREVNTSGFAPARPPPQPGSTTFWAW SVLRVPAPPSPQPATYTCVVSHEDSRTLLNASRSLEVSYVTDH	661
IgD Hinge	RWPESPKAQASSVPTAQPQAEGSLAKATTAPATTRNTGRGGEEKKKE KEKEEQEERETKTPECPSHTQPLGVYLLTPAVQDLWLRDKATFTCFVV GSDLKDAHLTWEVAGKVPTGGVEEGLLERHSNGSQSQHSRLTLPRSL WNAGTSVTCTLNHPSLPPQRLMALREPAAQAPVKLSLNLLASSDPPEA ASWLLCEVSGFSPPNILLMVVLEDQREVNTSGFAPARPPPQPGSTTFWA WSVLRVPAPPSPQPATYTCVVSHEDSRTLLNASRSLEVSYVTDH	662
IgD Hinge	ESPKAQASSVPTAQPQAEGSLAKATTAPATTRNTGRGGEEKKKEKEKE EQEERETKTP	663
IgD Hinge	RWPESPKAQASSVPTAQPQAEGSLAKATTAPATTRNTGRGGEEKKKE KEKEEQEERETKTPECPSHTQPLGVYLLTPAVQDLWLRDKATFTCFVV GSDLKDAHLTWEVAGKVPTGGVEEGLLERHSNGSQSQHSRLTLPRSL WNAGTSVTCTLNHPSLPPQRLMALREPAAQAPVKLSLNLLASSDPPEA ASWLLCEVSGFSPPNILLMWLEDQREVNTSGFAPARPPPQPGSTTFWA WSVLRVPAPPSPQPATYTCVVSHEDSRTLLNASRSLEVSYVTDH	664

IgD Hinge	RWPESPKAQASSVPTAQPQAEGSLAKATTAPATTRNTGRGGEEKKKE KEKEEQEERETKTPECPSHTQPLGVYLLTPAVQDLWLRDKATFTCFVV GSDLKDAHLTWEVAGKVPTGGVEEGLLERHSNGSQSQHSRLTLPRSL WNAGTSVTCTLNHPSLPPQRLMALREPAAQAPVKLSLNLLASSDPPEA ASWLLCEVSGFSPPNILLMWLEDQREVNTSGFAPARPPPQPGSTTFWA WSVLRVPAPPSPQPATYTCVVSHEDSRTLLNASRSLEVSYVTDH	665
IgD Hinge	RWPESPKAQASSVPTAQPQAEGSLAKATTAPATTRNTGRGGEEKKKE KEKEEQEERETKTPECPSHTQPLGVYLLTPAVQDLWLRDKATFTCFVV GSDLKDAHLTWEVAGKVPTGGVEEGLLERHSNGSQSQHSRLTLPRSL WNAGTSVTCTLNHPSLPPQRLMALREPAAQAPVKLSLNLLASSDPPEA ASWLLCEVSGFSPPNILLMWLEDQREVNTSGFAPARPPPQPGSTTFWAW SVLRVPAPPSPQPATYTCVVSHEDSRTLLNASRSLEVSYVTDH	666
IgD Hinge	RWPESPKAQASSVPTAQPQAEGSLAKATTAPATTRNTGRGGEEKKKE KEKEEQEERETKTPECPSHTQPLGVYLLTPAVQDLWLRDKATFTCFVV GSDLKDAHLTWEVAGKVPTGGVEEGLLERHSNGSQSQHSRLTLPRSL WNAGTSVTCTLNHPSLPPQRLMALREPAAQAPVKLSLNLLASSDPPEA ASWLLCEVSGFSPPNILLMWLEDQREVNTSGFAPARPPPQPGSTTFWA WSVLRVPAPPSPQPATYTCVVSHEDSRTLLNASRSLEVSYVTDH	667
IgG1 (CH2CH3) Hinge domain	AEPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIARTPEVTCVVV DVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLH QDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFL YSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKKD	668
IgG1 Hinge	AEPKSPDKTHTCPPCPKDPK	669
IgG1 Hinge	EPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIARTPEVTCVVVD VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQ DWLNGKEVKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELT KNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLV SKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKKD	670
IgG1 Hinge	SVFLFPPKPKDTL	671
IgG1 Hinge	EPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIARTPEVTCVVVD VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQ DWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELT KNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLY SKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK	672
IgG1 Hinge	EPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIARTPEVTCVVVD VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQ DWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELT KNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLY SKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKKDPK	673
IgG1 Hinge	VECPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEV KFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKE YKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTC LVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKS RWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK	674
IgG1 Hinge (CH2CH3 domain)	DPAEPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIARTPEVTCV VVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTV LHQDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRD ELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSF FLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKK	675
IgG3 Hinge	ELKTPLGDTTHTCPRCP	676
IgG3 Hinge	ELKTPLGDTHTCPRCPEPKSCDTPPPCPRCPEPKSCDTPPPCPRCPEPKS CDTPPPCPRCP	677
IgG4 (CH2 and CH3)	ESKYGPPCPPCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQ EDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWL NGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQ VSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLT VDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGKM	678

IgG4 (CH2 and CH3)	ESKYGPPCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQ EDPEVQFNWYVDGVEVHNAKTKPREEQFQSTYRVVSVLTVLHQDWL NGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQ VSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLT VDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGKM	679
IgG4 Hinge	SPNMVPHAHHAQ	680
IgG4 Hinge	GQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPE NNYKTTPPVLDSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNH YTQKSLSLSLGK	681
IgG4 Hinge	ESKYGPPCPPCPGGGSSGGGSGGQPREPQVYTLPPSQEEMTKNQVSLT CLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDK SRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGK	682
IgG4 Hinge	ESKYGPPCPSCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQ EDPEVQFNWYVDGVEVHQAKTKPREEQFNSTYRVVSVLTVLHQDWL NGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQ VSLTCLVKGFVPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLT VDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGK	683
IgG4 Hinge	ESKYGPPCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQ EDPEVQFNWYVDGVEVHQAKTKPREEQFNSTYRVVSVLTVLHQDWL NGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQ VSLTCLVKGFVPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLT VDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGK	684
IgG4 Hinge	GAATCTAAGTACGGACCGCCCTGCCCCCCTTGCCCT	685
IgG4 Hinge	ESKYGPPCPPCP	686
IgG4 Hinge	YGPPCPPCP	687
IgG4 Hinge	KYGPPCPPCP	688
IgG4 Hinge	EVVKYGPPCPPCP	689
IgG4 Hinge	ESKYGPPCPSCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSQ EDPEVQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWL NGKEYKCKVSNKGLPSSIEKTISKAKGQPREPQVYTLPPSQEEMTKNQ VSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSRLT VDLSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGK	690
IgG4 Hinge and Linker	ESKYGPPCPPCPGGGSSGGGSG	691
IgGl Hinge	EPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIARTPEVTCVVVD VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQ DWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELT KNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTIPPVLDSDGSFFLYS KLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK	692
IgGl Hinge	EPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIARTPEVTCVVVD VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQ DWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELT KNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTIPPVLDSDGSFFLYS KLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK	693
CH2CH3 spacer domain	EPKSCDKTHTCPPCPAPELLGGPSVFLFPPKPKDTLMISRTPEVTCVVV DVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLH QDWLNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDEL TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFL YSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKKDPK	694

[00235] In some embodiments, the CAR of the present invention may comprise one or more linkers between any of the domains of the CAR. The linker may be between 1-30 amino acids long. In this regard, the linker may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18,

19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 amino acids in length. In other embodiments, the linker may be flexible.

[00236] In some embodiments, the CH2CH3 may be preferentially excluded from the chimeric antigen structure to elicit a higher TNFa response as disclosed in WO2016149578 (the contents of which are herein incorporated by reference). In some constructs a CH2CH3 structural domain is included. This domain extends the scFV away from the plasma membrane extracellular surface, and allows for the efficient detection of transduced T cells with anti-IgG Fc-specific antibody. CAR constructs which include CH2CH3 domain are disclosed in WO2015069922A3 (the contents of which are incorporated herein by reference in its entirety).

[00237] In some embodiments, the components including the targeting moiety, transmembrane domain and intracellular signaling domains of the present invention may be constructed in a single fusion polypeptide. The fusion polypeptide may be the payload of an effector module of the invention. In some embodiments, more than one CAR fusion polypeptides may be included in an effector module, for example, two, three or more CARs may be included in the effector module under the control of a single SRE (e.g., a DD). Representative effector modules comprising the CAR payload are illustrated in Figures 2-6.

[00238] In some embodiments, the CAR sequences may be selected from Table 12.

Table 12: CAR sequences

Description	Source	Target	SEQ ID NO.
ALK CAR	SEQ ID NO. 37 in WO2015069922	ALK	695
ALK CAR	SEQ ID NO. 39 in WO2015069922	ALK	696
ALK CAR	SEQ ID NO. 41 in WO2015069922	ALK	697
ALK CAR	SEQ ID NO. 43 in WO2015069922	ALK	698
ALK CAR	SEQ ID NO. 44 in WO2015069922	ALK	699
ALK CAR	SEQ ID NO. 45 in WO2015069922	ALK	700
ALK CAR	SEQ ID NO. 46 in WO2015069922	ALK	701
ALK CAR	SEQ ID NO. 47 in WO2015069922	ALK	702
ALK CAR	SEQ ID NO. 48 in WO2015069922	ALK	703
ALK CAR	SEQ ID NO. 49 in WO2015069922	ALK	704
ALK CAR	SEQ ID NO. 50 in WO2015069922	ALK	705
ALK CAR	SEQ ID NO. 51 in WO2015069922	ALK	706
ALK CAR	SEQ ID NO. 52 in WO2015069922	ALK	707
ALK CAR	SEQ ID NO. 53 in WO2015069922	ALK	708
ALK CAR	SEQ ID NO. 54 in WO2015069922	ALK	709
ALK CAR	SEQ ID NO. 55 in WO2015069922	ALK	710
ALK CAR	SEQ ID NO. 56 in WO2015069922	ALK	711

ALK CAR	SEQ ID NO. 57 in WO2015069922	ALK	712
ALK CAR	SEQ ID NO. 58 in WO2015069922	ALK	713
ALK CAR	SEQ ID NO. 59 in WO2015069922	ALK	714
ALK CAR	SEQ ID NO. 60 in WO2015069922	ALK	715
ALK CAR	SEQ ID NO. 61 in WO2015069922	ALK	716
ALK CAR	SEQ ID NO. 62 in WO2015069922	ALK	717
ALK CAR	SEQ ID NO. 63 in WO2015069922	ALK	718
ALK CAR	SEQ ID NO. 64 in WO2015069922	ALK	719
ALK CAR	SEQ ID NO. 65 in WO2015069922	ALK	720
ALK CAR	SEQ ID NO. 66 in WO2015069922	ALK	721
ALK CAR	SEQ ID NO. 67 in WO2015069922	ALK	722
ALK CAR	SEQ ID NO. 68 in WO2015069922	ALK	723
ALK CAR	SEQ ID NO. 69 in WO2015069922	ALK	724
ALK CAR	SEQ ID NO. 70 in WO2015069922	ALK	725
ALK CAR	SEQ ID NO. 71 in WO2015069922	ALK	726
ALK CAR	SEQ ID NO. 72 in WO2015069922	ALK	727
ALK CAR	SEQ ID NO. 73 in WO2015069922	ALK	728
ALK CAR	SEQ ID NO. 74 in WO2015069922	ALK	729
ALK CAR	SEQ ID NO. 75 in WO2015069922	ALK	730
ALK CAR	SEQ ID NO. 76 in WO2015069922	ALK	731
ALK CAR	SEQ ID NO. 77 in WO2015069922	ALK	732
ALK CAR	SEQ ID NO. 78 in WO2015069922	ALK	733
ALK CAR	SEQ ID NO. 79 in WO2015069922	ALK	734
ALK CAR	SEQ ID NO. 80 in WO2015069922	ALK	735
ALK CAR	SEQ ID NO. 81 in WO2015069922	ALK	736
ALK CAR	SEQ ID NO. 82 in WO2015069922	ALK	737
ALK CAR	SEQ ID NO. 83 in WO2015069922	ALK	738
ALK CAR	SEQ ID NO. 84 in WO2015069922	ALK	739
ALK CAR	SEQ ID NO. 85 in WO2015069922	ALK	740
ALK CAR	SEQ ID NO. 86 in WO2015069922	ALK	741
ALK CAR	SEQ ID NO. 87 in WO2015069922	ALK	742
ALK CAR	SEQ ID NO. 88 in WO2015069922	ALK	743
ALK CAR	SEQ ID NO. 89 in WO2015069922	ALK	744
ALK CAR	SEQ ID NO. 90 in WO2015069922	ALK	745
CD22 (m971) third generation CAR	SEQ ID NO. 22 in WO2014065961	CD22	746
CD22 (m971) third generation CAR	SEQ ID NO. 23 in WO2014065961	CD22	747
CD22 (m971) third generation CAR	SEQ ID NO. 24 in WO2014065961	CD22	748
CD22 (CARsHA22 28z) CAR	SEQ ID NO. 15 in WO2013059593	CD22	749
CD22 (HA22 28BBz) CAR	SEQ ID NO. 16 in WO2013059593	CD22	750
CD22 (HASH22 28z) CAR	SEQ ID NO. 17 in WO2013059593	CD22	751
CD22 (HASH22 28BBz) CAR	SEQ ID NO. 18 in WO2013059593	CD22	752
CD22 (BL22 28z) CAR	SEQ ID NO. 19 in WO2013059593	CD22	753
CD22 (BL22 28BBz) CAR	SEQ ID NO. 20 in WO2013059593	CD22	754
CD22 (HA22SH-CAR-second generation, version 2) CAR	SEQ ID NO. 32 in WO2013059593	CD22	755

CD276 CAR (CD276.6 second generation)	SEQ ID NO. 39 in WO2014160627	CD276	756
CD276 CAR (CD276.1 second generation, version 1)	SEQ ID NO. 42 in WO2014160627	CD276	757
CD276 CAR (CD276.17 second generation, version 1)	SEQ ID NO. 45 in WO2014160627	CD276	758
CD276 CAR (CD276.6 CAR second generation, version 1)	SEQ ID NO. 122 in WO2014160627	CD276	759
CD276 CAR (CD276.6 CAR second generation, version 2)	SEQ ID NO. 123 in WO2014160627	CD276	760
CD276 CAR (CD276.6 CAR third generation)	SEQ ID NO. 124 in WO2014160627	CD276	761
CD276 CAR (CD276.1 CAR second generation, version 1)	SEQ ID NO. 125 in WO2014160627	CD276	762
CD276 CAR (CD276.1 CAR second generation, version 2)	SEQ ID NO. 126 in WO2014160627	CD276	763
CD276 CAR (CD276.1 CAR third generation)	SEQ ID NO. 127 in WO2014160627	CD276	764
CD276 CAR (CD276.17 CAR second generation, version 1)	SEQ ID NO. 128 in WO2014160627	CD276	765
CD276 CAR (CD276.17 CAR second generation, version 2)	SEQ ID NO. 129 in WO2014160627	CD276	766
CD276 CAR (CD276.17 CAR third generation)	SEQ ID NO. 130 in WO2014160627	CD276	767
CD 276 CAR	SEQ ID NO. 20 in WO2017044699	CD276	768
CD276.MG.BB.Z CAR	SEQ ID NO. 12 in WO2017044699	CD276	769

[00239] In one embodiment of the present invention, the payload of the invention is a CD33 specific CAR. The CD33 heavy and light chain may be combined with any of the signal peptides, transmembrane domains, costimulatory domains, intracellular domains and destabilizing domains described herein.

[00240] In one embodiment of the present invention, the payload of the invention is a GD2 specific CAR. The GD2 heavy and light chain may be combined with any of the signal peptides, transmembrane domains, costimulatory domains, intracellular domains and destabilizing domains described herein.

[00241] In one embodiment of the present invention, the payload of the invention is a Her2 specific CAR. The Her2 heavy and light chain may be combined with any of the signal peptides, transmembrane domains, costimulatory domains, intracellular domains and destabilizing domains described herein. Exemplary BCMA CAR sequences and its components are described in Table 13A. The amino acid sequences in Table 13A may comprise a stop codon which is denoted in the table with a "*" at the end of the amino acid sequence

Table 13A: DD-Her2 construct sequences

Description	Amino Acid Sequence	Amino Acid SEQ ID NO	Nucleic Acid SEQ ID NO/ Sequence

GMCSF Leader	LLLVTSLLLCELPHPAFLLIP	778	922
Linker	ASFE	920	921
Linker	GS	-	GGTTCC, GGATCC
Linker	TS	-	ACTAGT
Linker	HM	-	ATGCAC
4-1BB Intracellular Domain	KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGG CEL	773	919
4D5 scFV	DIQMTQSPSSLSASVGDRVTITCRASQDVNTAVAWYQQ KPGKAPKLLIYSASFLYSGVPSRFSGSRSGTDFTLTISSLQ PEDFATYYCQQHYTTPPTFGQGTKVEIKGSTSGSGKPGS GEGSGEVQLVESGGGLVQPGGSLRLSCAASGFNIKDTYI HWVRQAPGKGLEWVARIYPTNGYTRYADSVKGRFTISA DTSKNTAYLQMNSLRAEDTAVYYCSRWGGDGFYAMD VWGQGTLVTVSS	923	924
CD8a Hinge	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGL DFACD	925	926
Transmembrane Domain	IYIWAPLAGTCGVLLLSLVITLYC	927	928
CD3 Zeta signaling Domain	RVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDK RRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEI GMKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPP R	772	918
hDHFR (Amino acid 2-187 of WT; Y122I)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQRMT TTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVLSR ELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWIV GGSSVIKEAMNHPGHLKLFVTRIMQDFESDTFFPEIDLEK YKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	861	903-907
OT-Her2-004 (Met – GMCSF Leader - 4D5 scFv – Linker (ASFE) – CD8a hinge – Linker (GS) – Transmembrane Domain – Linker (TS) – 4-1BB intracellular signaling domain – Linker (HM) - CD3 zeta – Linker (GS) - hDHFR (Amino acid 2-187 of WT; Y122I) – stop)	MLLLVTSLLLCELPHPAFLLIPDIQMTQSPSSLSASVGDR VTITCRASQDVNTAVAWYQQKPGKAPKLLIYSASFLYS GVPSRFSGSRSGTDFTLTISSLQPEDFATYYCQQHYTTPP TFGQGTKVEIKGSTSGSGKPGSGEGSGEVQLVESGGGLV QPGGSLRLSCAASGFNIKDTYIHWVRQAPGKGLEWVAR IYPTNGYTRYADSVKGRFTISADTSKNTAYLQMNSLRAE DTAVYYCSRWGGDGFYAMDVWGQGTLVTVSSASFETT TPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDF ACDGSIYIWAPLAGTCGVLLLSLVITLYCTSKRGRKKLL YIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELHMRVK FSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKRRG RDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMK GERRRGKGHDGLYQGLSTATKDTYDALHMQALPPRGS VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQRMT TTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVLSR ELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWIV GGSSVIKEAMNHPGHLKLFVTRIMQDFESDTFFPEIDLEK YKLLPEYPGVLSDVQEEKGIKYKFEVYEKND*	906	907

[00242] In one embodiment, the CAR of the present invention is a BCMA (B-cell maturation antigen) CAR, also referred to as the CD269. The BCMA heavy and light chains may be combined with any of the signal peptides, transmembrane domains, costimulatory domains, intracellular domains and destabilizing domains described herein. Exemplary BCMA CAR sequences and its components are described in Table 13B. The amino acid sequences in Table

13B may comprise a stop codon which is denoted in the table with a "*" at the end of the amino acid sequence.

Table 13B: BCMA CAR

Description	Amino Acid Sequence	Amino Acid SEQ ID NO.	Nucleic Acid SEQ ID NO.
BCMA scFv (C11D5.3)	DIVLTQSPASLAMSLGKRATISCRASESVSVIGAHL IHWYQQKPGQPPKLLIYLASNLETGVPARFSGSGS GTDFTLTIDPVEEDDVAIYSCLQSRIFPRTFGGGTK LEIKGSTSGSGKPGSGEGSTKGQIQLVQSGPELKK PGETVKISCKASGYTFTDYSINWVKRAPGKGLKW MGWINTETREPAYAYDFRGRFAFSLETSASTAYL QINNLKYEDTATYFCALDYSYAMDYWGQGTSVT VSS	770	916
CD8α hinge--TM	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVH TRGLDFACDIYIWAPLAGTCGVLLLSLVITLYC	771	917
CD3 zeta signaling domain	RVKFSRSADAPAYKQGQNQLYNELNLGRREEYD VLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDK MAEAYSEIGMKGERRRGKGHDGLYQGLSTATKD TYDALHMQALPPR	772	918
4-1BB intracellular signaling domain	KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEE EEGGCEL	773	919
CD8α leader	MALPVTALLLPLALLLHAARP	131	132-136, 915
ecDHFR (Amino acid 2-159 of WT) (R12Y, Y100I)	ISLIAALAVDYVIGMENAMPWNLPADLAWFKRN TLNKPVIMGRHTWESIGRPLPGRKNIILSSQPGTDD RVTWVKSVDEAIAACGDVPEIMVIGGGRVIEQFLP KAQKLYLTHIDAEVEGDTHFPDYEPDDWESVFSE FHDADAQNSHSYCFEILERR	9	61, 869-874
FKBP (E31G, F36V, R71G, K105E)	GVQVETISPGDGRTFPKRGQTCVVHYTGMLGDG KKVDSSRDRNKPFKFMLGKQEVIRGWEEGVAQM SVGQGAKLTISPDYAYGATGHPGIIPPHATLVFDV ELLELE	12	88, 883-889
OT-BCMA-001 (CD8a leader- BCMA scFv - CD8a hinge—Tm – 4-1BB intracellular domain – CD3 zeta - stop)	MALPVTALLLPLALLLHAARPDIVLTQSPASLAMS LGKRATISCRASESVSVIGAHLIHWYQQKPGQPPK LLIYLASNLETGVPARFSGSGSGTDFTLTIDPVEED DVAIYSCLQSRIFPRTFGGGTKLEIKGSTSGSGKPG SGEGSTKGQIQLVQSGPELKKPGETVKISCKASGY TFTDYSINWVKRAPGKGLKWMGWINTETREPAY AYDFRGRFAFSLETSASTAYLQINNLKYEDTATYF CALDYSYAMDYWGQGTSVTVSSTTTPAPRPPTPA PTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYI WAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQP FMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRS ADAPAYKQGQNQLYNELNLGRREEYDVLDKRRG RDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSE IGMKGERRRGKGHDGLYQGLSTATKDTYDALHM QALPPR*	775	833
OT-BCMA-002 (CD8a leader - BCMA scFv - CD8a hinge—Tm – 4-1BB intracellular domain - CD3 zeta - Linker (SG) - FKBP (E31G, F36V, R71G, K105E) - stop)	MALPVTALLLPLALLLHAARPDIVLTQSPASLAMS LGKRATISCRASESVSVIGAHLIHWYQQKPGQPPK LLIYLASNLETGVPARFSGSGSGTDFTLTIDPVEED DVAIYSCLQSRIFPRTFGGGTKLEIKGSTSGSGKPG SGEGSTKGQIQLVQSGPELKKPGETVKISCKASGY TFTDYSINWVKRAPGKGLKWMGWINTETREPAY AYDFRGRFAFSLETSASTAYLQINNLKYEDTATYF CALDYSYAMDYWGQGTSVTVSSTTTPAPRPPTPA PTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYI	776	834

	WAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQP FMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRS ADAPAYKQGQNQLYNELNLGRREEYDVLDKRRG RDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSE IGMKGERRRGKGHDGLYQGLSTATKDTYDALHM QALPPRSGGVQVETISPGDGRTFPKRGQTCVVHY TGMLGDGKKVDSSRDRNKPFKFMLGKQEVIRGW EEGVAQMSVGQGAKLTISPDYAYGATGHPGIIPPH ATLVFDVELLELE*		
OT-BCMA-003 (CD8a leader - BCMA scFv - CD8a hinge---Tm -- 4-1BB intracellular domain - CD3 zeta - Linker (SG) - ecDHFR (Amino acid 2-159 of WT) (R12Y, Y100I) - stop)	MALPVTALLLPLALLLHAARPDIVLTQSPASLAMS LGKRATISCRASESVSVIGAHLIHWYQQKPGQPPK LLIYLASNLETGVPARFSGSGSGTDFTLTIDPVEED DVAIYSCLQSRIFPRTFGGGTKLEIKGSTSGSGKPG SGEGSTKGQIQLVQSGPELKKPGETVKISCKASGY TFTDYSINWVKRAPGKGLKWMGWINTETREPAY AYDFRGRFAFSLETSASTAYLQINNLKYEDTATYF CALDYSYAMDYWGQGTSVTVSSTTTPAPRPPTPA PTIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYI WAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQP FMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRS ADAPAYKQGQNQLYNELNLGRREEYDVLDKRRG RDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSE IGMKGERRRGKGHDGLYQGLSTATKDTYDALHM QALPPRSGISLIAALAVDYVIGMENAMPWNLPAD LAWFKRNTLNKPVIMGRHTWESIGRPLPGRKNIIL SSQPGTDDRVTWVKSVDEAIAACGDVPEIMVIGG GRVIEQFLPKAQKLYLTHIDAEVEGDTHFPDYEPD DWESVFSEFHDADAQNSHSYCFEILERR*	777	835

Tandem CAR (TanCAR)

[00243] In some embodiments, the CAR of the present invention may be a tandem chimeric antigen receptor (TanCAR) which is able to target two, three, four, or more tumor specific antigens. In some aspects, The CAR is a bispecific TanCAR including two targeting domains which recognize two different TSAs on tumor cells. The bispecific CAR may be further defined as comprising an extracellular region comprising a targeting domain (e.g., an antigen recognition domain) specific for a first tumor antigen and a targeting domain (e.g., an antigen recognition domain) specific for a second tumor antigen. In other aspects, the CAR is a multispecific TanCAR that includes three or more targeting domains configured in a tandem arrangement. The space between the targeting domains in the TanCAR may be between about 5 and about 30 amino acids in length, for example, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 and 30 amino acids.

Split CAR

[00244] In some embodiments, the components including the targeting moiety, transmembrane domain and intracellular signaling domains of the present invention may be split into two or more parts such that it is dependent on multiple inputs that promote assembly of the intact functional receptor. In one embodiment, the split synthetic CAR system can be constructed in

which the assembly of an activated CAR receptor is dependent on the binding of a ligand to the SRE (e.g. a small molecule) and a specific antigen to the targeting moiety. As a non-limiting example, the split CAR consists of two parts that assemble in a small molecule-dependent manner; one part of the receptor features an extracellular antigen binding domain (e.g. scFv) and the other part has the intracellular signaling domains, such as the CD3ζ intracellular domain.

[00245] In other aspects, the split parts of the CAR system can be further modified to increase signal. In one example, the second part of cytoplasmic fragment may be anchored to the plasma membrane by incorporating a transmembrane domain (e.g., CD8α transmembrane domain) to the construct. An additional extracellular domain may also be added to the second part of the CAR system, for instance an extracellular domain that mediates homo-dimerization. These modifications may increase receptor output activity, i.e., T cell activation.

[00246] In some aspects, the two parts of the split CAR system contain heterodimerization domains that conditionally interact upon binding of a heterodimerizing small molecule. As such, the receptor components are assembled in the presence of the small molecule, to form an intact system which can then be activated by antigen engagement. Any known heterodimerizing components can be incorporated into a split CAR system. Other small molecule dependent heterodimerization domains may also be used, including, but not limited to, gibberellin-induced dimerization system (GID1-GAI), trimethoprim-SLF induced ecDHFR and FKBP dimerization (Czlapinski et al., *J Am Chem Soc.*, 2008, 130(40): 13186-13187) and ABA (abscisic acid) induced dimerization of PP2C and PYL domains (Cutler et al., *Annu Rev Plant Biol.* 2010, 61: 651-679). The dual regulation using inducible assembly (e.g., ligand dependent dimerization) and degradation (e.g., destabilizing domain induced CAR degradation) of the split CAR system may provide more flexibility to control the activity of the CAR modified T cells.

Switchable CAR

[00247] In some embodiments, the CAR of the invention may be a switchable CAR. Juillerat et al (Juilerat et al., *Sci. Rep.*, 2016, 6: 18950; the contents of which are incorporated herein by reference in their entirety) recently reported controllable CARs that can be transiently switched on in response to a stimulus (e.g. a small molecule). In this CAR design, a system is directly integrated in the hinge domain that separate the scFv domain from the cell membrane domain in the CAR. Such system is possible to split or combine different key functions of a CAR such as activation and costimulation within different chains of a receptor complex, mimicking the complexity of the TCR native architecture. This integrated system can switch the scFv and antigen interaction between on/off states controlled by the absence/presence of the stimulus.

Reversible CAR

[00248] In other embodiments, the CAR of the invention may be a reversible CAR system. In this CAR architecture, a LID domain (ligand-induced degradation) is incorporated into the CAR system. The CAR can be temporarily down-regulated by adding a ligand of the LID domain. The combination of LID and DD mediated regulation provides tunable control of continuingly activated CAR T cells, thereby reducing CAR mediated tissue toxicity.

Activation-conditional CAR

[00249] In some embodiments, payloads of the invention may be an activation-conditional chimeric antigen receptor, which is only expressed in an activated immune cell. The expression of the CAR may be coupled to activation conditional control region which refers to one or more nucleic acid sequences that induce the transcription and/or expression of a sequence e.g. a CAR under its control. Such activation conditional control regions may be promoters of genes that are upregulated during the activation of the immune effector cell e.g. IL2 promoter or NFAT binding sites. In some embodiments, activation of the immune cell may be achieved by a constitutively expressed CAR (International Publication NO. WO2016126608; the contents of which are incorporated herein by reference in their entirety).

4. Additional effector module features

[00250] The effector module of the present invention may further comprise a signal sequence which regulates the distribution of the payload of interest, a cleavage and/or processing feature which facilitate cleavage of the payload from the effector module construct, a targeting and/or penetrating signal which can regulate the cellular localization of the effector module, a tag, and/or one or more linker sequences which link different components of the effector module.

Signal sequences

[00251] In addition to the SRE (e.g., DD) and payload region, effector modules of the invention may further comprise one or more signal sequences. Signal sequences (sometimes referred to as signal peptides, targeting signals, target peptides, localization sequences, transit peptides, leader sequences or leader peptides) direct proteins (e.g., the effector module of the present invention) to their designated cellular and/or extracellular locations. Protein signal sequences play a central role in the targeting and translocation of nearly all secreted proteins and many integral membrane proteins.

[00252] A signal sequence is a short (5-30 amino acids long) peptide present at the N-terminus of the majority of newly synthesized proteins that are destined towards a particular location. Signal sequences can be recognized by signal recognition particles (SRPs) and cleaved using type I and type II signal peptide peptidases. Signal sequences derived from human proteins can be incorporated as a regulatory module of the effector module to direct the effector module to a

particular cellular and/or extracellular location. These signal sequences are experimentally verified and can be cleaved (Zhang et al., *Protein Sci.* 2004, 13:2819-2824).

[00253] In some embodiments, a signal sequence may be, although not necessarily, located at the N-terminus or C-terminus of the effector module, and may be, although not necessarily, cleaved off the desired effector module to yield a "mature" payload, i.e., an immunotherapeutic agent as discussed herein.

[00254] In some examples, a signal sequence may be a secreted signal sequence derived from a naturally secreted protein, and its variant thereof. In some instances, the secreted signal sequences may be cytokine signal sequences such as, but not limited to, IL2 signal sequence (amino acid of SEQ ID NO. 49, encoded by the nucleic acid sequence of SEQ ID NO. 55-56 and/or 117-118), p40 signal sequence (amino acid sequence of SEQ ID NO. 119, encoded by the nucleic acid sequence of SEQ ID NO. 120-128), or a GMCSF leader sequence (SEQ ID NO. 778 (encoded by SEQ ID NO. 922), 779, 780).

[00255] In some instances, signal sequences directing the payload of interest to the surface membrane of the target cell may be used. Expression of the payload on the surface of the target cell may be useful to limit the diffusion of the payload to non-target *in vivo* environments, thereby potentially improving the safety profile of the payloads. Additionally, the membrane presentation of the payload may allow for physiologically and qualitative signaling as well as stabilization and recycling of the payload for a longer half-life. Membrane sequences may be the endogenous signal sequence of the N terminal component of the payload of interest. Optionally, it may be desirable to exchange this sequence for a different signal sequence. Signal sequences may be selected based on their compatibility with the secretory pathway of the cell type of interest so that the payload is presented on the surface of the T cell. In some embodiments, the signal sequence may be IgE signal sequence (amino acid SEQ ID NO. 129 and nucleotide sequence of SEQ ID NO. 130), CD8a signal sequence (also referred to as CD8a leader) (amino acid SEQ ID NO. 131 and nucleotide sequence of SEQ ID NO. 132-136, and/or 915) or an IL15Ra signal sequence (amino acid SEQ ID NO. 781, encoded by SEQ ID NO. 782).

[00256] Other examples of signal sequences include, a variant may be a modified signal sequence discussed in U.S. Pat. NOs. 8, 148, 494; 8,258,102; 9,133,265; 9,279,007; and U.S. patent application publication NOS. 20070141666; and International patent application publication NOS. WO1993018181; the contents of each of which are incorporated herein by reference in their entirety.

[00257] In other examples, a signal sequence may be a heterogeneous signal sequence from other organisms such as virus, yeast and bacteria, which can direct an effector module to a

particular cellular site, such as a nucleus (e.g., EP 1209450). Other examples may include Aspartic Protease (NSP24) signal sequences from *Trichoderma* that can increase secretion of fused protein such as enzymes (e.g., U. S. Pat. NOS. 8,093,016 to Cervin and Kim), bacterial lipoprotein signal sequences (e.g., PCT application publication NOS. WO199109952 to Lau and Rioux), *E.coli* enterotoxin II signal peptides (e.g., U.S. Pat. NOS. 6,605,697 to Kwon et al.), *E.coli* secretion signal sequence (e.g., U.S. patent publication NOS. US2016090404 to Malley et al.), a lipase signal sequence from a methylotrophic yeast (e.g., U.S. Pat. NOS. 8,975,041), and signal peptides for DNases derived from *Coryneform bacteria* (e.g., U.S. Pat. NOS. 4,965,197); the contents of each of which are incorporated herein by reference in their entirety.

[00258] Signal sequences may also include nuclear localization signals (NLSs), nuclear export signals (NESs), polarized cell tubulo-vesicular structure localization signals (See, e.g., U.S. Pat. NOS. 8, 993,742; Cour et al., *Nucleic Acids Res*. 2003, 31(1): 393-396; the contents of each of which are incorporated herein by reference in their entirety),extracellular localization signals, signals to subcellular locations (e.g. lysosome, endoplasmic reticulum, golgi, mitochondria, plasma membrane and peroxisomes, etc.) (See, e.g., U.S. Pat. NOS. 7,396,811; and Negi et al., *Database,* 2015, 1-7; the contents of each of which are incorporated herein by reference in their entirety).

[00259] In some embodiments, signal sequences of the present invention, include without limitation, any of those taught in Table 7 of copending commonly owned U.S. Provisional Patent Application No. 62/320,864, filed on 4/11/2016, or in US Provisional Application No. 62/466,596 filed March 3, 2017 and the International Publication WO2017/180587, the contents of which are incorporated herein by reference in their entirety.

Cleavage sites

[00260] In some embodiments, the effector module comprises a cleavage and/or processing feature. The effector module of the present invention may include at least one protein cleavage signal/site. The protein cleavage signal/site may be located at the N-terminus, the C-terminus, at any space between the N- and the C- termini such as, but not limited to, half-way between the N- and C-termini, between the N-terminus and the half-way point, between the half-way point and the C-terminus, and combinations thereof.

[00261] The effector module may include one or more cleavage signal(s)/site(s) of any proteinases. The proteinases may be a serine proteinase, a cysteine proteinase, an endopeptidase, a dipeptidase, a metalloproteinase, a glutamic proteinase, a threonine proteinase and an aspartic proteinase. In some aspects, the cleavage site may be a signal sequence of furin, actinidain, calpain-1, carboxypeptidase A, carboxypeptidase P, carboxypeptidase Y, caspase-1, caspase-2,

caspase-3, caspase-4, caspase-5, caspase-6, caspase-7, caspase-8, caspase-9, caspase-10, cathepsin B, cathepsin C, cathepsin G, cathepsin H, cathepsin K, cathepsin L, cathepsin S, cathepsin V, clostripain, chymase, chymotrypsin, elastase, endoproteinase, enterokinase, factor Xa, formic acid, granzyme B, Matrix metallopeptidase-2, Matrix metallopeptidase-3, pepsin, proteinase K, SUMO protease, subtilisin, TEV protease, thermolysin, thrombin, trypsin and TAGZyme.

[00262] In one embodiment, the cleavage site is a furin cleavage site comprising the amino acid sequence SARNRQKRS (SEQ ID NO. 137), encoded by nucleotide sequence of SEQ ID NO. 138; or a revised furin cleavage site comprising the amino acid sequence ARNRQKRS (SEQ ID NO. 139), encoded by nucleotide sequence of SEQ ID NO. 140; modified furin site comprising the amino acid sequence ESRRVRRNKRSK (SEQ ID NO. 141), encoded by nucleotide sequence of SEQ ID NO. 142-144; or a SGESRRVRRNKRSK (SEQ ID NO. 785), encoded by the nucleotide sequence of SEQ ID NO. 784. In some instances, the cleavage site is a P2A cleavage site (ATNFSLLKQAGDVEENPGP (SEQ ID NO. 783), encoded by SEQ ID NO. 786, or GATNFSLLKQAGDVEENPGP (SEQ ID NO.. 864), encoded by SEQ ID NO. 865), wherein NPGP (SEQ ID NO. 866) is the P2A site.

[00263] In some embodiments, cleavage sites of the present invention, include without limitation, any of those taught in Table 7 of copending commonly owned U.S. Provisional Patent Application No. 62/320,864, filed on 4/11/2016, or in US Provisional Application No. 62/466,596 filed March 3, 2017 and the International Publication WO2017/180587, the contents of which are incorporated herein by reference in their entirety.

Protein tags

[00264] In some embodiments, the effector module of the invention may comprise a protein tag. The protein tag may be used for detecting and monitoring the process of the effector module. The effector module may include one or more tags such as an epitope tag (e.g., a FLAG or hemagglutinin (HA) tag). A large number of protein tags may be used for the present effector modules. They include, but are not limited to, self-labeling polypeptide tags (e.g., haloalkane dehalogenase (halotag2 or halotag7), ACP tag, clip tag, MCP tag, snap tag), epitope tags (e.g., FLAG, HA, His, and Myc), fluorescent tags (e.g., green fluorescent protein (GFP), red fluorescent protein (RFP), yellow fluorescent protein (YFP), and its variants), bioluminescent tags (e.g. luciferase and its variants), affinity tags (e.g., maltose-binding protein (MBP) tag, glutathione-S-transferase (GST) tag), immunogenic affinity tags (e.g., protein A/G, IRS, AU1, AU5, glu-glu, KT3, S-tag, HSV, VSV-G, Xpress and V5), and other tags (e.g., biotin (small

molecule), StrepTag (StrepII), SBP, biotin carboxyl carrier protein (BCCP), eXact, CBP, CYD, HPC, CBD intein-chitin binding domain, Trx, NorpA, and NusA.

[00265] In other embodiments, a tag may also be selected from those disclosed in U.S. Pat. NOs. 8,999,897; 8,357,511; 7,094, 568; 5,011,912; 4,851,341; and 4,703,004; U.S patent application publication NOs. US2013115635 and US2013012687; and International application publication NOS. WO2013091661; the contents of each of which are incorporated herein by reference in their entirety.

[00266] In some aspects, a multiplicity of protein tags, either the same or different tags, may be used; each of the tags may be located at the same N- or C-terminus, whereas in other cases these tags may be located at each terminus.

[00267] In one embodiment, the protein tag is an HA tag. A non-limiting example of an HA tag is YPYDVPDYA (SEQ ID NO. 852, encoded by SEQ ID NO. 853, 867, and/or 868).

[00268] In some embodiments, protein tags of the present invention, include without limitation, any of those taught in Table 8 of copending commonly owned U.S. Provisional Patent Application No. 62/320,864, filed on 4/11/2016, or in US Provisional Application No. 62/466,596 filed March 3, 2017 and the International Publication WO2017/180587, the contents of which are incorporated herein by reference in their entirety.

Targeting peptides

[00269] In some embodiments, the effector module of the invention may further comprise a targeting and/or penetrating peptide. Small targeting and/or penetrating peptides that selectively recognize cell surface markers (e.g. receptors, trans-membrane proteins, and extra-cellular matrix molecules) can be employed to target the effector module to the desired organs, tissues or cells. Short peptides (5-50 amino acid residues) synthesized *in vitro* and naturally occurring peptides, or analogs, variants, derivatives thereof, may be incorporated into the effector module for homing the effector module to the desired organs, tissues and cells, and/or subcellular locations inside the cells.

[00270] In some embodiments, a targeting sequence and/or penetrating peptide may be included in the effector module to drive the effector module to a target organ, or a tissue, or a cell (e.g., a cancer cell). In other embodiments, a targeting and/or penetrating peptide may direct the effector module to a specific subcellular location inside a cell.

[00271] A targeting peptide has any number of amino acids from about 6 to about 30 inclusive. The peptide may have 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29 or 30 amino acids. Generally, a targeting peptide may have 25 or fewer amino acids, for example, 20 or fewer, for example 15 or fewer.

[00272] Exemplary targeting peptides may include, but are not limited to, those disclosed in the art, e.g., U.S. Pat. NOs. 9,206,231; 9,110,059; 8,706,219; and 8,772,449; and U.S. application publication NOs. 2016089447; 2016060296; 2016060314; 2016060312; 2016060311; 2016009772; 2016002613; 2015314011 and 2015166621; and International application publication NOs. WO2015179691 and WO2015183044; the contents of each of which are incorporated herein by reference in their entirety.

[00273] In some embodiments, targeting peptides of the present invention, include without limitation, any of those taught in Table 9 of copending commonly owned U.S. Provisional Patent Application No. 62/320,864, filed on 4/11/2016, the contents of which are incorporated herein by reference in their entirety.

Linkers

[00274] In some embodiments, the effector module of the invention may further comprise a linker sequence. The linker region serves primarily as a spacer between two or more polypeptides within the effector module. The "linker" or "spacer", as used herein, refers to a molecule or group of molecules that connects two molecules, or two parts of a molecule such as two domains of a recombinant protein.

[00275] In some embodiments, "Linker" (L) or "linker domain" or "linker region" or "linker module" or "peptide linker" as used herein refers to an oligo- or polypeptide region of from about 1 to 100 amino acids in length, which links together any of the domains/regions of the effector module (also called peptide linker). The peptide linker may be 1-40 amino acids in length, or 2-30 amino acids in length, or 20-80 amino acids in length, or 50-100 amino acids in length. Linker length may also be optimized depending on the type of payload utilized and based on the crystal structure of the payload. In some instances, a shorter linker length may be preferably selected. In some aspects, the peptide linker is made up of amino acids linked together by peptide bonds, preferably from 1 to 20 amino acids linked by peptide bonds, wherein the amino acids are selected from the 20 naturally occurring amino acids: Glycine (G), Alanine (A), Valine (V), Leucine (L), Isoleucine (I), Serine (S), Cysteine (C), Threonine (T), Methionine (M), Proline (P), Phenylalanine (F), Tyrosine (Y), Tryptophan (W), Histidine (H), Lysine (K), Arginine (R), Aspartate (D), Glutamic acid (E), Asparagine (N), and Glutamine (Q). One or more of these amino acids may be glycosylated, as is understood by those in the art. In some aspects, amino acids of a peptide linker may be selected from Alanine (A), Glycine (G), Proline (P), Asparagine (R), Serine (S), Glutamine (Q) and Lysine (K).

[00276] In one example, an artificially designed peptide linker may preferably be composed of a polymer of flexible residues like Glycine (G) and Serine (S) so that the adjacent protein domains

are free to move relative to one another. Longer linkers may be used when it is desirable to ensure that two adjacent domains do not interfere with one another. The choice of a particular linker sequence may concern if it affects biological activity, stability, folding, targeting and/or pharmacokinetic features of the fusion construct. Examples of peptide linkers include, but are not limited to: MH, SG, GGSG (SEQ ID NO. 145; encoded by the nucleotide sequence SEQ ID NO. 146), GGSGG (SEQ ID NO. 147; encoded by any of the nucleotide sequences SEQ ID NO. 148-152), GGSGGG (SEQ ID NO. 153; encoded by any of the nucleotide sequences SEQ ID NO. 154-155) , SGGGS (SEQ ID NO. 68; encoded by the nucleotide sequence SEQ ID NO. 85, 69, 86), GGSGGGSGG (SEQ ID NO. 156; encoded by the nucleotide sequence SEQ ID NO. 157), GGGGG (SEQ ID NO. 158), GGGGS (SEQ ID NO. 159) or (GGGGS)n (n=1 (SEQ ID NO. 159), 2 (SEQ ID NO. 160), 3 (SEQ ID NO. 161, encoded by 174, 175, 171, 219, 774, 837), 4 (SEQ ID NO. 162), 5 (SEQ ID NO. 163), or 6 (SEQ ID NO. 164)), SSSSG (SEQ ID NO. 165) or (SSSSG)n (n=1 (SEQ ID NO. 165), 2 (SEQ ID NO. 166), 3 (SEQ ID NO. 167), 4 (SEQ ID NO. 168), 5 (SEQ ID NO. 169), or 6 (SEQ ID NO. 170)), SGGGSGGGGSGGGGSGGGGSGGGSLQ (SEQ ID NO. 171; encoded by the nucleotide sequence SEQ ID NO. 172, 838-843), EFSTEF (SEQ ID NO. 50; encoded by any of the nucleotide sequences SEQ ID NO. 57, 173), GKSSGSGSESKS (SEQ ID NO. 176), GGSTSGSGKSSEGKG (SEQ ID NO. 177), GSTSGSGKSSSEGSGSTKG (SEQ ID NO. 178), GSTSGSGKPGSGEGSTKG (SEQ ID NO. 179), VDYPYDVPDYALD (SEQ ID NO. 67; encoded by nucleotide sequence SEQ ID NO. 84), EGKSSGSGSESKEF (SEQ ID NO. 180), SG3-(SG4)3-SG3-SLQ- YPYDVPDYA (SEQ ID NO. 787), encoded by the nucleotide sequence of SEQ ID NO. 788; DYKDDDDK (SEQ ID NO.789), encoded by the nucleotide sequence of SEQ ID NO. 790; SG3-(SG4)5-SG3-S (SEQ ID NO. 791), encoded by SEQ ID NO. 792; SGGGSGGGGSGGGGSGGGGSYPYDVPDYASGGGS (SEQ ID NO. 793), encoded by SEQ ID NO. 794; GSGATNFSLLKQAGDVEENPGP (SEQ ID NO. 795), encoded by SEQ ID NO.796; SGGGSGGGGSGGGGSGGGGS (SEQ ID NO. 844), encoded by the nucleotide sequence of SEQ ID NO. 845; QLIGMLQGLMRDL (SEQ ID NO. 908), encoded by SEQ ID NO. 909; ASFE (SEQ ID NO. 920), encoded by SEQ ID NO. 921; GS (encoded by GGTTCC), SG (encoded by AGCGGC), EF (encoded by GAGTTC), TS (encoded by ACTAGT), HM (encoded by CACATG), MH (encoded by ATGCAC) or GSG (encoded by GGATCCGGA or GGATCCGGT).

[00277] In other examples, a peptide linker may be made up of a majority of amino acids that are sterically unhindered, such as Glycine (G) and Alanine (A). Exemplary linkers are polyglycines (such as (G)4 (SEQ ID NO. 929), (G)5 (SEQ ID NO. 930), (G)8 (SEQ ID NO.

931)), poly(GA), and polyalanines. The linkers described herein are exemplary, and linkers that are much longer and which include other residues are contemplated by the present invention.

[00278] A linker sequence may be a natural linker derived from a multi-domain protein. A natural linker is a short peptide sequence that separates two different domains or motifs within a protein.

[00279] In some aspects, linkers may be flexible or rigid. In other aspects, linkers may be cleavable or non- cleavable. As used herein, the terms "cleavable linker domain or region" or "cleavable peptide linker" are used interchangeably. In some embodiments, the linker sequence may be cleaved enzymatically and/or chemically. Examples of enzymes (e.g., proteinase/peptidase) useful for cleaving the peptide linker include, but are not limited, to Arg-C proteinase, Asp-N endopeptidase, chymotrypsin, clostripain, enterokinase, Factor Xa, glutamyl endopeptidase, Granzyme B, Achromobacter proteinase I, pepsin, proline endopeptidase, proteinase K, Staphylococcal peptidase I, thermolysin, thrombin, trypsin, and members of the Caspase family of proteolytic enzymes (e.g. Caspases 1-10). Chemical sensitive cleavage sites may also be included in a linker sequence. Examples of chemical cleavage reagents include, but are not limited to, cyanogen bromide, which cleaves methionine residues; N-chloro succinimide, iodobenzoic acid or BNPS-skatole (2-(2-nitrophenylsulfenyl)-3-methylindole), which cleaves tryptophan residues; dilute acids, which cleave at aspartyl-prolyl bonds; and e aspartic acid-proline acid cleavable recognition sites (i.e., a cleavable peptide linker comprising one or more D-P dipeptide moieties). The fusion module may include multiple regions encoding peptides of interest separated by one or more cleavable peptide linkers.

[00280] In other embodiments, a cleavable linker may be a "self-cleaving" linker peptide, such as 2A linkers (for example T2A), 2A-like linkers or functional equivalents thereof and combinations thereof. In some embodiments, the linkers include the picornaviral 2A-like linker, CHYSEL sequences of porcine teschovirus (P2A), Thosea asigna virus (T2A) or combinations, variants and functional equivalents thereof. Other linkers will be apparent to those skilled in the art and may be used in connection with alternate embodiments of the invention.

[00281] In some embodiments, the biocircuits of the present invention may include 2A peptides. The 2A peptide is a sequence of about 20 amino acid residues from a virus that is recognized by a protease (2A peptidases) endogenous to the cell. The 2A peptide was identified among picornaviruses, a typical example of which is the Foot-and Mouth disease virus (Robertson BH, et. al., J Virol 1985, 54:651-660). 2A-like sequences have also been found in Picornaviridae like equine rhinitis A virus, as well as unrelated viruses such as porcine teschovirus-1 and the insect *Thosea asigna* virus (TaV). In such viruses, multiple proteins are derived from a large

polyprotein encoded by an open reading frame. The 2A peptide mediates the co-translational cleavage of this polyprotein at a single site that forms the junction between the virus capsid and replication polyprotein domains. The 2A sequences contain the consensus motif D-V/I-E-X-N-P-G-P (SEQ ID NO. 932). These sequences are thought to act co-translationally, preventing the formation of a normal peptide bond between the glycine and last proline, resulting in the ribosome skipping of the next codon (Donnelly ML et al. (2001). *J Gen Virol,* 82:1013-1025). After cleavage, the short peptide remains fused to the C -terminus of the protein upstream of the cleavage site, while the proline is added to the N-terminus of the protein downstream of the cleavage site. Of the 2A peptides identified to date, four have been widely used namely FMDV 2A (abbreviated herein as F2A); equine rhinitis A virus (ERAV) 2A (E2A); porcine teschovirus-1 2A (P2A) and *Thoseaasigna* virus 2A (T2A). In some embodiments, the 2A peptide sequences useful in the present invention are selected from SEQ ID NO.8-11 of International Patent Publication WO2010042490, the contents of which are incorporated by reference in its entirety.

[00282] The linkers of the present invention may also be non-peptide linkers. For example, alkyl linkers such as ----NH----(CH_2) a-C(O)----, wherein a=2-20 can be used. These alkyl linkers may further be substituted by any non-sterically hindering group such as lower alkyl (e.g., C_1-C_6) lower acyl, halogen (e.g., Cl, Br), CN, NH_2, phenyl, etc.

[00283] In some aspects, the linker may be an artificial linker from U.S. Pat. NOs. 4,946,778; 5, 525, 491; 5,856,456; and International patent publication NOs. WO2012/083424; the contents of each of which are incorporated herein by reference in their entirety.

[00284] In some embodiments, linkers of the present invention, include without limitation, any of those taught in Table 11 of copending commonly owned U.S. Provisional Patent Application No. 62/320,864, filed on 4/11/2016, or in US Provisional Application No. 62/466,596 filed March 3, 2017 and the International Publication WO2017/180587, the contents of which are incorporated herein by reference in their entirety.

[00285] In some embodiments, compositions of the invention may include optional proteasome adaptors. As used herein, the term "proteasome adaptor" refers to any nucleotide/ amino acid sequence that targets the appended payload for degradation. In some aspects, the adaptors target the payload for degradation directly thereby circumventing the need for ubiquitination reactions. Proteasome adaptors may be used in conjunction with destabilizing domains to reduce the basal expression of the payload. Exemplary proteasome adaptors include the UbL domain of Rad23 or hHR23b, HPV E7 which binds to both the target protein Rb and the S4 subunit of the proteasome with high affinity, which allows direct proteasome targeting, bypassing the

ubiquitination machinery; the protein gankyrin which binds to Rb and the proteasome subunit S6.

[00286] In one embodiment, the linker may be a spacer region of one or more nucleotides. Non-limiting examples of spacers are TCTAGATAATACGACTCACTAGAGATCC (SEQ ID NO. 846), TATGGCCACAACCATG (SEQ ID NO. 847), AATCTAGATAATACGACTCACTAGAGATCC (SEQ ID NO. 848), TCGCGAATG, TCGCGA, GCTTGCCACAACCCACAAGGAGACGACCTTCC (SEQ ID NO. 849), or ATNFSLLKQAGDVEENPGP (SEQ ID NO. 850, encoded by SEQ ID NO. 851).

[00287] In one embodiment, the linker may be a BamHI site. As a non-limiting example, the BamHI site has the amino acid sequence GS and/or the DNA sequence GGATCC.

Embedded stimulus, signals and other regulatory features

[00288] In some embodiments, the effector module of the present invention may further comprise one or more microRNAs, microRNA binding sites, promotors and tunable elements. In one embodiment, microRNA may be used in support of the creation of tunable biocircuits. Each aspect or tuned modality may bring to the effector module or biocircuit a differentially tuned feature. For example, a destabilizing domain may alter cleavage sites or dimerization properties or half-life of the payload, and the inclusion of one or more microRNA or microRNA binding site may impart cellular detargeting or trafficking features. Consequently, the present invention embraces biocircuits which are multifactorial in their tenability. Such biocircuits and effector modules may be engineered to contain one, two, three, four or more tuned features. In some embodiments, micro RNA sequences of the present invention, include without limitation, any of those taught in Table 13 of copending commonly owned U.S. Provisional Patent Application No. 62/320,864, filed on 4/11/2016, or in US Provisional Application No. 62/466,596 filed March 3, 2017 and the International Publication WO2017/180587, the contents of which are incorporated herein by reference in their entirety.

Polynucleotides

[00289] The term "polynucleotide" or "nucleic acid molecule" in its broadest sense, includes any compound and/or substance that comprise a polymer of nucleotides, e.g., linked nucleosides. These polymers are often referred to as polynucleotides. Exemplary nucleic acids or polynucleotides of the invention include, but are not limited to, ribonucleic acids (RNAs), deoxyribonucleic acids (DNAs), threose nucleic acids (TNAs), glycol nucleic acids (GNAs), peptide nucleic acids (PNAs), locked nucleic acids (LNAs, including LNA having a β- D-ribo configuration, α-LNA having an α-L-ribo configuration (a diastereomer of LNA), 2′-amino-LNA

having a 2′-amino functionalization, and 2′-amino- α-LNA having a 2′-amino functionalization) or hybrids thereof.

[00290] In some embodiments, polynucleotides of the invention may be a messenger RNA (mRNA) or any nucleic acid molecule and may or may not be chemically modified. In one aspect, the nucleic acid molecule is a mRNA. As used herein, the term "messenger RNA (mRNA)" refers to any polynucleotide which encodes a polypeptide of interest and which is capable of being translated to produce the encoded polypeptide of interest *in vitro*, *in vivo*, *in situ* or *ex vivo*.

[00291] Traditionally, the basic components of an mRNA molecule include at least a coding region, a 5′UTR, a 3′UTR, a 5′ cap and a poly-A tail. Building on this wild type modular structure, the present invention expands the scope of functionality of traditional mRNA molecules by providing payload constructs which maintain a modular organization, but which comprise one or more structural and/or chemical modifications or alterations which impart useful properties to the polynucleotide, for example tenability of function. As used herein, a "structural" feature or modification is one in which two or more linked nucleosides are inserted, deleted, duplicated, inverted or randomized in a polynucleotide without significant chemical modification to the nucleosides themselves. Because chemical bonds will necessarily be broken and reformed to effect a structural modification, structural modifications are of a chemical nature and hence are chemical modifications. However, structural modifications will result in a different sequence of nucleotides. For example, the polynucleotide "ATCG" may be chemically modified to "AT-5meC-G". The same polynucleotide may be structurally modified from "ATCG" to "ATCCCG". Here, the dinucleotide "CC" has been inserted, resulting in a structural modification to the polynucleotide.

[00292] In some embodiments, polynucleotides of the present invention may harbor 5'UTR sequences which play a role in translation initiation. 5'UTR sequences may include features such as Kozak sequences which are commonly known to be involved in the process by which the ribosome initiates translation of genes, Kozak sequences have the consensus XCCR(A/G) CCAUG, where R is a purine (adenine or guanine) three bases upstream of the start codon (AUG) and X is any nucleotide. In one embodiment, the Kozak sequence is ACCGCC. By engineering the features that are typically found in abundantly expressed genes of target cells or tissues, the stability and protein production of the polynucleotides of the invention can be enhanced.

[00293] Further provided are polynucleotides, which may contain an internal ribosome entry site (IRES) which play an important role in initiating protein synthesis in the absence of 5' cap

structure in the polynucleotide. An IRES may act as the sole ribosome binding site, or may serve as one of the multiple binding sites. Polynucleotides of the invention containing more than one functional ribosome binding site may encode several peptides or polypeptides that are translated independently by the ribosomes giving rise to bicistronic and/or multicistronic nucleic acid molecules.

[00294] In some embodiments, polynucleotides encoding biocircuits, effector modules, SREs and payloads of interest such as immunotherapeutic agents may include from about 30 to about 100,000 nucleotides (e.g., from 30 to 50, from 30 to 100, from 30 to 250, from 30 to 500, from 30 to 1,000, from 30 to 1,500, from 30 to 3,000, from 30 to 5,000, from 30 to 7,000, from 30 to 10,000, from 30 to 25,000, from 30 to 50,000, from 30 to 70,000, from 100 to 250, from 100 to 500, from 100 to 1,000, from 100 to 1,500, from 100 to 3,000, from 100 to 5,000, from 100 to 7,000, from 100 to 10,000, from 100 to 25,000, from 100 to 50,000, from 100 to 70,000, from 100 to 100,000, from 500 to 1,000, from 500 to 1,500, from 500 to 2,000, from 500 to 3,000, from 500 to 5,000, from 500 to 7,000, from 500 to 10,000, from 500 to 25,000, from 500 to 50,000, from 500 to 70,000, from 500 to 100,000, from 1,000 to 1,500, from 1,000 to 2,000, from 1,000 to 3,000, from 1,000 to 5,000, from 1,000 to 7,000, from 1,000 to 10,000, from 1,000 to 25,000, from 1,000 to 50,000, from 1,000 to 70,000, from 1,000 to 100,000, from 1,500 to 3,000, from 1,500 to 5,000, from 1,500 to 7,000, from 1,500 to 10,000, from 1,500 to 25,000, from 1,500 to 50,000, from 1,500 to 70,000, from 1,500 to 100,000, from 2,000 to 3,000, from 2,000 to 5,000, from 2,000 to 7,000, from 2,000 to 10,000, from 2,000 to 25,000, from 2,000 to 50,000, from 2,000 to 70,000, and from 2,000 to 100,000 nucleotides). In some aspects, polynucleotides of the invention may include more than 10,000 nucleotides.

[00295] Regions of the polynucleotides which encode certain features such as cleavage sites, linkers, trafficking signals, tags or other features may range independently from 10-1,000 nucleotides in length (e.g., greater than 20, 30, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, and 900 nucleotides or at least 10, 15, 20, 25, 30, 35, 40, 45, 50, 55, 60, 70, 80, 90, 100, 120, 140, 160, 180, 200, 250, 300, 350, 400, 450, 500, 600, 700, 800, 900, and 1,000 nucleotides).

[00296] In some embodiments, polynucleotides of the present invention may further comprise embedded regulatory moieties such as microRNA binding sites within the 3′UTR of nucleic acid molecules which when bind to microRNA molecules, down-regulate gene expression either by reducing nucleic acid molecule stability or by inhibiting translation. Conversely, for the purposes of the polynucleotides of the present invention, microRNA binding sites can be engineered out of (i.e. removed from) sequences in which they naturally occur in order to increase protein

expression in specific tissues. For example, miR-142 and miR-146 binding sites may be removed to improve protein expression in the immune cells. In some embodiments, any of the encoded payloads may be may be regulated by an SRE and then combined with one or more regulatory sequences to generate a dual or multi-tuned effector module or biocircuit system.

[00297] In some embodiments, polynucleotides of the present invention may encode fragments, variants, derivatives of polypeptides of the inventions. In some aspects, the variant sequence may keep the same or a similar activity. Alternatively, the variant may have an altered activity (e.g., increased or decreased) relative to the start sequence. Generally, variants of a particular polynucleotide or polypeptide of the invention will have at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% but less than 100% sequence identity to that particular reference polynucleotide or polypeptide as determined by sequence alignment programs and parameters described herein and known to those skilled in the art. Such tools for alignment include those of the BLAST suite (Stephen et al., Gapped BLAST and PSI-BLAST: a new generation of protein database search programs, *Nucleic Acids Res.*, 1997, 25:3389-3402.)

[00298] In some embodiments, polynucleotides of the present invention may be modified. As used herein, the terms "modified", or as appropriate, "modification" refers to chemical modification with respect to A, G, U (T in DNA) or C nucleotides. Modifications may be on the nucleoside base and/or sugar portion of the nucleosides which comprise the polynucleotide. In some embodiments, multiple modifications are included in the modified nucleic acid or in one or more individual nucleoside or nucleotide. For example, modifications to a nucleoside may include one or more modifications to the nucleobase and the sugar. Modifications to the polynucleotides of the present invention may include any of those taught in, for example, International Publication NO. WO2013052523, the contents of which are incorporated herein by reference in its entirety.

[00299] As described herein "nucleoside" is defined as a compound containing a sugar molecule (e.g., a pentose or ribose) or a derivative thereof in combination with an organic base (e.g., a purine or pyrimidine) or a derivative thereof (also referred to herein as "nucleobase"). As described herein, "nucleotide" is defined as a nucleoside including a phosphate group.

[00300] In some embodiments, the modification may be on the internucleoside linkage (e.g., phosphate backbone). Herein, in the context of the polynucleotide backbone, the phrases "phosphate" and "phosphodiester" are used interchangeably. Backbone phosphate groups can be modified by replacing one or more of the oxygen atoms with a different substituent. Further, the modified nucleosides and nucleotides can include the wholesale replacement of an unmodified

phosphate moiety with another internucleoside linkage. Examples of modified phosphate groups include, but are not limited to, phosphorothioate, phosphoroselenates, boranophosphates, boranophosphate esters, hydrogen phosphonates, phosphoramidates, phosphorodiamidates, alkyl or aryl phosphonates, and phosphotriesters. Phosphorodithioates have both non-linking oxygens replaced by sulfur. The phosphate linker can also be modified by the replacement of a linking oxygen with nitrogen (bridged phosphoramidates), sulfur (bridged phosphorothioates), and carbon (bridged methylene-phosphonates). Other modifications which may be used are taught in, for example, International Application NO. WO2013052523, the contents of which are incorporated herein by reference in their entirety.

[00301] Chemical modifications and/or substitution of the nucleotides or nucleobases of the polynucleotides of the invention which are useful in the present invention include any modified substitutes known in the art, for example, (±)1-(2-Hydroxypropyl)pseudouridine TP, (2R)-1-(2-Hydroxypropyl)pseudouridine TP, 1-(4-Methoxy-phenyl)pseudo-UTP, ,2′-O-dimethyladenosine, 1,2′-O-dimethylguanosine, 1,2′-O-dimethylinosine, 1-Hexyl-pseudo-UTP , 1-Homoallylpseudouridine TP, 1-Hydroxymethylpseudouridine TP, 1-iso-propyl-pseudo-UTP , 1-Me-2-thio-pseudo-UTP, 1-Me-4-thio-pseudo-UTP, 1-Me-alpha-thio-pseudo-UTP , 1-Me-GTP, 2'-Amino-2'-deoxy-ATP, 2'-Amino-2'-deoxy-CTP, 2'-Amino-2'-deoxy-GTP, 2'-Amino-2'-deoxy-UTP, 2'-Azido-2'-deoxy-ATP, tubercidine, under modified hydroxywybutosine, uridine 5-oxyacetic acid, uridine 5-oxyacetic acid methyl ester, wybutosine, wyosine, xanthine, Xanthosine-5'-TP, xylo-adenosine, zebularine, α-thio-adenosine, α-thio-cytidine, α-thio-guanosine, and/or α-thio-uridine.

[00302] Polynucleotides of the present invention may comprise one or more of the modifications taught herein. Different sugar modifications, base modifications, nucleotide modifications, and/or internucleoside linkages (e.g., backbone structures) may exist at various positions in the polynucleotide of the invention. One of ordinary skill in the art will appreciate that the nucleotide analogs or other modification(s) may be located at any position(s) of a polynucleotide such that the function of the polynucleotide is not substantially decreased. A modification may also be a 5′ or 3′ terminal modification. The polynucleotide may contain from about 1% to about 100% modified nucleotides (either in relation to overall nucleotide content, or in relation to one or more types of nucleotide, i.e. any one or more of A, G, U or C) or any intervening percentage (e.g., from 1% to 20%, from 1% to 25%, from 1% to 50%, from 1% to 60%, from 1% to 70%, from 1% to 80%, from 1% to 90%, from 1% to 95%, from 10% to 20%, from 10% to 25%, from 10% to 50%, from 10% to 60%, from 10% to 70%, from 10% to 80%, from 10% to 90%, from 10% to 95%, from 10% to 100%, from 20% to 25%, from 20% to 50%,

from 20% to 60%, from 20% to 70%, from 20% to 80%, from 20% to 90%, from 20% to 95%, from 20% to 100%, from 50% to 60%, from 50% to 70%, from 50% to 80%, from 50% to 90%, from 50% to 95%, from 50% to 100%, from 70% to 80%, from 70% to 90%, from 70% to 95%, from 70% to 100%, from 80% to 90%, from 80% to 95%, from 80% to 100%, from 90% to 95%, from 90% to 100%, and from 95% to 100%).

[00303] In some embodiments, one or more codons of the polynucleotides of the present invention may be replaced with other codons encoding the native amino acid sequence to tune the expression of the SREs, through a process referred to as codon selection. Since mRNA codon, and tRNA anticodon pools tend to vary among organisms, cell types, sub cellular locations and over time, the codon selection described herein is a spatiotemporal (ST) codon selection.

[00304] In some embodiments of the invention, certain polynucleotide features may be codon optimized. Codon optimization refers to a process of modifying a nucleic acid sequence for enhanced expression in the host cell by replacing at least 1, 2, 3, 4, 5, 10, 15, 20, 25, 50 or more codons of the native sequence with codons that are most frequently used in the genes of that host cell while maintaining the native amino acid sequence. Codon usage may be measured using the Codon Adaptation Index (CAI) which measures the deviation of a coding polynucleotide sequence from a reference gene set. Codon usage tables are available at the Codon Usage Database (http://www.kazusa.or.jp/codon/) and the CAI can be calculated by EMBOSS CAI program (http://emboss.sourceforge.net/). Codon optimization methods are known in the art and may be useful in efforts to achieve one or more of several goals. These goals include to match codon frequencies in target and host organisms to ensure proper folding, bias nucleotide content to alter stability or reduce secondary structures, minimize tandem repeat codons or base runs that may impair gene construction or expression, customize transcriptional and translational control regions, insert or remove protein signaling sequences, remove/add post translation modification sites in encoded protein (e.g. glycosylation sites), add, remove or shuffle protein domains, insert or delete restriction sites, modify ribosome binding sites and degradation sites, to adjust translational rates to allow the various domains of the protein to fold properly, or to reduce or eliminate problem secondary structures within the polynucleotide. Codon optimization tools, algorithms and services are known in the art, and non-limiting examples include services from GeneArt (Life Technologies), DNA2.0 (Menlo Park CA), OptimumGene (GenScript, Piscataway, NJ), algorithms such as but not limited to, DNAWorks v3.2.3, Mr. Gene (GmBH, Regensburg, Germany) and/or proprietary methods. In one embodiment, a polynucleotide sequence or portion thereof is codon optimized using optimization algorithms. Codon options for

each amino acid are well-known in the art as are various species table for optimizing for expression in that particular species.

[00305] In some embodiments of the invention, certain polynucleotide features may be codon optimized. For example, a preferred region for codon optimization may be upstream (5') or downstream (3') to a region which encodes a polypeptide. These regions may be incorporated into the polynucleotide before and/or after codon optimization of the payload encoding region or open reading frame (ORF).

[00306] After optimization (if desired), the polynucleotide components are reconstituted and transformed into a vector such as, but not limited to, plasmids, viruses, cosmids, and artificial chromosomes.

[00307] Spatiotemporal codon selection may impact the expression of the polynucleotides of the invention, since codon composition determines the rate of translation of the mRNA species and its stability. For example, tRNA anticodons to optimized codons are abundant, and thus translation may be enhanced. In contrast, tRNA anticodons to less common codons are fewer and thus translation may proceed at a slower rate. Presnyak et al. have shown that the stability of an mRNA species is dependent on the codon content, and higher stability and thus higher protein expression may be achieved by utilizing optimized codons (Presnyak et al. (2015) Cell 160, 1111–1124; the contents of which are incorporated herein by reference in their entirety). Thus, in some embodiments, ST codon selection may include the selection of optimized codons to enhance the expression of the SRES, effector modules and biocircuits of the invention. In other embodiments, spatiotemporal codon selection may involve the selection of codons that are less commonly used in the genes of the host cell to decrease the expression of the compositions of the invention. The ratio of optimized codons to codons less commonly used in the genes of the host cell may also be varied to tune expression.

[00308] In some embodiments, certain regions of the polynucleotide may be preferred for codon selection. For example, a preferred region for codon selection may be upstream (5') or downstream (3') to a region which encodes a polypeptide. These regions may be incorporated into the polynucleotide before and/or after codon selection of the payload encoding region or open reading frame (ORF).

[00309] The stop codon of the polynucleotides of the present invention may be modified to include sequences and motifs to alter the expression levels of the SREs, payloads and effector modules of the present invention. Such sequences may be incorporated to induce stop codon readthrough, wherein the stop codon may specify amino acids e.g. selenocysteine or pyrrolysine. In other instances, stop codons may be skipped altogether to resume translation through an

alternate open reading frame. Stop codon read through may be utilized to tune the expression of components of the effector modules at a specific ratio (e.g.as dictated by the stop codon context). Examples of preferred stop codon motifs include UGAN, UAAN, and UAGN, where N is either C or U. Polynucleotide modifications and manipulations can be accomplished by methods known in the art such as, but not limited to, site directed mutagenesis and recombinant technology. The resulting modified molecules may then be tested for activity using *in vitro* or *in vivo* assays such as those described herein or any other suitable screening assay known in the art.

[00310] In some embodiments, polynucleotides of the invention may comprise two or more effector module sequences, or two or more payloads of interest sequences, which are in a pattern such as ABABAB or AABBAABBAABB or ABCABCABC or variants thereof repeated once, twice, or more than three times. In these patterns, each letter, A, B, or C represent a different effector module component.

[00311] In yet another embodiment, polynucleotides of the invention may comprise two or more effector module component sequences with each component having one or more SRE sequences (DD sequences), or two or more payload sequences. As a non-limiting example, the sequences may be in a pattern such as ABABAB or AABBAABBAABB or ABCABCABC or variants thereof repeated once, twice, or more than three times in each of the regions. As another non-limiting example, the sequences may be in a pattern such as ABABAB or AABBAABBAABB or ABCABCABC or variants thereof repeated once, twice, or more than three times across the entire polynucleotide. In these patterns, each letter, A, B, or C represent a different sequence or component.

[00312] According to the present invention, polynucleotides encoding distinct biocircuits, effector modules, SREs and payload constructs may be linked together through the 3′-end using nucleotides which are modified at the 3′-terminus. Chemical conjugation may be used to control the stoichiometry of delivery into cells. Polynucleotides can be designed to be conjugated to other polynucleotides, dyes, intercalating agents (e.g. acridines), cross-linkers (e.g. psoralene, mitomycin C), porphyrins (TPPC4, texaphyrin, sapphyrin), polycyclic aromatic hydrocarbons (e.g., phenazine, dihydrophenazine), artificial endonucleases (e.g. EDTA), alkylating agents, phosphate, amino, mercapto, PEG (e.g., PEG-40K), MPEG, $(MPEG)_2$, polyamino, alkyl, substituted alkyl, radiolabeled markers, enzymes, haptens (e.g. biotin), transport/absorption facilitators (e.g., aspirin, vitamin E, folic acid), synthetic ribonucleases, proteins, e.g., glycoproteins, or peptides, e.g., molecules having a specific affinity for a co-ligand, or antibodies e.g., an antibody, that binds to a specified cell type such as a cancer cell, endothelial cell, or bone cell, hormones and hormone receptors, non-peptidic species, such as lipids, lectins,

carbohydrates, vitamins, cofactors, or a drug. As non-limiting examples, they may be conjugates with other immune conjugates.

[00313] In some embodiments, the compositions of the polynucleotides of the invention may be generated by combining the various components of the effector modules using the Gibson assembly method. The Gibson assembly reaction consists of three isothermal reactions, each relying on a different enzymatic activity including a 5' exonuclease which generates long overhangs, a polymerase which fills in the gaps of the annealed single strand regions and a DNA ligase which seals the nicks of the annealed and filled-in gaps. Polymerase chain reactions performed prior to Gibson assembly may be used to generate PCR products with overlapping sequence. These methods can be repeated sequentially, to assemble larger and larger molecules. For example, the method can comprise repeating the steps described as above to join a second set of two or more DNA molecules of interest to one another, and then repeating the method again to join the first and second set DNA molecules of interest, and so on. At any stage during these multiple rounds of assembly, the assembled DNA can be amplified by transforming it into a suitable microorganism, or it can be amplified in vitro (e.g., with PCR).

[00314] In some embodiments, polynucleotides of the present invention may encode a fusion polypeptide comprising a destabilizing domain (DD) and at least one immunotherapeutic agent taught herein. The DD domain may be a FKBP mutant encoded by nucleotide sequence of SEQ ID NOS. 60, 87-88, and/or 878-889, an ecDHFR mutant encoded by nucleotide sequence of SEQ ID NO. 61, 89, 90, and/or 869-877, hDHFR mutant encoded by nucleotide sequence of SEQ ID NO. 91-93, 182-192, SEQ ID NO. 797-832, and/or 890-905.

[00315] In some embodiments, the polynucleotides of the invention may encode effector modules comprising IL2 as the payload comprising the nucleotide sequence of SEQ ID NO. 62-64, or caspase 9 as the payload comprising the nucleotide sequence of SEQ ID NO. 94-102, or FOXP3 as the payload, comprising the nucleotide sequence of SEQ ID NO. 193-202 or luciferase as the payload comprising the nucleotide sequence of SEQ ID NO. 203-208, or BCMA CAR as the payload comprising the nucleotide sequence of SEQ ID NO. 833-835 or Her2 as the payload comprising the nucleotide sequence of 907.

Cells

[00316] In accordance with the present invention, cells genetically modified to express at least one biocircuit, SRE (e. g, DD), effector module and immunotherapeutic agent of the invention, are provided. Cells of the invention may include, without limitation, immune cells, stem cells and tumor cells. In some embodiments, immune cells are immune effector cells, including, but not limiting to, T cells such as $CD8^+$ T cells and $CD4^+$ T cells (e.g., Th1, Th2, Th17 , Foxp3+

cells), memory T cells such as T memory stem cells, central T memory cells, and effector memory T cells, terminally differentiated effector T cells, natural killer (NK) cells, NK T cells, tumor infiltrating lymphocytes (TILs), cytotoxic T lymphocytes (CTLs), regulatory T cells (Tregs), and dendritic cells (DCs), other immune cells that can elicit an effector function, or the mixture thereof. T cells may be Tαβ cells and Tγδ cells. In some embodiments, stem cells may be from human embryonic stem cells, mesenchymal stem cells, and neural stem cells. In some embodiments, T cells may be depleted endogenous T cell receptors (See US Pat. NOs. 9, 273, 283; 9, 181, 527; and 9,028, 812; the contents of each of which are incorporated herein by reference in their entirety).

[00317] In some embodiments, cells of the invention may be autologous, allogeneic, syngeneic, or xenogeneic in relation to a particular individual subject.

[00318] In some embodiments, cells of the invention may be mammalian cells, particularly human cells. Cells of the invention may be primary cells or immortalized cell lines.

[00319] In some embodiments, cells of the invention may be expanded using expansion factors to trigger proliferation and expansion of the cells. Exemplary expansion factors include RAS such as KRAS, NRAS, RRAS, RRAS2, MRAS, ERAS, and HRAS, DIRAS such as DIRAS1, DIRAS2, and DIRAS3, NKIRAS such as NKIRAS1, and NKIRAS2, RAL such as RALA, and RALB, RAP such as RAP1A, RAP1B, RAP2A, RAP2B, and RAP2C, RASD such as RASD1, and RASD2, RASL such as RASL10A, RASL10B, RASL11A, RASL11B, and RASL12, REM such as REM1, and REM2, GEM, RERG, RERGL, and RRAD.

[00320] Engineered immune cells can be accomplished by transducing a cell compositions with a polypeptide of a biocircuit, an effector module, a SRE and/or a payload of interest (i.e., immunotherapeutic agent), or a polynucleotide encoding said polypeptide, or a vector comprising said polynucleotide. The vector may be a viral vector such as a lentiviral vector, a gamma-retroviral vector, a recombinant AAV, an adenoviral vector and an oncolytic viral vector. In other aspects, non-viral vectors for example, nanoparticles and liposomes may also be used. In some embodiments, immune cells of the invention are genetically modified to express at least one immunotherapeutic agent of the invention which is tunable using a stimulus. In some examples, two, three or more immunotherapeutic agents constructed in the same biocircuit and effector module are introduced into a cell. In other examples, two, three, or more biocircuits, effector modules, each of which comprises an immunotherapeutic agent, may be introduced into a cell.

[00321] In some embodiments, T cells expressing Chimeric antigen receptors or T cells receptors may be further modified to express another one, two, three or more immunotherapeutic

agents of the present invention. The immunotherapeutic agents may be another a cytokine such as IL2, IL12, IL15 and IL18; a regulatory switch; or a safety switch gene (e.g., a suicide gene) that kills activated T cells when a severe event is observed after adoptive cell transfer or when the transferred immune cells are no-longer needed. These molecules may be included in the same effector module or in separate effector modules.

[00322] In some embodiments, immune cells of the invention may be NK cells modified to express payloads of the invention.

[00323] Natural killer (NK) cells are members of the innate lymphoid cell family and characterized in humans by expression of the phenotypic marker CD56 (neural cell adhesion molecule) in the absence of CD3 (T-cell co-receptor). NK cells are potent effector cells of the innate immune system which mediate cytotoxic attack without the requirement of prior antigen priming, forming the first line of defense against diseases including cancer malignancies and viral infection.

[00324] Several pre-clinical and clinical trials have demonstrated that adoptive transfer of NK cells is a promising treatment approach against cancers such as acute myeloid leukemia (Ruggeri et al., *Science*; 2002, 295: 2097–2100; and Geller et al., *Immunotherapy*, 2011, 3: 1445-1459).

[00325] NK cell activation is characterized by an array of receptors with activating and inhibitory functions. The important activation receptors on NK cells include CD94/NKG2C and NKG2D (the C-type lectin-like receptors), and the natural cytotoxicity receptors (NCR) NKp30, NKp44 and NKp46, which recognize ligands on tumor cells or virally infected cells. NK cell inhibition is essentially mediated by interactions of the polymorphic inhibitory killer cell immunoglobulin-like receptors (KIRs) with their cognate human–leukocyte–antigen (HLA) ligands via the alpha-1 helix of the HLA molecule. The balance between signals that are generated from activating receptors and inhibitory receptors mainly determines the immediate cytotoxic activation.

[00326] NK cells may be isolated from peripheral blood mononuclear cells (PBMCs), or derived from human embryonic stem (ES) cells and induced pluripotent stem cells (iPSCs). The primary NK cells isolated from PBMCs may be further expanded for adoptive immunotherapy. Strategies and protocols useful for the expansion of NK cells may include interleukin 2 (IL2) stimulation and the use of autologous feeder cells, or the use of genetically modified allogeneic feeder cells. In some aspects, NK cells can be selectively expanded with a combination of stimulating ligands including IL15, IL21, IL2, 41BBL, IL12, IL18, MICA, 2B4, LFA-1, and BCM1/SLAMF2 (e.g., US patent publication NO. US20150190471).

[00327] In some embodiments, cells of the present invention may be dendritic cells that are genetically modified to express the compositions of the invention.

[00328] In some embodiments, cells of the invention may be Treg cells. Payloads of the invention may be used to promote the proliferation, survival, activation and /or function of T regulatory cells. Tregs are a distinct population of cells that are positively selected on high affinity ligands in the thymus and play an important role in the tolerance to self-antigens. In addition, T regs have also been shown to play a role in peripheral tolerance to foreign antigens. The ability of Tregs to induce tolerance may be utilized to tune immune responses to the immunotherapeutic agents described herein. Methods for expanding Tregs for immunotherapy have been described by Tang et al., 2004, J. Exp. Med. 199: 1455-65; Battaglia et al., 2005, Blood 105: 4743-48; Earle et al., 2005, Clin. Immunol. 115: 3-9; Godfrey et al., 2004, Blood 104: 453-61; Hoffmann et al., 2004, Blood 104: 895-903.

III. PHARMACEUTICAL COMPOSITIONS AND FORMULATIONS

[00329] The present invention further provides pharmaceutical compositions comprising one or more biocircuits, effector modules, SREs (e.g., DDs), stimuli and payloads of interest (i.e., immunotherapeutic agents), vectors, cells and other components of the invention, and optionally at least one pharmaceutically acceptable excipient or inert ingredient.

[00330] As used herein the term "pharmaceutical composition" refers to a preparation of biocircuits, SREs, stimuli and payloads of interest (i.e., immunotherapeutic agents), other components, vectors, cells and described herein, or pharmaceutically acceptable salts thereof, optionally with other chemical components such as physiologically suitable carriers and excipients. The pharmaceutical compositions of the invention comprise an effective amount of one or more active compositions of the invention. The preparation of a pharmaceutical composition that contains at least one composition of the present invention and/or an additional active ingredient will be known to those skilled in the art in light of the present disclosure, as exemplified by Remington's Pharmaceutical Sciences, 18th Ed. Mack Printing Company, 1990, incorporated herein by reference.

[00331] The term "excipient" or "inert ingredient" refers to an inactive substance added to a pharmaceutical composition and formulation to further facilitate administration of an active ingredient. For the purposes of the present disclosure, the phrase "active ingredient" generally refers to any one or more biocircuits, effector modules, SREs, stimuli and payloads of interest (i.e., immunotherapeutic agents), other components, vectors, and cells to be delivered as described herein. The phrases "pharmaceutically acceptable" refers to molecular entities and

compositions that do not produce an adverse, allergic or other untoward reaction when administered to an animal, such as, for example, a human, as appropriate.

[00332] In some embodiments, pharmaceutical compositions and formulations are administered to humans, human patients or subjects. Although the descriptions of pharmaceutical compositions provided herein are principally directed to pharmaceutical compositions which are suitable for administration to humans, it will be understood by the skilled artisan that such compositions are generally suitable for administration to any other animal, e.g., to non-human animals, e.g. non-human mammals. Subjects to which administration of the pharmaceutical compositions is contemplated include, but are not limited to, non-human mammals, including agricultural animals such as cattle, horses, chickens and pigs, domestic animals such as cats, dogs, or research animals such as mice, rats, rabbits, dogs and non-human primates. It will be understood that, for human administration, preparations should meet sterility, pyrogenicity, general safety and purity standards as required by FDA Office of Biological Standards.

[00333] A pharmaceutical composition and formulation in accordance with the invention may be prepared, packaged, and/or sold in bulk, as a single unit dose, and/or as a plurality of single unit doses. As used herein, a "unit dose" is discrete amount of the pharmaceutical composition comprising a predetermined amount of the active ingredient. The amount of the active ingredient is generally equal to the dosage of the active ingredient which would be administered to a subject and/or a convenient fraction of such a dosage such as, for example, one-half or one-third of such a dosage.

[00334] The compositions of the present invention may be formulated in any manner suitable for delivery. The formulation may be, but is not limited to, nanoparticles, poly (lactic-co-glycolic acid) (PLGA) microspheres, lipidoids, lipoplex, liposome, polymers, carbohydrates (including simple sugars), cationic lipids and combinations thereof.

[00335] In one embodiment, the formulation is a nanoparticle which may comprise at least one lipid. The lipid may be selected from, but is not limited to, DLin-DMA, DLin-K-DMA, 98N12-5, C12-200, DLin-MC3-DMA, DLin-KC2-DMA, DODMA, PLGA, PEG, PEG-DMG and PEGylated lipids. In another aspect, the lipid may be a cationic lipid such as, but not limited to, DLin-DMA, DLin-D-DMA, DLin-MC3-DMA, DLin-KC2-DMA and DODMA.

[00336] For polynucleotides of the invention, the formulation may be selected from any of those taught, for example, in International Application PCT/US2012/069610, the contents of which are incorporated herein by reference in its entirety.

[00337] Relative amounts of the active ingredient, the pharmaceutically acceptable excipient or inert ingredient, and/or any additional ingredients in a pharmaceutical composition in accordance

with the invention will vary, depending upon the identity, size, and/or condition of the subject treated and further depending upon the route by which the composition is to be administered. By way of example, the composition may comprise between 0.1 and 100, e.g., between 0.5 and 50, between 1-30, between 5-80, at least 80 (w/w) active ingredient.

[00338] Efficacy of treatment or amelioration of disease can be assessed, for example by measuring disease progression, disease remission, symptom severity, reduction in pain, quality of life, dose of a medication required to sustain a treatment effect, level of a disease marker or any other measurable parameter appropriate for a given disease being treated or targeted for prevention. It is well within the ability of one skilled in the art to monitor efficacy of treatment or prevention by measuring any one of such parameters, or any combination of parameters. In connection with the administration of compositions of the present invention, "effective against" for example a cancer, indicates that administration in a clinically appropriate manner results in a beneficial effect for at least a statistically significant fraction of patients, such as an improvement of symptoms, a cure, a reduction in disease load, reduction in tumor mass or cell numbers, extension of life, improvement in quality of life, or other effect generally recognized as positive by medical doctors familiar with treating the particular type of cancer.

[00339] A treatment or preventive effect is evident when there is a statistically significant improvement in one or more parameters of disease status, or by a failure to worsen or to develop symptoms where they would otherwise be anticipated. As an example, a favorable change of at least 10 in a measurable parameter of disease, and preferably at least 20, 30, 40, 50 or more can be indicative of effective treatment. Efficacy for a given composition or formulation of the present invention can also be judged using an experimental animal model for the given disease as known in the art. When using an experimental animal model, efficacy of treatment is evidenced when a statistically significant change is observed.

[00340] Preferably, the compositions of the invention are administered by injection, e.g., intravenously. When the inventive CAR material is a host cell (or a population thereof) expressing the inventive CAR, the pharmaceutically acceptable carrier for the cells for injection may include any isotonic carrier such as, for example, normal saline (about 0.90% w/v of NaCl in water, about 300 mOsm/L NaCl in water, or about 9.0 g NaCl per liter of water), NORMOSOL R electrolyte solution (Abbott, Chicago, IL), PLASMA- LYTE A (Baxter, Deerfield, IL), about 5% dextrose in water, or Ringer's lactate. In an embodiment, the pharmaceutically acceptable carrier may be supplemented with human serum albumen. Any of the carriers taught in WO2016149578A1 may be useful in the present invention.

IV. APPLICATIONS

[00341] In one aspect of the present invention, methods for reducing a tumor volume or burden are provided. The methods comprise administering a pharmaceutically effective amount of a pharmaceutical composition comprising at least one biocircuit system, effector module, DD, and/or payload of interest (i.e., an immunotherapeutic agent), at least one vector, or cells to a subject having a tumor. The biocircuit system and effector module having any immunotherapeutic agent as described herein may be in forms of a polypeptide, or a polynucleotide such as mRNA, or a viral vector comprising the polynucleotide, or a cell modified to express the biocircuit, effector module, DD, and payload of interest (i.e., immunotherapeutic agent).

[00342] In another aspect of the present invention, methods for inducing an anti-tumor immune response in a subject are provided. The methods comprise administering a pharmaceutically effective amount of a pharmaceutical composition comprising at least one biocircuit system, effector module, DD, and/or payload of interest (i.e., an immunotherapeutic agent), at least one vector, or cells to a subject having a tumor. The biocircuit and effector module having any immunotherapeutic agent as described herein may be in forms of a polypeptide, or a polynucleotide such as mRNA, or a viral vector comprising the polynucleotide, or a cell modified to express the biocircuit, effector module, DD, and payload of interest (i.e., immunotherapeutic agent).

[00343] The methods, according to the present invention, may be adoptive cell transfer (ACT) using genetically engineered cells such as immune effector cells of the invention, cancer vaccines comprising biocircuit systems, effector modules, DDs, payloads of interest (i.e., immunotherapeutic agents) of the invention, or compositions that manipulate the tumor immunosuppressive microenvironment, or the combination thereof. These treatments may be further employed with other cancer treatment such as chemotherapy and radiotherapy.

[00344] In some embodiments, the safety switches described herein may be useful in the treatment of diseases of protein proliferation and/or protein aggregation e.g. renal diseases and/or neurological diseases such as Alzheimer's diseases, prior diseases etc. In one embodiment, safety switches of the present invention may be expressed in phagocytic cells that are engineered to target aggregated proteins such as amyloid proteins, wherein the safety switches described herein may be used to eliminate the phagocytic cells after the clearance of the aggregated proteins.

1. Adoptive cell transfer (adoptive immunotherapy)

[00345] In some embodiments, cells which are genetically modified to express at least one biocircuit system, effector module, DD, and/or payload of interest (immunotherapeutic agent) may be used for adoptive cell therapy (ACT). As used herein, Adoptive cell transfer refers to the administration of immune cells (from autologous, allogenic or genetically modified hosts) with direct anticancer activity. ACT has shown promise in clinical application against malignant and infectious disease. For example, T cells genetically engineered to recognize CD19 have been used to treat follicular B cell lymphoma (Kochenderfer et al., *Blood*, 2010, 116:4099-4102; and Kochenderfer and Rosenberg, *Nat Rev Clin Oncol.*, 2013, 10(5): 267-276) and ACT using autologous lymphocytes genetically-modified to express anti-tumor T cell receptors has been used to treat metastatic melanoma (Rosenberg and Dudley, *Curr. Opin. Immunol.* 2009, 21: 233-240).

[00346] According to the present invention, the biocircuits and systems may be used in the development and implementation of cell therapies such as adoptive cell therapy. Certain effector modules useful in cell therapy are given in Figures 7-12. The biocircuits, their components, effector modules and their SREs and payloads may be used in cell therapies to regulate epitope tagged receptors, in APC platforms for stimulating T cells, as a tool to enhance *ex vivo* APC stimulation, to improve methods of T cell expansion, in *ex vivo* stimulation with antigen, in TCR/CAR combinations, in the manipulation or regulation of TILs, in allogeneic cell therapy, in combination T cell therapy with other treatment lines (e.g. radiation, cytokines).

[00347] Provided herein are methods for use in adoptive cell therapy. The methods involve preconditioning a subject in need thereof, modulating immune cells with SRE, biocircuits and compositions of the present invention, administering to a subject, engineered immune cells expressing compositions of the invention and the successful engraftment of engineered cells within the subject.

[00348] In some embodiments, SREs, biocircuits and compositions of the present invention may be used to minimize preconditioning regimens associated with adoptive cell therapy. As used herein "preconditioning" refers to any therapeutic regimen administered to a subject to improve the outcome of adoptive cell therapy. Preconditioning strategies include, but are not limited to total body irradiation and/or lymphodepleting chemotherapy. Adoptive therapy clinical trials without preconditioning have failed to demonstrate any clinical benefit, indicating its importance in ACT. Yet, preconditioning is associated with significant toxicity and limits the subject cohort that is suitable for ACT. In some instances, immune cells for ACT may be

engineered to express cytokines such as IL2 as payload using SREs of the present invention to reduce the need for preconditioning.

[00349] In some embodiments, immune cells for ACT may be dendritic cells, T cells such as $CD8^+$ T cells and $CD4^+$ T cells, natural killer (NK) cells, NK T cells, Cytotoxic T lymphocytes (CTLs), tumor infiltrating lymphocytes (TILs), lymphokine activated killer (LAK) cells, memory T cells, regulatory T cells (Tregs), helper T cells, cytokine-induced killer (CIK) cells, and any combination thereof. In other embodiments, immune stimulatory cells for ACT may be generated from embryonic stem cell (ESC) and induced pluripotent stem cell (iPSC). In some embodiments, autologous or allogeneic immune cells are used for ACT.

[00350] In some embodiments, NK cells engineered to express the present compositions may be used for ACT. NK cell activation induces perforin/granzyme-dependent apoptosis in target cells. NK cell activation also induces cytokine secretion such as IFN-γ, TNF-α and GM-CSF. These cytokines enhance the phagocytic function of macrophages and their antimicrobial activity, and augment the adaptive immune response via up-regulation of antigen presentation by antigen presenting cells such as dendritic cells (DCs) (Reviewed by Vivier et al., *Nat. Immunol.*, 2008, 9(5): 503-510).

[00351] NK cells may also be genetically reprogrammed to circumvent NK cell inhibitory signals upon interaction with tumor cells. For example, using CRISPR, ZFN, or TALEN to genetically modify NK cells to silence their inhibitory receptors may enhance the anti-tumor capacity of NK cells.

[00352] Immune cells can be isolated and expanded *ex vivo* using a variety of methods known in the art. For example, methods of isolating and expanding cytotoxic T cells are described in U.S. Pat. NOs. 6,805,861 and 6,531, 451; US Patent Publication NOs. US20160348072A1 and International Patent Publication NO. WO2016168595A1; the contents of each of which are incorporated herein by reference in their entirety. Isolation and expansion of NK cells is described in US Patent Publication NOS. US20150152387A1, U.S. Patent NOS. 7,435, 596; and Oyer, J.L. (2016). Cytotherapy.18(5):653-63; the contents of each of which are incorporated by reference herein in its entirety. Specifically, human primary NK cells may be expanded in the presence of feeder cells e.g. a myeloid cell line that has been genetically modified to express membrane bound IL15, IL21, IL12 and 4-1BBL.

[00353] In some instances, sub populations of immune cells may be enriched for ACT. Methods for immune cell enrichment are taught in International Patent Publication NOS. WO2015039100A1. In another example, T cells positive for B and T lymphocyte attenuator marker BTLA) may be used to enrich for T cells that are anti-cancer reactive as described in U.S.

Pat. NOS. 9,512,401 (the content of each of which are incorporated herein by reference in their entirety).

[00354] In some embodiments, immune cells for ACT may be depleted of select sub populations to enhance T cell expansion. For example, immune cells may be depleted of Foxp3+ T lymphocytes to minimize the ant-tumor immune response using methods taught in US Patent Publication NOS. US 20160298081A1; the contents of which are incorporated by reference herein in their entirety.

[00355] In some embodiments, immune cells may be enriched for FOXP3+ cells to enrich for T cells that are critical for immune tolerance to reduce graft versus host disease.

[00356] In some embodiments, activation and expansion of T cells for ACT is achieved antigenic stimulation of a transiently expressed Chimeric Antigen Receptor (CAR) on the cell surface. Such activation methods are taught in International Patent NOS. WO2017015427, the content of which are incorporated herein by reference in their entirety.

[00357] In some embodiments, immune cells may be activated by antigens associated with antigen presenting cells (APCs). In some embodiments, the APCs may be dendritic cells, macrophages or B cells that antigen specific or nonspecific. The APCs may autologous or homologous in their organ. In some embodiments, the APCs may be artificial antigen presenting cells (aAPCs) such as cell based aAPCs or acellular aAPCs. Cell based aAPCs are may be selected from either genetically modified allogeneic cells such as human erythroleukemia cells or xenogeneic cells such as murine fibroblasts and Drosophila cells. Alternatively, the APCs maybe be acellular wherein the antigens or costimulatory domains are presented on synthetic surfaces such as latex beads, polystyrene beads, lipid vesicles or exosomes.

[00358] In some embodiments, cells of the invention, specifically T cells may be expanded using artificial cell platforms. In one embodiment, the mature T cells may be generated using artificial thymic organoids (ATOs) described by Seet CS et al. 2017. *Nat Methods*. 14, 521–530 (the contents of which are incorporated herein by reference in their entirety). ATOs are based on a stromal cell line expressing delta like canonical notch ligand (DLL1). In this method, stromal cells are aggregated with hematopoietic stem and progenitor cells by centrifugation and deployed on a cell culture insert at the air–fluid interface to generate organoid cultures. ATO-derived T cells exhibit naive phenotypes, a diverse T cell receptor (TCR) repertoire and TCR-dependent function.

[00359] In some embodiments, the T cells of the invention may be separated from peripheral blood by a process known as apheresis, which separates lymphocytes from plasma, platelets and RBCs, and granulocytes. Lymphocyte in peripheral blood cells may further be separated from

monocytes using a semi-automated elutriation device. T cells may also be enriched by magnetic selection with anti CD3/CD28 beads. In one embodiment, an additional step of using a plastic adherent surface to deplete monocytes from the PBMCs may be utilized. Methods of T cell enrichment are disclosed in Stroncek DF et al. (2017) Journal of Translational Medicine.15:59; the contents of which are incorporated by reference in its entirety.

[00360] In some embodiments, adoptive cell therapy is carried out by autologous transfer, wherein the cells are derived from a subject in need of a treatment and the cells, following isolation and processing are administered to the same subject. In other instances, ACT may involve allogenic transfer wherein the cells are isolated and/or prepared from a donor subject other than the recipient subject who ultimately receives cell therapy. The donor and recipient subject may be genetically identical, or similar or may express the same HLA class or subtype.

[00361] In some embodiments, the multiple immunotherapeutic agents introduced into the immune cells for ACT (e.g., T cells and NK cells) may be controlled by the same biocircuit system. In one example, a cytokine such as IL2 and a Caspase 9 are linked to the same hDHFR destabilizing domain. The expression of IL2 and Caspase 9 is tuned using TMP simultaneously. In other embodiments, the multiple immunotherapeutic agents introduced into the immune cells for ACT (e.g., T cells and NK cells) may be controlled by different biocircuit systems. In one example, a cytokine such as IL2 and Caspase 9 constructs are linked to different DDs in two separate effector modules, and can be tuned separately using different stimuli. In another example, a suicide gene and a CAR construct may be linked to two separate effector modules.

[00362] Following genetic modulation using SREs, biocircuits and compositions of the invention, cells are administered to the subject in need thereof. Methods for administration of cells for adoptive cell therapy are known and may be used in connection with the provided methods and compositions. For example, adoptive T cell therapy methods are described, e.g., in US Patent Application Publication No. 2003/0170238 to Gruenberg et al; US Patent No. 4,690,915 to Rosenberg; Rosenberg (2011) Nat Rev Clin Oncol. 8(10):577-85). See, e.g., Themeli et al. (2013) Nat Biotechnol. 31(10): 928-933; Tsukahara et al. (2013) Biochem Biophys Res Commun 438(1): 84-9; Davila et al. (2013) PLoS ONE 8(4): e61338; the contents of each of which are incorporated herein by reference in their entirety.

[00363] In some embodiments, immune cells for ACT may be modified to express one or more immunotherapeutic agents which facilitate immune cells activation, infiltration, expansion, survival and anti-tumor functions.

[00364] In some embodiments, immune cells used for adoptive cell transfer can be genetically manipulated to improve their persistence, cytotoxicity, tumor targeting capacity, and ability to

home to disease sites *in vivo*, with the overall aim of further improving upon their capacity to kill tumors in cancer patients. One example is to introduce effector modules of the invention comprising cytokines such as gamma-cytokines (IL2) into immune cells to promote immune cell proliferation and survival. Transduction of cytokine genes (e.g., gamma-cytokines IL2) into cells will be able to propagate immune cells without addition of exogenous cytokines and cytokine expressing NK cells have enhanced tumor cytotoxicity.

[00365] In some embodiments, biocircuits, their components, SREs or effector modules may be utilized to prevent T cell exhaustion. As used herein, "T cell exhaustion" refers to the stepwise and progressive loss of T cell function caused by chronic T cell activation. T cell exhaustion is a major factor limiting the efficacy of antiviral and antitumor immunotherapies. Exhausted T cells have low proliferative and cytokine producing capabilities concurrent with high rates of apoptosis and high surface expression of multiple inhibitory receptors. T cell activation leading to exhaustion may occur either in the presence or absence of the antigen.

[00366] In some embodiments, effector modules of the present invention, useful for immunotherapy may be placed under the transcriptional control of the T cell receptor alpha locus constant (TRAC) locus in the T cells. Eyquem et al. have shown that expression of the CAR from the TRAC locus prevents T cell exhaustion and the accelerated differentiation of T cells caused by excessive T cell activation (Eyquem J. et al (2017) Nature.543(7643):113-117; the contents of which are incorporated herein by reference in their entirety).

[00367] In some embodiments, payloads of the invention may be used in conjunction with antibodies or fragments that target T cell surface markers associated with T cell exhaustion. T-cell surface markers associated with T cell exhaustion that may be used include, but are not limited to, CTLA-1, PD-1, TGIT, LAG-3, 2B4, BTLA, TIM3, VISTA, and CD96.

[00368] T cells that are specific to certain tumor antigens, may be subject to chronic antigen exposure. Persistent antigen expression can lead to immune check-point expression, which in turn, induces a state of exhaustion among cognate antigen specific T cells. Constant expression of the chimeric antigen receptors of the invention may result in chronic interaction with the antigen, which leads to exhaustion. The compositions disclosed herein may be used to prevent T cell exhaustion by modulating surface CAR expression using the stimulus specific to the invention. In one embodiment, the SREs of the present invention may be used to achieve pulsatile expression of the compositions of the invention. As used here, "pulsatile" refers to a plurality of payload expression at spaced apart time intervals. Generally, upon administration of the stimulus, the expression of the payload is increased causing the first pulse; following the withdrawal of the stimulus, the expression of the payload decreases and this represents the

interval time between the first exposure and the next exposure to the stimulus, after which the second exposure to the stimulus is initiated.

[00369] Also provided herein, is a method of preventing or reversing T cell exhaustion in a subject in need thereof, where the method comprising administering to the subject a therapeutically effective amount of a composition comprising at least one effector module. In some embodiments, the effector module includes a stimulus response element (SRE) operably linked to at least one immunotherapeutic agent, such that the SRE responds to a stimulus and tunes the expression and/or function of the immunotherapeutic agent, thereby preventing or reversing T cell exhaustion. In some embodiments, the immunotherapeutic agent may be a chimeric antigen receptor. Examples of chimeric antigen receptors include, but are not limited to GD2 CAR, BCMA CAR, CD33 CAR, Her2 CAR, ALK CAR, CD22 CAR, or a CD276 CAR. In some embodiments, the CAR may be a bispecific CAR comprising an extracellular domain which recognizes at least one antigen such as GD2, BCMA, CD33, Her2, ALK, CD22 or a CD276. In some embodiments, the methods described herein may include pulsatile expression of the compositions of the invention to prevent T cell exhaustion. In some instances, T cell exhaustion may be reversed by the addition of the stimulus. In other instances, T cell exhaustion may be reversed by the withdrawal of the stimulus.

[00370] In some embodiments, the compositions of the present invention may be utilized to alter TIL (tumor infiltrating lymphocyte) populations in a subject. In one embodiment, any of the payloads described herein may be utilized to change the ratio of CD4 positive cells to CD8 positive populations. In some embodiments, TILs may be sorted ex vivo and engineered to express any of the cytokines described herein. Payloads of the invention may be used to expand CD4 and/or CD8 populations of TILs to enhance TIL mediated immune response.

2. Cancer vaccines

[00371] In some embodiments, biocircuits, effector modules, payloads of interest (immunotherapeutic agents), vectors, cells and compositions of the present invention may be used in conjunction with cancer vaccines. In one aspect, dendritic cells are modified to express the compositions of the invention and used as cancer vaccines.

[00372] In some embodiments, cancer vaccine may comprise peptides and/or proteins derived from tumor associated antigen (TAA). Such strategies may be utilized to evoke an immune response in a subject, which in some instances may be a cytotoxic T lymphocyte (CTL) response. Peptides used for cancer vaccines may also be modified to match the mutation profile of a subject. For example, EGFR derived peptides with mutations matched to the mutations found in the subject in need of therapy have been successfully used in patients with lung cancer

(Li F et al. (2016) Oncoimmunology. Oct 7;5(12): e1238539; the contents of which are incorporated herein by reference in their entirety).

[00373] In one embodiment, cancer vaccines of the present invention may superagonist altered peptide ligands (APL) derived from TAAs. These are mutant peptide ligands deviate from the native peptide sequence by one or more amino acids, which activate specific CTL clones more effectively than native epitopes. These alterations may allow the peptide to bind better to the restricting Class I MHC molecule or interact more favorably with the TCR of a given tumor-specific CTL subset. APLs may be selected using methods taught in US Patent Publication NOS. US20160317633A1, the contents of which are incorporated herein by reference in their entirety.

[00374] Relapse of hematologic malignancies is the primary cause of treatment failure after allogeneic hematopoietic stem cell transplantation (HCT). The Wilm's tumor (WT1) gene product is a tumor associated antigen that is expressed in acute leukemia and other hematological malignancies, with limited expression in normal tissues. The compositions of the present invention may be co-administered with donor derived WT1 peptide loaded dendritic cell vaccine to prevent relapse of disease following immunotherapy (Shah NN et al. (2016) Biol Blood Marrow Transplant. 22(12):2149-215; the contents of which are incorporated herein by reference in their entirety.

3. Combination treatments

[00375] In some embodiments, it is desirable to combine compositions, vectors and cells of the invention for administration to a subject. Compositions of the invention comprising different immunotherapeutic agents may be used in combination or in conjunction with known immunotherapeutic agents for enhancement of immunotherapy.

[00376] In some embodiments, it is desirable to combine compositions of the invention with adjuvants, that can enhance the potency and longevity of antigen-specific immune responses. Adjuvants used as immunostimulants in combination therapy include biological molecules or delivery carriers that deliver antigens. As non-limiting examples, the compositions of the invention may be combined with biological adjuvants such as cytokines, Toll Like Receptors, bacterial toxins, and/or saponins. In other embodiments, the compositions of the present invention may be combined with delivery carriers. Exemplary delivery carriers include, polymer microspheres, immune stimulating complexes, emulsions (oil-in-water or water-in-oil), aluminum salts, liposomes and virosomes.

[00377] In some embodiments, immune effector cells modified to express biocircuits, effector modules, DDs and payloads of the invention may be combined with the biological adjuvants described herein.

[00378] In some embodiments, immune effector cells modified to expressed biocircuits, effector modules, DDs and payloads of the invention may be combined with cancer vaccines.

[00379] In some embodiments, an effector module comprising a cytokine may be used in combination with an effector module encoding a safety switch or a regulatory switch.

[00380] In some embodiments, methods of the invention may include combination of the compositions of the invention with other agents effective in the treatment of cancers, infection diseases and other immunodeficient disorders, such as anti-cancer agents. As used herein, the term "anti-cancer agent" refers to any agent which is capable of negatively affecting cancer in a subject, for example, by killing cancer cells, inducing apoptosis in cancer cells, reducing the growth rate of cancer cells, reducing the incidence or number of metastases, reducing tumor size, inhibiting tumor growth, reducing the blood supply to a tumor or cancer cells, promoting an immune response against cancer cells or a tumor, preventing or inhibiting the progression of cancer, or increasing the lifespan of a subject with cancer.

[00381] In some embodiments, anti-cancer agent or therapy may be a chemotherapeutic agent, or radiotherapy, immunotherapeutic agent, surgery, or any other therapeutic agent which, in combination with the present invention, improves the therapeutic efficacy of treatment.

[00382] In some embodiments, compositions of the present invention may be used in combination with immunotherapeutics other than the inventive therapy described herein, such as antibodies specific to some target molecules on the surface of a tumor cell.

[00383] Exemplary chemotherapies include, without limitation, Acivicin; Aclarubicin; Acodazole hydrochloride; Acronine; Adozelesin; Aldesleukin; Altretamine; Ambomycin; Ametantrone acetate; Amsacrine; Anastrozole; Anthramycin; Asparaginase; Asperrin, Sulindac, Curcumin, alkylating agents including: Nitrogen mustards such as mechlor-ethamine, cyclophosphamide, ifosfamide, melphalan and chlorambucil; nitrosoureas such as carmustine (BC U), lomustine (CCNU), and semustine (methyl-CC U); thylenimines/methylmelamine such as thriethylenemelamine (TEM), triethylene, thiophosphoramide (thiotepa), hexamethylmelamine (HMM, altretamine); alkyl sulfonates such as busulfan; triazines such as dacarbazine (DTIC); antimetabolites including folic acid analogs such as methotrexate and trimetrexate, pyrrolidine analogs such as 5- fluorouracil, fluorodeoxyuridine, gemcitabine, cytosine arabinoside (AraC, cytarabine), 5-azacytidine, 2,2'- difluorodeoxycytidine, purine analogs such as 6-mercaptopurine, 6-thioguanine, azathioprine, 2'-deoxycoformycin (pentostatin), erythrohydroxynonyladenine (EHNA), fludarabine phosphate, and 2-chlorodeoxyadenosine (cladribine, 2- CdA); natural products including antimitotic drugs such as paclitaxel, vinca alkaloids including vinblastine (VLB), vincristine, and vinorelbine, taxotere,

estramustine, and estramustine phosphate; epipodophylotoxins such as etoposide and teniposide; antibiotics, such as actimomycin D, daunomycin (rubidomycin), doxorubicin, mitoxantrone, idarubicin, bleomycins, plicamycin (mithramycin), mitomycinC, and actinomycin; enzymes such as L-asparaginase, cytokines such as interferon (IFN)-gamma, tumor necrosis factor (TNF)-alpha, TNF-beta and GM-CSF, anti-angiogenic factors, such as angiostatin and endostatin, inhibitors of FGF or VEGF such as soluble forms of receptors for angiogenic factors, including soluble VGF/VEGF receptors, platinum coordination complexes such as cisplatin and carboplatin, anthracenediones such as mitoxantrone, substituted urea such as hydroxyurea, methylhydrazine derivatives including N- methylhydrazine (MIFf) and procarbazine, adrenocortical suppressants such as mitotane (o,p'-DDD) and aminoglutethimide; hormones and antagonists including adrenocorticosteroid antagonists such as prednisone and equivalents, dexamethasone and aminoglutethimide; progestin such as hydroxyprogesterone caproate, medroxyprogesterone acetate and megestrol acetate; estrogen such as diethylstilbestrol and ethinyl estradiol equivalents; antiestrogen such as tamoxifen; androgens including testosterone propionate and fluoxymesterone/equivalents; antiandrogens such as flutamide, gonadotropin-releasing hormone analogs and leuprolide; non-steroidal antiandrogens such as flutamide; kinase inhibitors, histone deacetylase inhibitors, methylation inhibitors, proteasome inhibitors, monoclonal antibodies, oxidants, anti-oxidants, telomerase inhibitors, BH3 mimetics, ubiquitin ligase inhibitors, stat inhibitors and receptor tyrosin kinase inhibitors such as imatinib mesylate (marketed as Gleevac or Glivac) and erlotinib (an EGF receptor inhibitor) now marketed as Tarveca; anti-virals such as oseltamivir phosphate, Amphotericin B, and palivizumab; Sdi 1 mimetics; Semustine; Senescence derived inhibitor 1; Sparfosic acid; Spicamycin D; Spiromustine; Splenopentin; Spongistatin 1; Squalamine; Stipiamide; Stromelysin inhibitors; Sulfinosine; Superactive vasoactive intestinal peptide antagonist; Velaresol; Veramine; Verdins; Verteporfin; Vinorelbine; Vinxaltine; Vitaxin; Vorozole; Zanoterone; Zeniplatin; Zilascorb; and Zinostatin stimalamer; PI3Kβ small-molecule inhibitor, GSK2636771; pan-PI3K inhibitor (BKM120); BRAF inhibitors. Vemurafenib (Zelboraf) and dabrafenib (Tafinlar); or any analog or derivative and variant of the foregoing.

[00384] Radiotherapeutic agents and factors include radiation and waves that induce DNA damage for example, γ-irradiation, X-rays, UV-irradiation, microwaves, electronic emissions, radioisotopes, and the like. Therapy may be achieved by irradiating the localized tumor site with the above described forms of radiations. It is most likely that all of these factors effect a broad range of damage DNA, on the precursors of DNA, the replication and repair of DNA, and the assembly and maintenance of chromosomes. Dosage ranges for X-rays range from daily doses of

50 to 200 roentgens for prolonged periods of time (3 to 4 weeks), to single doses of 2000 to 6000 roentgens. Dosage ranges for radioisotopes vary widely, and depend on the half-life of the isotope, the strength and type of radiation emitted, and the uptake by the neoplastic cells.

[00385] In some embodiments, the chemotherapeutic agent may be an immunomodulatory agent such as lenalidomide (LEN). Recent studies have demonstrated that lenalidomide can enhance antitumor functions of CAR modified T cells (Otahal et al., *Oncoimmunology*, 2015, 5(4): e1115940). Some examples of anti-tumor antibodies include tocilizumab, siltuximab.

[00386] Other agents may be used in combination with compositions of the invention may also include, but not limited to, agents that affect the upregulation of cell surface receptors and their ligands such as Fas/Fas ligand, DR4 or DR5/TRAIL and GAP junctions, cytostatic and differentiation agents, inhibitors of cell adhesion such as focal adhesion kinase (FAKs) inhibitors and Lovastatin, or agents that increase the sensitivity of the hyper proliferative cells to apoptotic inducers such as the antibody C225.

[00387] The combinations may include administering the compositions of the invention and other agents at the same time or separately. Alternatively, the present immunotherapy may precede or follow the other agent/therapy by intervals ranging from minutes, days, weeks to months.

[00388] In some embodiments, CAR-T cells of the invention may be co-administered with retinoids to eradicate myeloid derived suppressor cells. Myeloid-derived suppressor cells (MDSCs) are a heterogeneous population of early myeloid progenitors, immature granulocytes, macrophages, and dendritic cells at different stages of differentiation. MDSCs have the capacity to suppress both the cytotoxic activities of natural killer (NK) and NKT cells, and the adaptive immune response mediated by CD4+ and CD8+ T cells. Long AH et al. (2016) Cancer Immunol Res.;4(10):869-880 have described the co-treatment of CARs with all trans retinoic acid (ATRA) for the successful treatment of solid tumors (the contents of which are incorporated by reference in its entirety).

[00389] Adjuvant therapy as used herein refers to the treatment that is given in addition to primary therapy to kill any cancer cells, even if the cancer is undetectable by standard laboratory tests. Experimental data have demonstrated that the lymphocyte depletion induced by cytotoxic regimens for the treatment of cancer could contribute to relapse. Relapse to cancer immunotherapy may be minimized by adjuvant immunotherapy. In some embodiments, adjuvant therapy may include the co-administration of recombinant human Interleukin2 in conjunction with dendritic cells pulsed with peptides derived from tumor cells. In some embodiments, dendritic cells pulsed with autologous tumor cell lysate and keyhole limpet hemocyanin (KLH).

Immune cells may be further depleted of CD25 positive T cells. Interleukin 7 may also be co-administered as immunotherapy. In some embodiments, the risk of reinfusing donor- derived tumor cells may be purged with monoclonal antibody 8H9, which interacts with tumor cell surface antigens. Any of the adjuvant therapy methods taught in Merchant et al. (2016), Clin Cancer Res. 1;22(13):3182-91 may be utilized (the contents of which are incorporated by reference in their entirety).

[00390] In some embodiments, compositions of the invention can be combined with CXCR2 inhibitors or anti – CXCR2 antibodies. Highfill SL et al. (2014) found that CXCR2 positive MDSC cells limit the efficacy of immunotherapy by mediating local immunosuppression (Highfill SL, et al. Sci Transl Med. 2014 May 21;6(237):237ra67; the contents of which are incorporated herein by reference in its entirety).

4. Diseases

[00391] Provided in the present invention is a method of reducing a tumor volume or burden in a subject in need, the method comprising introducing into the subject a composition of the invention.

[00392] The present invention also provides methods for treating a cancer in a subject, comprising administering to the subject an effective amount of an immune effector cell genetically modified to express at least one effector module of the invention.

Cancer

[00393] Various cancers may be treated with pharmaceutical compositions, biocircuits, biocircuit components, effector modules including their SREs or payloads of the present invention. As used herein, the term "cancer" refers to any of various malignant neoplasms characterized by the proliferation of anaplastic cells that tend to invade surrounding tissue and metastasize to new body sites and also refers to the pathological condition characterized by such malignant neoplastic growths. Cancers may be tumors or hematological malignancies, and include but are not limited to, all types of lymphomas/leukemias, carcinomas and sarcomas, such as those cancers or tumors found in the anus, bladder, bile duct, bone, brain, breast, cervix, colon/rectum, endometrium, esophagus, eye, gallbladder, head and neck, liver, kidney, larynx, lung, mediastinum (chest), mouth, ovaries, pancreas, penis, prostate, skin, small intestine, stomach, spinal marrow, tailbone, testicles, thyroid and uterus.

[00394] Types of carcinomas which may be treated with the compositions of the present invention include, but are not limited to, papilloma/carcinoma, choriocarcinoma, endodermal sinus tumor, teratoma, adenoma/adenocarcinoma, melanoma, fibroma, lipoma, leiomyoma, rhabdomyoma, mesothelioma, angioma, osteoma, chondroma, glioma, lymphoma/leukemia,

squamous cell carcinoma, small cell carcinoma, large cell undifferentiated carcinomas, basal cell carcinoma and sinonasal undifferentiated carcinoma.

[00395] Types of carcinomas which may be treated with the compositions of the present invention include, but are not limited to, soft tissue sarcoma such as alveolar soft part sarcoma, angiosarcoma, dermatofibrosarcoma, desmoid tumor, desmoplastic small round cell tumor, extraskeletal chondrosarcoma, extraskeletal osteosarcoma, fibrosarcoma, hemangiopericytoma, hemangiosarcoma, Kaposi's sarcoma, leiomyosarcoma, liposarcoma, lymphangiosarcoma, lymphosarcoma, malignant fibrous histiocytoma, neurofibrosarcoma, rhabdomyosarcoma, synovial sarcoma, and Askin's tumor, Ewing's sarcoma (primitive neuroectodermal tumor), malignant hemangioendothelioma, malignant schwannoma, osteosarcoma, and chondrosarcoma.

[00396] As a non-limiting example, the carcinoma which may be treated may be Acute granulocytic leukemia, Acute lymphocytic leukemia, Acute myelogenous leukemia, Adenocarcinoma, Adenosarcoma, Adrenal cancer, Adrenocortical carcinoma, Anal cancer, Anaplastic astrocytoma, Angiosarcoma, Appendix cancer, Astrocytoma, Basal cell carcinoma, B-Cell lymphoma), Bile duct cancer, Bladder cancer, Bone cancer, Bowel cancer, Brain cancer, Brain stem glioma, Brain tumor, Breast cancer, Carcinoid tumors, Cervical cancer, Cholangiocarcinoma, Chondrosarcoma, Chronic lymphocytic leukemia, Chronic myelogenous leukemia, Colon cancer, Colorectal cancer, Craniopharyngioma, Cutaneous lymphoma, Cutaneous melanoma, Diffuse astrocytoma, Ductal carcinoma in situ, Endometrial cancer, Ependymoma, Epithelioid sarcoma, Esophageal cancer, Ewing sarcoma, Extrahepatic bile duct cancer, Eye cancer, Fallopian tube cancer, Fibrosarcoma, Gallbladder cancer, Gastric cancer, Gastrointestinal cancer, Gastrointestinal carcinoid cancer, Gastrointestinal stromal tumors, General, Germ cell tumor, Glioblastoma multiforme, Glioma, Hairy cell leukemia, Head and neck cancer, Hemangioendothelioma, Hodgkin lymphoma, Hodgkin's disease, Hodgkin's lymphoma, Hypopharyngeal cancer, Infiltrating ductal carcinoma, Infiltrating lobular carcinoma, Inflammatory breast cancer, Intestinal Cancer, Intrahepatic bile duct cancer, Invasive / infiltrating breast cancer, Islet cell cancer, Jaw cancer, Kaposi sarcoma, Kidney cancer, Laryngeal cancer, Leiomyosarcoma, Leptomeningeal metastases, Leukemia, Lip cancer, Liposarcoma, Liver cancer, Lobular carcinoma in situ, Low-grade astrocytoma, Lung cancer, Lymph node cancer, Lymphoma, Male breast cancer, Medullary carcinoma, Medulloblastoma, Melanoma, Meningioma, Merkel cell carcinoma, Mesenchymal chondrosarcoma, Mesenchymous, Mesothelioma, Metastatic breast cancer, Metastatic melanoma, Metastatic squamous neck cancer, Mixed gliomas, Mouth cancer, Mucinous carcinoma, Mucosal melanoma, Multiple myeloma, Nasal cavity cancer, Nasopharyngeal cancer, Neck cancer,

Neuroblastoma, Neuroendocrine tumors, Non-Hodgkin lymphoma, Non-Hodgkin's lymphoma, Non-small cell lung cancer, Oat cell cancer, Ocular cancer, Ocular melanoma, Oligodendroglioma, Oral cancer, Oral cavity cancer, Oropharyngeal cancer, Osteogenic sarcoma, Osteosarcoma, Ovarian cancer, Ovarian epithelial cancer, Ovarian germ cell tumor, Ovarian primary peritoneal carcinoma, Ovarian sex cord stromal tumor, Paget's disease, Pancreatic cancer, Papillary carcinoma, Paranasal sinus cancer, Parathyroid cancer, Pelvic cancer, Penile cancer, Peripheral nerve cancer, Peritoneal cancer, Pharyngeal cancer, Pheochromocytoma, Pilocytic astrocytoma, Pineal region tumor, Pineoblastoma, Pituitary gland cancer, Primary central nervous system lymphoma, Prostate cancer, Rectal cancer, Renal cell cancer, Renal pelvis cancer, Rhabdomyosarcoma, Salivary gland cancer, Sarcoma, Sarcoma, bone, Sarcoma, soft tissue, Sarcoma, uterine, Sinus cancer, Skin cancer, Small cell lung cancer, Small intestine cancer, Soft tissue sarcoma, Spinal cancer, Spinal column cancer, Spinal cord cancer, Spinal tumor, Squamous cell carcinoma, Stomach cancer, Synovial sarcoma, T-cell lymphoma), Testicular cancer, Throat cancer, Thymoma / thymic carcinoma, Thyroid cancer, Tongue cancer, Tonsil cancer, Transitional cell cancer, Transitional cell cancer, Transitional cell cancer, Triple-negative breast cancer, Tubal cancer, Tubular carcinoma, Ureteral cancer, Ureteral cancer, Urethral cancer, Uterine adenocarcinoma, Uterine cancer, Uterine sarcoma, Vaginal cancer, and Vulvar cancer.

[00397] In some embodiments, the CARs of the present invention may be a CAR useful in the treatment of multiple myeloma such as a CS1 CAR, a CD38 CAR, a CD138 CAR, and a BCMA CAR. In some embodiments, the CARs of the present invention may be a CAR useful in the treatment of acute myeloid leukemia such as a CD33 CAR, a CD123 CAR, and a CLL1 CAR. In some embodiments, the CARs of the present invention may be a CAR useful in the treatment of T cell leukemia such as a CD5 CAR, and a CD7 CAR. In some embodiments, the CARs of the present invention may be a CAR useful in the treatment of solid tumors such a mesothelin CAR, a GD2 CAR, a GPC3 CAR, a Her2 CAR, an EGFR CAR, a Muc1 CAR, an EpCAM CAR, a PD-L1 CAR, a CEA CAR, a Muc16 CAR, a CD133 CAR, a CD171 CAR, a CD70 CAR, a CLD18 CAR, a cMET CAR, a EphA2 CAR, a FAP CAR, a Folate Receptor CAR, an IL13Ra2 CAR, an MG7 CAR, a PSMA CAR, a ROR1 CAR, and a VEGFR2 CAR.

Infectious diseases

[00398] In some embodiment, biocircuits of the invention may be used for the treatment of infectious diseases. Biocircuits of the invention may be introduced in cells suitable for adoptive cell transfer such as macrophages, dendritic cells, natural killer cells, and or T cells. Infectious diseases treated by the biocircuits of the invention may be diseases caused by viruses, bacteria,

fungi, and/or parasites. IL15-IL15Ra payloads of the invention may be used to increase immune cell proliferation and/or persistence of the immune cells useful in treating infectious diseases.

[00399] "Infection diseases" herein refer to diseases caused by any pathogen or agent that infects mammalian cells, preferably human cells and causes a disease condition. Examples thereof include bacteria, yeast, fungi, protozoans, *mycoplasma*, viruses, prions, and parasites. Examples include those involved in (a) viral diseases such as, for example, diseases resulting from infection by an adenovirus, a herpesvirus (e.g., HSV-I, HSV-II, CMV, or VZV), a poxvirus (e-g-, an orthopoxvirus such as variola or vaccinia, or molluscum contagiosum), a picornavirus (e.g., rhinovirus or enterovirus), an orthomyxovirus (e.g., influenzavirus), a paramyxovirus (e.g., parainfluenza virus, mumps virus, measles virus, and respiratory syncytial virus (RSV)), a coronavirus (e.g., SARS), a papovavirus (e.g., papillomaviruses, such as those that cause genital warts, common warts, or plantar warts), a hepadnavirus (e.g., hepatitis B virus), a flavivirus (e.g., hepatitis C virus or Dengue virus), or a retrovirus (e.g., a lentivirus such as HIV); (b) bacterial diseases such as, for example, diseases resulting from infection by bacteria of, for example, the genus *Escherichia, Enterobacter, Salmonella, Staphylococcus, Shigella, Listeria, Aerobacter, Helicobacter, Klebsiella, Proteus, Pseudomonas, Streptococcus, Chlamydia, Mycoplasma, Pneumococcus, Neisseria, Clostridium, Bacillus, Corynebacterium, Mycobacterium, Campylobacter, Vibrio, Serratia, Providencia, Chromobacterium, Brucella, Yersinia, Haemophilus*, or *Bordetella*; (c) other infectious diseases, such *chlamydia*, fungal diseases including but not limited to candidiasis, aspergillosis, histoplasmosis, cryptococcal meningitis, parasitic diseases including but not limited to malaria, *Pneumocystis carnii* pneumonia, leishmaniasis, cryptosporidiosis, toxoplasmosis, and trypanosome infection and prions that cause human disease such as Creutzfeldt-Jakob Disease (CJD), variant Creutzfeldt-Jakob Disease (vCJD), Gerstmann-Straüssler-Scheinker syndrome, Fatal Familial Insomnia and kuru.

5. Microbiome

[00400] Alterations in the composition of the microbiome may impact the action of anti-cancer therapies. A diverse community of symbiotic, commensal and pathogenic microorganisms exist in all environmentally exposed sites in the body and is herein referred to as the "Microbiome." Environmentally exposed sites of the body that may be inhabited by a microbiome include the skin, nasopharynx, the oral cavity, the respiratory tract, the gastrointestinal tract, and the reproductive tract.

[00401] In some embodiments, microbiome native or engineered with immunotherapeutic agents may be used to improve the efficacy of the anti-cancer immunotherapies. Methods of using microbiome to improve responsive to immunotherapeutic agents have been described by

Sivan et al. (Sivan A., et al.2015. Science; 350:1084--9; the contents of which are incorporated herein by reference in their entirety). In other embodiments, the microorganisms may be delivered along with immunotherapeutic compositions of the present invention to improve the efficacy of immunotherapy.

6. Tools and agents for making therapeutics

[00402] Provided in the present invention are tools and agents that may be used in generating immunotherapeutics for reducing a tumor volume or burden in a subject in need. A considerable number of variables are involved in producing a therapeutic agent, such as structure of the payload, types of cell, methods of gene transfer, method and time of ex vivo expansion, pre-conditioning and the amount and type of tumor burden in the subject. Such parameters may be optimized using tools and agents described herein.

Cell lines

[00403] The present disclosure provides a mammalian cell that has been genetically modified with the compositions of the invention. Suitable mammalian cells include primary cells and immortalized cell lines. Suitable mammalian cell lines include, but are not limited to Human embryonic kidney cell line 293, fibroblast cell line NIH 3T3, human colorectal carcinoma cell line HCT116, ovarian carcinoma cell line SKOV-3, immortalized T cell lines Jurkat cells and SupT1 cells, lymphoma cell line Raji cells, NALM-6 cells, K562 cells, HeLa cells, PC12 cells, HL-60 cells, NK cell lines (e.g., NKL, NK92, NK962, and YTS), REH, SEM, KOPN8, Daudi, Raji, and the like. In some instances, the cell is not an immortalized cell line, but instead a cell obtained from an individual and is herein referred to as a primary cell. For example, the cell is a T lymphocyte obtained from an individual. Other examples include, but are not limited to cytotoxic cells, stem cells, peripheral blood mononuclear cells or progenitor cells obtained from an individual.

Tracking SREs, biocircuits and cell lines

[00404] In some embodiments, it may be desirable to track the compositions of the invention or the cells modified by the compositions of the invention. Tracking may be achieved by using payloads such as reporter moieties, which, as used herein, refers to any protein capable of creating a detectable signal, in response to an input. Examples include alkaline phosphatase, β-galactosidase, chloramphenicol acetyltransferase, β-glucuronidase, peroxidase, β-lactamase, catalytic antibodies, bioluminescent proteins e.g. luciferase, and fluorescent proteins such as Green fluorescent protein (GFP).

[00405] Reporter moieties may be used to monitor the response of the DD upon addition of the ligand corresponding to the DD. In other instances, reporter moieties may be used to track cell survival, persistence, cell growth, and/or localization *in vitro, in vivo, or ex vivo.*

[00406] In some embodiments, the preferred reporter moiety may be luciferase proteins. In one embodiment, the reporter moiety is the Renilla luciferase, or a firefly luciferase. Table 14 provides the sequences of the reporter moieties. The amino acid sequences in Table 14 may comprise a stop codon which is denoted in the table with a "*" at the end of the amino acid sequence

Table 14: DD-luciferase constructs

Description	Amino acid sequence	Amino Acid SEQ ID NO.	Nucleic Acid SEQ ID NO.
Linker	EF	-	GAGTTC
Linker	SG	-	AGCGGC
Renilla luciferase	MTSKVYDPEQRKRMITGPQWWARCKQMNVLDSFIN YYDSEKHAENAVIFLHGNAASSYLWRHVVPHIEPVA RCIIPDLIGMGKSGKSGNGSYRLLDHYKYLTAWFELL NLPKKIIFVGHDWGACLAFHYSYEHQDKIKAIVHAES VVDVIESWDEWPDIEEDIALIKSEEGEKMVLENNFFV ETMLPSKIMRKLEPEEFAAYLEPFKEKGEVRRPTLSW PREIPLVKGGKPDVVQIVRNYNAYLRASDDLPKMFIE SDPGFFSNAIVEGAKKFPNTEFVKVKGLHFSQEDAPD EMGKYIKSFVERVLKNEQ	209	217
Firefly Luciferase	MEDAKNIKKGPAPFYPLEDGTAGEQLHKAMKRYAL VPGTIAFTDAHIEVDITYAEYFEMSVRLAEAMKRYGL NTNHRIVVCSENSLQFFMPVLGALFIGVAVAPANDIY NERELLNSMGISQPTVVFVSKKGLQKILNVQKKLPIIQ KIIIMDSKTDYQGFQSMYTFVTSHLPPGFNEYDFVPES FDRDKTIALIMNSSGSTGLPKGVALPHRTACVRFSHA RDPIFGNQIIPDTAILSVVPFHHGFGMFTTLGYLICGFR VVLMYRFEEELFLRSLQDYKIQSALLVPTLFSFFAKST LIDKYDLSNLHEIASGGAPLSKEVGEAVAKRFHLPGI RQGYGLTETTSAILITPEGDDKPGAVGKVVPFFEAKV VDLDTGKTLGVNQRGELCVRGPMIMSGYVNNPEAT NALIDKDGWLHSGDIAYWDEDEHFFIVDRLKSLIKY KGYQVAPAELESILLQHPNIFDAGVAGLPDDDAGELP AAVVVLEHGKTMTEKEIVDYVASQVTTAKKLRGGV VFVDEVPKGLTGKLDARKIREILIKAKKGGKSKL	210	218
FKBP (F36V, L106P)	GVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKK VDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQ RAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKPE	11	60, 878-882
FKBP (E31G, F36V, R71G, K105E)	GVQVETISPGDGRTFPKRGQTCVVHYTGMLGDGKK VDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQ GAKLTISPDYAYGATGHPGIIPPHATLVFDVELLELE	12	88, 883-889
ecDHFR (R12Y, Y100I)	MISLIAALAVDYVIGMENAMPWNLPADLAWFKRNT LNKPVIMGRHTWESIGRPLPGRKNIILSSQPGTDDRVT WVKSVDEAIAACGDVPEIMVIGGGRVIEQFLPKAQK LYLTHIDAEVEGDTHFPDYEPDDWESVFSEFHDADA QNSHSYCFEILERR	8	89
OT-Rluc-001 (Renilla Luc - stop)	MTSKVYDPEQRKRMITGPQWWARCKQMNVLDSFIN YYDSEKHAENAVIFLHGNAASSYLWRHVVPHIEPVA RCIIPDLIGMGKSGKSGNGSYRLLDHYKYLTAWFELL NLPKKIIFVGHDWGACLAFHYSYEHQDKIKAIVHAES	211	203

	VVDVIESWDEWPDIEEDIALIKSEEGEKMVLENNFFV ETMLPSKIMRKLEPEEFAAYLEPFKEKGEVRRPTLSW PREIPLVKGGKPDVVQIVRNYNAYLRASDDLPKMFIE SDPGFFSNAIVEGAKKFPNTEFVKVKGLHFSQEDAPD EMGKYIKSFVERVLKNEQ*		
OT-Fluc-002 (Met - FKBP (F36V, L106P) – Linker (EF) – Firefly Luc - stop)	MGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGK KVDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVG QRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKPE EFMEDAKNIKKGPAPFYPLEDGTAGEQLHKAMKRY ALVPGTIAFTDAHIEVDITYAEYFEMSVRLAEAMKRY GLNTNHRIVVCSENSLQFFMPVLGALFIGVAVAPAN DIYNERELLNSMGISQPTVVFVSKKGLQKILNVQKKL PIIQKIIIMDSKTDYQGFQSMYTFVTSHLPPGFNEYDF VPESFDRDKTIALIMNSSGSTGLPKGVALPHRTACVR FSHARDPIFGNQIIPDTAILSVVPFHHGFGMFTTLGYLI CGFRVVLMYRFEEELFLRSLQDYKIQSALLVPTLFSFF AKSTLIDKYDLSNLHEIASGGAPLSKEVGEAVAKRFH LPGIRQGYGLTETTSAILITPEGDDKPGAVGKVVPFFE AKVVDLDTGKTLGVNQRGELCVRGPMIMSGYVNNP EATNALIDKDGWLHSGDIAYWDEDEHFFIVDRLKSLI KYKGYQVAPAELESILLQHPNIFDAGVAGLPDDDAG ELPAAVVVLEHGKTMTEKEIVDYVASQVTTAKKLR GGVVFVDEVPKGLTGKLDARKIREILIKAKKGGKSK L*	212	204
OT-Rluc-003 (Met - FKBP (F36V, L106P) – Linker (SG) - Amino acid 2 – 311 of Renilla Luciferase – stop)	MGVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGK KVDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVG QRAKLTISPDYAYGATGHPGIIPPHATLVFDVELLKPE SGTSKVYDPEQRKRMITGPQWWARCKQMNVLDSFI NYYDSEKHAENAVIFLHGNAASSYLWRHVVPHIEPV ARCIIPDLIGMGKSGKSGNGSYRLLDHYKYLTAWFE LLNLPKKIIFVGHDWGACLAFHYSYEHQDKIKAIVHA ESVVDVIESWDEWPDIEEDIALIKSEEGEKMVLENNF FVETMLPSKIMRKLEPEEFAAYLEPFKEKGEVRRPTL SWPREIPLVKGGKPDVVQIVRNYNAYLRASDDLPKM FIESDPGFFSNAIVEGAKKFPNTEFVKVKGLHFSQED APDEMGKYIKSFVERVLKNEQ*	213	205
OT-Rluc-004 (ecDHFR (R12Y, Y100I) – Linker (SG) - Amino acid 2 – 311 of Renilla Luciferase - stop)	MISLIAALAVDYVIGMENAMPWNLPADLAWFKRNT LNKPVIMGRHTWESIGRPLPGRKNIILSSQPGTDDRVT WVKSVDEAIAACGDVPEIMVIGGGRVIEQFLPKAQK LYLTHIDAEVEGDTHFPDYEPDDWESVFSEFHDADA QNSHSYCFEILERRSGTSKVYDPEQRKRMITGPQWW ARCKQMNVLDSFINYYDSEKHAENAVIFLHGNAASS YLWRHVVPHIEPVARCIIPDLIGMGKSGKSGNGSYRL LDHYKYLTAWFELLNLPKKIIFVGHDWGACLAFHYS YEHQDKIKAIVHAESVVDVIESWDEWPDIEEDIALIKS EEGEKMVLENNFFVETMLPSKIMRKLEPEEFAAYLEP FKEKGEVRRPTLSWPREIPLVKGGKPDVVQIVRNYN AYLRASDDLPKMFIESDPGFFSNAIVEGAKKFPNTEF VKVKGLHFSQEDAPDEMGKYIKSFVERVLKNEQ*	214	206
OT-Rluc-005 (Renilla Luc - Linker (SG) - FKBP (E31G, F36V, R71G, K105E) - stop)	MTSKVYDPEQRKRMITGPQWWARCKQMNVLDSFIN YYDSEKHAENAVIFLHGNAASSYLWRHVVPHIEPVA RCIIPDLIGMGKSGKSGNGSYRLLDHYKYLTAWFELL NLPKKIIFVGHDWGACLAFHYSYEHQDKIKAIVHAES VVDVIESWDEWPDIEEDIALIKSEEGEKMVLENNFFV ETMLPSKIMRKLEPEEFAAYLEPFKEKGEVRRPTLSW PREIPLVKGGKPDVVQIVRNYNAYLRASDDLPKMFIE SDPGFFSNAIVEGAKKFPNTEFVKVKGLHFSQEDAPD EMGKYIKSFVERVLKNEQSGGVQVETISPGDGRTFPK RGQTCVVHYTGMLGDGKKVDSSRDRNKPFKFMLGK	215	207

	QEVIRGWEEGVAQMSVGQGAKLTISPDYAYGATGH PGIIPPHATLVFDVELLELE*		
OT-Rluc-006 (Renilla Luc - Linker (SG) - ecDHFR (Amino acid 2-159 of WT) (R12Y, Y100I) - stop)	MTSKVYDPEQRKRMITGPQWWARCKQMNVLDSFIN YYDSEKHAENAVIFLHGNAASSYLWRHVVPHIEPVA RCIIPDLIGMGKSGKSGNGSYRLLDHYKYLTAWFELL NLPKKIIFVGHDWGACLAFHYSYEHQDKIKAIVHAES VVDVIESWDEWPDIEEDIALIKSEEGEKMVLENNFFV ETMLPSKIMRKLEPEEFAAYLEPFKEKGEVRRPTLSW PREIPLVKGGKPDVVQIVRNYNAYLRASDDLPKMFIE SDPGFFSNAIVEGAKKFPNTEFVKVKGLHFSQEDAPD EMGKYIKSFVERVLKNEQSGISLIAALAVDYVIGMEN AMPWNLPADLAWFKRNTLNKPVIMGRHTWESIGRP LPGRKNIILSSQPGTDDRVTWVKSVDEAIAACGDVPE IMVIGGGRVIEQFLPKAQKLYLTHIDAEVEGDTHFPD YEPDDWESVFSEFHDADAQNSHSYCFEILERR*	216	208

Animal models

[00407] The utility and efficacy of the compositions of the present invention may be tested *in vivo* animal models, preferably mouse models. Mouse models used to may be syngeneic mouse models wherein mouse cells are modified with compositions of the invention and tested in mice of the same genetic background. Examples include pMEL-1 and 4T1 mouse models. Alternatively, xenograft models where human cells such as tumor cells and immune cells are introduced into immunodeficient mice may also be utilized in such studies. Immunodeficient mice used may be CByJ.Cg-Foxn1^{nu}/J, B6;129S7-*Rag1*^{tm1Mom}/J, B6.129S7-*Rag1*^{tm1Mom}/J, B6.CB17-*Prkdc*^{scid}/SzJ, NOD.129S7(B6)-*Rag1*^{tm1Mom}/J, NOD.Cg-*Rag1*^{tm1Mom}*Prf1*^{tm1Sd}z/Sz, NOD.CB17-*Prkdc*^{scid}/SzJ, NOD.Cg-*Prkdc*^{scid}*B2m*^{tm1Unc}/J, NOD-*scid* IL2Rg^{null}, Nude (nu) mice, SCID mice, NOD mice, RAG1/RAG2 mice, NOD-Scid mice, IL2rg*null* mice, b2m*null* mice, NOD-*scid* IL2rγnull mice, NOD-*scid*-B2m*null* mice, and HLA transgenic mice.

Cellular assays

[00408] In some embodiments, the effectiveness of the compositions of the inventions as immunotherapeutic agents may be evaluated using cellular assays. Levels of expression and/or identity of the compositions of the invention may be determined according to any methods known in the art for identifying proteins and/or quantitating proteins levels. In some embodiments, such methods may include Western Blotting, flow cytometry, and immunoassays.

[00409] Provided herein are methods for functionally characterizing cells expressing SRE, biocircuits and compositions of the invention. In some embodiments, functional characterization is carried out in primary immune cells or immortalized immune cell lines and may be determined by expression of cell surface markers. Examples of cell surface markers for T cells include, but are not limited to, CD3, CD4, CD8, CD 14, CD20, CD11b, CD16, CD45 and HLA-DR, CD 69, CD28, CD44, IFNgamma, PD1, TIM3, LAG3. Examples of cell surface markers for antigen presenting cells include, but are not limited to, MHC class I, MHC Class II, CD40, CD45, B7-1,

B7-2, IFN-γ receptor and IL2 receptor, ICAM-1 and/or Fcγ receptor. Examples of cell surface markers for dendritic cells include, but are not limited to, MHC class I, MHC Class II, B7-2, CD18, CD29, CD31, CD43, CD44, CD45, CD54, CD58, CD83, CD86, CMRF-44, CMRF-56, DCIR and/or Dectin-1 and the like; while in some cases also having the absence of CD2, CD3, CD4, CD8, CD14, CD15, CD16, CD 19, CD20, CD56, and/or CD57. Examples of cell surface markers for NK cells include, but are not limited to, CCL3, CCL4, CCL5, CCR4, CXCR4, CXCR3, NKG2D, CD71, CD69, CCR5, Phospho JAK/STAT, phospho ERK, phospho p38/ MAPK, phospho AKT, phospho STAT3, Granulysin, Granzyme B, Granzyme K, IL10, IL22, IFNg, LAP, Perforin, and TNFa.

V. DELIVERY MODALITIES AND/OR VECTORS

Vectors

[00410] The present invention also provides vectors that package polynucleotides of the invention encoding biocircuits, effector modules, SREs (DDs) and payload constructs, and combinations thereof. Vectors of the present invention may also be used to deliver the packaged polynucleotides to a cell, a local tissue site or a subject. These vectors may be of any kind, including DNA vectors, RNA vectors, plasmids, viral vectors and particles. Viral vector technology is well known and described in Sambrook et al. (2001, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory, New York). Viruses, which are useful as vectors include, but are not limited to lentiviral vectors, adenoviral vectors, adeno-associated viral (AAV) vectors, herpes simplex viral vectors, retroviral vectors, oncolytic viruses, and the like.

[00411] In general, vectors contain an origin of replication functional in at least one organism, a promoter sequence and convenient restriction endonuclease site, and one or more selectable markers e.g. a drug resistance gene.

[00412] As used herein a promoter is defined as a DNA sequence recognized by transcription machinery of the cell, required to initiate specific transcription of the polynucleotide sequence of the present invention. Vectors can comprise native or non-native promoters operably linked to the polynucleotides of the invention. The promoters selected may be strong, weak, constitutive, inducible, tissue specific, development stage-specific, and/or organism specific. One example of a suitable promoter is the immediate early cytomegalovirus (CMV) promoter sequence. This promoter sequence is a strong constitutive promoter sequence capable of driving high levels of expression of polynucleotide sequence that is operatively linked to it. Another example of a preferred promoter is the Elongation Growth Factor-1. Alpha (EF-1. alpha) promoter. Other constitutive promoters may also be used, including, but not limited to simian virus 40 (SV40)

promoter, the mouse mammary tumor virus (MMTV) promoter, human immunodeficiency virus (HIV) promoter, long terminal repeat (LTR), promoter, an avian leukemia virus promoter, an Epstein-Barr virus immediate early promoter, a Rous sarcoma virus promoter as well as human gene promoters including, but not limited to the phosphoglycerate kinase (PGK) promoter, the actin promoter, the myosin promoter, the hemoglobin promoter, the Ubiquitin C (Ubc) promoter, the human U6 small nuclear protein promoter and the creatine kinase promoter. In some instances, inducible promoters such as but not limited to the metallothionine promoter, the glucocorticoid promoter, the progesterone promoter, and the tetracycline promoter may be used. In some embodiments, the promoter may be selected from the SEQ ID NO. 220-222, SEQ ID NO. 836.

[00413] In some embodiments, the optimal promoter may be selected based on its ability to achieve minimal expression of the SREs and payloads of the invention in the absence of the ligand and detectable expression in the presence of the ligand.

[00414] Additional promoter elements e.g. enhancers may be used to regulate the frequency of transcriptional initiation. Such regions may be located 10-100 base pairs upstream or downstream of the start site. In some instances, two or more promoter elements may be used to cooperatively or independently activate transcription.

[00415] Suitable vectors include those designed for propagation and expansion or for expression or both, such as plasmids and viruses. The vector can be selected from the group consisting of the pUC series (Fermentas Life Sciences, Glen Burnie, MD), the pBluescript series (Stratagene, La Jolla, CA), the pET series (Novagen, Madison, WI), the pGEX series (Pharmacia Biotech, Uppsala, Sweden), and the pEX series (Clontech, Palo Alto, CA). Bacteriophage vectors, such as λGT10, λGT1 1, λZapII (Stratagene), λEMBL4, and λNM1 149, also can be used. Examples of plant expression vectors include pBI01, pBI101.2, pBI101.3, pBI121 and pBIN19 (Clontech). Examples of animal expression vectors include pEUK-Cl, pMAM, and pMAMneo (Clontech). The recombinant expression vector may be a viral vector, e.g., a retroviral vector or a lenti viral vector. In some embodiments, the vector can be a transposon. Any of the vectors disclosed in the International Patent Publication WO2014065961, may be useful in the present invention (the contents of which are incorporated herein by reference in their entirety).

[00416] In some embodiments, the recombinant expression vector may comprise regulatory sequences, such as transcription and translation initiation and termination codons, which are specific to the type of the host cell into which the vector is to be introduced.

1. Lentiviral vectors

[00417] In some embodiments, lentiviral vectors/particles may be used as vehicles and delivery modalities. Lentiviruses are subgroup of the *Retroviridae* family of viruses, named because reverse transcription of viral RNA genomes to DNA is required before integration into the host genome. As such, the most important features of lentiviral vehicles/particles are the integration of their genetic material into the genome of a target/host cell. Some examples of lentivirus include the Human Immunodeficiency Viruses: HIV-1 and HIV-2, the Simian Immunodeficiency Virus (SIV), feline immunodeficiency virus (FIV), bovine immunodeficiency virus (BIV), Jembrana Disease Virus (JDV), equine infectious anemia virus (EIAV), equine infectious anemia virus, visna-maedi and caprine arthritis encephalitis virus (CAEV).

[00418] Typically, lentiviral particles making up the gene delivery vehicle are replication defective on their own (also referred to as "self-inactivating"). Lentiviruses are able to infect both dividing and non-dividing cells by virtue of the entry mechanism through the intact host nuclear envelope (Naldini L et al., *Curr. Opin. Biotechnol*, 1998, 9: 457-463). Recombinant lentiviral vehicles/particles have been generated by multiply attenuating the HIV virulence genes, for example, the genes Env, Vif, Vpr, Vpu, Nef and Tat are deleted making the vector biologically safe. Correspondingly, lentiviral vehicles, for example, derived from HIV-1/HIV-2 can mediate the efficient delivery, integration and long-term expression of transgenes into non-dividing cells. As used herein, the term "recombinant" refers to a vector or other nucleic acid containing both lentiviral sequences and non-lentiviral retroviral sequences.

[00419] Lentiviral particles may be generated by co-expressing the virus packaging elements and the vector genome itself in a producer cell such as human HEK293T cells. These elements are usually provided in three (in second generation lentiviral systems) or four separate plasmids (in third generation lentiviral systems). The producer cells are co-transfected with plasmids that encode lentiviral components including the core (i.e. structural proteins) and enzymatic components of the virus, and the envelope protein(s) (referred to as the packaging systems), and a plasmid that encodes the genome including a foreign transgene, to be transferred to the target cell, the vehicle itself (also referred to as the transfer vector). In general, the plasmids or vectors are included in a producer cell line. The plasmids/vectors are introduced via transfection, transduction or infection into the producer cell line. Methods for transfection, transduction or infection are well known by those of skill in the art. As non-limiting example, the packaging and transfer constructs can be introduced into producer cell lines by calcium phosphate transfection, lipofection or electroporation, generally together with a dominant selectable marker, such as neo,

DHFR, Gln synthetase or ADA, followed by selection in the presence of the appropriate drug and isolation of clones.

[00420] The producer cell produces recombinant viral particles that contain the foreign gene, for example, the effector module of the present invention. The recombinant viral particles are recovered from the culture media and titrated by standard methods used by those of skill in the art. The recombinant lentiviral vehicles can be used to infect target cells.

[00421] Cells used to produce high-titer lentiviral particles may include, but are not limited to, HEK293T cells, 293G cells, STAR cells (Relander et al., *Mol. Ther.*, 2005, 11: 452-459), FreeStyle™ 293 Expression System (ThermoFisher, Waltham, MA), and other HEK293T-based producer cell lines (e.g., Stewart et al., *Hum Gene Ther.* 2011, 22(3):357-369; Lee et al., *Biotechnol Bioeng*, 2012, 10996): 1551-1560; Throm et al., *Blood.* 2009, 113(21): 5104-5110; the contents of each of which are incorporated herein by reference in their entirety).

[00422] In some aspects, the envelope proteins may be heterologous envelop proteins from other viruses, such as the G protein of vesicular stomatitis virus (VSV G) or baculoviral gp64 envelop proteins. The VSV-G glycoprotein may especially be chosen among species classified in the vesiculovirus genus: *Carajas virus* (CJSV), *Chandipura virus* (CHPV), *Cocal virus* (COCV), *Isfahan virus* (ISFV), *Maraba virus* (MARAV), *Piry virus* (PIRYV), *Vesicular stomatitis Alagoas virus* (VSAV), *Vesicular stomatitis Indiana virus* (VSIV) *and Vesicular stomatitis New Jersey virus* (VSNJV) and/or stains provisionally classified in the vesiculovirus genus as *Grass carp rhabdovirus*, *BeAn 157575 virus* (BeAn 157575), *Boteke virus* (BTKV), *Calchaqui virus* (CQIV), *Eel virus American* (EVA), *Gray Lodge virus* (GLOV), *Jurona virus* (JURY), *Klamath virus* (KLAV), *Kwatta virus* (KWAV), *La Joya virus* (LJV), *Malpais Spring virus* (MSPV), *Mount Elgon bat virus* (MEBV), *Perinet virus* (PERV), *Pike fry rhabdovirus* (PFRV), *Porton virus* (PORV), *Radi virus* (RADIV), *Spring viremia of carp virus* (SVCV), *Tupaia virus* (TUPV), *Ulcerative disease rhabdovirus* (UDRV) and *Yug Bogdanovac virus* (YBV). The gp64 or other baculoviral env protein can be derived from *Autographa californica* nucleopolyhedrovirus (AcMNPV), *Anagrapha falcifera* nuclear polyhedrosis virus, *Bombyx mori* nuclear polyhedrosis virus, *Choristoneura fumiferana* nucleopolyhedrovirus, *Orgyia pseudotsugata* single capsid nuclear polyhedrosis virus, *Epiphyas postvittana* nucleopolyhedrovirus, *Hyphantria cunea* nucleopolyhedrovirus, *Galleria mellonella* nuclear polyhedrosis virus, Dhori virus, Thogoto virus, *Antheraea pemyi* nucleopolyhedrovirus or Batken virus.

[00423] Additional elements provided in lentiviral particles may comprise retroviral LTR (long-terminal repeat) at either 5' or 3' terminus, a retroviral export element, optionally a lentiviral

reverse response element (RRE), a promoter or active portion thereof, and a locus control region (LCR) or active portion thereof. Other elements include central polypurine tract (cPPT) sequence to improve transduction efficiency in non-dividing cells, Woodchuck Hepatitis Virus (WHP) Posttranscriptional Regulatory Element (WPRE) which enhances the expression of the transgene, and increases titer. The effector module is linked to the vector.

[00424] Methods for generating recombinant lentiviral particles are discussed in the art, for example, U.S. Pat. NOs. 8, 846, 385; 7,745, 179; 7,629,153; 7,575,924; 7,179, 903; and 6, 808, 905; the contents of each of which are incorporated herein by reference in their entirety.

[00425] Lentivirus vectors used may be selected from, but are not limited to pLVX, pLenti, pLenti6, pLJM1, FUGW, pWPXL, pWPI, pLenti CMV puro DEST, pLJM1-EGFP, pULTRA, pInducer20, pHIV-EGFP, pCW57.1, pTRPE, pELPS, pRRL, and pLionII.

[00426] Lentiviral vehicles known in the art may also be used (See, U.S. Pat. NOs. 9, 260, 725; 9,068,199; 9,023,646; 8,900,858; 8,748,169; 8,709,799; 8,420,104; 8,329,462; 8,076,106; 6,013,516; and 5,994,136; International Patent Publication NOS. WO2012079000; the contents of each of which are incorporated herein by reference in their entirety).

2. Retroviral vectors (γ-retroviral vectors)

[00427] In some embodiments, retroviral vectors may be used to package and deliver the biocircuits, biocircuit components, effector modules, SREs or payload constructs of the present invention. Retroviral vectors (RVs) allow the permanent integration of a transgene in target cells. In addition to lentiviral vectors based on complex HIV-1/2, retroviral vectors based on simple gamma-retroviruses have been widely used to deliver therapeutic genes and demonstrated clinically as one of the most efficient and powerful gene delivery systems capable of transducing a broad range of cell types. Example species of Gamma retroviruses include the murine leukemia viruses (MLVs) and the feline leukemia viruses (FeLV).

[00428] In some embodiments, gamma-retroviral vectors derived from a mammalian gamma-retrovirus such as murine leukemia viruses (MLVs), are recombinant. The MLV families of gamma retroviruses include the ecotropic, amphotropic, xenotropic and polytropic subfamilies. Ecotropic viruses can infect only murine cells using mCAT-1 receptor. Examples of ecotropic viruses are Moloney MLV and AKV. Amphotropic viruses infect murine, human and other species through the Pit-2 receptor. One example of an amphotropic virus is the 4070A virus. Xenotropic and polytropic viruses utilize the same (Xpr1) receptor, but differ in their species tropism. Xenotropic viruses such as NZB-9-1 infect human and other species but not murine species, whereas polytropic viruses such as focus-forming viruses (MCF) infect murine, human and other species.

[00429] Gamma-retroviral vectors may be produced in packaging cells by co-transfecting the cells with several plasmids including one encoding the retroviral structural and enzymatic (gag-pol) polyprotein, one encoding the envelope (env) protein, and one encoding the vector mRNA comprising polynucleotide encoding the compositions of the present invention that is to be packaged in newly formed viral particles.

[00430] In some aspects, the recombinant gamma-retroviral vectors are pseudotyped with envelope proteins from other viruses. Envelope glycoproteins are incorporated in the outer lipid layer of the viral particles which can increase/alter the cell tropism. Exemplary envelop proteins include the gibbon ape leukemia virus envelope protein (GALV) or vesicular stomatitis virus G protein (VSV-G), or Simian endogenous retrovirus envelop protein, or Measles Virus H and F proteins, or Human immunodeficiency virus gp120 envelope protein, or cocal vesiculovirus envelop protein (See, e.g., U.S. application publication NOS. 2012/164118; the contents of which are incorporated herein by reference in its entirety). In other aspects, envelope glycoproteins may be genetically modified to incorporate targeting/binding ligands into gamma-retroviral vectors, binding ligands including, but not limited to, peptide ligands, single chain antibodies and growth factors (Waehler et al., *Nat. Rev. Genet.* 2007, 8(8):573–587; the contents of which are incorporated herein by reference in its entirety). These engineered glycoproteins can retarget vectors to cells expressing their corresponding target moieties. In other aspects, a "molecular bridge" may be introduced to direct vectors to specific cells. The molecular bridge has dual specificities: one end can recognize viral glycoproteins, and the other end can bind to the molecular determinant on the target cell. Such molecular bridges, for example ligand-receptor, avidin-biotin, and chemical conjugations, monoclonal antibodies and engineered fusogenic proteins, can direct the attachment of viral vectors to target cells for transduction (Yang et al., *Biotechnol. Bioeng.*, 2008, 101(2): 357-368; and Maetzig et al., *Viruses*, 2011, 3, 677-713; the contents of each of which are incorporated herein by reference in their entirety).

[00431] In some embodiments, the recombinant gamma-retroviral vectors are self-inactivating (SIN) gammaretroviral vectors. The vectors are replication incompetent. SIN vectors may harbor a deletion within the 3' U3 region initially comprising enhancer/promoter activity. Furthermore, the 5' U3 region may be replaced with strong promoters (needed in the packaging cell line) derived from Cytomegalovirus or RSV, or an internal promoter of choice, and/or an enhancer element. The choice of the internal promoters may be made according to specific requirements of gene expression needed for a particular purpose of the invention.

[00432] In some embodiments, polynucleotides encoding the biocircuit, biocircuit components, effector module, and SRE are inserted within the recombinant viral genome. The other

components of the viral mRNA of a recombinant gamma-retroviral vector may be modified by insertion or removal of naturally occurring sequences (e.g., insertion of an IRES, insertion of a heterologous polynucleotide encoding a polypeptide or inhibitory nucleic acid of interest, shuffling of a more effective promoter from a different retrovirus or virus in place of the wild-type promoter and the like). In some examples, the recombinant gamma-retroviral vectors may comprise modified packaging signal, and/or primer binding site (PBS), and/or 5'-enhancer/promoter elements in the U3-region of the 5'- long terminal repeat (LTR), and/or 3'-SIN elements modified in the U3-region of the 3'-LTR. These modifications may increase the titers and the ability of infection.

[00433] Gamma retroviral vectors suitable for delivering biocircuit components, effector modules, SREs or payload constructs of the present invention may be selected from those disclosed in U.S. Pat. NOs. 8,828,718; 7,585,676; 7,351,585; U.S. application publication NOS. 2007/048285; PCT application publication NOs. WO2010/113037; WO2014/121005; WO2015/056014; and EP Pat. NOs. EP1757702; EP1757703 (the contents of each of which are incorporated herein by reference in their entirety).

3. Adeno-associated viral vectors (AAV)

[00434] In some embodiments, polynucleotides of present invention may be packaged into recombinant adeno-associated viral (rAAV) vectors. Such vectors or viral particles may be designed to utilize any of the known serotype capsids or combinations of serotype capsids. The serotype capsids may include capsids from any identified AAV serotypes and variants thereof, for example, AAV1, AAV2, AAV2G9, AAV3, AAV4, AAV4-4, AAV5, AAV6, AAV7, AAV8, AAV9, AAV10, AAV11, AAV12 and AAVrh10.

[00435] In one embodiment, the AAV serotype may be or have a sequence as described in United States Publication No. US20030138772, herein incorporated by reference in its entirety, such as, but not limited to, AAV1 (SEQ ID NO. 6 and 64 of US20030138772), AAV2 (SEQ ID NO. 7 and 70 of US20030138772), AAV3 (SEQ ID NO. 8 and 71 of US20030138772), AAV4 (SEQ ID NO. 63 of US20030138772), AAV5 (SEQ ID NO. 114 of US20030138772), AAV6 (SEQ ID NO. 65 of US20030138772), AAV7 (SEQ ID NO. 1-3 of US20030138772), AAV8 (SEQ ID NO. 4 and 95 of US20030138772), AAV9 (SEQ ID NO. 5 and 100 of US20030138772), AAV10 (SEQ ID NO. 117 of US20030138772), AAV11 (SEQ ID NO. 118 of US20030138772), AAV12 (SEQ ID NO. 119 of US20030138772), AAVrh10 (amino acids 1 to 738 of SEQ ID NO. 81 of US20030138772) or variants thereof. Non-limiting examples of variants include SEQ ID NOs. 9, 27-45, 47-62, 66-69, 73-81, 84-94, 96, 97, 99, 101-113 of US20030138772, the contents of which are herein incorporated by reference in their entirety.

[00436] In one embodiment, the AAV serotype may have a sequence as described in Pulicherla et al. (*Molecular Therapy*, 2011, 19(6):1070-1078), U.S. Pat. NOs. 6,156,303; 7,198,951; U.S. Patent Publication NOs. US2015/0159173 and US2014/0359799; and International Patent Publication NOs. WO1998/011244, WO2005/033321 and WO2014/14422; the contents of each of which are incorporated herein by reference in their entirety.

[00437] AAV vectors include not only single stranded vectors but self-complementary AAV vectors (scAAVs). scAAV vectors contain DNA which anneals together to form double stranded vector genome. By skipping second strand synthesis, scAAVs allow for rapid expression in the cell.

[00438] The rAAV vectors may be manufactured by standard methods in the art such as by triple transfection, in sf9 insect cells or in suspension cell cultures of human cells such as HEK293 cells.

[00439] The biocircuits, biocircuit components, effector modules, SREs or payload constructs may be encoded in one or more viral genomes to be packaged in the AAV capsids taught herein.

[00440] Such vectors or viral genomes may also include, in addition to at least one or two ITRs (inverted terminal repeats), certain regulatory elements necessary for expression from the vector or viral genome. Such regulatory elements are well known in the art and include for example promoters, introns, spacers, stuffer sequences, and the like.

[00441] In some embodiments, more than one effector module or SRE (e.g. DD) may be encoded in a viral genome.

4. Oncolytic viral vector

[00442] In some embodiments, polynucleotides of present invention may be packaged into oncolytic viruses, such as vaccine viruses. Oncolytic vaccine viruses may include viral particles of a thymidine kinase (TK)-deficient, granulocyte macrophage (GM)-colony stimulating factor (CSF)-expressing, replication-competent vaccinia virus vector sufficient to induce oncolysis of cells in the tumor (e.g., US Pat. NOS. 9,226,977; the contents of which are incorporated by reference in their entirety).

5. Messenger RNA (mRNA)

[00443] In some embodiments, the effector modules of the invention may be designed as a messenger RNA (mRNA). As used herein, the term "messenger RNA" (mRNA) refers to any polynucleotide which encodes a polypeptide of interest and which is capable of being translated to produce the encoded polypeptide of interest *in vitro, in vivo, in situ* or *ex vivo*. Such mRNA molecules may have the structural components or features of any of those taught in International

Application number PCT/US2013/030062, the contents of which are incorporated herein by reference in its entirety.

[00444] Polynucleotides of the invention may also be designed as taught in, for example, Ribostem Limited in United Kingdom patent application serial number 0316089.2 filed on July 9, 2003 now abandoned, PCT application number PCT/GB2004/002981 filed on July 9, 2004 published as WO2005005622, United States patent application national phase entry serial number 10/563,897 filed on June 8, 2006 published as US20060247195 now abandoned, and European patent application national phase entry serial number EP2004743322 filed on July 9, 2004 published as EP1646714 now withdrawn; Novozymes, Inc. in PCT application number PCT/US2007/88060 filed on December 19, 2007 published as WO2008140615, United States patent application national phase entry serial number 12/520,072 filed on July 2, 2009 published as US20100028943 and European patent application national phase entry serial number EP2007874376 filed on July 7, 2009 published as EP2104739; University of Rochester in PCT application number PCT/US2006/46120 filed on December 4, 2006 published as WO2007064952 and United States patent application serial number 11/606,995 filed on December 1, 2006 published as US20070141030; BioNTech AG in European patent application serial number EP2007024312 filed December 14, 2007 now abandoned, PCT application number PCT/EP2008/01059 filed on December 12, 2008 published as WO2009077134, European patent application national phase entry serial number EP2008861423 filed on June 2, 2010 published as EP2240572, United States patent application national phase entry serial number 12/,735,060 filed November 24, 2010 published as US20110065103, German patent application serial number DE 10 2005 046 490 filed September 28, 2005, PCT application PCT/EP2006/0448 filed September 28, 2006 published as WO2007036366, national phase European patent EP1934345 published March, 21, 2012 and national phase US patent application serial number 11/992,638 filed August 14, 2009 published as 20100129877; Immune Disease Institute Inc. in United States patent application serial number 13/088,009 filed April 15, 2011 published as US20120046346 and PCT application PCT/US2011/32679 filed April 15, 2011 published as WO20110130624; Shire Human Genetic Therapeutics in United States patent application serial number 12/957,340 filed on November 20, 2010 published as US20110244026; Sequitur Inc. in PCT application PCT/US1998/019492 filed on September 18, 1998 published as WO1999014346; The Scripps Research Institute in PCT application number PCT/US2010/00567 filed on February 24, 2010 published as WO2010098861, and United States patent application national phase entry serial number 13/203,229 filed November 3, 2011 published as US20120053333; Ludwig-Maximillians University in PCT application number PCT/EP2010/004681 filed on July 30, 2010

published as WO2011012316; Cellscript Inc. in United States patent number 8,039,214 filed June 30, 2008 and granted October 18, 2011, United States patent application serial numbers 12/962,498 filed on December 7, 2010 published as US20110143436, 12/962,468 filed on December 7, 2010 published as US20110143397, 13/237,451 filed on September 20, 2011 published as US20120009649, and PCT applications PCT/US2010/59305 filed December 7, 2010 published as WO2011071931 and PCT/US2010/59317 filed on December 7, 2010 published as WO2011071936; The Trustees of the University of Pennsylvania in PCT application number PCT/US2006/32372 filed on August 21, 2006 published as WO2007024708, and United States patent application national phase entry serial number 11/990,646 filed on March 27, 2009 published as US20090286852; Curevac GMBH in German patent application serial numbers DE10 2001 027 283.9 filed June 5, 2001, DE10 2001 062 480.8 filed December 19, 2001, and DE 20 2006 051 516 filed October 31, 2006 all abandoned, European patent numbers EP1392341 granted March 30, 2005 and EP1458410 granted January 2, 2008, PCT application numbers PCT/EP2002/06180 filed June 5, 2002 published as WO2002098443, PCT/EP2002/14577 filed on December 19, 2002 published as WO2003051401, PCT/EP2007/09469 filed on December 31, 2007 published as WO2008052770, PCT/EP2008/03033 filed on April 16, 2008 published as WO2009127230, PCT/EP2006/004784 filed on May 19, 2005 published as WO2006122828, PCT/EP2008/00081 filed on January 9, 2007 published as WO2008083949, and United States patent application serial numbers 10/729,830 filed on December 5, 2003 published as US20050032730, 10/870,110 filed on June 18, 2004 published as US20050059624, 11/914,945 filed on July 7, 2008 published as US20080267873, 12/446,912 filed on October 27, 2009 published as US2010047261 now abandoned, 12/522,214 filed on January 4, 2010 published as US20100189729, 12/787,566 filed on May 26, 2010 published as US20110077287, 12/787,755 filed on May 26, 2010 published as US20100239608, 13/185,119 filed on July 18, 2011 published as US20110269950, and 13/106,548 filed on May 12, 2011 published as US20110311472 all of which are herein incorporated by reference in their entirety.

[00445] In some embodiments, the effector modules may be designed as self-amplifying RNA. "Self-amplifying RNA" as used herein refers to RNA molecules that can replicate in the host resulting in the increase in the amount of the RNA and the protein encoded by the RNA. Such self-amplifying RNA may have structural features or components of any of those taught in International Patent Application Publication No. WO2011005799 (the contents of which are incorporated herein by reference in their entirety).

VI. DOSING, DELIVERY AND ADMINISTRATIONS

[00446] The compositions of the invention may be delivered to a cell or a subject through one or more routes and modalities. The viral vectors containing one or more effector modules, SREs, immunotherapeutic agents and other components described herein may be used to deliver them to a cell and/or a subject. Other modalities may also be used such as mRNAs, plasmids, and as recombinant proteins.

1. Delivery to cells

[00447] In another aspect of the invention, polynucleotides encoding biocircuits, effector modules, SREs (e.g., DDs), payloads of interest (immunotherapeutic agents) and compositions of the invention and vectors comprising said polynucleotides may be introduced into cells such as immune effector cells.

[00448] In one aspect of the invention, polynucleotides encoding biocircuits, effector modules, SREs (e.g., DDs), payloads of interest (immunotherapeutic agents) and compositions of the invention, may be packaged into viral vectors or integrated into viral genomes allowing transient or stable expression of the polynucleotides. Preferable viral vectors are retroviral vectors including lentiviral vectors. In order to construct a retroviral vector, a polynucleotide molecule encoding a biocircuit, an effector module, a DD or a payload of interest (i.e. an immunotherapeutic agent) is inserted into the viral genome in the place of certain viral sequences to produce a virus that is replication-defective. The recombinant viral vector is then introduced into a packaging cell line containing the gag, pol, and env genes, but without the LTR and packaging components. The recombinant retroviral particles are secreted into the culture media, then collected, optionally concentrated, and used for gene transfer. Lentiviral vectors are especially preferred as they are capable of infecting both dividing and non-dividing cells.

[00449] Vectors may also be transferred to cells by non-viral methods by physical methods such as needles, electroporation, sonoporation, hyrdoporation; chemical carriers such as inorganic particles (e.g. calcium phosphate, silica, gold) and/or chemical methods. In some embodiments, synthetic or natural biodegradable agents may be used for delivery such as cationic lipids, lipid nano emulsions, nanoparticles, peptide based vectors, or polymer based vectors.

[00450] In some embodiments, the polypeptides of the invention may be delivered to the cell directly. In one embodiment, the polypeptides of the invention may be delivered using synthetic peptides comprising an endosomal leakage domain (ELD) fused to a cell penetration domain (CLD). The polypeptides of the invention are co introduced into the cell with the ELD-CLD-synthetic peptide. ELDs facilitate the escape of proteins that are trapped in the endosome, into the cytosol. Such domains are derived proteins of microbial and viral origin and have been

described in the art. CPDs allow the transport of proteins across the plasma membrane and have also been described in the art. The ELD-CLD fusion proteins synergistically increase the transduction efficiency when compared to the co-transduction with either domain alone. In some embodiments, a histidine rich domain may optionally be added to the shuttle construct as an additional method of allowing the escape of the cargo from the endosome into the cytosol. The shuttle may also include a cysteine residue at the N or C terminus to generate multimers of the fusion peptide. Multimers of the ELD-CLD fusion peptides generated by the addition of cysteine residue to the terminus of the peptide show even greater transduction efficiency when compared to the single fusion peptide constructs. The polypeptides of the invention may also be appended to appropriate localization signals to direct the cargo to the appropriate sub-cellular location e.g. nucleus. In some embodiments any of the ELDs, CLDs or the fusion ELD-CLD synthetic peptides taught in the International Patent Publication, WO2016161516 and WO2017175072 may be useful in the present invention (the contents of each of which are herein incorporated by reference in their entirety).

2. Dosing

[00451] The present invention provides methods comprising administering any one or more compositions for immunotherapy to a subject in need thereof. These may be administered to a subject using any amount and any route of administration effective for preventing or treating a clinical condition such as cancer, infection diseases and other immunodeficient diseases.

[00452] Compositions in accordance with the invention are typically formulated in dosage unit form for ease of administration and uniformity of dosage. It will be understood, however, that the total daily usage of the compositions of the present invention may be decided by the attending physician within the scope of sound medical judgment. The specific therapeutically effective, or prophylactically effective dose level for any particular patient will depend upon a variety of factors including the disorder being treated and the severity of the disorder; the activity of the specific compound employed; the specific composition employed; the age, body weight, general health, sex and diet of the patient; the time of administration, route of administration, previous or concurrent therapeutic interventions and rate of excretion of the specific compound employed; the duration of the treatment; drugs used in combination or coincidental with the specific compound employed; and like factors well known in the medical arts.

[00453] Compositions of the invention may be used in varying doses to avoid T cell energy, prevent cytokine release syndrome and minimize toxicity associated with immunotherapy. For example, low doses of the compositions of the present invention may be used to initially treat patients with high tumor burden, while patients with low tumor burden may be treated with high

and repeated doses of the compositions of the invention to ensure recognition of a minimal tumor antigen load. In another instance, the compositions of the present invention may be delivered in a pulsatile fashion to reduce tonic T cell signaling and enhance persistence *in vivo*. In some aspects, toxicity may be minimized by initially using low doses of the compositions of the invention, prior to administering high doses. Dosing may be modified if serum markers such as ferritin, serum C-reactive protein, IL-6, IFN-γ, and TNF-α are elevated.

3. Administration

[00454] In some embodiments, the compositions for immunotherapy may be administered to cells *ex vivo* and subsequently administered to the subject. Immune cells can be isolated and expanded *ex vivo* using a variety of methods known in the art. For example, methods of isolating cytotoxic T cells are described in U.S. Pat. NOs. 6,805,861 and 6,531,451; the contents of each of which are incorporated herein by reference in their entirety. Isolation of NK cells are described in U.S. Pat. NOs. 7,435,596; the contents of which are incorporated by reference herein in its entirety.

[00455] In some embodiments, compositions of the present invention, may be administered by any of the methods of administration taught in the copending commonly owned U.S. Provisional Patent Application No. 62/320,864, filed on 4/11/2016, or in US Provisional Application No. 62/466,596 filed March 3, 2017 and the International Publication WO2017/180587, the contents of which are incorporated herein by reference in their entirety.

[00456] In some embodiments, depending upon the nature of the cells, the cells may be introduced into a host organism e.g. a mammal, in a wide variety of ways including by injection, transfusion, infusion, local instillation or implantation. In some aspects, the cells of the invention may be introduced at the site of the tumor. The number of cells that are employed will depend upon a number of circumstances, the purpose for the introduction, the lifetime of the cells, the protocol to be used, for example, the number of administrations, the ability of the cells to multiply, or the like. The cells may be in a physiologically-acceptable medium.

[00457] In some embodiments, the cells of the invention may be administrated in multiple doses to subjects having a disease or condition. The administrations generally effect an improvement in one or more symptoms of cancer or a clinical condition and/or treat or prevent cancer or clinical condition or symptom thereof.

[00458] In some embodiments, the compositions for immunotherapy may be administered *in vivo*. In some embodiments, polypeptides of the present invention comprising biocircuits, effector molecules, SREs, payloads of interest (immunotherapeutic agents) and compositions of the invention may be delivered in vivo to the subject. In vivo delivery of immunotherapeutic

agents is well described in the art. For example, methods of delivery of cytokines are described in the E.P. Pat. NOs. EP0930892 A1, the contents of which are incorporated herein by reference.

[00459] In one embodiment, the payloads of the present invention may be administered in conjunction with inhibitors of SHP-1 and/or SHP-2. The tyrosine-protein phosphatase SHP1 (also known as PTPN6) and SHP2 (also known as PTPN11) are involved in the Programmed Cell Death (PD1) inhibitory signaling pathway. The intracellular domain of PD1 contains an immunoreceptor tyrosine-based inhibitory motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM). ITSM has been shown to recruit SHP-1 and 2. This generates negative costimulatory micro clusters that induce the dephosphorylation of the proximal TCR signaling molecules, thereby resulting in suppression of T cell activation, which can lead to T cell exhaustion. In one embodiment, inhibitors of SHP-1 and 2 may include expressing dominant negative versions of the proteins in T cells, TILs or other cell types to relieve exhaustion. Such mutants can bind to the endogenous, catalytically active proteins, and inhibit their function. In one embodiment, the dominant negative mutant of SHP-1 and/ or SHP-2 lack the phosphatase domain required for catalytic activity. In some embodiments, any of the dominant negative SHP-1 mutants taught Bergeron S et al. (2011). Endocrinology. 2011 Dec;152(12):4581-8.; Dustin JB et al. (1999) J Immunol. Mar 1;162(5):2717-24.; Berchtold S (1998) Mol Endocrinol. Apr;12(4):556-67 and Schram et al. (2012) Am J Physiol Heart Circ Physiol. 1;302(1):H231-43.; may be useful in the invention (the contents of each of which are incorporated by reference in their entirety).

Routes of delivery

[00460] The pharmaceutical compositions, biocircuits, biocircuit components, effector modules including their SREs (e.g., DDs), payloads (i.e. immunotherapeutic agents), vectors and cells of the present invention may be administered by any route to achieve a therapeutically effective outcome.

[00461] These include, but are not limited to enteral (into the intestine), gastroenteral, epidural (into the dura matter), oral (by way of the mouth), transdermal, peridural, intracerebral (into the cerebrum), intracerebroventricular (into the cerebral ventricles), epicutaneous (application onto the skin), intradermal, (into the skin itself), subcutaneous (under the skin), nasal administration (through the nose), intravenous (into a vein), intravenous bolus, intravenous drip, intra-arterial (into an artery), intramuscular (into a muscle), intracranial (into the heart), intraosseous infusion (into the bone marrow), intrathecal (into the spinal canal), intraperitoneal, (infusion or injection into the peritoneum), intrasinal infusion, intravitreal, (through the eye), intravenous injection

(into a pathologic cavity) intracavitary (into the base of the penis), intravaginal administration, intrauterine, extra-amniotic administration, transdermal (diffusion through the intact skin for systemic distribution), transmucosal (diffusion through a mucous membrane), transvaginal, insufflation (snorting), sublingual, sublabial, enema, eye drops (onto the conjunctiva), in ear drops, auricular (in or by way of the ear), buccal (directed toward the cheek), conjunctival, cutaneous, dental (to a tooth or teeth), electro-osmosis, endocervical, endosinusial, endotracheal, extracorporeal, hemodialysis, infiltration, interstitial, intra-abdominal, intra-amniotic, intra-articular, intrabiliary, intrabronchial, intrabursal, intracartilaginous (within a cartilage), intracaudal (within the cauda equine), intracisternal (within the cisterna magna cerebellomedularis), intracorneal (within the cornea), dental intracornal, intracoronary (within the coronary arteries), intracorporus cavernosum (within the dilatable spaces of the corporus cavernosa of the penis), intradiscal (within a disc), intraductal (within a duct of a gland), intraduodenal (within the duodenum), intradural (within or beneath the dura), intraepidermal (to the epidermis), intraesophageal (to the esophagus), intragastric (within the stomach), intragingival (within the gingivae), intraileal (within the distal portion of the small intestine), intralesional (within or introduced directly to a localized lesion), intraluminal (within a lumen of a tube), intralymphatic (within the lymph), intramedullary (within the marrow cavity of a bone), intrameningeal (within the meninges), intramyocardial (within the myocardium), intraocular (within the eye), intraovarian (within the ovary), intrapericardial (within the pericardium), intrapleural (within the pleura), intraprostatic (within the prostate gland), intrapulmonary (within the lungs or its bronchi), intrasinal (within the nasal or periorbital sinuses), intraspinal (within the vertebral column), intrasynovial (within the synovial cavity of a joint), intratendinous (within a tendon), intratesticular (within the testicle), intrathecal (within the cerebrospinal fluid at any level of the cerebrospinal axis), intrathoracic (within the thorax), intratubular (within the tubules of an organ), intratumor (within a tumor), intratympanic (within the aurus media), intravascular (within a vessel or vessels), intraventricular (within a ventricle), iontophoresis (by means of electric current where ions of soluble salts migrate into the tissues of the body), irrigation (to bathe or flush open wounds or body cavities), laryngeal (directly upon the larynx), nasogastric (through the nose and into the stomach), occlusive dressing technique (topical route administration which is then covered by a dressing which occludes the area), ophthalmic (to the external eye), oropharyngeal (directly to the mouth and pharynx), parenteral, percutaneous, periarticular, peridural, perineural, periodontal, rectal, respiratory (within the respiratory tract by inhaling orally or nasally for local or systemic effect), retrobulbar (behind the pons or behind the eyeball), intramyocardial (entering the myocardium), soft tissue, subarachnoid, subconjunctival,

submucosal, topical, transplacental (through or across the placenta), transtracheal (through the wall of the trachea), transtympanic (across or through the tympanic cavity), ureteral (to the ureter), urethral (to the urethra), vaginal, caudal block, diagnostic, nerve block, biliary perfusion, cardiac perfusion, photopheresis or spinal.

VII. DEFINITIONS

[00462] At various places in the present specification, features or functions of the compositions of the present disclosure are disclosed in groups or in ranges. It is specifically intended that the present disclosure include each and every individual sub combination of the members of such groups and ranges. The following is a non-limiting list of term definitions.

[00463] *Activity*: As used herein, the term "activity" refers to the condition in which things are happening or being done. Compositions of the invention may have activity and this activity may involve one or more biological events. In some embodiments, biological events may include cell signaling events. In some embodiments, biological events may include cell signaling events associated protein interactions with one or more corresponding proteins, receptors, small molecules or any of the biocircuit components described herein.

[00464] *Adoptive cell therapy (ACT)*: The terms "Adoptive cell therapy" or "Adoptive cell transfer", as used herein, refer to a cell therapy involving in the transfer of cells into a patient, wherein cells may have originated from the patient, or from another individual, and are engineered (altered) before being transferred back into the patient. The therapeutic cells may be derived from the immune system, such as Immune effector cells: CD4+ T cell; CD8+ T cell, Natural Killer cell (NK cell); and B cells and tumor infiltrating lymphocytes (TILs) derived from the resected tumors. Most commonly transferred cells are autologous anti-tumor T cells after *ex vivo* expansion or manipulation. For example, autologous peripheral blood lymphocytes can be genetically engineered to recognize specific tumor antigens by expressing T-cell receptors (TCR) or chimeric antigen receptor (CAR).

[00465] *Agent*: As used herein, the term "agent" refers to a biological, pharmaceutical, or chemical compound. Non-limiting examples include simple or complex organic or inorganic molecule, a peptide, a protein, an oligonucleotide, an antibody, an antibody derivative, antibody fragment, a receptor, and soluble factor.

[00466] *Agonist*: the term "agonist" as used herein, refers to a compound that, in combination with a receptor, can produce a cellular response. An agonist may be a ligand that directly binds to the receptor. Alternatively, an agonist may combine with a receptor indirectly by, for example, (a) forming a complex with another molecule that directly binds to the receptor, or (b) otherwise resulting in the modification of another compound so that the other compound directly binds to

the receptor. An agonist may be referred to as an agonist of a particular receptor or family of receptors, e.g., agonist of a co-stimulatory receptor.

[00467] *Antagonist*: the term "antagonist" as used herein refers to any agent that inhibits or reduces the biological activity of the target(s) it binds.

[00468] *Antigen*: the term "antigen" as used herein is defined as a molecule that provokes an immune response when it is introduced into a subject or produced by a subject such as tumor antigens which arise by the cancer development itself. This immune response may involve either antibody production, or the activation of specific immunologically-competent cells such as cytotoxic T lymphocytes and T helper cells, or both. An antigen can be derived from organisms, subunits of proteins/antigens, killed or inactivated whole cells or lysates. In the context of the invention, the terms "antigens of interest" or "desired antigens" refers to those proteins and/or other biomolecules provided herein that are immunospecifically bound or interact with antibodies of the present invention and/or fragments, mutants, variants, and/or alterations thereof described herein. In some embodiments, antigens of interest may comprise any of the polypeptides or payloads or proteins described herein, or fragments or portions thereof.

[00469] *Approximately*: As used herein, the term "approximately" or "about," as applied to one or more values of interest, refers to a value that is similar to a stated reference value. In certain embodiments, the term "approximately" or "about" refers to a range of values that fall within 25, 20, 19, 18, 17, 16, 15, 14, 13, 12, 11, 10, 9, 8, 7, 6, 5, 4, 3, 2, 1, or less in either direction (greater than or less than) of the stated reference value unless otherwise stated or otherwise evident from the context (except where such number would exceed 100 of a possible value).

[00470] *Associated with*: As used herein, the terms "associated with," "conjugated," "linked," "attached," and "tethered," when used with respect to two or more moieties, mean that the moieties are physically associated or connected with one another, either directly or via one or more additional moieties that serve as linking agents, to form a structure that is sufficiently stable so that the moieties remain physically associated under the conditions in which the structure is used, e.g., physiological conditions. An "association" need not be strictly through direct covalent chemical bonding. It may also suggest ionic or hydrogen bonding or a hybridization based connectivity sufficiently stable such that the "associated" entities remain physically associated.

[00471] *Autologous*: the term "autologous" as used herein is meant to refer to any material derived from the same individual to which it is later to be re-introduced into the individual.

[00472] *Barcode*: the term "barcode" as used herein refers to polynucleotide or amino acid sequence that distinguishes one polynucleotide or amino acid from another.

[00473] *Cancer*: the term "cancer" as used herein refers a broad group of various diseases characterized by the uncontrolled growth of abnormal cells in the body. Unregulated cell division and growth results in the formation of malignant tumors that invade neighboring tissues ultimately metastasize to distant parts of the body through the lymphatic system or bloodstream.

[00474] *Co-stimulatory molecule*: As used herein, in accordance with its meaning in immune T cell activation, refers to a group of immune cell surface receptor/ligands which engage between T cells and APCs and generate a stimulatory signal in T cells which combines with the stimulatory signal in T cells that results from T cell receptor (TCR) recognition of antigen/MHC complex (pMHC) on APCs

[00475] *Cytokines*: the term "cytokines", as used herein, refers to a family of small soluble factors with pleiotropic functions that are produced by many cell types that can influence and regulate the function of the immune system.

[00476] *Delivery*: the term "delivery" as used herein refers to the act or manner of delivering a compound, substance, entity, moiety, cargo or payload. A "delivery agent" refers to any agent which facilitates, at least in part, the in vivo delivery of one or more substances (including, but not limited to a compound and/or compositions of the present invention) to a cell, subject or other biological system cells.

[00477] *Destabilized*: As used herein, the term "destable," "destabilize," "destabilizing region" or "destabilizing domain" means a region or molecule that is less stable than a starting, reference, wild-type or native form of the same region or molecule.

[00478] *Engineered*: As used herein, embodiments of the invention are "engineered" when they are designed to have a feature or property, whether structural or chemical, that varies from a starting point, wild type or native molecule.

[00479] *Expression*: As used herein, "expression" of a nucleic acid sequence refers to one or more of the following events: (1) production of an RNA template from a DNA sequence (e.g., by transcription); (2) processing of an RNA transcript (e.g., by splicing, editing, 5′ cap formation, and/or 3′ end processing); (3) translation of an RNA into a polypeptide or protein; (4) folding of a polypeptide or protein; and (5) post-translational modification of a polypeptide or protein.

[00480] *Feature*: As used herein, a "feature" refers to a characteristic, a property, or a distinctive element.

[00481] *Formulation*: As used herein, a "formulation" includes at least a compound and/or composition of the present invention and a delivery agent.

[00482] *Fragment*: A "fragment," as used herein, refers to a portion. For example, fragments of proteins may comprise polypeptides obtained by digesting full-length protein. In some

embodiments, a fragment of a protein includes at least 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90, 95, 100, 150, 200, 250 or more amino acids. In some embodiments, fragments of an antibody include portions of an antibody.

[00483] *Functional*: As used herein, a "functional" biological molecule is a biological entity with a structure and in a form in which it exhibits a property and/or activity by which it is characterized.

[00484] *Immune cells*: the term "an immune cell", as used herein, refers to any cell of the immune system that originates from a hematopoietic stem cell in the bone marrow, which gives rise to two major lineages, a myeloid progenitor cell (which give rise to myeloid cells such as monocytes, macrophages, dendritic cells, megakaryocytes and granulocytes) and a lymphoid progenitor cell (which give rise to lymphoid cells such as T cells, B cells and natural killer (NK) cells). Exemplary immune system cells include a CD4+ T cell, a CD8+ T cell, a CD4− CD8− double negative T cell, a T γδ cell, a Tαβ cell, a regulatory T cell, a natural killer cell, and a dendritic cell. Macrophages and dendritic cells may be referred to as "antigen presenting cells" or "APCs," which are specialized cells that can activate T cells when a major histocompatibility complex (MHC) receptor on the surface of the APC complexed with a peptide interacts with a TCR on the surface of a T cell.

[00485] *Immunotherapy*: the term "immunotherapy" as used herein, refers to a type of treatment of a disease by the induction or restoration of the reactivity of the immune system towards the disease.

[00486] *Immunotherapeutic agent:* the term "immunotherapeutic agent" as used herein, refers to the treatment of disease by induction or restoration of the reactivity of the immune system towards the disease with a biological, pharmaceutical, or chemical compound.

[00487] *In vitro:* As used herein, the term "in vitro" refers to events that occur in an artificial environment, e.g., in a test tube or reaction vessel, in cell culture, in a Petri dish, etc., rather than within an organism (e.g., animal, plant, or microbe).

[00488] *In vivo*: As used herein, the term "in vivo" refers to events that occur within an organism (e.g., animal, plant, or microbe or cell or tissue thereof).

[00489] *Linker*: As used herein, a linker refers to a moiety that connects two or more domains, moieties or entities. In one embodiment, a linker may comprise 10 or more atoms. In a further embodiment, a linker may comprise a group of atoms, e.g., 10-1,000 atoms, and can be comprised of the atoms or groups such as, but not limited to, carbon, amino, alkylamino, oxygen, sulfur, sulfoxide, sulfonyl, carbonyl, and imine. In some embodiments, a linker may comprise

one or more nucleic acids comprising one or more nucleotides. In some embodiments, the linker may comprise an amino acid, peptide, polypeptide or protein. In some embodiments, a moiety bound by a linker may include, but is not limited to an atom, a chemical group, a nucleoside, a nucleotide, a nucleobase, a sugar, a nucleic acid, an amino acid, a peptide, a polypeptide, a protein, a protein complex, a payload (e.g., a therapeutic agent). or a marker (including, but not limited to a chemical, fluorescent, radioactive or bioluminescent marker). The linker can be used for any useful purpose, such as to form multimers or conjugates, as well as to administer a payload, as described herein. Examples of chemical groups that can be incorporated into the linker include, but are not limited to, alkyl, alkenyl, alkynyl, amido, amino, ether, thioether, ester, alkylene, heteroalkylene, aryl, or heterocyclyl, each of which can be optionally substituted, as described herein. Examples of linkers include, but are not limited to, unsaturated alkanes, polyethylene glycols (e.g., ethylene or propylene glycol monomeric units, e.g., diethylene glycol, dipropylene glycol, triethylene glycol, tripropylene glycol, tetraethylene glycol, or tetraethylene glycol), and dextran polymers, Other examples include, but are not limited to, cleavable moieties within the linker, such as, for example, a disulfide bond (-S-S-) or an azo bond (-N=N-), which can be cleaved using a reducing agent or photolysis. Non-limiting examples of a selectively cleavable bonds include an amido bond which may be cleaved for example by the use of tris(2-carboxyethyl) phosphine (TCEP), or other reducing agents, and/or photolysis, as well as an ester bond which may be cleaved for example by acidic or basic hydrolysis.

[00490] *Checkpoint/factor*: As used herein, a checkpoint factor is any moiety or molecule whose function acts at the junction of a process. For example, a checkpoint protein, ligand or receptor may function to stall or accelerate the cell cycle.

[00491] *Metabolite:* Metabolites are the intermediate products of metabolic reactions catalyzed by enzymes that naturally occur within cells. This term is usually used to describe small molecules, fragments of larger biomolecules or processed products.

[00492] *Modified*: As used herein, the term "modified" refers to a changed state or structure of a molecule or entity as compared with a parent or reference molecule or entity. Molecules may be modified in many ways including chemically, structurally, and functionally. In some embodiments, compounds and/or compositions of the present invention are modified by the introduction of non-natural amino acids.

[00493] *Mutation*: As used herein, the term "mutation" refers to a change and/or alteration. In some embodiments, mutations may be changes and/or alterations to proteins (including peptides and polypeptides) and/or nucleic acids (including polynucleic acids). In some embodiments, mutations comprise changes and/or alterations to a protein and/or nucleic acid sequence. Such

changes and/or alterations may comprise the addition, substitution and or deletion of one or more amino acids (in the case of proteins and/or peptides) and/or nucleotides (in the case of nucleic acids and or polynucleic acids e.g., polynucleotides). In some embodiments, wherein mutations comprise the addition and/or substitution of amino acids and/or nucleotides, such additions and/or substitutions may comprise 1 or more amino acid and/or nucleotide residues and may include modified amino acids and/or nucleotides. The resulting construct, molecule or sequence of a mutation, change or alteration may be referred to herein as a mutant.

[00494] *Neoantigen*: the term "neoantigen", as used herein, refers to a tumor antigen that is present in tumor cells but not normal cells and do not induce deletion of their cognate antigen specific T cells in thymus (i.e., central tolerance). These tumor neoantigens may provide a "foreign" signal, like pathogens, to induce an effective immune response needed for cancer immunotherapy. A neoantigen may be restricted to a specific tumor. A neoantigen be a peptide/protein with a missense mutation (missense neoantigen), or a new peptide with long, completely novel stretches of amino acids from novel open reading frames (neoORFs). The neoORFs can be generated in some tumors by out-of-frame insertions or deletions (due to defects in DNA mismatch repair causing microsatellite instability), gene-fusion, read-through mutations in stop codons, or translation of improperly spliced RNA (e.g., Saeterdal et al., *Proc Natl Acad Sci USA*, 2001, 98: 13255-13260).

[00495] *Off-target*: As used herein, "off target" refers to any unintended effect on any one or more target, gene, cellular transcript, cell, and/or tissue.

[00496] *Operably linked*: As used herein, the phrase "operably linked" refers to a functional connection between two or more molecules, constructs, transcripts, entities, moieties or the like.

[00497] *Payload or payload of interest (POI)*: the terms "payload" and "payload of interest (POI)", as used herein, are used interchangeable. A payload of interest (POI) refers to any protein or compound whose function is to be altered. In the context of the present invention, the POI is a component in the immune system, including both innate and adaptive immune systems. Payloads of interest may be a protein, a fusion construct encoding a fusion protein, or non-coding gene, or variant and fragment thereof. Payload of interest may, when amino acid based, may be referred to as a protein of interest.

[00498] *Pharmaceutically acceptable excipients*: the term "pharmaceutically acceptable excipient," as used herein, refers to any ingredient other than active agents (e.g., as described herein) present in pharmaceutical compositions and having the properties of being substantially nontoxic and non-inflammatory in subjects. In some embodiments, pharmaceutically acceptable excipients are vehicles capable of suspending and/or dissolving active agents. Excipients may

include, for example: antiadherents, antioxidants, binders, coatings, compression aids, disintegrants, dyes (colors), emollients, emulsifiers, fillers (diluents), film formers or coatings, flavors, fragrances, glidants (flow enhancers), lubricants, preservatives, printing inks, sorbents, suspending or dispersing agents, sweeteners, and waters of hydration. Exemplary excipients include, but are not limited to: butylated hydroxytoluene (BHT), calcium carbonate, calcium phosphate (dibasic), calcium stearate, croscarmellose, crosslinked polyvinyl pyrrolidone, citric acid, crospovidone, cysteine, ethylcellulose, gelatin, hydroxypropyl cellulose, hydroxypropyl methylcellulose, lactose, magnesium stearate, maltitol, mannitol, methionine, methylcellulose, methyl paraben, microcrystalline cellulose, polyethylene glycol, polyvinyl pyrrolidone, povidone, pregelatinized starch, propyl paraben, retinyl palmitate, shellac, silicon dioxide, sodium carboxymethyl cellulose, sodium citrate, sodium starch glycolate, sorbitol, starch (corn), stearic acid, sucrose, talc, titanium dioxide, vitamin A, vitamin E, vitamin C, and xylitol.

[00499] *Pharmaceutically acceptable salts*: Pharmaceutically acceptable salts of the compounds described herein are forms of the disclosed compounds wherein the acid or base moiety is in its salt form (e.g., as generated by reacting a free base group with a suitable organic acid). Examples of pharmaceutically acceptable salts include, but are not limited to, mineral or organic acid salts of basic residues such as amines; alkali or organic salts of acidic residues such as carboxylic acids; and the like. Representative acid addition salts include acetate, adipate, alginate, ascorbate, aspartate, benzenesulfonate, benzoate, bisulfate, borate, butyrate, camphorate, camphorsulfonate, citrate, cyclopentanepropionate, digluconate, dodecylsulfate, ethanesulfonate, fumarate, glucoheptonate, glycerophosphate, hemisulfate, heptonate, hexanoate, hydrobromide, hydrochloride, hydroiodide, 2-hydroxy-ethanesulfonate, lactobionate, lactate, laurate, lauryl sulfate, malate, maleate, malonate, methanesulfonate, 2-naphthalenesulfonate, nicotinate, nitrate, oleate, oxalate, palmitate, pamoate, pectinate, persulfate, 3-phenylpropionate, phosphate, picrate, pivalate, propionate, stearate, succinate, sulfate, tartrate, thiocyanate, toluenesulfonate, undecanoate, valerate salts, and the like. Representative alkali or alkaline earth metal salts include sodium, lithium, potassium, calcium, magnesium, and the like, as well as nontoxic ammonium, quaternary ammonium, and amine cations, including, but not limited to ammonium, tetramethylammonium, tetraethylammonium, methylamine, dimethylamine, trimethylamine, triethylamine, ethylamine, and the like. Pharmaceutically acceptable salts include the conventional non-toxic salts, for example, from non-toxic inorganic or organic acids. In some embodiments, a pharmaceutically acceptable salt is prepared from a parent compound which contains a basic or acidic moiety by conventional chemical methods. Generally, such salts can be prepared by reacting the free acid or base forms of these compounds with a stoichiometric

amount of the appropriate base or acid in water or in an organic solvent, or in a mixture of the two; generally, nonaqueous media like ether, ethyl acetate, ethanol, isopropanol, or acetonitrile are preferred. Lists of suitable salts are found in Remington's Pharmaceutical Sciences, 17th ed., Mack Publishing Company, Easton, Pa., 1985, p. 1418, Pharmaceutical Salts: Properties, Selection, and Use, P.H. Stahl and C.G. Wermuth (eds.), Wiley-VCH, 2008, and Berge et al., Journal of Pharmaceutical Science, 66, 1-19 (1977), each of which is incorporated herein by reference in its entirety. Pharmaceutically acceptable solvate: The term "pharmaceutically acceptable solvate," as used herein, refers to a crystalline form of a compound wherein molecules of a suitable solvent are incorporated in the crystal lattice. For example, solvates may be prepared by crystallization, recrystallization, or precipitation from a solution that includes organic solvents, water, or a mixture thereof. Examples of suitable solvents are ethanol, water (for example, mono-, di-, and tri-hydrates), N-methylpyrrolidinone (NMP), dimethyl sulfoxide (DMSO), N, N'-dimethylformamide (DMF), N, N'-dimethylacetamide (DMAC), 1,3-dimethyl-2-imidazolidinone (DMEU), 1,3-dimethyl-3,4,5,6-tetrahydro-2-(1H)-pyrimidinone (DMPU), acetonitrile (ACN), propylene glycol, ethyl acetate, benzyl alcohol, 2-pyrrolidone, benzyl benzoate, and the like. When water is the solvent, the solvate is referred to as a "hydrate." In some embodiments, the solvent incorporated into a solvate is of a type or at a level that is physiologically tolerable to an organism to which the solvate is administered (e.g., in a unit dosage form of a pharmaceutical composition).

[00500] *Stable*: As used herein "stable" refers to a compound or entity that is sufficiently robust to survive isolation to a useful degree of purity from a reaction mixture, and preferably capable of formulation into an efficacious therapeutic agent.

[00501] *Stabilized*: As used herein, the term "stabilize", "stabilized," "stabilized region" means to make or become stable. In some embodiments, stability is measured relative to an absolute value. In some embodiments, stability is measured relative to a secondary status or state or to a reference compound or entity.

[00502] *Standard CAR*: As used herein, the term "standard CAR" refers to the standard design of a chimeric antigen receptor. The components of a CAR fusion protein including the extracellular scFv fragment, transmembrane domain and one or more intracellular domains are linearly constructed as a single fusion protein.

[00503] *Stimulus response element (SRE)*: the term "stimulus response element (SRE), as used herein, is a component of an effector module which is joined, attached, linked to or associated with one or more payloads of the effector module and in some instances, is responsible for the responsive nature of the effector module to one or more stimuli. As used herein, the "responsive"

nature of an SRE to a stimulus may be characterized by a covalent or non-covalent interaction, a direct or indirect association or a structural or chemical reaction to the stimulus. Further, the response of any SRE to a stimulus may be a matter of degree or kind. The response may be a partial response. The response may be a reversible response. The response may ultimately lead to a regulated signal or output. Such output signal may be of a relative nature to the stimulus, e.g., producing a modulatory effect of between 1 and 100 or a factored increase or decrease such as 2-fold, 3-fold, 4-fold, 5-fold, 10-fold or more. One non-limiting example of an SRE is a destabilizing domain (DD).

[00504] *Subject*: As used herein, the term "subject" or "patient" refers to any organism to which a composition in accordance with the invention may be administered, e.g., for experimental, diagnostic, prophylactic, and/or therapeutic purposes. Typical subjects include animals (e.g., mammals such as mice, rats, rabbits, non-human primates, and humans) and/or plants.

[00505] *T cell*: A T cell is an immune cell that produces T cell receptors (TCRs). T cells can be naïve (not exposed to antigen; increased expression of CD62L, CCR7, CD28, CD3, CD127, and CD45RA, and decreased expression of CD45RO as compared to T_{CM}), memory T cells (T_M) (antigen-experienced and long-lived), and effector cells (antigen-experienced, cytotoxic). T_M can be further divided into subsets of central memory T cells (T_{CM}, increased expression of CD62L, CCR7, CD28, CD127, CD45RO, and CD95, and decreased expression of CD54RA as compared to naïve T cell and effector memory T cells (T_{EM}, decreased expression of CD62L, CCR7, CD28, CD45RA, and increased expression of CD127 as compared to naïve T cells or T_{CM}). Effector T cells (T_E) refers to antigen-experienced CD8+ cytotoxic T lymphocytes that have decreased expression of CD62L, CCR7, CD28, and are positive for granzyme and perforin as compared to T_{CM}. Other exemplary T cells include regulatory T cells, such as CD4+ CD25+ (Foxp3+) regulatory T cells and Treg17 cells, as well as Tr1, Th3, CD8+CD28−, and Qa-1 restricted T cells.

[00506] *T cell receptor*: T cell receptor (TCR) refers to an immunoglobulin superfamily member having a variable antigen binding domain, a constant domain, a transmembrane region, and a short cytoplasmic tail, which is capable of specifically binding to an antigen peptide bound to a MHC receptor. A TCR can be found on the surface of a cell or in soluble form and generally is comprised of a heterodimer having α and β chains (also known as TCRα and TCRβ, respectively), or γ and δ chains (also known as TCRγ and TCRδ, respectively). The extracellular portion of TCR chains (e.g., α-chain, β-chain) contains two immunoglobulin domains, a variable domain (e.g., α-chain variable domain or V_α, β-chain variable domain or V_β) at the N-terminus, and one constant domain (e.g., α-chain constant domain or C_α and β-chain constant domain or

C_{β},) adjacent to the cell membrane. Similar to immunoglobulin, the variable domains contain complementary determining regions (CDRs) separated by framework regions (FRs). A TCR is usually associated with the CD3 complex to form a TCR complex. As used herein, the term "TCR complex" refers to a complex formed by the association of CD3 with TCR. For example, a TCR complex can be composed of a CD3γ chain, a CD3δ chain, two CD3ε chains, a homodimer of CD3ζ chains, a TCRα chain, and a TCRβ chain. Alternatively, a TCR complex can be composed of a CD3γ chain, a CD3δ chain, two CD3ε chains, a homodimer of CD3ζ chains, a TCRγ chain, and a TCRδ chain. A "component of a TCR complex," as used herein, refers to a TCR chain (i.e., TCRα, TCRβ, TCRγ or TCRδ), a CD3 chain (i.e., CD3γ, CD3δ, CD3ε or CD3ζ), or a complex formed by two or more TCR chains or CD3 chains (e.g., a complex of TCRα and TCRβ, a complex of TCRγ and TCRδ, a complex of CD3ε and CD3δ, a complex of CD3γ and CD3ε, or a sub-TCR complex of TCRα, TCRβ, CD3γ, CD3δ, and two CD3ε chains.

[00507] *Therapeutically effective amount*: As used herein, the term "therapeutically effective amount" means an amount of an agent to be delivered (e.g., nucleic acid, drug, therapeutic agent, diagnostic agent, prophylactic agent, etc.) that is sufficient, when administered to a subject suffering from or susceptible to an infection, disease, disorder, and/or condition, to treat, improve symptoms of, diagnose, prevent, and/or delay the onset of the infection, disease, disorder, and/or condition. In some embodiments, a therapeutically effective amount is provided in a single dose. In some embodiments, a therapeutically effective amount is administered in a dosage regimen comprising a plurality of doses. Those skilled in the art will appreciate that in some embodiments, a unit dosage form may be considered to comprise a therapeutically effective amount of a particular agent or entity if it comprises an amount that is effective when administered as part of such a dosage regimen.

[00508] *Treatment or treating:* As used herein, the terms "treatment" or "treating" denote an approach for obtaining a beneficial or desired result including and preferably a beneficial or desired clinical result. Such beneficial or desired clinical results include, but are not limited to, one or more of the following: reducing the proliferation of (or destroying) cancerous cells or other diseased, reducing metastasis of cancerous cells found in cancers, shrinking the size of the tumor, decreasing symptoms resulting from the disease, increasing the quality of life of those suffering from the disease, decreasing the dose of other medications required to treat the disease, delaying the progression of the disease, and/or prolonging survival of individuals.

[00509] *Tune:* As used herein, the term "tune" means to adjust, balance or adapt one thing in response to a stimulus or toward a particular outcome. In one non-limiting example, the SREs and/or DDs of the present invention adjust, balance or adapt the function or structure of

compositions to which they are appended, attached or associated with in response to particular stimuli and/or environments.

EQUIVALENTS AND SCOPE

[00510] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments in accordance with the invention described herein. The scope of the present invention is not intended to be limited to the above Description, but rather is as set forth in the appended claims.

[00511] In the claims, articles such as "a," "an," and "the" may mean one or more than one unless indicated to the contrary or otherwise evident from the context. Claims or descriptions that include "or" between one or more members of a group are considered satisfied if one, more than one, or all of the group members are present in, employed in, or otherwise relevant to a given product or process unless indicated to the contrary or otherwise evident from the context. The invention includes embodiments in which exactly one member of the group is present in, employed in, or otherwise relevant to a given product or process. The invention includes embodiments in which more than one, or the entire group members are present in, employed in or otherwise relevant to a given product or process.

[00512] It is also noted that the term "comprising" is intended to be open and permits but does not require the inclusion of additional elements or steps. When the term "comprising" is used herein, the term "consisting of" is thus also encompassed and disclosed.

[00513] Where ranges are given, endpoints are included. Furthermore, it is to be understood that unless otherwise indicated or otherwise evident from the context and understanding of one of ordinary skill in the art, values that are expressed as ranges can assume any specific value or subrange within the stated ranges in different embodiments of the invention, to the tenth of the unit of the lower limit of the range, unless the context clearly dictates otherwise.

[00514] In addition, it is to be understood that any particular embodiment of the present invention that falls within the prior art may be explicitly excluded from any one or more of the claims. Since such embodiments are deemed to be known to one of ordinary skill in the art, they may be excluded even if the exclusion is not set forth explicitly herein. Any particular embodiment of the compositions of the invention (e.g., any antibiotic, therapeutic or active ingredient; any method of production; any method of use; etc.) can be excluded from any one or more claims, for any reason, whether or not related to the existence of prior art.

[00515] It is to be understood that the words which have been used are words of description rather than limitation, and that changes may be made within the purview of the appended claims without departing from the true scope and spirit of the invention in its broader aspects.

EXAMPLES

Example 1. Generation of novel ligand responsive SREs or DDs by mutagenesis screening

Study design

[00516] To engineer constructs that display ligand dependent stability, a candidate ligand binding domain (LBD) is selected and a cell-based screen using yellow fluorescent protein (YFP) as a reporter for protein stability is designed to identify mutants of the candidate LBD possessing the desired characteristics of a destabilizing domain: low protein levels in the absence of a ligand of the LBD, (i.e., low basal stability), large dynamic range, robust and predictable dose-response behavior, and rapid kinetics of degradation (Banaszynski, *et al.*, (2006) *Cell*; 126(5): 995–1004). The candidate LBD binds to a desired ligand but not endogenous signaling molecules.

[00517] The candidate LBD sequence (as a template) is first mutated using a combination of nucleotide analog mutagenesis and error-prone PCR, to generate libraries of mutants based on the template candidate domain sequence. The libraries generated are cloned in-frame at either the 5′- or 3′-ends of the YFP gene, and a retroviral expression system is used to stably transduce the libraries of YFP fusions into NIH3T3 fibroblasts.

[00518] The transduced NIH3T3 cells are subjected to three to four rounds of sorting using fluorescence-activated cell sorting (FACS) to screen the libraries of candidate DDs. Transduced NIH3T3 cells are cultured in the absence of the high affinity ligand of the ligand binding domain (LBD), and cells that exhibit low levels of YFP expression are selected through FACS.

Screening Strategy I

[00519] The selected cell population is cultured in the presence of the high affinity ligand of the ligand binding domain for a period of time (e.g., 24 hours), at which point cells are sorted again by FACS. Cells that exhibit high levels of YFP expression are selected through FACS and the selected cell population is split into two groups and treated again with the high affinity ligand of the ligand binding domain at different concentrations; one group is treated with the lower concentration of the ligand and the other is treated with a high concentration of the ligand, for a period of time (e.g., 24 hours), at which point cells are sorted again by FACS. Cells expressing mutants that are responsive to lower concentrations of the ligand are isolated.

[00520] The isolated cells responsive to the lower concentration of the ligand are treated with the ligand again and cells exhibiting low fluorescence levels are collected 4 hours following removal of the ligand from the media. This fourth sorting is designed to enrich cells that exhibit fast kinetics of degradation (Iwamoto *et al.*, *Chem Biol.* 2010 Sep 24; 17(9): 981–988).

Screening Strategy II

[00521] The selected cell population is subject to additional one or more sorts by FACS in the absence of high affinity ligand of LBD and cells that exhibit low levels of YFP expression are selected for further analysis. Cells are treated with high affinity ligand of the ligand binding domain, for a period of time (e.g. 24 hours), and sorted again by FACS. Cells expressing high levels of YFP are selected for through FACS. Cells with high expression of YFP are treated with ligand again and cells exhibiting low fluorescence levels are collected 4 hours following removal of the ligand from the media to enrich cells that exhibit fast kinetics of degradation. Any of the sorting steps may be repeated to identify DDs with ligand dependent stability.

[00522] The cells are recovered after sorting. The identified candidate cells are harvested and the genomic DNA is extracted. The candidate DDs are amplified by PCR and isolated. The candidate DDs are sequenced and compared to the LBD template to identify the mutations in candidate DDs.

Example 2. DD regulated recombinant IL2 expression

[00523] FKBP (L106P) and ecDHFR (R12Y, Y100I) are well-characterized destabilizing domains which can confer instability to fusion partners (e.g., a POI). The instability is reversed by a synthetic ligand named Shield-1 that binds to FKBP DDs and TMP or MTX that binds to DHFR DD. An IL2 polypeptide was linked to either FKBP (F36V; L106P) or ecDHFR (R12Y, Y100I). IL2 constructs were packaged into pLVX-IRES-Puro lentiviral vectors. An IL2 signal sequence was inserted at the N terminus of the construct and linkers were placed between the signal sequence, the DD and IL2.

[00524] OT-IL2-001 (FKBP) was transduced into human colorectal carcinoma line, HCT-116. To measure the dependence of IL2 levels on Shield-1 dose, cells were plated onto a 96 well plate and treated with varying concentrations of Shield-1. Media was then collected from cells and IL2 levels were quantified using IL2 ELISA (Figure 13). IL2 increased with increase in Shield-1 concentration and plateaued at higher Shield-1 dose levels. The half maximal effective concentration or EC_{50} of Shield-1 was determined to be 50 nM.

Example 3. *In vitro* T cell assay development

[00525] The goal of the study was to determine the T cell stimulation regimen and dose of IL12 required to maximize T cell persistence and T cell differentiation *in vitro*. The study recapitulates the design of the adoptive cell therapy regimen wherein the T cells were initially exposed to the antigen *in vitro* which results in activation followed by a resting phase and finally *in vivo* transfer where the T cells encounter the antigen again. T cells were stimulated CD3/CD28 beads or soluble CD3/CD28 on day 0 and the CD3/CD28 stimulus was washed off at the end of 48 hours.

Cells were treated with a dose of IL12 ranging from 0.01- 1000 ng/mL. On day 9, the Th1 phenotype of the cells was evaluated by examining the frequency of IFNgamma positive CD4+ cells and CD8+ cells. On day 14, cells were divided into two groups- one group received a second CD3/CD28 stimulation and a second group that was not stimulated. On day 16, the Th1 phenotype was evaluated in both groups using FACS. The results for day 16 are presented in Figure 14. IFN gamma expression was higher in cells that received a CD3/CD28 restimulation on day 14 compared to cells that did not receive second stimulation. This indicates that both antigen restimulation and IL12 exposure were required for the Th1 phenotype. Further, as little as 0.1 ng/mL of IL12 was able to cause Th1- skewing and IFN gamma production from T cells *in vitro*, and higher doses of IL12 further improved this effect.

Example 4. Measuring human T cell responses *in vitro* and *in vivo*

[00526] IL12 promotes the differentiation of naïve T cells into Th1 cells which results in the secretion of IFN gamma from T cells. Human T cells were treated with IL12 or left untreated and analyzed by flow cytometry for the expression of IFN gamma and T cell marker CD3. Treatment with IL12 resulted in the differentiation of T cells as measured by an increase in the percentage of IFN gamma positive T cells from 0.21 to 22.3 (see inset of Figure 15A).

[00527] To test if membrane bound IL15/IL15Ra fusion protein (OT-IL15-008) can induce human T cell expansion, human T cells were transduced with the construct. T cell proliferation was measured by evaluating forward and side scatter of the T cell population using flow cytometry. Transduction with membrane bound IL15/IL15Ra fusion construct resulted in the expansion of human T cells (58.9) compared to control untransfected cells (37.8) (Figure 15B).

[00528] Tracking T cells following their adoptive transfer is critical to determine their distribution at different sites in the host, their identity and persistence over time. Human T cells were stimulated with CD3/CD28 beads and incubated with 50U/ml of IL2. Cells were expanded *in vitro* for 7 days with IL2 supplementation on day 3 and day 5. On day 5, the CD3/CD28 beads were removed and the cells were cultured for two days. On day 7, cells were washed to remove IL2 and 5 million human T cells were injected intravenously into immune compromised, NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ mice. Blood samples were obtained 4, 24, 120 and 168 hours after cell transfer. Mice were euthanized 168 hours after cell transfer and the bone marrow and spleen were harvested. Immune cells were isolated from all samples and analyzed for the presence of human T cells using CD3 and CD45 cell surface markers. As shown in Figure 15C, the percentage of CD3 positive, CD45 positive human T cells in the blood was higher in animals injected with human T cells, especially at 120 and 168 hours. CD3 positive, CD45 positive human T cells were also detected in the spleen and bone marrow of animals injected with human

T cells. As expected no CD3 positive, CD45 positive human T cells were detected in control animals that were not injected with human T cells.

[00529] To determine the identity of the T cells following adoptive transfer, blood samples were collected from mice 48 hours after injection. CD4 and CD8 T cells were analyzed for surface expression of CD45RA and CD62L. Both markers are highly expressed in naïve T cells but are lost as the T cells become antigen exposure. As shown in Figure 15D, human CD4 and CD8 T cells showed high surface expression of both markers prior to injecting into mice, but was lost 48 hours after *in vivo* cell transfer indicating that the human T cells are exposed to the antigen *in vivo*.

Example 5. DD regulated luciferase

[00530] A luciferase polypeptide was linked to either FKBP (F36V, L106P or F36V, E31G, R71G, K105E) or ecDHFR (R12Y, Y100I) and cloned into pLVX.IRES. Puro vector.

[00531] DD regulated luciferase can be used to track cells *in vivo* e.g. T cells. Firefly luciferase or Renilla luciferase may be utilized as the payload. HCT-116 cells were stably transduced with the constitutive (OT-RLuc-001) or DD regulated constructs (OT-RLuc-002, OT-RLuc-003, OT-RLuc-004, OT-RLuc-005 and OT-RLuc-006). Cells were treated with 1μM Shield-1, 10μM Trimethoprim or vehicle control for 24 hours and luciferase expression was measured via western blotting using anti-Renilla luciferase and anti-Firefly luciferase antibodies (Abcam, Cambridge, UK). Blots were also probed with anti-GAPDH antibody to ensure uniform protein loading in all samples. As shown in Figure 16A, OT-RLuc-003 showed strong Shield-1 dependent stabilization of Renilla luciferase. OT-RLuc-004, 005 and 006 showed modest stabilization of Renilla luciferase in the presence of their corresponding ligand, while OT-FLuc-002 showed modest stabilization of firefly luciferase with the addition of Shield-1. As expected, the constitutive luciferase construct (OT-RLuc-001) showed expression of Renilla luciferase both in the presence and absence of ligand. (Figure 16A).

[00532] Ligand dependent activity of Renilla and firefly luciferase constructs was measured using coelentrazine and luciferin substrates respectively. Cells were treated with1μM Shield-1, 10μM Trimethoprim, or vehicle control for 24 hours, lysed with assay lysis buffer and incubated with the luciferase substrate. Luciferase activity was measured as luminescence reading using a luminometer and the values were compared to control samples consisting of lysis buffer and substrate. All DD regulated showed ligand dependent increase in luciferase activity compared to control. As expected, the constitutive construct OT-RLuc-001 showed high luciferase activity both in the presence and absence of ligand (Figure 16B).

Example 6. DD regulated IL2 and IL2 mediated functions

[00533] DD-IL2 function is characterized *in vivo* by evaluating the ability of tumor cells expressing these constructs to establish tumors and proliferate under the treatment of corresponding synthetic ligands e.g. Shield-1, Trimethoprim or Methotrexate. 2-10 million HCT-116 cells stably transduced with the constructs are subcutaneously xenografted with matrigel into mice that capable of producing functional B and NK cells. Approximately, two weeks after injection, when the tumors reach a size of approximately 300 cubic mm, mice are dosed with corresponding stabilizing ligands e.g. Shield-1, Trimethoprim or Methotrexate at varying concentrations every two days. Shield-1 is injected with a carrier consisting of 10%Dimethylacetamide, 10% Solutol HS15, and 80% saline. Tumor volume and body weight are monitored twice a week and the experiment is terminated once the tumors reach 1000 cubic mm in size. Plasma and tumor samples are collected 8 hours after the last dose of the ligand and IL2 as well as the ligand levels are measured.

[00534] To evaluate the ability of IL2 expressing cells to form tumors, HCT-116 cells stably transduced with DD-IL2 constructs are pretreated with corresponding stabilizing ligands, Shield-1, Trimethoprim or Methotrexate and subsequently xenografted into mice. Reduction in tumor growth and a concomitant increase in IL2 levels in ligand treated mice compared to untreated controls is indicative conditional regulation of IL2 *in vivo*.

Example 7. DD regulated Caspase 9

[00535] A caspase 9 polypeptide was linked to the N or C-terminus of FKBP, ecDHFR and hDHFR DDs and cloned into pLVX.IRES. Puro vectors.

[00536] To test ligand dependent Caspase 9 production, 1 million HEK-293T cells are plated in a 6-well plate in growth media containing DMEM and 10 FBS and incubated overnight at 37°C, 5% CO2. Cells are transiently transfected with 100ng of DD-Caspase 9 constructs using Lipofectamine 2000 and incubated for 48 hrs. Following the incubation, growth media is exchanged for media containing ligands (Trimethoprim, Methotrexate, or Shield-1, depending on the construct used). Following 24-hour incubation with ligand, cells are lysed and immunoblotted for caspase 9. Increase in Caspase 9 levels with increase in ligand concentrations is indicative of DD mediated regulation of Caspase 9.

Example 8. Evaluation of antitumor response of DD regulated payloads in syngeneic mouse models

[00537] The efficacy of cancer immunotherapy in organisms with intact immune cells is evaluated using syngeneic mouse models e.g. pMEL-1 and 4T1 mouse models. Immune cells such as T cells and NK cells are isolated from syngeneic mice and transduced with DD regulated

payloads such as DD-IL2, and DD caspase9. Cells are then injected into mice bearing subcutaneous syngeneic tumors and treated with varying concentrations of ligand, Shield-1, Trimethoprim or Methotrexate, depending on the DD used. Mice treated with immune cells transduced with DD regulated payload are expected to have a reduced tumor burden when compared to control animals.

Example 9. Co-expression of DD regulated payloads

[00538] Cells are co-transfected with DD-Interleukin e.g. DD-IL2 and DD Caspase9 or DD FOXP3 constructs. Transfected cells are treated with stabilizing ligands depending on the DD utilized. DD-IL2, expression in the media is measured by ELISA. FOXP3 and caspase 9 expression are evaluated by immunoblotting for FOXP3 and caspase 9 respectively.

Example 10. *In vivo* tracking of DD luciferase cells

[00539] DD luciferase constructs can be utilized as an optical reporter to asses if the ligand and/or the cells expressing the DD constructs reach the targeted tissue. It may also be utilized to study pharmacokinetic and pharmacodynamic (PK/PD) relationships in the context of DD. PK/PD depends on (i) the PK of the stabilizing ligand (ii) behavior of the DD in a specific cell type (iii) cargo protein behavior; some of which may be studied by utilizing the DD regulated luciferase constructs. DD luciferase constructs are expressed or co-expressed in cells of interest such as primary T cells or cell lines e.g. HCT-116, SKOV-3 cells and injected into immune compromised mice e.g. via tail vein, intra peritoneal, or subcutaneous injections. Mice are treated with the corresponding ligand or vehicle control. 8-24 hours following ligand injection, mice are injected with D-Luciferin when the payload is firefly luciferase and Coelentrazin when the payload is Renilla luciferase. Animals are anesthetized and imaged using the Bioluminescence imager (PerkinElmer, Massachusetts). The luciferase output of ligand injected mice is compared to control mice and the signal is quantitated using image analysis software (PerkinElmer, Massachusetts). Luciferase signal is expected to be much higher than background in mice treated with ligand compared to mice treated with vehicle control.

Example 11. Effect of cytokines on immune cell proliferation and activation

[00540] Immune cells such as Natural Killer cells depend on cytokines such as IL2 for their proliferation and survival. This dependence on cytokines can be used to test the functionality of DD regulated or constitutively expressed cytokines and cytokine fusion proteins.

[00541] The dependency of the NK-92 cells on cytokines for activation was tested. Cells were initially cultured for 3 days with IL2, following which cells were washed twice and cultured in media without IL2 for 7 hours. The cells were cultured for 18 hours in the presence of IL2 (100

μg/ml). NK-92 cell activation in response to cytokine treatment was evaluated by FACS analysis of a panel of markers whose increased expression is associated with NK activation. These include NKG2D, CD71, CD69; chemokine receptors such as CCR5, CXCR4, and CXCR3, Perforin, Granzyme B and Interferon gamma (IFNg). Prior to FACS for IFNg, cells were cultured for 4 hours with Brefeldin A. IFNg levels were also measured in the media. NK cells respond to external stimuli such as cytokines in their environment through the phosphorylation of proteins JAK/STAT, ERK, and p38/MAPK pathways which are important for cell activation, signalling and differentiation pathways. The phosphorylation of AKT, STAT3 and STAT5 in response to cytokine addition was measured by FACS. Since phosphorylation events are transient NK-92 cells were treated with the cytokines for 15 or 60 minutes, prior to the analysis. The fold change in mean fluorescence intensities compared to untreated for IL2 treatment for select markers are presented in Table 15.

Table 15: IL2-induced markers

Marker	Fold change
CD69	1.60
CXCR4	3.56
Perforin	1.72
Granzyme	1.62
IFNg (cellular)	1.15
IFNg (Supernatant)	7.43
pAKT (15 mins)	1.23
pAKT (60 mins)	1.34
pSTAT3 (15 mins)	1.19
pSTAT3 (60 mins)	1.38
pSTAT5 (15 mins)	1.90

[00542] Treatment with IL2 resulted in an increase in an increase in the expression of CD69, CXCR4, Perforin, Granzyme B, and IFNg. Further, IFNg levels secreted by NK-92 cells into the media higher than untreated controls upon treatment with IL2. Phosphorylation of STAT5 was increased both at 15 and 60 minutes after the addition of IL2. A modest increase in phospho AKT and STAT3 was observed. Taken together, these results show that cytokines can activate NK cells.

[00543] The dependency of immune cells on cytokines for proliferation was tested in NK-92 cells (natural killer cell line) and T cell lines Jurkat cells and SupT1 cells. Cells were seeded at 40,000 per well and cultured in the presence of IL2 or IL15 for 3 days. The number of NK-92 cells decreased with time in the untreated cells. However, treatment with IL2 increased cell

numbers. In contrast, the cell numbers obtained with cytokine treatment in SupT1 and Jurkat cells was comparable to the untreated control. Thus, NK-92 cells depend on cytokines for survival.

Example 12. DD regulated FOXP3 expression

[00544] A fusion molecule is generated by fusing full length or truncated (FOXP3Δ2) and DDs such as ecDHFR (DD) or FKBP (DD). These fusion molecules were cloned into pLVX-IRES-Puro vectors.

[00545] To test ligand dependent FOXP3 production, 1 million HEK-293T cells were plated in a 6-well plate in growth media containing DMEM and 10% FBS and incubated overnight at 37°C, 5% CO2. Cells were transfected with the constructs using Lipofectamine 2000 and incubated for 24 hrs. Following the incubation, media was exchanged for growth medium with or without 10μM Trimethoprim or 1μM Shield-1 and further incubated for 24 hrs. Cells were harvested and FOXP3 levels were analyzed via western blotting using anti FOXP3 antibody (Abcam, Cambridge, UK). OT-FOXP3-003, and OT-FOXP3-007 showed the strongest Shield-1 dependent stabilization of FOXP3, while OT-FOXP3-008 showed the strongest Trimethoprim dependent stabilization of FOXP3 (Figure 17A and Figure 17B). Constructs OT-FOXP3-004 and OT-FOXP3-009 showed modest TMP and shield-1 dependent stabilization.

Example 13. Effect of ligand on T cell proliferation

[00546] The effect of ligands specific to the SREs of the invention on immune cell proliferation was measured to identify concentrations of the ligand that did not inhibit T cell growth or survival. T cells derived from two different donors were stimulated with CD3/CD28 and treated with ligand TMP at doses ranging from 0.04 μM to 160 μM or with control vehicle (DMSO). The percentage of divided cells within the CD4 and the CD8 populations of T cells was measured using FACS. Concentrations of TMP ranging from 0.04 μM to 40 μM showed no effect on the percentage of divided cells within the CD8 and CD4 populations, while 160 μM concentration of TMP resulted in an 70-90% reduction in the percentage of divided cells. Thus, the optimal concentration of TMP for T cell-based experiments was determined to be less than 160 μM.

Example 14. Promoter selection for expression of SREs in T cells

[00547] The expression of SREs expressed in a vector can be driven either by the retroviral long terminal repeat (LTR) or by cellular or viral promoters located upstream of the SRE. The activity

of the promoter may vary with the cell type and thus promoter selection must be optimized for each cell type. To identify optimal promoters, AcGFP (SEQ ID No. 223) was cloned into pLVX. IRES Puro construct with a CMV or an EF1a promoter. Patient-derived T cells and Sup T1 cells were transduced with the constructs and GFP expression was measured at day 3 and day 5 after transduction using FACS. As shown in Figure 18, both the CMV promoter and the EF1a promoter can drive the expression of GFP in SupT1 cells and T cells. The percentage of GFP positive T cells was higher when the GFP expression was driven by CMV promoter compared to an EF1a promoter, both at 3 days and 6 days after transduction. In contrast, the percentage of GFP positive cells was much higher when GFP expression was driven by the EF1a promoter compared to the CMV promoter. Thus, the optimum promoter suitable for expression differs based on the cell type.

Example 15. EBV tumor antigen mediated TCR re-stimulation *invivo*

[00548] Human T cells engineered to express DD regulated cytokines are not antigen specific. However, functionality of T cells *in vivo* requires their restimulation which occurs upon engagement with the antigen. This requirement for antigen mediated restimulation can be mimicked *in vivo* in mice, the Epstein Barr Virus (EBV) antigen may be utilized. Approximately 90% adults have a current or a previous EBV infection. Additionally, the major histocompatibility group HLA-A02 has been associated with the decreased risk of developing EBV positive Hodgkin's lymphoma, suggesting that the CTL peptide epitopes that promote viral clearance are presented by HLA-A02. Several tumor cell lines that are HLA-A02 positive, e.g. Raji cells, are used for *in vivo* studies. Primary human T cells obtained from various donors are expanded with CD3/CD28 dynabeads. To test reactivity of T cells the EBV antigen, EBV positive Raji cells and EBV negative Ramos cells are used. The involvement of HLA-A02 in antigen recognition is tested using anti-HLA antibodies to test assay specificity. Cell killing assays are performed by incubating T cells with fluorescently labelled Raji cells or Ramos cells and the ability of the donor T cells to preferentially kill Raji cells is evaluated. The activation of T cells in response to interaction with EBV antigen is measured by culturing mitomycin treated Raji or Ramos cells with fluorescently labelled T cells. The activation and proliferation status of T cells is examined by measuring expression of IFNg, CD107a, Granzyme, and Perforin. Since most humans have been exposed to EBV, the donor T cells in most instances are expected to be immunoreactive to Raji cells, but not Ramos cells. It is likely that T cells reactive to Raji cells will be positive for markers of T cell activation such as Granzyme and Perforin.

Example 16. Ligand dependent IL2 stabilization *in vivo*

[00549] An IL2 polypeptide was linked to either FKBP (F36V; L106P) or ecDHFR (R12Y, Y100I). IL2 constructs were packaged into pLVX-IRES-Puro lentiviral vectors. An IL2 signal sequence was inserted at the N terminus of the construct and linkers were placed between the signal sequence, the DD and IL2.

[00550] OT-IL2-001 (FKBP) was transduced into human colorectal carcinoma line, HCT-116. IL2 expression *in vitro* was confirmed by ELISA. To test *in vivo* ligand mediated regulation, 5 million HCT 116 cells transduced with IL2 constructs were injected subcutaneously into the flanks of immunocompromised mice. Approximately, two weeks after injection, when the tumors reach a size of approximately 300 cubic mm, mice are dosed with corresponding stabilizing ligands or corresponding vehicle control. Shield-1 is injected with a carrier consisting of 10%Dimethylacetamide, 10% Solutol HS15, and 80% saline at a concentration of 10mg/kg body weight. Mice were euthanized at tumor and plasma samples were collected and analyzed for IL2 levels. Tumor IL2 levels were measured by ELISA as picograms per mg of protein. As shown in Figure 19, tumor IL2 levels detected with Shield-1 treatment was higher than levels detected with vehicle control. These data demonstrate a dose dependent IL2 secretion from HCT116 cells stably expressing FKP-IL2 *in vivo*.

Example 17. Optimization of T cell transduction and expansion

[00551] The T cell expansion and transduction protocol consists of stimulating donor derived human T cells on day zero with aCD3/aCD28 bead stimulation to promote T cell proliferation. On day one, T cells were transduced with the construct of choice and expanded for up to day 11 with occasional media changes. On day 11, the beads were washed out and the cells were frozen in aliquots for use in various functional assays. The process of T cell expansion and transduction requires optimization to ensure the exponential proliferation of T cells, following the initial 4-day lag in growth of the T cells which is caused by the viral transduction. Unoptimized expansion protocol may result in static growth for up to 10 days requiring instead 21 days to achieve similar expected T cell number and a concomitant reduction in viability and abnormal CD4 to CD8 T cell ratio. The source of αCD3/αCD28 used for T cell stimulation and the ratio of the beads were tested to identify optimal conditions. αCD3/αCD28 beads from different sources were utilized. These included dynabeads (ThermoFisher, Waltham, MA), CD3/CD28 +/- CD2 with anti-Biotin MACSiBead (Miltenyi Biotec, Germany), Macrobeads – 3μm magnetic polymer beads with αCD3/αCD28 antibody coating, soluble tetrameric antibody complexes of

αCD3/αCD28 (StemCell, Canada). Bead and T cells were mixed at 3:1, 1:1, 1:3 and 1:9 ratios to identify optimal ratios for co-culture. Assays were also performed using two separate donors to account for donor related variabilities. Percentage cell viability was measured at 0, 4 and 7 days. As shown in Figure 20, both donors showed improved cell viability counts at 1:3 bead to cell ratio compared to all other conditions, especially at day 4. One of the donors also showed improved cell viability at with 1:9 bead to cell ratio. Cell number counts were performed using both the cellometer and flow cytometry analysis. For this analysis, cells were infected with viruses at MOI of 10 and compared to mock (Lentiboost (LB) transduced cells to examine the effects of viral transduction on T cell counts. The fold change in cell growth at day 4 and day 7 compared to growth at day 0 was analyzed and the results from the cellometer counts are represented in Table 16.

Table 16. T cell proliferation

Experimental condition	Donor1		Donor 2	
	Day 4	Day 7	Day 4	Day 7
MOI 3:1	1.70	5.00	1.94	3.90
MOI 1:1	2.15	3.95	2.55	4.30
MOI 1:3	1.69	4.25	2.75	8.80
MOI 1:9	1.65	3.70	3.91	6.35
MOI Immuno Cult	0.98	2.30	0.95	3.05
LB 3:1	2.10	4.25	1.45	5.00
LB 1:1	2.20	6.35	2.35	6.65
LB 1:3	2.05	9.45	2.97	10.50
LB 1:9	1.55	5.60	2.77	3.75
LB Immuno Cult	1.05	3.10	0.86	2.30

[00552] As shown in Table 16, the overall proliferation in both donors was greater at day 7 than at day 4. For donor 1, the proliferation at day 4 among the virally transduced cells was highest when an MOI of 10 was used in conjunction with a bead to cell ratio of 3:1, while donor 2 T cells proliferated the most at a ratio of 1:9. At day 7, 1:1 ratio for donor 1 and 1:3 ratio for donor 2 showed highest cell proliferation. Based on these results a bead to cell ratio of 1:3 was identified as optimal for promoting T cell proliferation.

[00553] T cells transduced at various multiplicity of infection (MOI) were also tested using flow cytometry. The results for donor 1 are shown in Table 17.

Table 17. Effect of viral titer and bead to cell ratio

MOI	Bead to cell ratio				Immunocult
	3:1	1:1	1:3	1:9	
10 MOI	0.81	0.93	0.85	1.28	1.42
2 MOI	0.37	0.50	0.51	1.00	0.65
0.4 MOI	0.33	0.46	0.50	0.72	0.86

[00554] As shown in Table 17, bead ratio of 1: 9 showed the highest cell viability among all conditions tested for donor1. Similar results were obtained for donor 2.

[00555] Ratio of CD4 to CD8 within the proliferating T cell populations. At the start of the experiment, the CD4 to CD8 ratio was determined to be 3 and the skewing of populations. The results are shown in Table 18.

Table 18. CD4 to CD8 cell ratio

MOI	**Donor 1**					**Donor 2**				
	3:1	**1:1**	**1:3**	**1:9**	**Immunocult**	**3:1**	**1:1**	**1:3**	**1:9**	**Immunocult**
10	8.63	8.73	5.84	3.7	7.44	4.53	3.25	2.56	1.65	2.75
2	9.6	9.91	7.18	4.11	6.45	5.25	3.16	2.04	1.56	3.23
0.4	10.85	11.33	7.51	4.23	5	6.22	4.71	2.59	1.43	3.55
LB	6.95	7.24	5.24	3.21	4.96	3.86	3.27	2.18	1.23	2.78

[00556] Donor 1 showed CD4 to CD8 ratio close to 3 when a bead to cell ratio of 1:9 was used. Similar results were obtained for donor 2 when a bead to cell ratio of 1:3 was used.

[00557] Taken together, these experiments show that activation induced cell death may occur when T cells are overstimulated by beads, and therefore optimal bead to T cell ratio is required. The experiments suggest that the optimal bead to cell ratio was 1:3 across various MOIs.

Example 18. Analysis of BCMA CAR expression

[00558] A BCMA CAR fusion polypeptide was linked to either to the N terminus of the FKBP-DD, ecDHFR-DD or human DHFR-DD and the constructs were cloned into pLVX-IRES-Puro vector under the transcriptional control of EF1a promoter.

[00559] To test ligand dependent expression of DD-BCMA CAR constructs, 1 million HEK HCT116 cells were cultured in growth medium containing DMEM and 10% FBS and transfected with CAR constructs using Lipofectamine 2000 or LentiBoost. 48 hours after transfection, cells were treated with 1μM or10μM Shield-1, 10μM Trimethoprim, 1μM Methotrexate, or vehicle control and incubated for 24 hours. Surface expression of DD-BCMA CAR constructs in HCT116 cells was measured using Fluorescence activated cell sorting (FACS) with Protein L-Biotin-Strepavidin-Allophycocyanin which binds to the kappa light chain of the CAR (ThermoFisher Scientific, Waltham, MA). As shown in Figure 21, surface expression of OT-BCMA-002 with FKBP-DD was elevated in the presence of Shield-1, while OT-BCMA-003 with ecDHFR-DD showed elevated surface expression in the presence of Trimethoprim. As expected, constitutively expressed construct OT-BCMA-001 showed high expression even in the absence of ligand.

Example 19. Analysis of CAR expression and function

[00560] The expression of chimeric antigen receptors described herein such as, but not limited to CD22 CAR, ALK CAR, CD33 CAR, HER2 CAR and GD2 CAR constructs are fused to destabilizing domains such as ecDHFR DD, FKBP DD and hDHFR DDs. To test ligand dependent expression of the constructs, immune cells are cultured in growth medium containing DMEM and 10% and transduced are transduced with the CAR constructs. 48 hours after transduction, cells are treated with the ligand corresponding to the DD such as 1μM or10μM Shield-1, 10μM Trimethoprim, 1μM Methotrexate, or vehicle control and incubated for 24 hours. Cells are then analyzed by western blot using the CD3 Zeta, a component of the CAR. Surface expression of the CAR is analyzed by FACS using Protein L. Intracellular and surface expression of CARs is expected to be undetectable in the absence of ligand, but strongly induced by the presence of the ligand.

[00561] The efficacy of T cells expressing DD regulated CAR constructs in functionally interacting with target cells is evaluated. To interact with the CAR T cells, the chosen target cells express the antigen related to the CAR either naturally or ectopically. For example, target cells which have high endogenous expression of BCMA include KMS11, MM-1S, RPMI-8226 cells; target cells expressing CD33 include HL-60, MOLM13, MOLM14 cells. Alternatively, target

cell lines may be engineered to ectopically express the antigen in cell lines that have low endogenous expression of the antigen.

[00562] Multiple assays are used to measure functionality. Prior to co culture, the target cells are optionally cultured in the presence of mitomycin C to prevent target cell proliferation. This ensures that target cell growth does not out compete T cell growth. Cytotoxicity assays are used to measure the ability of T cells induce target cell death. Target cells are engineered to express Renilla or Firefly luciferase and co cultured with T cells expressing DD regulated CAR constructs for 18 to 24 hours in the presence of the ligand related to the DD or vehicle control. At the end of co culture, cells are lysed and luciferase activity is measured using appropriate substrate. Luciferase activity is expected to increase when DD regulated CAR expressing T cells are co cultured with antigen expressing target cells in the presence of ligand. Cytotoxicity is not expected in vehicle control cells or when the target cells do not express the antigen are utilized.

[00563] Cytolytic potential of DD CAR expressing cells is evaluated in primary human T cells or human cell lines (e.g. NALM6, K562 and Raji) using Chromium-51 Release Assay. Target cells are loaded with of $Na_2{}^{51}CrO_4$, washed twice and resuspended in phenol red-free growth medium. Untreated or ligand treated DD CAR and mock transduced cells are co-incubated with cognate antigen expressing target cells at various effector: target cell ratios, and chromium release into the supernatant is measured using a liquid scintillation counter. Cells with DD CAR are expected to demonstrate specific cytolysis only in the presence of ligand. Cells with DDCAR in the absence of ligand or mock transfected cells are expected to show minimal cytolytic activity.

[00564] Activation of T cells results in degranulation, an exocytic process by which cytotoxic T cells release molecules like perforin and granzymes which enable target cell killing. Degranulation is measured by analysis of media for indications of exocytosis e.g. CD107 by FACS and by markers of degranulation such as perforin and granzyme using immunoassays.

[00565] Engagement of the CAR with its cognate antigen results in the activation of T cells is measured 24 hours after co culture of CAR expressing T cells and target cells. Activation of T cells is evaluated by measuring levels of IFNg, IL2, and CD69. T cell proliferation in response to antigen mediated T cell activation is measured by labelling T cells with Carboxyfluorescein succinimidyl ester, which is used to trace cells across multiple generations. Labelled T cells are cultured with Mitomycin treated target cells and cell proliferation is tracked over a period of 3 to 5 days. T cell proliferation and activation is expected to increase when DD regulated CAR expressing T cells are co cultured with cognate antigen expressing target cells in the presence of

ligand. Both parameters are not expected in to change in vehicle control cells or when the target cells do not express antigen are utilized.

[00566] To measure the ability of DD regulated CARs to promote tumor-free survival, mice are injected intravenously with antigen positive target cells. Following injections, mice are treated with untransduced mock T cells or T cells that are transduced with antigen specific chimeric antigen receptors. Mice are then split into two cohorts; one cohort is treated with the ligand specific to the SRE and the second cohort which is treated with the vehicle control. Survival of the mice is monitored for up to 80 days after the administration of target cells. Tumor bearing mice treated with CAR transduced T cells and the ligand, are expected to survive longer than mice treated with untransduced cells; vector control transduced T cells or vehicle control.

[00567] To measure ligand induced reduction in tumor growth, mice are injected orthotopically with antigen positive target cells on day 0. The mice are then treated with cyclophosphamide intraperitoneally on day 3. Cells are treated with antigen specific CAR construct. The mice are treated with recombinant interleukin 7 or interleukin 7 complexed with IL7 antibody, two-three times a week for three weeks to promote T cell persistence. Mice are then split into two cohorts; one cohort is treated with the ligand specific to the SRE and the second cohort which is treated with the vehicle control. The size of the tumors is measured at various time points after inoculation. Mice treated with antigen specific CAR and the ligand are expected to be tumor free while mice treated with mock transduced cells, or vehicle controlled cells are predicted to succumb to the tumors

[00568] To test if DD regulated CARs can cause regression of established tumors in a ligand dependent fashion, mice are orthotopically inoculated with antigen positive T cells expressing luciferase. Tumors are allowed to grow for 8 days, and the growth is monitored using bioluminescent imaging. On day 8, after tumor inoculation, CAR transduced cells are injected intravenously with or without cytokine and cytotoxic therapy augmentation. Concurrent ligand or vehicle control treatment is also initiated. Mice treated with CAR T cells are expected to show tumor regression and long term disease control in the presence of ligand, whereas all mice treated with mock transduced cells are expected to succumb to progressive tumor growth.

Example 20. Measuring T cell exhaustion phenotype and its reversal

[00569] Primary T cells are activated using soluble CD3/CD28 or CD3/CD28 dynabeads. 24 hours later, cells are transduced with DD regulated CD19 CAR constructs and allowed to rest for 24 hours. Cells are then treated with the ligand specific to the SRE or vehicle control. At day 4, the CD3/CD28 beads are removed and the cells are cultured for another 3 days. At day 7, cells

are washed extensively to remove the ligand and replated in the absence of the ligand and cultured for 3 days. At day 10, cells were washed and replated in the absence of ligand. A sample of the cells are analyzed at day 10 for phenotypic and functional markers associated with exhaustion. The rest of the cells are cultured for 3 days in the absence of ligand and analyzed for phenotypic and functional exhaustion markers at day 14. T cells cultured for the duration of the experiment either in the presence or absence of ligand are included as controls. Phenotypic markers for exhaustion such as PD1, TIM3, LAG3, BTLA, CD160, 2B4, and CD39a are measured both in CD4 and CD8 T cell populations. Since chronic T cell activation has been shown to result in T cell exhaustion, T cells cultured under the continuous presence of ligand throughout the experiment are expected to be positive for multiple markers of exhaustion. T cells cultured in the absence of ligand throughout the experiment are expected to negative for multiple markers of exhaustion, since the expression of the CAR will be undetectable/low in the absence of ligand. At day 10, cells where the ligand is removed at day 7 are expected to have a lower percentage of cells that are positive for multiple exhaustion markers when compared to cells that have been treated with ligand throughout the duration of the experiment. This result is expected in both CD8 and CD4 population of T cells.

Example 21. Dual specific chimeric antigen receptor

[00570] Human T cells are transduced with a lentiviral vector encoding dual specific chimeric antigen receptors. Dual specific CARs targets include, but not limited to GD2, CD33, BCMA, Her2, ALK, CD22 and CD276. Cells are cultured in the presence of ligand or vehicle control for 24-48 hours. Additional controls such as human T cells that are mock transduced or transduced with a vector encoding septicity to one antigen only are included. CAR expression is evaluated by flow cytometry using anti-idiotype CAR antibody. Expression of dual specific CAR is expected only in cells transduced with dual specific CAR construct in the presence of ligand, while the controls transduced with the CAR constructs showing single specificity are expected to show expression of the single CAR construct, and in the presence of ligand.

[00571] T cells are also analyzed for proliferation, markers of apoptosis and memory phenotype. Cells are cultured per methods described above. Ligand is removed either at day 7 or day 10 and cells are analyzed at day 14 for apoptosis using Annexin V staining and for the memory phenotype using CD62L as the marker. When compared to untreated cells, cells continuously treated with ligand are expected to show an increase in apoptotic cells, a decrease in proliferation and a decrease in CD62L expression, which are indicative of T cell exhaustion.

Ligand withdrawal at day 7 or day 10 is expected to show low Annexin V and high CD62L expression, and an increase in proliferation similar to untreated cells.

Example 22. Measuring T cell exhaustion phenotype and its reversal

[00572] Primary T cells are activated using soluble CD3/CD28 or CD3/CD28 dynabeads. 24 hours later, cells are transduced with DD regulated CD19 CAR constructs and allowed to rest for 24 hours. Cells are then treated with the ligand specific to the SRE or vehicle control. At day 4, the CD3/CD28 beads are removed and the cells are cultured for another 3 days. At day 7, cells are washed extensively to remove the ligand and replated in the absence of the ligand and cultured for 3 days. At day 10, cells were washed and replated in the absence of ligand. A sample of the cells are analyzed at day 10 for phenotypic and functional markers associated with exhaustion. The rest of the cells are cultured for 3 days in the absence of ligand and analyzed for phenotypic and functional exhaustion markers at day 14. T cells cultured for the duration of the experiment either in the presence or absence of ligand are included as controls. Phenotypic markers for exhaustion such as PD1, TIM3, LAG3, BTLA, CD160, 2B4, and CD39a are measured both in CD4 and CD8 T cell populations. Since chronic T cell activation has been shown to result in T cell exhaustion, T cells cultured under the continuous presence of ligand throughout the experiment are expected to be positive for multiple markers of exhaustion. T cells cultured in the absence of ligand throughout the experiment are expected to be negative for multiple markers of exhaustion, since the expression of the CAR will be undetectable/low in the absence of ligand. At day 10, cells where the ligand is removed at day 7 are expected to have a lower percentage of cells that are positive for multiple exhaustion markers when compared to cells that have been treated with ligand throughout the duration of the experiment. This result is expected in both CD8 and CD4 population of T cells.

[00573] T cells are also analyzed for proliferation, markers of apoptosis and memory phenotype. Cells are cultured per methods described above. Ligand is removed either at day 7 or day 10 and cells are analyzed at day 14 for apoptosis using Annexin V staining and for the memory phenotype using CD62L as the marker. When compared to untreated cells, cells continuously treated with ligand are expected to show an increase in apoptotic cells, a decrease in proliferation and a decrease in CD62L expression, which are indicative of T cell exhaustion. Ligand withdrawal at day 7 or day 10 is expected to show low Annexin V and high CD62L expression, and an increase in proliferation similar to untreated cells.

Example 23. Functional analysis of reversal of T cell exhaustion

[00574] The functionality of T cells expressing DD regulated CAR constructs is evaluated by measuring cytokine release. T cells expressing DD CAR constructs are co cultured with target cells expressing the antigen (endogenously or ectopically). T cells are activated at day 0 using CD3/CD28 beads. 24 hours later, cells are transduced with DD regulated CAR constructs and allowed to rest for 24 hours. Cells are then treated with the ligand specific to the SRE or vehicle control. At day 4, the CD3/CD28 beads are removed and the cells are cultured for another 3 days. At day 7, cells are washed extensively to remove the ligand and replated in the absence of the ligand and cultured for 3 days. In an additional experimental condition, cells are treated with ligand till day 10 instead of day 7. T cells cultured for the duration of the experiment either in the presence or absence of ligand are included as controls. Supernatant was collected from cells after 24 hours and IL2 and IFNg levels were measured as a read out of T cell function. When compared to untreated cells, cells continuously treated with ligand are expected to show an increase in IL2 and IFNg expression at day 14, which are indicative of functional T cells. Ligand withdrawal at day 7 or day 10 is expected to minimally affect the levels of IFNg and IL2 levels.

[00575] The ability of biocircuits of the invention to functionally rescue the exhausted T cells is also evaluated. T cells treated with ligand till day 10 are sorted and selected for cells that are positive for chimeric antigen receptor as well as multiple exhaustion makers (e.g. PD1, TIM3, LAG3). The cells are divided into two groups, one group is treated with ligand, while the second group is treated with vehicle control. Functionality of T cells is measured using IL2 levels as a surrogate. Ligand withdrawal is predicted to reverse T cell exhaustion, and hence cells subject to ligand withdrawal at day 10 are expected to have higher IL2 levels compared to ligand treated cells.

Example 24. Optimizing biocircuit behavior

[00576] The biocircuits of the invention comprise multiple modules which can be optimized. Libraries of each of the components is generated to allow for the rapid generation of new constructs with desired behaviors. Ligand pharmacokinetics is a powerful tool for payload specific tuning *in vivo,* which can be used to shift the ligand response curve of the effector module to the left or right depending on the modulating factors. Several modulating factors are tested, including, but not limited to the ligand dose, concentrations, magnitude, duration, and route of administration. Destabilizing domains can also be modified to improve biocircuit behavior. The destabilizing domain is the core determinant of the dynamic range of the biocircuit. Depending on the DD selected, the ligand response curve of the effector module can

be shifted up or down. The nature, position of the DD within the effector module as well as the number of DDs within an effector module are modified. DD selection is also altered depending on its degradation kinetics desired. Promoters that transcriptionally control the expression of the SREs are optimized. Choice of promoter impacts the basal-off state and affects the dynamic range of stabilization. Further, promoter choice contributes to the extent of stabilized payload produced. Other optimizable elements of the biocircuits include vector, translational elements, leader sequence, placement of the components within the SRE, codon selection, protease sites, linkers, and mRNA stability.

[00577] While the present invention has been described at some length and with some particularity with respect to the several described embodiments, it is not intended that it should be limited to any such particulars or embodiments or any particular embodiment, but it is to be construed with references to the appended claims so as to provide the broadest possible interpretation of such claims in view of the prior art and, therefore, to effectively encompass the intended scope of the invention.

[00578] All publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present specification, including definitions, will control. In addition, section headings, the materials, methods, and examples are illustrative only and not intended to be limiting.

CLAIMS

1. A composition for inducing an immune response in a cell or a subject comprising a first effector module, said effector module comprising a first stimulus response element (SRE) operably linked to at least one immunotherapeutic agent, wherein said at least one immunotherapeutic agent is selected from a cytokine, a safety switch, a regulatory switch, a chimeric antigen receptor and combinations thereof.

2. The composition of claim 1, wherein said first SRE is responsive to or interacts with at least one stimulus.

3. The composition of claim 2, wherein said first SRE is a destabilizing domain (DD).

4. The composition of claim 3, wherein the DD is derived from a parent protein or a mutant protein having one, two, three or more amino acid mutations compared to said parent protein, wherein the parent protein is selected from:

(a) human protein FKBP comprising the amino acid sequence of SEQ ID NO. 3,
(b) human DHFR (hDHFR) comprising the amino acid sequence of SEQ ID NO. 1,
(c) E. coli DHFR (ecDHFR) comprising the amino acid sequence of SEQ ID NO. 2,
(d) PDE5 comprising the amino acid sequence of SEQ ID NO. 4,
(e) PPAR gamma comprising the amino acid sequence of SEQ ID NO. 5,
(f) CA2 comprising the amino acid sequence of SEQ ID NO. 6,
(g) NQO2 comprising the amino acid sequence of SEQ ID NO. 7, and
(h) human DPPIV comprising the amino acid sequence of SEQ ID NO. 224.

5. The composition of claim 4, wherein the parent protein is hDHFR and the DD comprises a mutant protein having at least one mutation selected from M1del, V2A, C7R, I8V, V9A, A10T, A10V, Q13R, N14S, G16S, I17N, I17V, K19E, N20D, G21T, G21E, D22S, L23S, P24S, L28P, N30D, N30H, N30S, E31G, E31D, F32M, R33G, R33S, F35L, Q36R, Q36S, Q36K, Q36F, R37G, M38V, M38T, T40A, V44A, K47R, N49S, N49D, M53T, G54R, K56E, K56R, T57A, F59S, I61T, K64R, N65A, N65S, N65D, N65F, L68S, K69E, K69R, R71G, I72T, I72A, I72V, N73G, L74N, V75F, R78G, L80P, K81R, E82G, H88Y, F89L, R92G, S93G, S93R, L94A, D96G, A97T, L98S, K99G, K99R, L100P, E102G, Q103R, P104S, E105G, A107T, A107V, N108D, K109E, K109R, V110A, D111N, M112T, M112V, V113A, W114R, I115V, V116I,

G117D, V121A, Y122C, Y122D, Y122I, K123R, K123E, A125F, M126I, N127R, N127S, N127Y, H128R, H128Y, H131R, L132P, K133E, L134P, F135P, F135L, F135S, F135V, V136M, T137R, R138G, R138I, I139T, I139V, M140I, M140V, Q141R, D142G, F143S, F143L, E144G, D146G, T147A, F148S, F148L, F149L, P150L, E151G, I152V, D153A, D153G, E155G, K156R, Y157R, Y157C, K158E, K158R, L159P, L160P, E162G, Y163C, V166A, S168C, D169G, V170A, Q171R, E172G, E173G, E173A, K174R, I176A, I176F, I176T, K177E, K177R, Y178C, Y178H, F180L, E181G, V182A, Y183C, Y183H, E184R, E184G, K185R, K185del, K185E, N186S, N186D, D187G, and D187N.

6. The composition of claim 5, wherein the stimulus is selected from Trimethoprim (TMP) and Methotrexate (MTX).

7. The composition of claim 1, wherein the immunotherapeutic agent is a cytokine.

8. The composition of claim 7, wherein the cytokine is an interleukin, an interferon, a tumor necrosis factor, a transforming growth factor B, a CC chemokine, a CXC chemokine, a CX3C chemokine or a growth factor.

9. The composition of claim 8, wherein the cytokine is an interleukin and the interleukin is selected from a group consisting of IL1, IL1-alpha, IL1-beta, IL1-delta, IL1-epsilon, IL1-eta, IL1-zeta, IL-RA, IL2, IL3, IL4, IL5, IL6, IL7, IL8, IL9, IL10, IL10C, IL10D, IL11a, IL11b, IL13, IL14, IL16, IL17, IL-17A, IL17B, IL17C, IL17E, IL17F, IL18, IL19, IL20, IL20L, IL21, IL22, IL23, IL23A, IL24, IL25, IL26, IL27, IL28, IL29, IL30, IL31, IL32, IL33, IL34, IL36α, IL36β, IL36γ, IL36RN, IL37, IL37a, IL37b, IL37c, Il37d, IL37e, and IL38.

10. The composition of claim 9, wherein the interleukin is IL2, comprising the amino acid sequence of SEQ ID NO. 51.

11. The composition of claim 1, wherein the immunotherapeutic agent is a safety switch.

12. The composition of claim 11, wherein the safety switch is selected from a Caspase 9, an inducible FAS (iFAS), an inducible caspase 9 (icasp9), a CD20/anti-CD20 antibody pair, a protein tag/anti-tag antibody, and a compact suicide gene (RQR8).

13. The composition of claim 12, wherein the safety switch is a Caspase 9 comprising the amino acid sequence of SEQ ID NO. 65.

14. The composition of claim 1, wherein the immunotherapeutic agent encodes a regulatory switch.

15. The composition of claim 14, wherein the regulatory switch is selected from a FOXP3, a Nr4a, a FOXO, and a NF-κB.

16. The composition of claim 15, wherein the regulatory switch is a FOXP3, comprising the amino acid sequence of SEQ ID NO. 103-106.

17. The composition of claim 1, wherein the immunotherapeutic agent is a chimeric antigen receptor (CAR) and is selected from a GD2 CAR, a Her2 CAR, a BCMA CAR, a CD33 CAR, an ALK CAR, a CD22 CAR, and a CD276 CAR, each of which comprises an extracellular moiety, a transmembrane domain, an intracellular signaling domain, and optionally, one or more co-stimulatory domains.

18. The composition of claim 17, wherein the CAR is designed as a standard CAR, a split CAR, an off-switch CAR, an on-switch CAR, a first-generation CAR, a second-generation CAR, a third-generation CAR, or a fourth-generation CAR.

19. The composition of claim 18, wherein the extracellular target moiety is selected from any of:

i. an Ig NAR,
ii. a Fab fragment,
iii. a Fab' fragment,
iv. a F(ab)'2 fragment,
v. a F(ab)'3 fragment,
vi. an Fv,
vii. a single chain variable fragment (scFv),
viii. a bis-scFv, a (scFv)2,
ix. a minibody,
x. a diabody,
xi. a triabody,

xii. a tetrabody,

xiii. an intrabody,

xiv. a disulfide stabilized Fv protein (dsFv),

xv. a unibody,

xvi. a nanobody, and

xvii. an antigen binding region derived from an antibody that specifically binds to any of a protein of interest, a ligand, a receptor, a receptor fragment or a peptide aptamer.

20. The composition of claim 17, wherein the extracellular target moiety is selected from an ALK target moiety, comprising the amino acid sequence of SEQ ID NO. 242- 257 and 422-429, a CD22 target moiety, comprising the amino acid sequence of SEQ ID NO.258-262 and 430-432, a CD276 target moiety, comprising the amino acid sequence of SEQ ID NO. 263-270 and 433-436, a GD2 target moiety, comprising the amino acid sequence of SEQ ID NO.271-349 and 437-465, a CD33 target moiety, comprising the amino acid sequence of SEQ ID NO. 350-357, a BCMA target moiety, comprising the amino acid sequence of SEQ ID NO. 358-365, and a Her2 target moiety, comprising the amino acid sequence of SEQ ID NO. 366-421 and 466-473.

21. The composition of claim 17, wherein

(a) the intracellular signaling domain of the CAR is the signaling domain derived from T cell receptor CD3zeta or a cell surface molecule selected from the group consisting of FcR gamma, FcR beta, CD3 gamma, CD3 delta, CD3 epsilon, CD5, CD22, CD79a, CD79b, and CD66d; and

(b) the co-stimulatory domain is present and is selected from the group consisting of 2B4, HVEM, ICOS, LAG3, DAP10, DAP12, CD27, CD28, 4-1BB (CD137), OX40 (CD134), CD30, CD40, ICOS (CD278), glucocorticoid-induced tumor necrosis factor receptor (GITR), lymphocyte function-associated antigen- 1 (LFA-1), CD2, CD7, LIGHT, NKG2C, and B7-H3.

22. The composition of claim 17, wherein the transmembrane domain comprises the amino acid sequence of SEQ ID NO. 527-624.

23. The composition of claim 17, wherein the transmembrane domain further comprises a hinge region comprising the amino acid sequence of SEQ ID NO.628-694.

24. The composition of any of claims 1-23 wherein said first effector module comprises one or more of:

(a) an IL2-DD, comprising the amino acid sequence of any of SEQ ID NOs. 52-54,

(b) a Caspase 9-DD, comprising the amino acid sequence of any of SEQ ID NOs. 72-80,

(c) a FOXP3-DD, comprising the amino acid sequence of any of SEQ ID NOs. 107-116,

(d) a BCMA CAR-DD, comprising the amino acid sequence of any of SEQ ID NOs. 775-777, and

(e) a HER2-DD, comprising the amino acid sequence of SEQ ID NO. 906.

25. A polynucleotide encoding any of the compositions of claims 1-24, wherein said at least one immunotherapeutic agent is selected from a cytokine, a safety switch, a regulatory switch, a chimeric antigen receptor and the combination thereof.

26. The polynucleotide of claim 25, wherein the polynucleotide is a DNA molecule, or a RNA molecule.

27. The polynucleotide of claim 26, wherein the polynucleotide is RNA and said RNA is a messenger RNA.

28. The polynucleotide of claim 27, which is chemically modified.

29. The polynucleotide of claim 26, which comprises spatiotemporally selected codons.

30. The polynucleotide of claim 27, wherein the polynucleotide encodes at least one additional feature selected from a promoter, a linker, a signal peptide, a tag, a cleavage site and a targeting peptide.

31. The polynucleotide of claim 25, wherein the chimeric antigen receptor is selected from a GD2 CAR, a Her2 CAR, a BCMA CAR, a CD33 CAR, an ALK CAR, CD22 CAR, and a CD276 CAR.

32. A vector comprising a polynucleotide of any of claims 25-31 wherein said at least one immunotherapeutic agent is selected from a cytokine, a safety switch, a regulatory switch, a chimeric antigen receptor and the combination thereof.

33. The vector of claim 32, wherein the vector is a viral vector, or a plasmid.

34. The vector of claim 33, wherein the vector is a viral vector and said viral vector is a retroviral vector, a lentiviral vector, a gamma retroviral vector, a recombinant AAV vector, an adeno viral vector, or an oncolytic viral vector.

35. The vector of claim 34, wherein the polynucleotide encodes any of the compositions of claim 1-24.

36. An immune cell for adoptive cell transfer (ACT), which expresses any of the compositions of any of claims 1-24, the polynucleotides of any of claims 25-31, and/or is infected or transfected with the vector of any of claims 32-35.

37. The immune cell of claim 36, wherein the immune cell is a CD8+ T cell, a CD4+ T cell, a helper T cell, a natural killer (NK) cell, a NKT cell, a cytotoxic T lymphocyte (CTL), a tumor infiltrating lymphocyte (TIL), a memory T cell, a regulatory T (Treg) cell, a cytokine-induced killer (CIK) cell, a dendritic cell, a human embryonic stem cell, a mesenchymal stem cell, a hematopoietic stem cell, or a mixture thereof.

38. The immune cell of claim 36, wherein the SRE is a destabilizing domain DD, wherein the DD is derived from human protein FKBP comprising the amino acid sequence of SEQ ID NO. 3, DHFR comprising the amino acid sequence of SEQ ID NO. 1-2, PDE5 comprising the amino acid sequence of SEQ ID NO. 4, PPAR gamma comprising the amino acid sequence of SEQ ID NO. 5, CA2 comprising the amino acid sequence of SEQ ID NO. 6 and NQO2 comprising the amino acid sequence of SEQ ID NO. 7.

39. The immune cell of claim 38, wherein the DD is derived from a parent protein and the parent protein is hDHFR and the DD comprises a mutant protein having at least one mutation selected from M1del, V2A, C7R, I8V, V9A, A10T, A10V, Q13R, N14S, G16S, I17N, I17V, K19E, N20D, G21T, G21E, D22S, L23S, P24S, L28P, N30D, N30H, N30S, E31G, E31D, F32M, R33G, R33S, F35L, Q36R, Q36S, Q36K, Q36F, R37G, M38V, M38T, T40A, V44A, K47R, N49S, N49D, M53T, G54R, K56E, K56R, T57A, F59S, I61T, K64R, N65A, N65S, N65D, N65F, L68S, K69E, K69R, R71G, I72T, I72A, I72V, N73G, L74N, V75F, R78G, L80P, K81R,

E82G, H88Y, F89L, R92G, S93G, S93R, L94A, D96G, A97T, L98S, K99G, K99R, L100P, E102G, Q103R, P104S, E105G, A107T, A107V, N108D, K109E, K109R, V110A, D111N, M112T, M112V, V113A, W114R, I115V, V116I, G117D, V121A, Y122C, Y122D, Y122I, K123R, K123E, A125F, M126I, N127R, N127S, N127Y, H128R, H128Y, H131R, L132P, K133E, L134P, F135P, F135L, F135S, F135V, V136M, T137R, R138G, R138I, I139T, I139V, M140I, M140V, Q141R, D142G, F143S, F143L, E144G, D146G, T147A, F148S, F148L, F149L, P150L, E151G, I152V, D153A, D153G, E155G, K156R, Y157R, Y157C, K158E, K158R, L159P, L160P, E162G, Y163C, V166A, S168C, D169G, V170A, Q171R, E172G, E173G, E173A, K174R, I176A, I176F, I176T, K177E, K177R, Y178C, Y178H, F180L, E181G, V182A, Y183C, Y183H, E184R, E184G, K185R, K185del, K185E, N186S, N186D, D187G, and D187N.

40. The immune cell of claim 39, which is autologous, allogeneic, syngeneic, or xenogeneic in relation to a particular individual subject.

41. A method of reducing a tumor volume or burden in a subject comprising contacting the subject with compositions of any of claims 1-24, the polynucleotides of any of claims 25-31, the vector of any of claims 32-35or the immune cells of any of claims 36-40.

42. A method of providing an anti-tumor immune response in a subject comprising administering to the subject an effective amount of the compositions of any of claims 1-24, the polynucleotides of any of claims 25-31, the vector of any of claims 32-35 or the immune cells of any of claims 36-40.

43. A method of inducing an immune response in a subject comprising administering to the subject an effective amount of any of the compositions of claims 1-24, the polynucleotides of any of claims 25-31, the vector of any of claims 32-35 or the immune cells of any of claims 36-40.

44. A method of preventing or reversing T cell exhaustion in a subject in need thereof, the method comprising administering to the subject, a therapeutically effective amount of compositions of any of claims 1-24, the polynucleotides of any of claims 25-31, the vector of any of claims 32-35 or the immune cells of any of claims 36-40, wherein the SRE responds to a

stimulus and tunes the expression and/or function of the immunotherapeutic agent, thereby preventing or reversing T cell exhaustion.

45. The method of claim 44, wherein the immunotherapeutic agent is a chimeric antigen receptor.

46. The method of claim 45, wherein the chimeric antigen receptor is a GD2 CAR, BCMA CAR, CD33 CAR, Her2 CAR, ALK CAR, CD22 CAR, or a CD276 CAR.

47. A method of detecting the presence of cancer in a mammal, comprising the steps of:

(a) contacting a sample comprising one or more cells from the mammal with the compositions of any of claims 1-24, the polynucleotides of any of claims 25-31, the vector of any of claims 32-35 or the immune cells of any of claims 36-40 and

(b) detecting the complex, wherein the detection of the complex is indicative of the presence of cancer in the mammal.

Figure 1

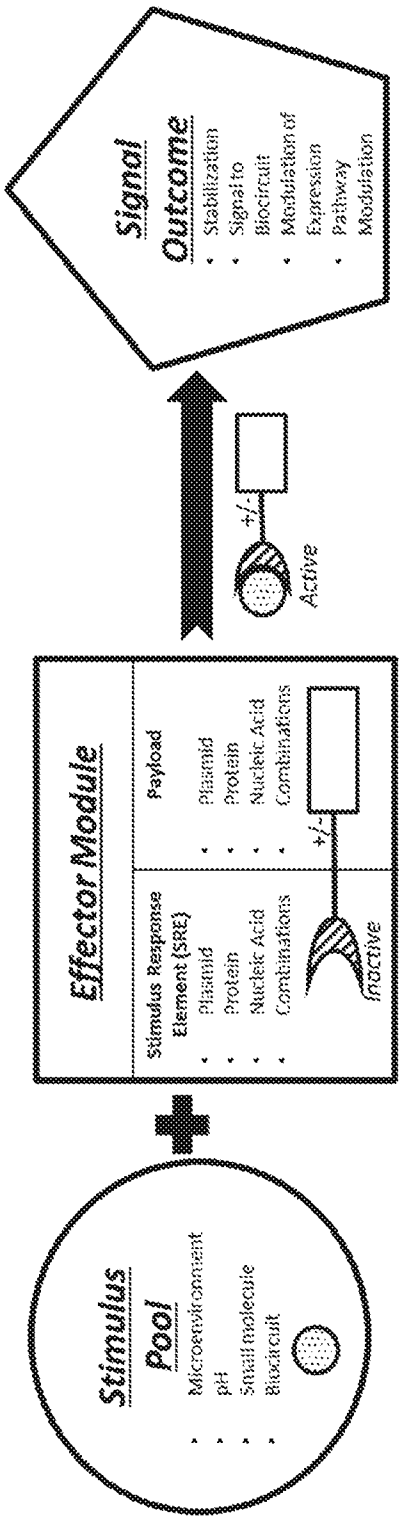

Signal Outcome
Stabilization
Signal to Biocircuit
Modulation of Expression
Pathway Modulation
+/-
Active
Effector Module
Stimulus Response Element (SRE)
Plasmid
Protein
Nucleic Acid
Combinations
Payload
Plasmid
Protein
Nucleic Acid
Combinations
+/-
Inactive
Stimulus Pool
Microenvironment
pH
Small molecule
Biocircuit

Figure 2

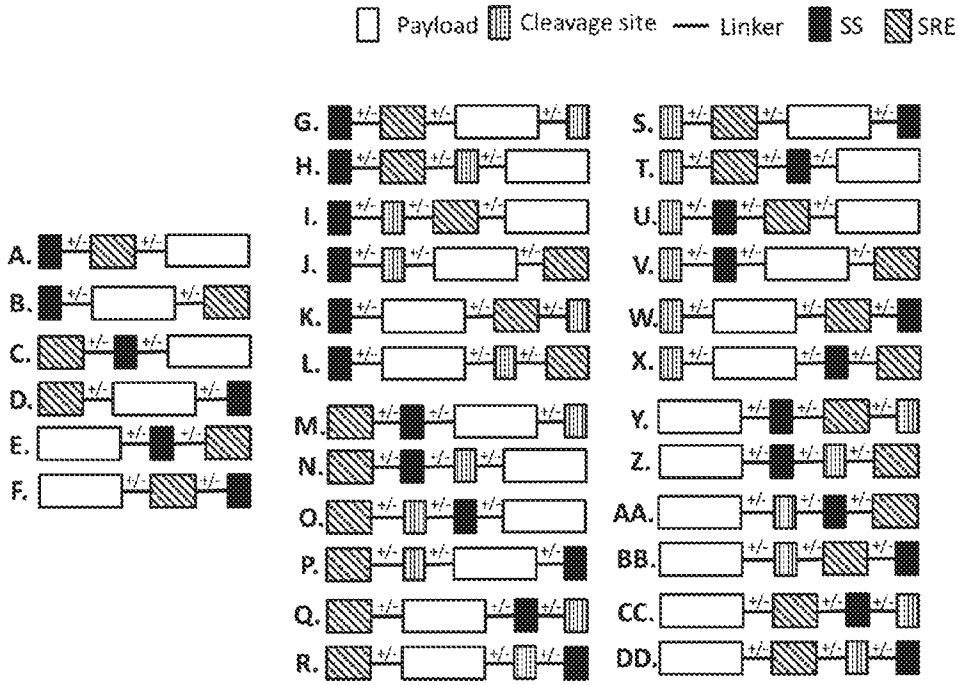


Figure 3

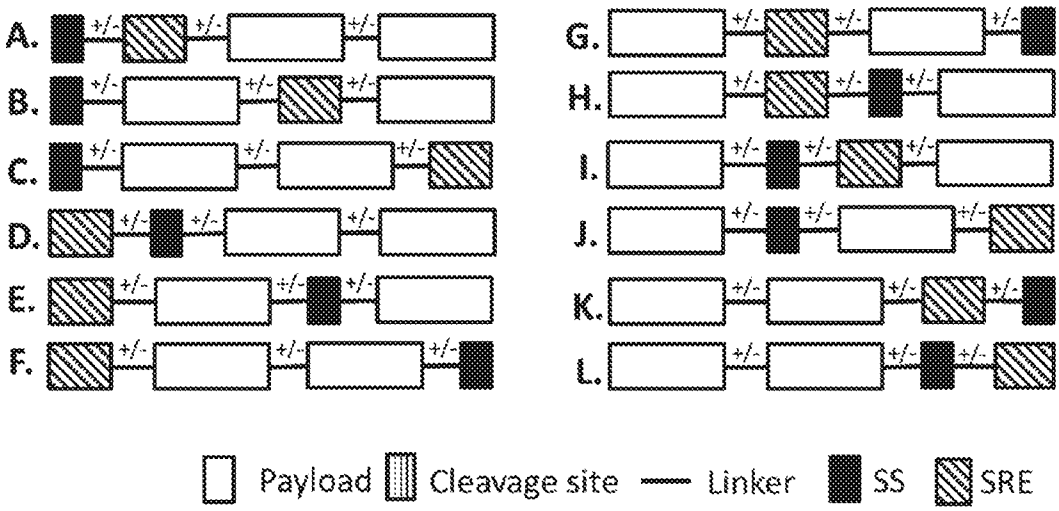


Figure 4

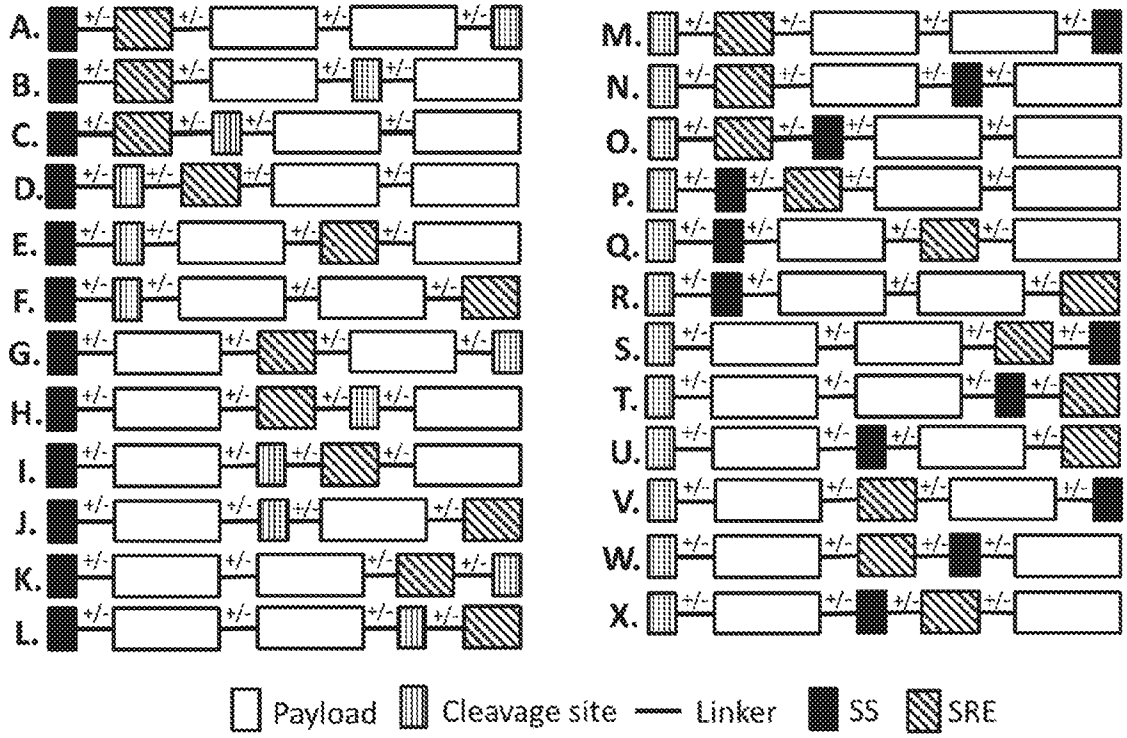


Figure 5

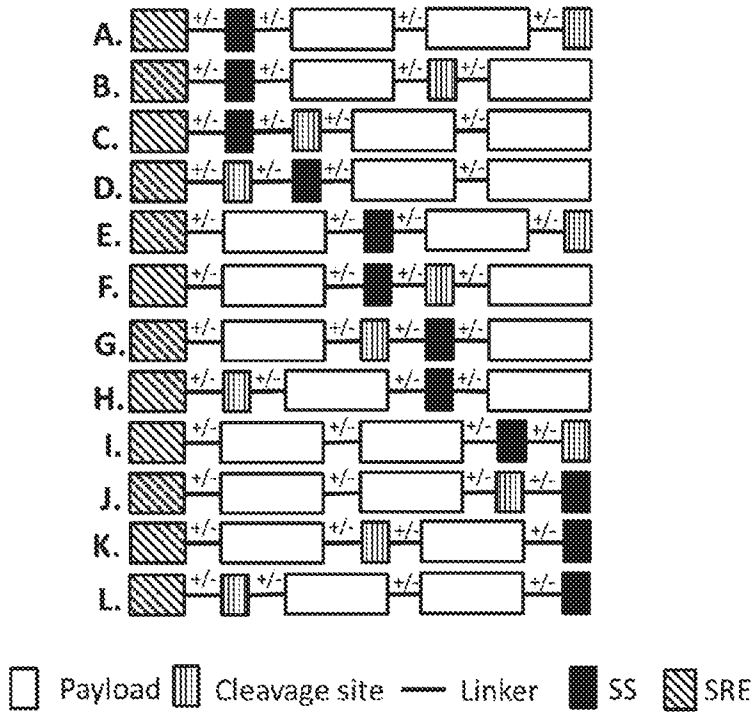


Figure 6

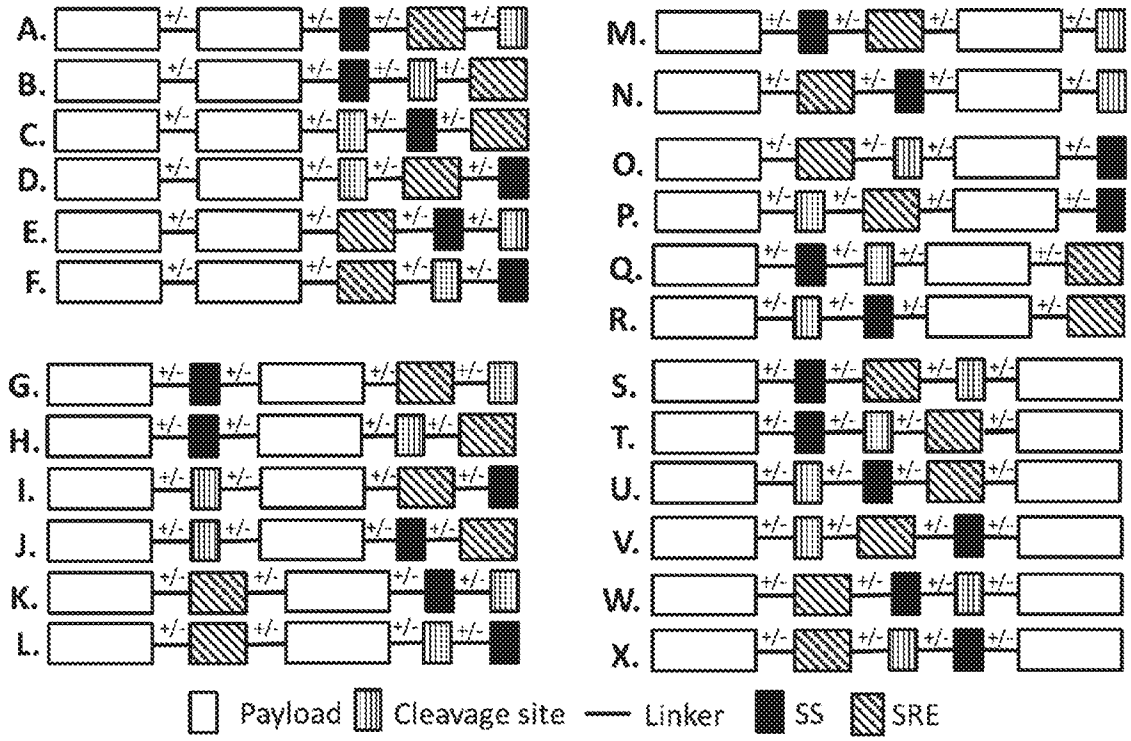


Figure 7

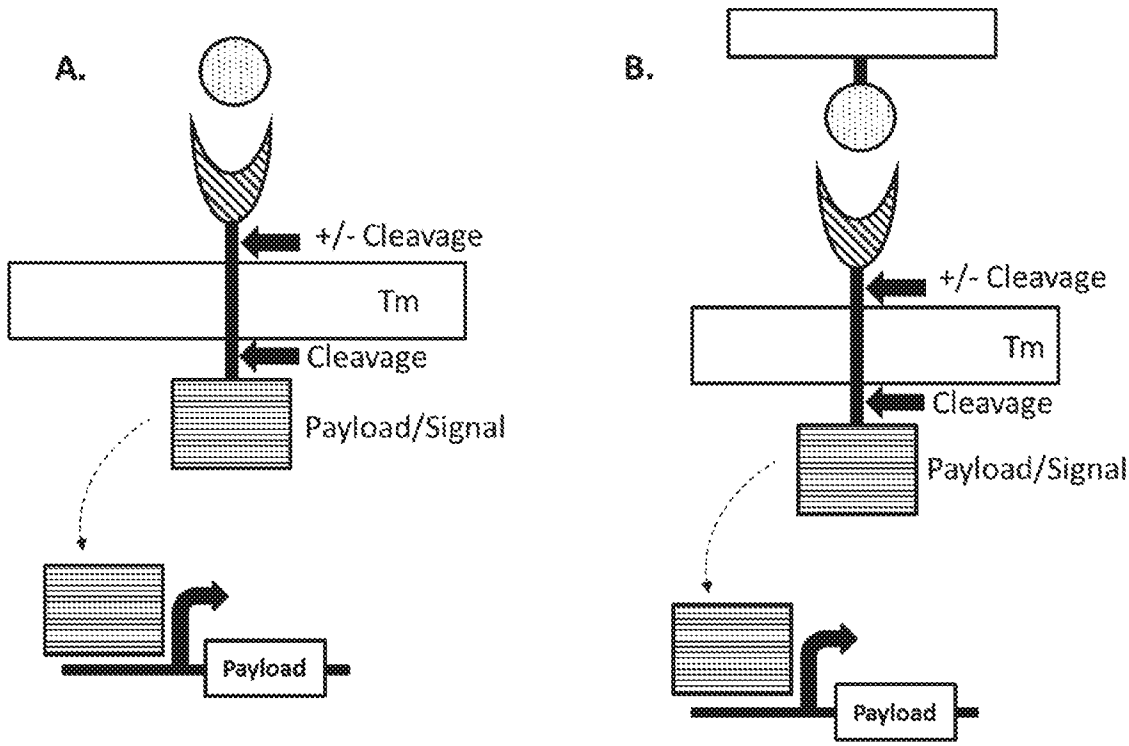


Figure 8

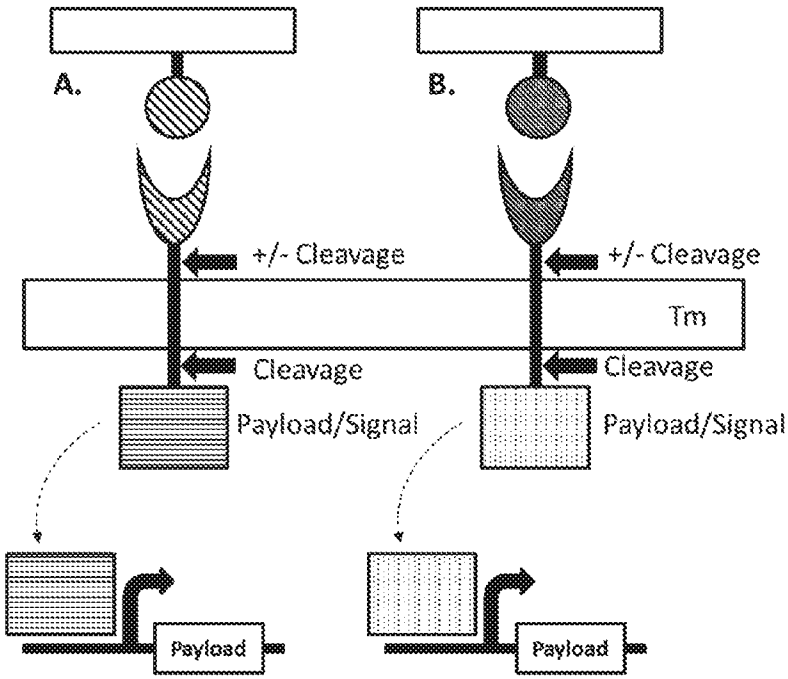


Figure 9

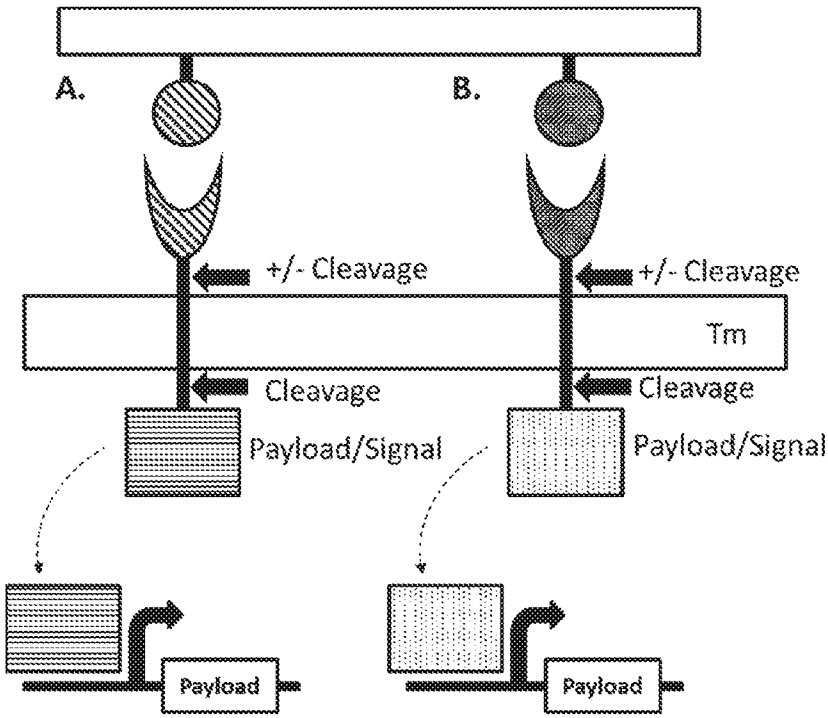


Figure 10

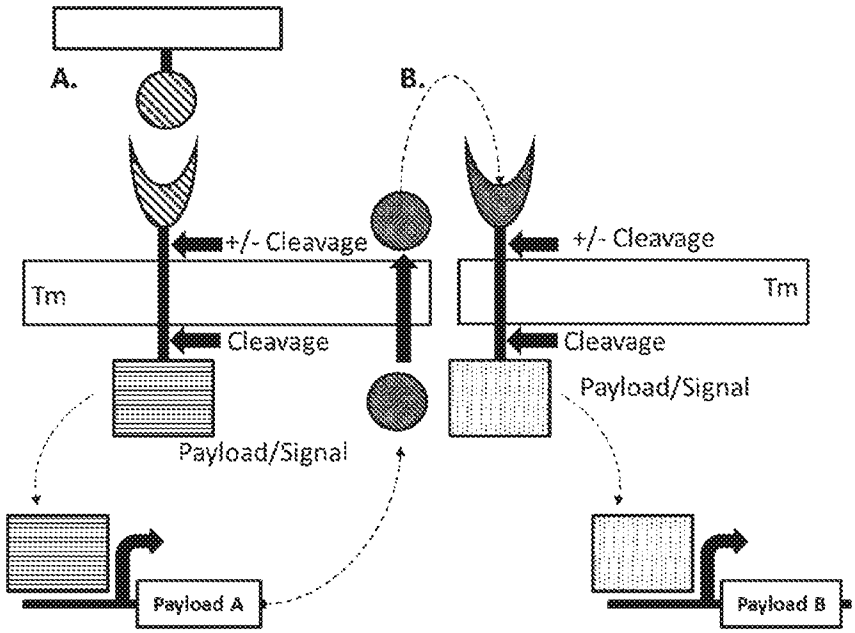


Figure 11

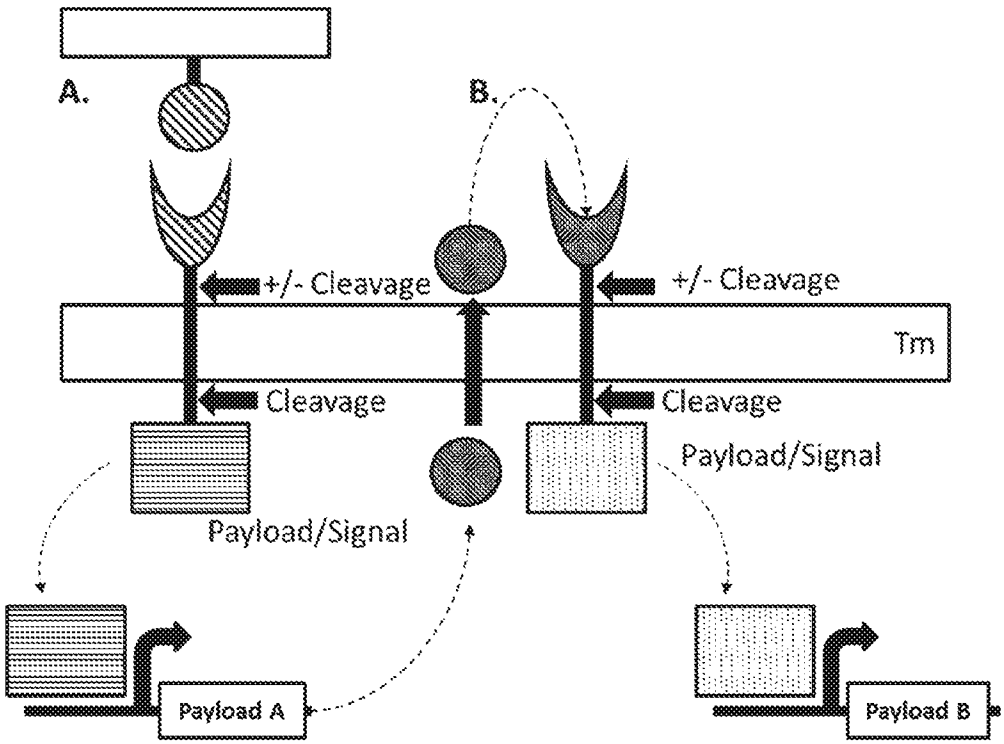


Figure 12

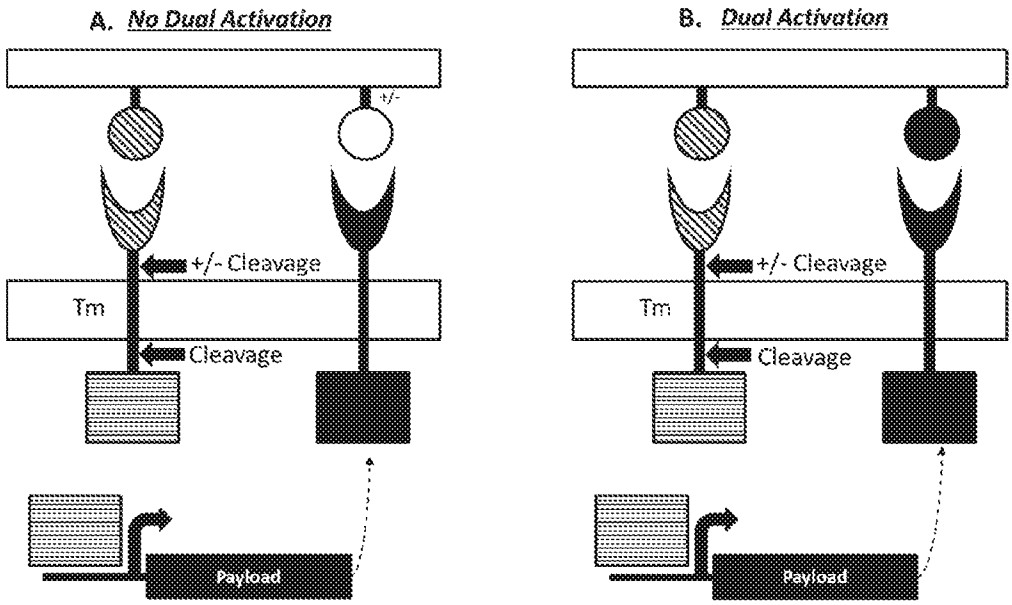


Figure 13

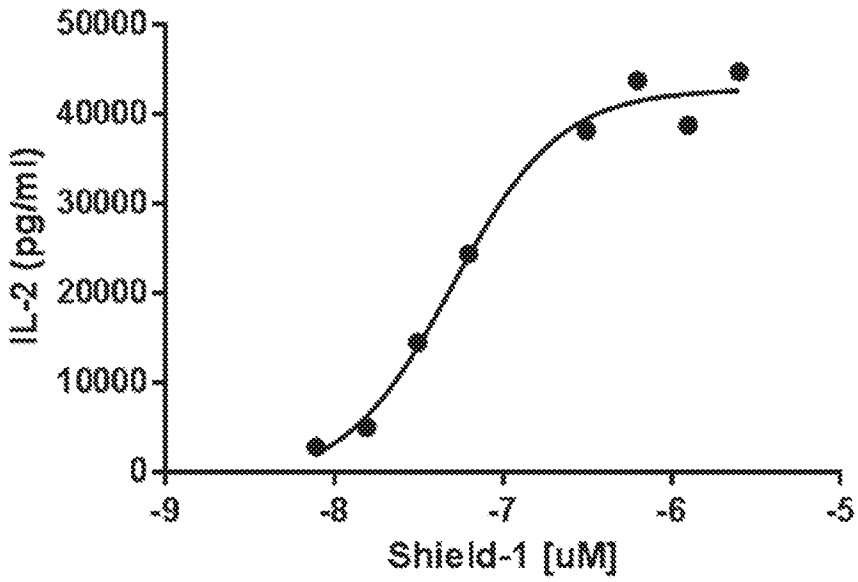


Figure 14

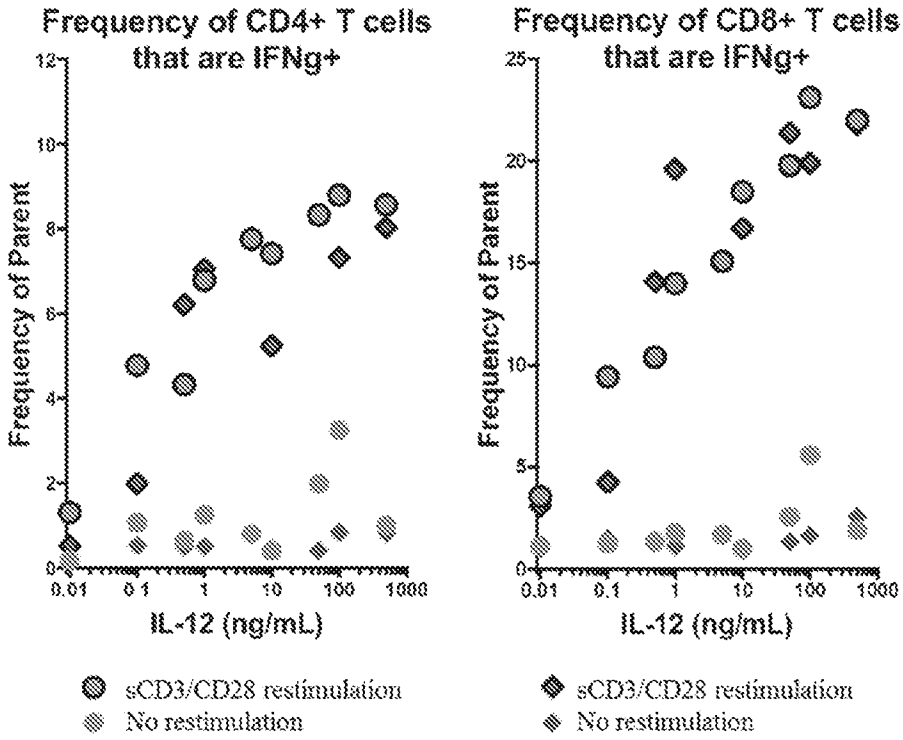
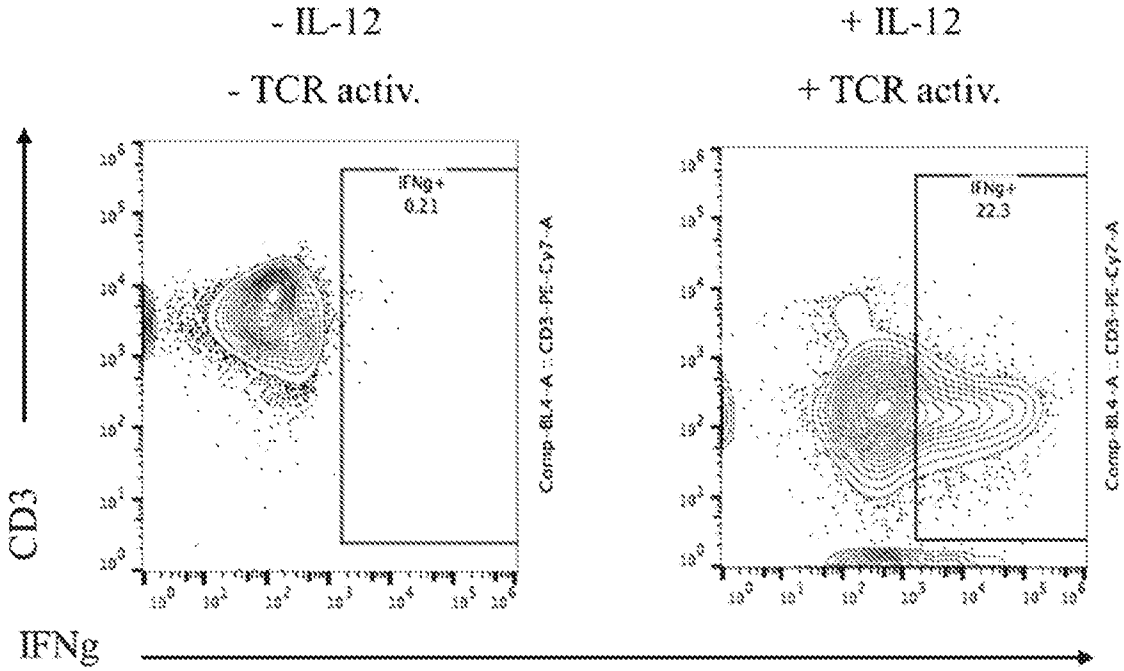


Figure 15A



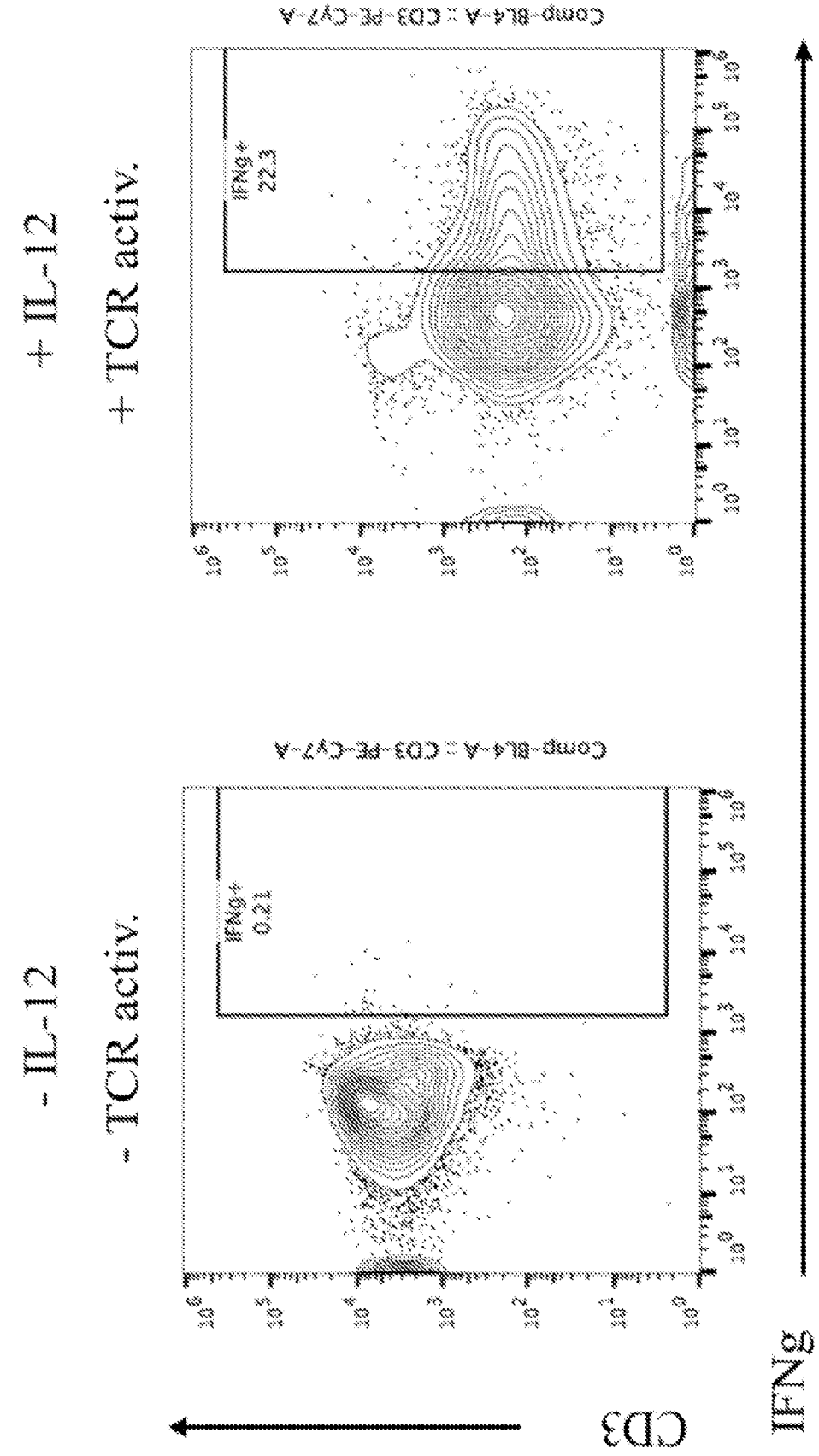

- IL-12
- TCR activ.
+ IL-12
+ TCR activ.
IFNg+
0.21
IFNg+
22.3
Comp-BL4-A :: CD3-PE-Cy7-A
Comp-BL4-A :: CD3-PE-Cy7-A
CD3
IFNg

Figure 15A

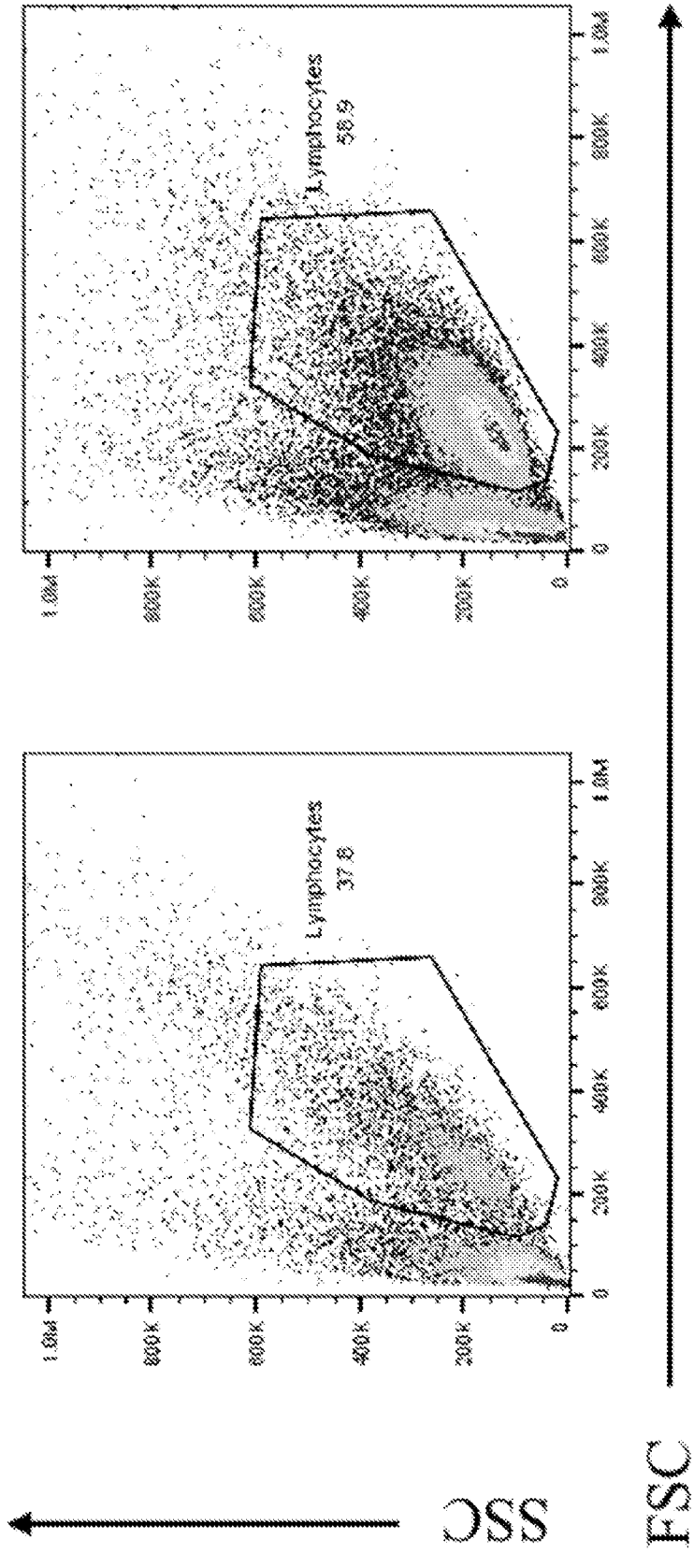

-IL-15/IL15Ra
+IL-15/IL15Ra
Lymphocytes
37.8
Lymphocytes
38.9
SSC
FSC

Figure 15B

Figure 15C

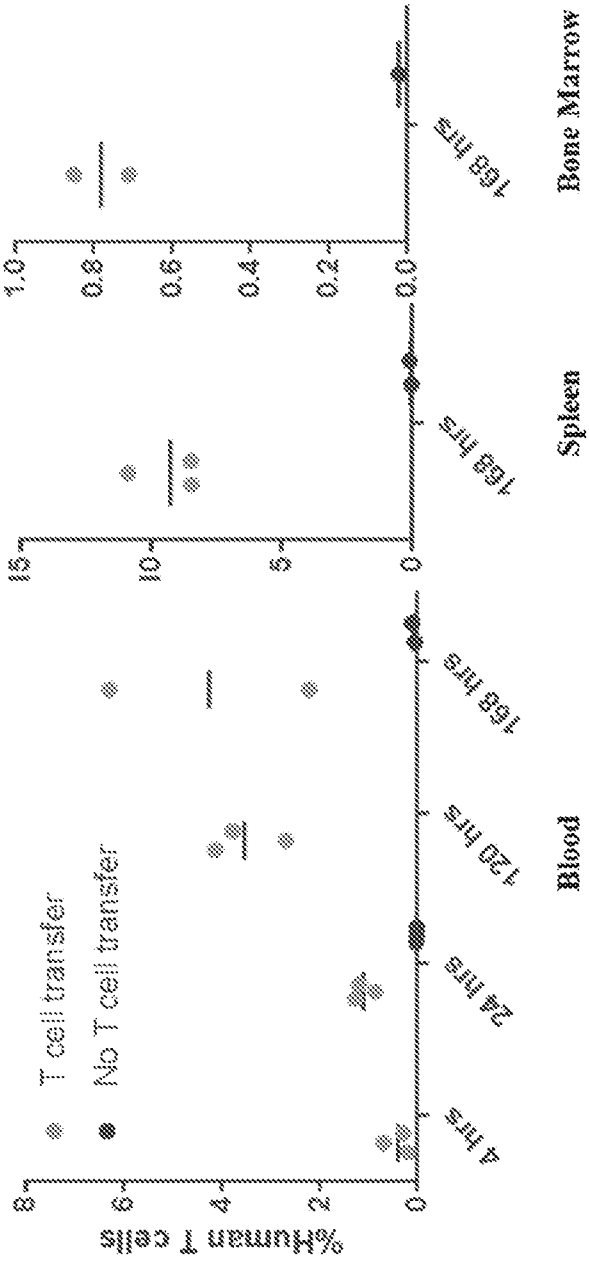

CD3+ CD45+ cells
%Human T cells
T cell transfer
No T cell transfer
Blood
Spleen
Bone Marrow
4 hrs
24 hrs
120 hrs
168 hrs

Figure 15D

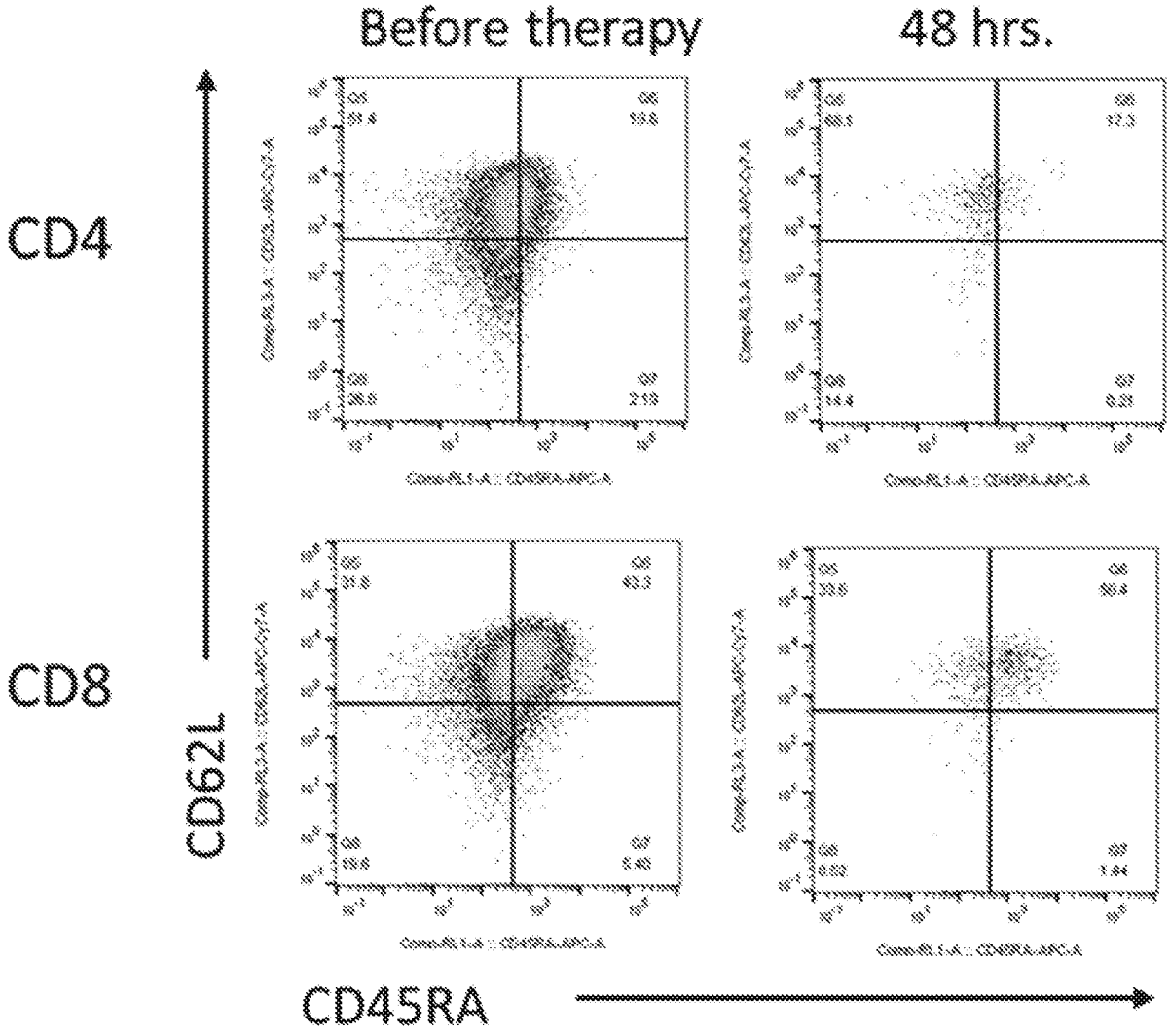


Figure 16A

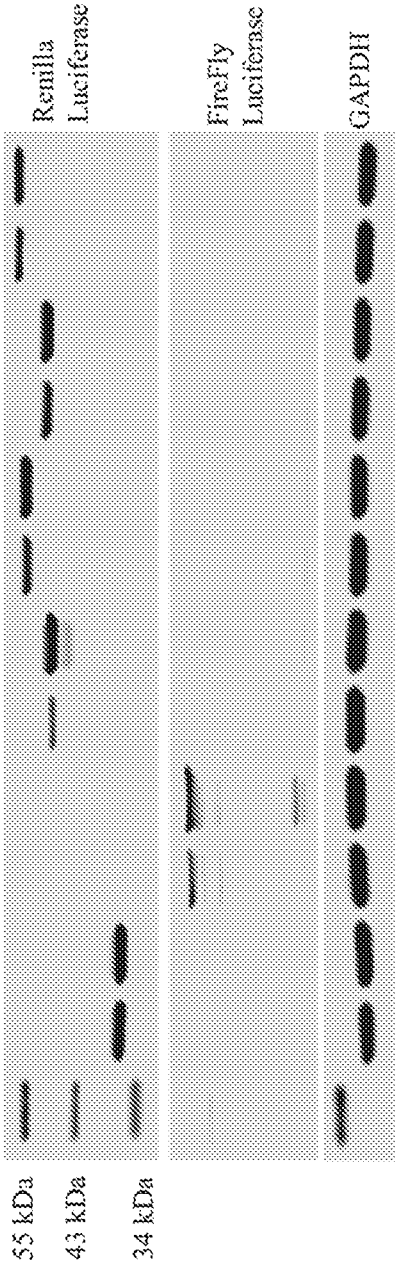

Marker
OT-RLuc-001 Ligand
OT-FLuc-002 Shield-1
OT-RLuc-003 Shield-1
OT-RLuc-004 TMP
OT-RLuc-005 Shield-1
OT-RLuc-006 TMP
55 kDa
43 kDa
34 kDa
Renilla Luciferase
FireFly Luciferase
GAPDH

Figure 16B

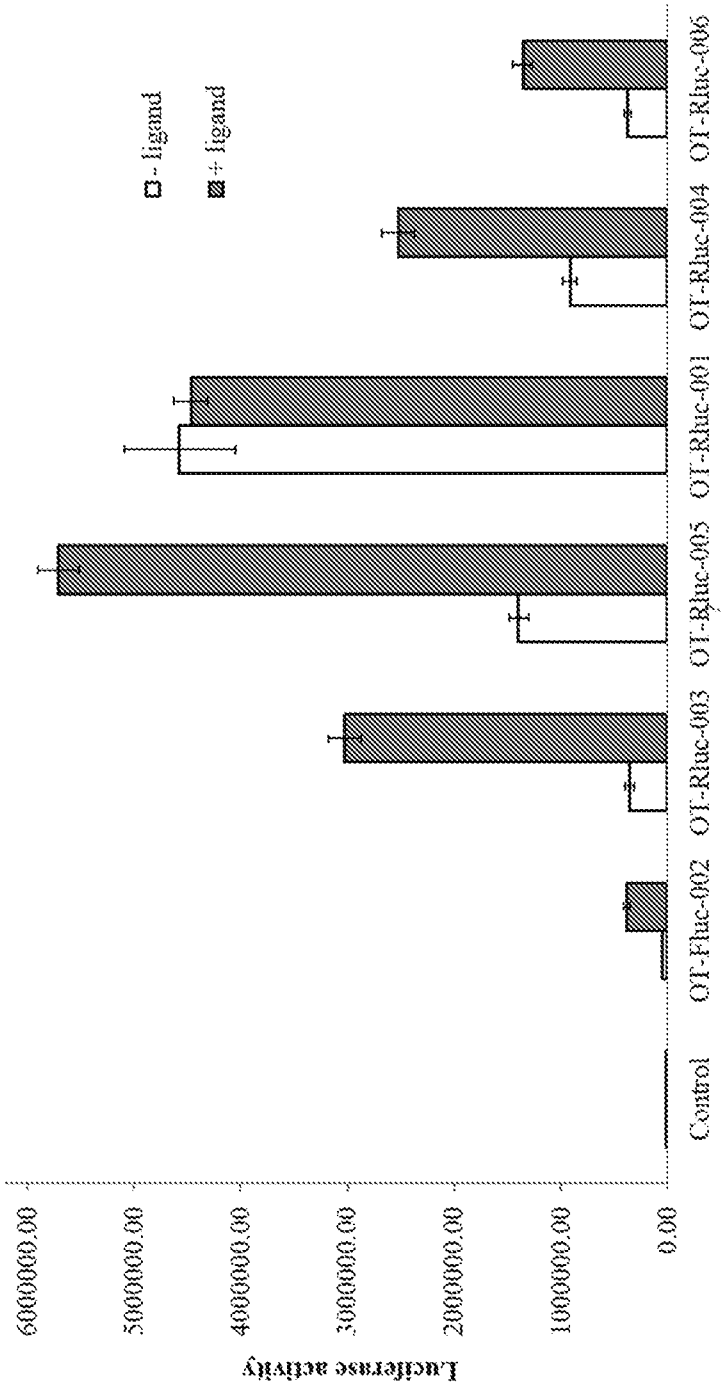

Luciferase activity
6000000.00
5000000.00
4000000.00
3000000.00
2000000.00
1000000.00
0.00
- ligand
+ ligand
Control
OT-Fluc-002
OT-Rluc-003
OT-Rluc-005
OT-Rluc-001
OT-Rluc-004
OT-Rluc-006

Figure 17A

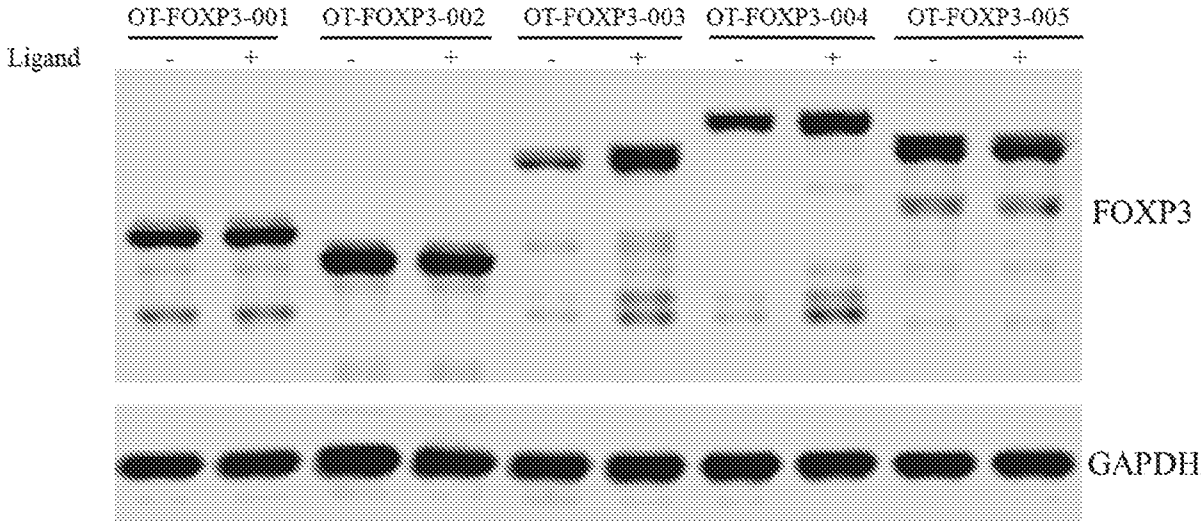


Figure 17B

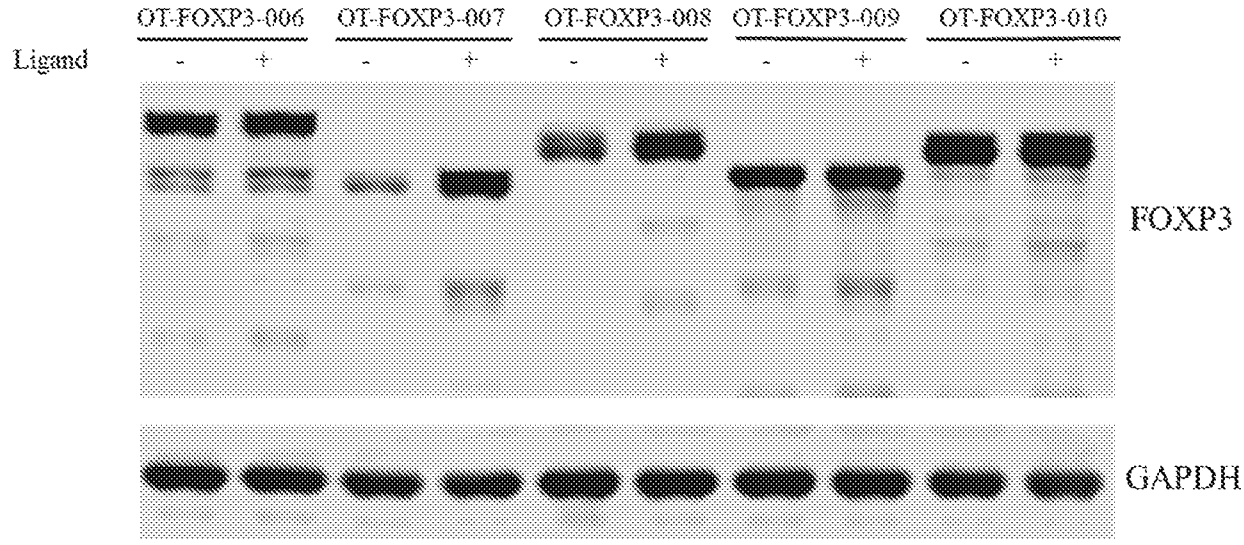


Figure 18

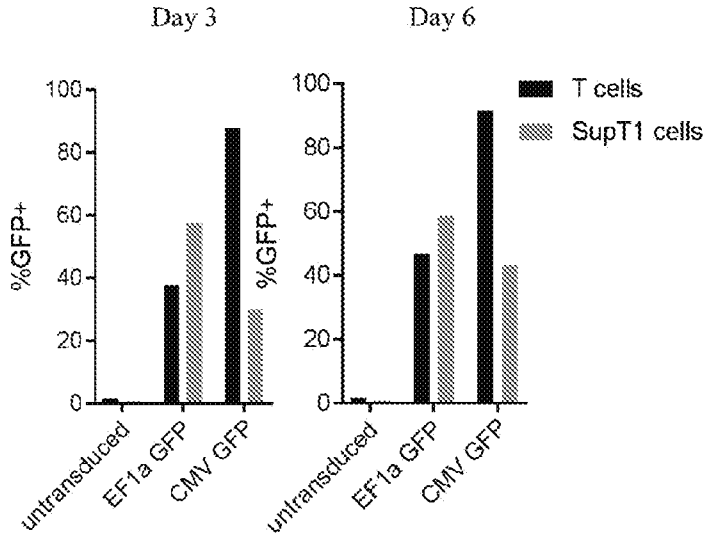


Figure 19

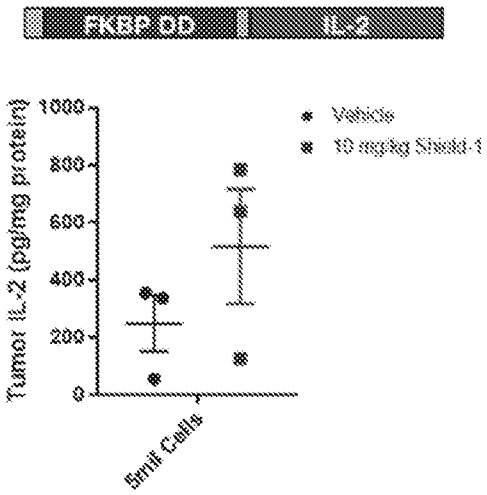


Figure 20

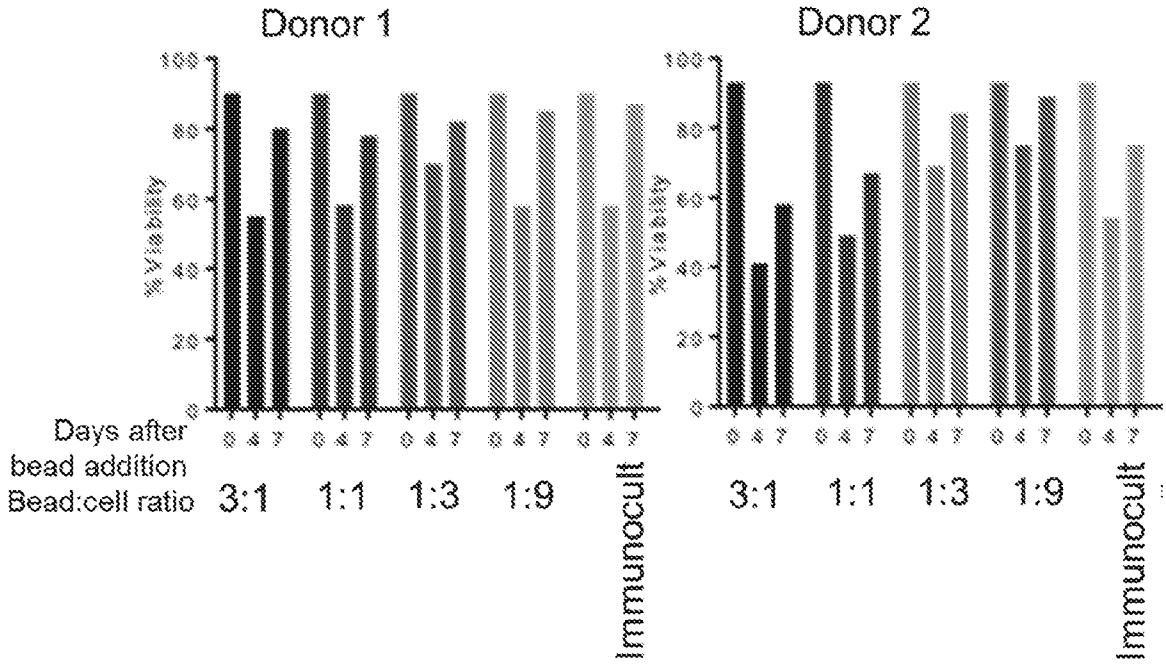
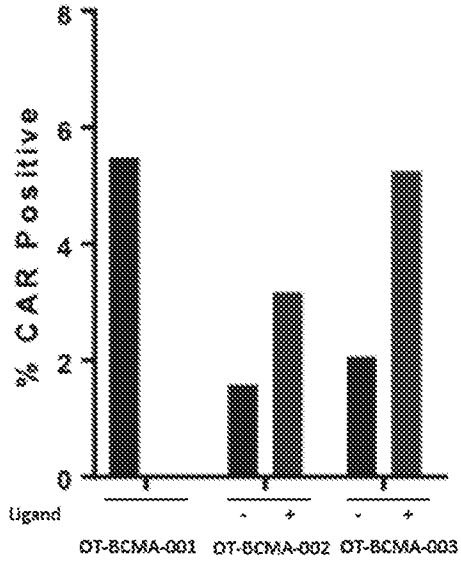


Figure 21



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/20704

A. CLASSIFICATION OF SUBJECT MATTER

IPC - A61P 37/02; C07K 14/54, 14/55; A61K 39/39 (2018.01)
CPC - A61P 37/02; C07K 14/54, 14/55; A61K 39/39

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
See Search History document

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
See Search History document

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X -- Y	US 2014/0010791 A1 (THE BOARD OF TRUSTEES OF THE LELAND STANFORD JUNIOR UNIVERSITY) 09 January 2014; paragraphs [0174]-[0176], [0214], [0217]; figure 28	1-3, 7-8 ---------- 4, 9
Y	(NIH) FKBP1A protein [Homo sapiens]. National Center for Biotechnology Information. Sequence Accession AAI19733. 04 October 2006 [retrieved on 18 April 2018]. Retrieved from the Internet: < https://www.ncbi.nlm.nih.gov/protein/AAI19733>Genbank Supplement pages 1-2	4
Y	WO 2015/007542 A1 (VIB VZW et al.) 22 January 2015; page 5, paragraph 4	9
P, X	WO 2017/180587 A2 (OBSIDIAN THERAPEUTICS, INC.) 19 October 2017; whole document	1-4, 7-9

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier application or patent but published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
29 May 2018 (29.05.2018)	13 JUN 2018
Name and mailing address of the ISA/ Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-8300	Authorized officer Shane Thomas PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (January 2015)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/20704

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 25-47
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:
-***- Please See Within the Next Supplemental Page-***-

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:
Group 1+ Claims 1-4, 7-9

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US18/20704

-***-Continued from Box III Observations where unity of invention is lacking -***-

This application contains the following inventions or groups of inventions which are not so linked as to form a single general inventive concept under PCT Rule 13.1. In order for all inventions to be examined, the appropriate additional examination fees must be paid.

Groups I+, Claims 1-36, an IL-1 cytokine (immunotherapeutic agent) and a destabilizing domain encompassing human FKBP (SEQ ID NO: 3) (SRE) are directed toward a composition for inducing an immune response in a cell or a subject comprising a first effector module, said effector module comprising a first stimulus response element (SRE) operably linked to at least one immunotherapeutic agent.

The composition and polynucleotide will be searched to the extent they encompass an IL-1 cytokine (immunotherapeutic agent), and a destabilizing domain encompassing human FKBP (SEQ ID NO: 3) (SRE). Applicant is invited to elect additional immunotherapeutic agent(s), and/or SRE(s), with, where applicable, specified SEQ ID NO: for each, and/or, where applicable, specified SEQ ID NO: for the construct, to be searched. Additional cytokine(s) and/or SRE(s), and sequence(s) associated therewith will be searched upon the payment of additional fees. It is believed that claims 1 (in-part), 2 (in-part), 3 (in-part), 4 (in-part), 7 (in-part), 8 (in-part), and 9 (in-part) encompass this first named invention and thus these claims will be searched without fee to the extent that they encompass an IL-1 cytokine (immunotherapeutic agent) and human FKBP (SEQ ID NO: 3) (SRE). Applicants must specify the claims that encompass any additionally elected immunotherapeutic agent(s) and/or SRE(s) and/or their associated sequence(s). Applicants must further indicate, if applicable, the claims which encompass the first named invention, if different than what was indicated above for this group. Failure to clearly identify how any paid additional invention fees are to be applied to the "+" group(s) will result in only the first claimed invention to be searched/examined. An exemplary election would be an immunotherapeutic agent encompassing an IL-1alpha cytokine (immunotherapeutic agent).

No technical features are shared between the effector modules of Groups I+ and, accordingly, these groups lack unity a priori.

Additionally, even if Groups I+ were considered to share the technical features including: a composition for inducing an immune response in a cell or a subject comprising a first effector module, said effector module comprising a first stimulus response element (SRE) operably linked to at least one immunotherapeutic agent, wherein said at least one immunotherapeutic agent is selected from a cytokine, a safety switch, a regulatory switch, a chimeric antigen receptor and combinations thereof; these shared technical features are previously disclosed by US 2014/0010791 A1 to The Board of Trustees of The Leland Stanford Junior University (hereinafter 'Stanford').

Stanford discloses a composition (a composition; abstract, paragraph [0127]) for inducing an immune response in a cell or a subject (for inducing an immune response in a cell or a subject; paragraph [0158]) comprising a first effector module (comprising a ligand-responsive stability-affecting protein (comprising a first effector module); paragraph [0012]), said effector module comprising a first stimulus response element (SRE) (said stability-affecting protein comprising a ligand-dependent stability affecting protein (said effector module comprising a first stimulus response element (SRE); paragraphs [0012], [0013]) operably linked to at least one immunotherapeutic agent (fused to a protein of interest, including TNF-a or IL-2 (operably linked to at least one immunotherapeutic agent); paragraphs [0012], [0013], [0016]), wherein said at least one immunotherapeutic agent is a cytokine (wherein said protein of interest is TNF-a or IL-2 (at least one immunotherapeutic agent is a cytokine); paragraph [0016]).

Since none of the special technical features of the Groups I+ inventions is found in more than one of the inventions, and since all of the shared technical features are previously disclosed by the Stanford reference, unity of invention is lacking.