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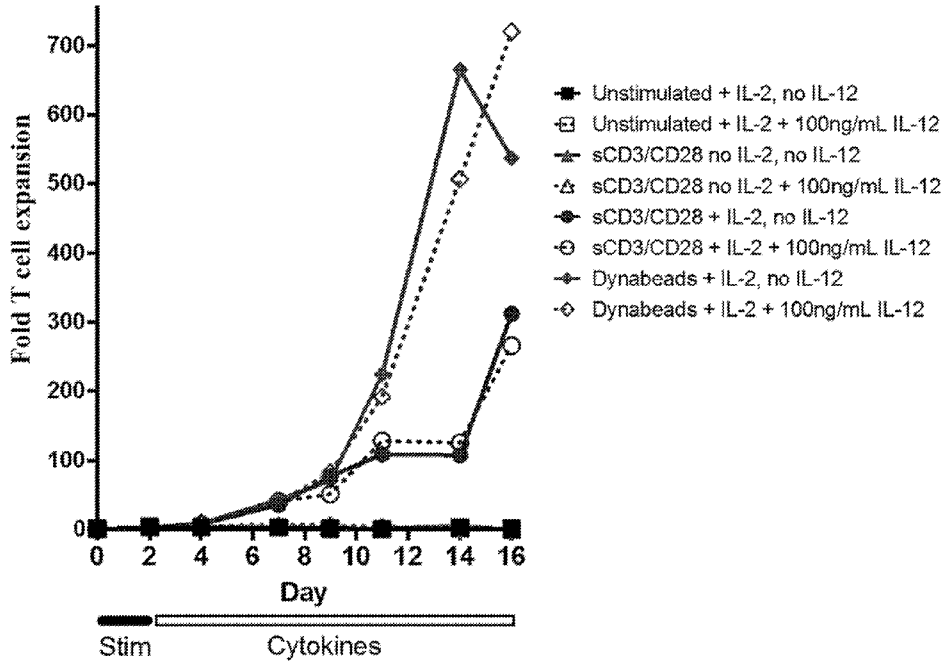
(54) Title: CD19 COMPOSITIONS AND METHODS FOR IMMUNOTHERAPY

Figure 28A



(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.

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[Continued on next page]

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CD19 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 and the US Provisional Patent Application No. 62/484,052, filed on April 11, 2017 entitled Anti CD19 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_1201PCT_SL.txt, created on March 2, 2018, which is 2,116,001 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 of each of which are herein incorporated by reference in their entirety).

[0010] In some embodiments, the composition for inducing an immune response may comprise an effector module. In some embodiments, the effector module may comprise a 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 chimeric antigen receptor (CAR) and an antibody.

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

[0013] In some embodiments, the 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. The mutant protein may comprise a single mutation and may be selected from, but not limited to hDHFR (I17V), hDHFR (F59S), hDHFR (N65D), hDHFR (K81R), hDHFR (A107V), hDHFR (Y122I), hDHFR (N127Y), hDHFR (M140I), hDHFR (K185E), hDHFR (N186D), and hDHFR (M140I), hDHFR (Amino acid 2-187 of WT; N127Y), hDHFR (Amino acid 2-187 of WT; I17V), hDHFR (Amino acid 2-187 of WT; Y122I), and hDHFR (Amino acid 2-187 of WT; K185E). In some embodiments, the mutant protein may comprise two mutations and may be selected from, but not limited to, hDHFR (C7R, Y163C), hDHFR (A10V, H88Y), hDHFR (Q36K, Y122I), hDHFR (M53T, R138I), hDHFR (T57A, I72A), hDHFR (E63G, I176F), hDHFR (G21T, Y122I), hDHFR (L74N, Y122I), hDHFR (V75F, Y122I), hDHFR (L94A, T147A), DHFR (V121A, Y22I), hDHFR (Y122I, A125F), hDHFR (H131R, E144G), hDHFR (T137R, F143L), hDHFR (Y178H, E18IG), and hDHFR (Y183H, K185E), hDHFR (E162G, I176F) hDHFR (Amino acid 2-187 of WT; I17V, Y122I), hDHFR (Amino acid 2-187 of WT; Y122I, M140I), hDHFR (Amino acid 2-187 of WT; N127Y, Y122I), hDHFR (Amino acid 2-187 of WT; E162G, I176F), and hDHFR (Amino acid 2-187 of WT; H131R, E144G), and hDHFR (Amino acid 2-187 of WT; Y122I, A125F). In some embodiments, the mutant may comprise three mutations and the mutant may be selected from hDHFR (V9A, S93R, P150L), hDHFR (I8V, K133E, Y163C), hDHFR (L23S, V121A, Y157C), hDHFR (K19E, F89L, E181G), hDHFR (Q36F, N65F, Y122I), hDHFR (G54R, M140V, S168C), hDHFR (V110A, V136M, K177R), hDHFR (Q36F, Y122I, A125F), hDHFR (N49D, F59S, D153G), and hDHFR (G21E, I72V, I176T), hDHFR (Amino acid 2-187 of WT; Q36F, Y122I, A125F), hDHFR (Amino acid

2-187 of WT; Y122I, H131R, E144G), hDHFR (Amino acid 2-187 of WT; E31D, F32M, V116I), and hDHFR (Amino acid 2-187 of WT; Q36F, N65F, Y122I). In some embodiments, the mutant may comprise four or more mutations and the mutant may be selected from hDHFR (V2A, R33G, Q36R, L100P, K185R), hDHFR (Amino acid 2-187 of WT; D22S, F32M, R33S, Q36S, N65S), hDHFR (I17N, L98S, K99R, M112T, E151G, E162G, E172G), hDHFR (G16S, I17V, F89L, D96G, K123E, M140V, D146G, K156R), hDHFR (K81R, K99R, L100P, E102G, N108D, K123R, H128R, D142G, F180L, K185E), hDHFR (R138G, D142G, F143S, K156R, K158E, E162G, V166A, K177E, Y178C, K185E, N186S), hDHFR (N14S, P24S, F35L, M53T, K56E, R92G, S93G, N127S, H128Y, F135L, F143S, L159P, L160P, E173A, F180L), hDHFR (F35L, R37G, N65A, L68S, K69E, R71G, L80P, K99G, G117D, L132P, I139V, M140I, D142G, D146G, E173G, D187G), hDHFR (L28P, N30H, M38V, V44A, L68S, N73G, R78G, A97T, K99R, A107T, K109R, D111N, L134P, F135V, T147A, I152V, K158R, E172G, V182A, E184R), 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), 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), and 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).

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

[0016] In some embodiments, the immunotherapeutic agent of the effector module is a chimeric antigen receptor (CAR). The chimeric antigen may comprise an extracellular target moiety; a transmembrane domain; an intracellular signaling domain; and optionally, one or more co-stimulatory domains.

[0017] 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.

[0018] 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.

[0019] In one aspect, the extracellular target moiety may be an scFv derived from an antibody. In one aspect, the scFv may specifically bind to a CD19 antigen

[0020] In one aspect, the scFv of the CAR may be a CD19 scFv. In some embodiments, the CD19 scFv may comprise a heavy chain variable region having an amino acid sequence independently selected from the group consisting of SEQ ID NO: 49-80, and a light chain variable region having an amino acid sequence independently selected from the group consisting of any of SEQ ID NOs: 81-122. In some embodiments, the CD19 scFv may comprise an amino acid sequence selected from the group consisting of any of SEQ ID NOs: 123-267 and 624.

[0021] In some embodiments, the intracellular signaling domain of the CAR may be a signaling domain derived from T cell receptor CD3zeta. In some embodiments, the intracellular signaling domain may be selected from 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. In one aspect, the CAR may include a co-stimulatory domain. In some embodiments, the co-stimulatory 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.

[0022] (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.

[0023] In some embodiments, the intracellular signaling domain of the CAR may be a T cell receptor CD3zeta signaling domain, which may comprise the amino acid sequence of SEQ ID NO: 339.

[0024] In some embodiments, T cell receptor CD3zeta signaling domain of the CAR, comprising the amino acid sequence of SEQ ID NO: 626 may further comprise at least one co-stimulatory domain. The co-stimulatory domain may comprise an amino acid sequence of SEQ ID NOs: 268-374.

[0025] In one embodiment, the transmembrane domain of the CAR may be derived from a transmembrane region of an alpha, beta or zeta chain of a T-cell receptor. In one aspect, the transmembrane domain may be derived from the CD3 epsilon chain of a T-cell receptor. In one embodiment, the transmembrane domain may be derived from a molecule selected from CD4,

CD5, CD8, CD8α, CD9, CD16, CD22, CD33, CD28, CD37, CD45, CD64, CD80, CD86, CD148, DAP 10, EpoRI, GITR, LAG3, ICOS, Her2, OX40 (CD134), 4-1BB (CD137), CD152, CD154, PD-1, or CTLA-4. In another embodiment, the transmembrane domain may be derived from an immunoglobulin selected from IgG1, IgD, IgG4, and an IgG4 Fc region. In one aspect, the transmembrane domain may comprise an amino acid sequence selected from the group consisting of any of SEQ ID NOs: 375-425 and 897-907.

[0026] 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: 426-504.

[0027] In some embodiments, the immunotherapeutic agent may be an antibody that is specifically immunoreactive to an antigen selected from a tumor specific antigen (TSA), a tumor associated antigen (TAA), or an antigenic epitope.

[0028] In one aspect, the antigen may be an antigenic epitope. In some embodiments, the antigenic epitope may be CD19.

[0029] In some embodiments, the antibody may comprise a heavy chain variable region having an amino acid sequence independently selected from the group consisting of any of SEQ ID NOs: 49-80 and a light chain variable region having an amino acid sequence independently selected from the group consisting of any of SEQ ID NOs: 81-122. In one aspect, the antibody may comprise an amino acid sequence selected from the group consisting of any of SEQ ID NOs: 123-267 and 624.

[0030] In one aspect, the first effector module may comprise the amino acid sequence of any of SEQ ID NO: 635-649, 1005-1010, 1015-1018 and 1215-1231.

[0031] In some embodiments, the first SRE of the effector module may stabilize the immunotherapeutic agent by a stabilization ratio of 1 or more, wherein the stabilization ratio may comprise the ratio of expression, function or level of the immunotherapeutic agent in the presence of the stimulus to the expression, function or level of the immunotherapeutic agent in the absence of the stimulus.

[0032] In some embodiments, the SRE may destabilize the immunotherapeutic agent by a destabilization ratio between 0, and 0.09, wherein the destabilization ratio may comprise the ratio of expression, function or level of the immunotherapeutic agent in the absence of the stimulus specific to the SRE to the expression, function or level of the immunotherapeutic agent that is expressed constitutively, and in the absence of the stimulus specific to the SRE.

[0033] The present invention also provides polynucleotides comprising 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 one aspect, the polynucleotides of the invention may be a DNA molecule. 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.

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

[0039] In some embodiments, the immune cell may further express a composition comprising a second effector module, said second effector module comprising a second SRE linked to a second immunotherapeutic agent. In one aspect, the second immunotherapeutic agent may be selected from a cytokine, and a cytokine- cytokine receptor fusion.

[0040] In one aspect, the second immunotherapeutic agent may be a cytokine. In one aspect, the cytokine may be IL12 or IL15.

[0041] In one aspect, the second immunotherapeutic agent may be a cytokine- cytokine receptor fusion polypeptide.

[0042] In some embodiments, the cytokine-cytokine receptor fusion polypeptide may be selected from, but is not limited to a IL12-IL12 receptor fusion polypeptide, a IL15-IL15 receptor fusion polypeptide, and a IL15-IL15 receptor sushi domain fusion polypeptide.

[0043] 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.

[0044] The present invention also provides methods for enhancing the expansion and/or survival of immune cells, comprising contacting the immune cells with the compositions of the invention, the polynucleotides of the invention, and/or the vectors of the invention.

[0045] 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.

[0046] The present invention also provides a method of identifying a domain of a CD19 antigen which will not bind the FMC63 antibody (FMC63-distinct CD19 binding domain). The method may comprise (a) preparing a composition comprising a CD19 antigen, (b) contacting the composition in (a) with saturating levels of FMC63 antibody, (c) contacting the composition of step (b) with one or more selected members of a library of potential CD19 binders; and (d) identifying a binding domain on the CD19 antigen based on the differential binding of the selected members of the library of CD19 binders compared to the binding of FMC63. In some embodiments, the binding domains of the library may be generated using phage display techniques with the CD19 antigen as the seed sequence. In one aspect, the binding domain may be selected from 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 unibody, a nanobody, or an antigen binding region of an antibody, and an antibody fragment. In one aspect, the CD19 antigen may be selected from a whole or a portion of a human CD19 antigen, and a whole or a portion of a Rhesus CD19 antigen.

[0047] The present invention also provides chimeric antigen receptors that may comprise the FMC63-distinct CD19 binding domain obtained according to the methods described herein. Also, provided herein is a stimulus response element (SRE) operably linked to the chimeric antigen receptors that include the FMC63-distinct CD19 binding domain.

[0048] In some embodiments, the effector module comprises a stimulus response element (SRE) and at least one payload comprising a protein of interest (POI).

[0049] 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.

[0050] The payload may be any immunotherapeutic agent used for cancer immunotherapy such as a chimeric agent receptor (CAR) such as CD19 CAR that targets any molecule of tumor cells, an antibody, an antigen binding domain or combination of antigen binding domains, a cytokine such as IL12, IL15 or IL15/IL15Ra fusion, 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.

[0051] 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.

[0052] In some embodiments, the DD may stabilize the immunotherapeutic agent with a stabilization ratio of at least one in the presence of the stimulus. According to the present invention, the DD may destabilize the immunotherapeutic agent in the absence of ligand with a destabilization ratio between 0, and 0.99.

[0053] 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.

[0054] 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.

[0055] In some embodiments, the vector of the invention may be a non-viral vector, such as a nanoparticles and liposomes.

[0056] 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).

[0057] 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.

[0058] Also provided herein are methods for identifying FMC63-distinct binding domains and using CD19 antigens in which the FMC63 binding epitope is masked or absent. In some embodiments, the FMC63 binding domain may be included in the payloads and effector modules of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

[0059] 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.

[0060] 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.

[0061] 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.

[0062] 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.

[0063] 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.

[0064] 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.

[0065] 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.

[0066] 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.

[0067] 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.

[0068] 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).

[0069] 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.

[0070] 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).

[0071] Figure 13 depicts a standard effector module of a chimeric antigen receptor (CAR) system which comprises an antigen binding domain as an SRE, and signaling domain(s) as payload.

[0072] Figure 14 depicts the structure design of a regulatable CAR system, where the trans-membrane effector modules comprise antigen binding domains sensing an antigen and a first switch domain and the intracellular module comprises a second switch domain and signaling domains. A stimulus (e.g., a dimerization small molecule) can dimerize the first and second switch domains and assemble an activated CAR system.

[0073] Figure 15 shows schematic representation of CAR systems having one (A) or two (B and C) SREs incorporated into the effector module.

[0074] Figure 16 depicts a split CAR design to control T cell activation by a dual stimulus (e.g., an antigen and small molecule). Figure 16A shows normal T cell activation which entails a dual activation of TCR and co-stimulatory receptor. The regular CAR design (Figure 16B) combines the antigen recognition domain with TCR signaling motif and co-stimulatory motif in a single molecule. The split CAR system separates the components of the regular CAR into two

separate effector modules which can be reassembled when a heterodimerizing small molecule (stimulus) is present.

[0075] Figure 17 depicts the positive and negative regulation of CAR engineered T cell activation. The absence or presence of a second stimulus can negatively (A) or positively (B) control T cell activation.

[0076] Figure 18 shows schematic representation of gated activation of CAR engineered T cells. If a normal cell that has no stimulus (e.g., an antigen) (Figure 18A) or an antigen that cannot bind to the trans-membrane effector module (Figure18B), or only an antigen that activates the trans-membrane effector module and primes the receiver T cell to express the second effector (Fig 18C), the receiver T cell remains inactive. When both stimuli (e.g. two antigens) that bind the trans-membrane effector module and the primed effector, are present on the presenter cell (e.g. a cancer cell), the T cell is activated (Figure 18D).

[0077] Figure 19A is a bar graph depicting IL12 levels in the various dilutions of media derived from cells expressing DD-IL12. Figure 19B is a bar graph depicting the Shield-1 dose responsive induction of DD- IL12. Figure 19C depicts plasma IL12 levels in mice implanted with SKOV3 cells. Figure 19D depicts plasma IL12 levels in mice in response to different Shield-1 dosing regimens.

[0078] Figure 20A is a western blot of IL15 protein levels in 293 cells. Figure 20B and 20C are histograms depicting surface expression of IL15 and IL15Ra. Figure 20 D is a western blot of IL15 and hDHFR in HCT116 cells.

[0079] Figure 21A and Figure 21B are western blots of depicting the protein levels of CD3 Zeta of the DD- CD19 CAR construct and actin. Figure 21C shows the expression of CD19 chimeric antigen receptors in a western blot using 4-1BB antibody. Figure 21D is a bar graph depicting the surface expression of CD19 CAR.

[0080] Figure 22 denotes the frequency of IFNgamma positive T cells.

[0081] Figure 23A depicts IFN gamma production in T cells. Figure 23B depicts T cell expansion with IL15/IL15Ra treatment. Figure 23C is a dot plot depicting percentage human cells after *in vivo* cell transfer. Figure 23D is scatter plot depicting CD4+/CD8+ T cells.

[0082] Figure 24A depicts T cell subpopulations expressing CD19 CAR. Figure 28B depicts cell death caused by CD19 CAR expressing T cells.

[0083] Figure 25A is a bar graph depicting IL15Ra positive cells with 24 hour TMP treatment. Figure 25B is a bar graph depicting IL15Ra positive cells with 48 hour TMP treatment. Figure 25C is a bar graph depicting IL15Ra positive cells in response to varying concentrations of TMP.

[0084] Figure 26 is a western blot of IL15Ra protein levels in HCT116 cells.

[0085] Figure 27A represents percentage of human T cells blood with respect to mouse T cells. Figure 27B represents the number of T cells in blood. Figure 27C represents ratio of CD4 to CD8 cells in the blood. Figure 27D represents the percentage of IL15Ra positive CD4 and CD8 T cells in the blood.

[0086] Figure 28A depicts the expansion of T cells in response to cytokine treatment. Figure 28B, Figure 28C and Figure 28D depict the frequency of IFN gamma positive cells with IL12 treatment.

[0087] Figure 29 is a bar graph representing the effect of promoters on transgene expression.

[0088] Figure 30A shows the expression of CD19 in parental K562 cells and K562-CD19 cells. Figure 30B shows the proliferation of K562 cells cocultured with T cells expressing DD regulated CAR constructs, in the presence or absence of ligand. Figure 30C shows the area of target cells killed by T cells expressing DD regulated CAR constructs, in the presence of ligand.

[0089] Figure 31A shows IFNgamma concentration. Figure 31B shows IL2 concentration.

[0090] Figure 32A provides the final IL12 concentration for each of the four groups tested. Figure 32B shows that IL12 is detectable in kidney and Figure 32C shows that IL12 is detectable in tumor.

[0091] Figure 33A shows the regulation of IL12 over 24 hours. Figure 33B shows the regulation in the plasma and Figure 33C shows the detection of flexi-IL12 in the kidneys.

[0092] Figure 34A shows that restimulation increased the expression of IL12. Figure 34B and Figure 34C show that ligand increased production of IL12.

[0093] Figure 35A shows the concentration-dependent induction of IL12 secretion of IL12 secretion from primary human T cells. Figure 35B shows the time course induction of IL12 secretion from primary human T cells.

[0094] Figure 36A shows the dose response of Aquashield-Induced DD-IL12 regulation *in vivo*. Figure 36B shows that plasma levels of IL12 remain high in animals transplanted with constitutive IL12 transduced T cells.

[0095] Figure 37A and 37B show the expression of IL12 *in vivo* over 7 days. Figure 37C and 37D show the expression of IL12 *in vivo* over 11 days. Figure 37E shows the Geometric MFI (GeoMFI) of Granzyme B (GrB) after 7 days in CD8+ T cells. Figure 37F shows the GeoMFI of Perforin at day 7 in CD8+ T cells.

[0096] Figure 38A shows the regulation of IL12 with PGK and EF1a promoters and FKBP domains. Figure 38B shows the relative expression of IL12.

[0097] Figure 39 depicts the kinetics of IL15Ra surface expression on CD4 T cells after TMP treatment.

[0098] Figure 40 represents a western blot of IL15-IL15Ra protein in HCT116 tumors from mice treated with TMP for 17 days in xenograft assays.

[0099] Figure 41 is a graph of the results of the MSD assay of IL15 protein levels in HEK293 cells.

[00100] Figure 42A provides FACS plots showing the expression of membrane bound IL15 after a dose response study of TMP. Figure 42B is two graphs showing the dose and time of exposure of TMP *in vitro* influences membrane bound IL15 expression.

[00101] Figures 43A- 43C show the regulation of membrane bound IL15 using IL15 (Figure 43A), IL15Ra (Figure 43B), or IL15/IL15Ra double ++ staining (Figure 43C). Figure 43D shows FACS plots of the expression of IL15. Figure 43E is a graph of the regulation of IL15 in blood and Figure 43F is a graph of the plasma TMP levels.

[00102] Figure 44 represents the regulation of membrane bound IL15 with PO or IP dosing of TMP.

DETAILED DESCRIPTION OF THE INVENTION

[00103] 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

[00104] 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.

[00105] 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.

[00106] 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.

[00107] 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.

[00108] 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.

[00109] 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.

[00110] 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

[00111] 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 can be found in U.S. Provisional Patent Application No. 62/320,864 filed April 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 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.

[00112] 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.

[00113] 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

[00114] 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.

[00115] 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.

[00116] 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%.

[00117] 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.

[00118] 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.

[00119] 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.

[00120] 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.

[00121] 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.

[00122] 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.

[00123] 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.

[00124] 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.

[00125] 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.

[00126] 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, protein engineering may be used 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 MHC proteins. For example, amino acid modifications may be engineered such that there are no or a minimal of number of immune epitopes that are predicted 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 used to score epitopes in the compositions of 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.

[00127] Epitope identification and subsequent sequence modification may be applied to reduce immunogenicity. The identification of immunogenic epitopes may be achieved either physically or computationally. Physical methods of epitope identification may include, for example, mass spectrometry and tissue culture/cellular techniques. Computational approaches that utilize information obtained on antigen processing, loading and display, structural and/or proteomic data toward identifying non-self-peptides that may result from antigen processing, and that are

likely to have good binding characteristics in the groove of the MHC may also be utilized. 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.

[00128] In some embodiments, protein modifications engineered into the structure of the compositions of the invention to interfere with antigen processing and peptide loading such as glycosylation and PEGylation, may also be useful in the present invention. Compositions of the invention may also be engineered to include non-classical amino acid sidechains 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).

[00129] 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.

[00130] In some embodiments, reduced immunogenicity may be achieved by limiting immuproteasome 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).

1. Destabilizing domains (DDs)

[00131] 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.

[00132] 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.

[00133] 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.

[00134] 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).

[00135] 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.

[00136] 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.

[00137] 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.

[00138] 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.

[00139] 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 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 each of 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 NO.:	Ligands
E. coli Dihydrofolate reductase (ecDHFR) (Uniprot ID: P0ABQ4)	MISLIAALAVDRVIGMENAMPWNLPADL AWFKRNTLNKPVIMGRHTWESIGRPLPGR KNIILSSQPGTDDRVTWVKSVDEAIAACG DVPEIMVIGGGRVYEQFLPKAQKLYLTHI DAEVEGDTHFPDYEPDDWESVFSEFHDA DAQNSHSYCFEILERR	1	Methotrexate (MTX) Trimethoprim (TMP)
Human Dihydrofolate reductase (hDHFR) (Uniprot ID: P00374)	MVGSLNCIVAVSQNMGIGKNGDLPWPPL RNEFRYFQRMTTTSSVEGKQNLVIMGKK TWFSIPEKNRPLKGRINLVLSRELKEPPQG AHFLSRSLDDALKLTEQPELANKVDMVW IVGGSSVYKEAMNHPGHLKLFVTRIMQDF ESDTFFPEIDLEKYKLLPEYPGVLSDVQEE KGIKYKFEVYEKND	2	Methotrexate (MTX) Trimethoprim (TMP)
FK506 binding protein (FKBP) (Uniprot ID: P62942)	GVQVETISPGDGRTFPKRGQTCVVHYTG MLEDGKKFDSSRDRNKPFKFMLGKQEVI RGWEEGVAQMSVGQRAKLTISPDYAYGA TGHPGIIPPHATLVFDVELLKLE	3	Shield-1
Phosphodiesterase 5 (PDE5), ligand binding domain (Uniprot ID: Uniprot ID O76074)	MEETRELQSLAAAVVPSAQTLKITDFSFS DFELSDLETALCTIRMFTDLNLVQNFQMK HEVLCRWILSVKKNYRKNVAYHNWRHA FNTAQCMFAALKAGKIQNKLTDLEILALL IAALSHDLDHRGVNNSYIQRSEHPLAQLY CHSIMEHHHFDQCLMILNSPGNQILSGLSI EEYKTTLKIIKQAILATDLALYIKRRGEFFE LIRKNQFNLEDPHQKELFLAMLMTACDLS AITKPWPIQQRIAELVATEFFDQGDRERKE LNIEPTDLMNREKKNKIPSMQVGFIDAICL QLYEALTHVSEDCFPLLDGCRKNRQKWQ ALAEQQ	4	Sildenafil; Vardenafil; Tadalafil
PPAR gamma, ligand binding domain (Uniprot ID: P37231; amino acids 317-505)	SVEAVQEITEYAKSIPGFVNLDLNDQVTL LKYGVHEIIYTMLASLMNKDGVLISEGQG FMTREFLKSLRKPFGDFMEPKFEFAVKFN ALELDDSDLAIFIAVIILSGDRPGLLNVKPI EDIQDNLLQALELQLKLNHPESSQLFAKL LQKMTDLRQIVTEHVQLLQVIKKTETDMS LHPLLQEIYKDLY	5	Posiglitazone Pioglitazone
Carbonic anhydrase II (CA2) (Uniprot ID: P00918)	MSHHWGYGKHNGPEHWHKDFPIAKGER QSPVDIDTHTAKYDPSLKPLSVSYDQATS LRILNNGHAFNVEFDDSQDKAVLKGGPL DGTYRLIQFHFHWGSLDGQGSEHTVDKK KYAAELHLVHWNTKYGDFGKAVQQPDG LAVLGIFLKVGSAKPGLQKVVDVLDSIKT KGKSADFTNFDPRGLLPESLDYWTYPGSL TTPPLLECVTWIVLKEPISVSSEQVLKFRK LNFNGEGEPEELMVDNWRPAQPLKNRQI KASFK	6	Celecoxib Acetazolamide
NRH: Quinone oxidoreductase 2 (NQO2) (Uniprot ID: P16083)	MAGKKVLIVYAHQEPKSFNGSLKNVAVD ELSRQGCTVTVSDLYAMNLEPRATDKDIT GTLSNPEVFNYGVETHEAYKQRSLASDIT DEQKKVREADLVIFQFPLYWFSVPAILKG	7	Imatinib Melatonin

	WMDRVLCQGFAFDIPGFYDSGLLQGKLA LLSVTTGGTAEMYTKTGVNGDSRYFLWP LQHGTLHFCGFKVLAPQISFAPEIASEEER KGMVAAWSQRLQTIWKEEPIPCTAHWHF GQ		

[00140] 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)	MISLIAALAVDYVIGMENAMPWNLPADLAWFKRNTL NKPVIMGRHTWESIGRPLPGRKNIILSSQPGTDDRVTW VKSVDEAIAACGDVPEIMVIGGGRVIEQFLPKAQKLY LTHIDAEVEGDTHFPDYEPDDWESVFSEFHDADAQNS HSYCFEILERR	8
ecDHFR (Amino acid 2-159 of WT) (R12Y, Y100I)	ISLIAALAVDYVIGMENAMPWNLPADLAWFKRNTLN KPVIMGRHTWESIGRPLPGRKNIILSSQPGTDDRVTWV KSVDEAIAACGDVPEIMVIGGGRVIEQFLPKAQKLYLT HIDAEVEGDTHFPDYEPDDWESVFSEFHDADAQNSHS YCFEILERR	9
ecDHFR (Amino acid 2-159 of WT) (R12H, E129K)	ISLIAALAVDHVIGMENAMPWNLPADLAWFKRNTLN KPVIMGRHTWESIGRPLPGRKNIILSSQPGTDDRVTWV KSVDEAIAACGDVPEIMVIGGGRVYEQFLPKAQKLYL THIDAEVEGDTHFPDYKPDDWESVFSEFHDADAQNSH SYCFEILERR	10
FKBP (F36V, L106P)	GVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKV DSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQRA KLTISPDYAYGATGHPGIIPPHATLVFDVELLKPE	11
FKBP (E31G, F36V, R71G, K105E)	GVQVETISPGDGRTFPKRGQTCVVHYTGMLGDGKKV DSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQGA KLTISPDYAYGATGHPGIIPPHATLVFDVELLELE	12

[00141] 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.

[00142] 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.

[00143] 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.

[00144] 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.

[00145] 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.

[00146] 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.

[00147] 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.

[00148] 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.In one aspect of the invention, the DDs of the invention may be hDHFR mutants including the single mutation hDHFR (Y122I), hDHFR (K81R), hDHFR (F59S), hDHFR (I17V), hDHFR (N65D), hDHFR (A107V), hDHFR (N127Y), hDHFR (K185E), hDHFR (N186D), and hDHFR (M140I); double mutations: hDHFR (M53T, R138I), hDHFR (V75F, Y122I), hDHFR (A125F, Y122I), hDHFR (L74N, Y122I), hDHFR (L94A, T147A), hDHFR (G21T, Y122I), hDHFR (V121A, Y122I), hDHFR (Q36K, Y122I), hDHFR (C7R, Y163C),hDHFR (Y178H, E18IG), hDHFR (A10V, H88Y), hDHFR (T137R, F143L), hDHFR (E63G, I176F), hDHFR (T57A, I72A), hDHFR (H131R, E144G), and hDHFR (Y183H, K185E); and triple mutations: hDHFR (Q36F, N65F, Y122I), hDHFR (G21E, I72V, I176T), hDHFR (I8V, K133E, Y163C), hDHFR (V9A, S93R, P150L), hDHFR (K19E, F89L, E181G), hDHFR (G54R, M140V, S168C), hDHFR (L23S, V121A, Y157C), hDHFR (V110A, V136M, K177R), and hDHFR (N49D, F59S, D153G).

[00149] In one embodiment, the stimulus is a small molecule that binds to a SRE 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, "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	871
hDHFR (V2A, R33G, Q36R, L100P, K185R)	MAGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFGYFRR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAHFLSRSLDDALKPTEQPELANKVDMVW IVGGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFPEID LEKYKLLPEYPGVLSDVQEEKGIKYKFEVYERND	872
hDHFR (G16S, I17V, F89L, D96G, K123E, M140V, D146G, K156R)	MVGSLNCIVAVSQNMSVGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAHLLSRSLDGALKLTEQPELANKVDMVW IVGGSSVYEEAMNHPGHLKLFVTRIVQDFESGTFFPEIDL ERYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	873
hDHFR (F35L, R37G, N65A, L68S, K69E, R71G, L80P, K99G, G117D, L132P, I139V, M140I, D142G, D146G, E173G, D187G)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYLQG MTTTSSVEGKQNLVIMGKKTWFSIPEKARPSEGGINLVL SREPKEPPQGAHFLSRSLDDALGLTEQPELANKVDMVW IVDGSSVYKEAMNHPGHPKLFVTRVIQGFESGTFFPEIDL EKYKLLPEYPGVLSDVQEGKGIKYKFEVYEKNG	874
hDHFR (I17N, L98S, K99R, M112T, E151G, E162G, E172G)	MVGSLNCIVAVSQNMGNGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAHFLSRSLDDASRLTEQPELANKVDTVWI VGGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFPGIDL EKYKLLPGYPGVLSDVQGEKGIKYKFEVYEKND	875
hDHFR (R138G, D142G, F143S, K156R, K158E, E162G, V166A, K177E, Y178C, K185E, N186S)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVW IVGGSSVYKEAMNHPGHLKLFVTGIMQGSESDTFFPEID LERYELLPGYPGALSDVQEEKGIECKFEVYEESD	876
hDHFR (K81R, K99R, L100P, E102G, N108D, K123R, H128R, D142G, F180L, K185E)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELREPPQGAHFLSRSLDDALRPTGQPELADKVDMVW IVGGSSVYREAMNRPGHLKLFVTRIMQGFESDTFFPEID LEKYKLLPEYPGVLSDVQEEKGIKYKLEVYEEND	877
hDHFR (N14S, P24S, F35L, M53T, K56E, R92G, S93G, N127S, H128Y, F135L, F143S, L159P, L160P, E173A, F180L)	MVGSLNCIVAVSQSMGIGKNGDLSWPPLRNEFRYLQRM TTTSSVEGKQNLVITGKETWFSIPEKNRPLKGRINLVLSR ELKEPPQGAHFLSGGLDDALKLTEQPELANKVDMVWIV GGSSVYKEAMSYPGHLKLLVTRIMQDSESDTFFPEIDLE KYKPPPEYPGVLSDVQEAKGIKYKLEVYEKND	878
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	879
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	880
hDHFR (A10T, Q13R, N14S, N20D, P24S, N30S, M38T, T40A,	MVGSLNCIVTVSRSMGIGKDGDLSWPPLRSEFRYFQRTT ATSSVEGRQSLVIMGKRTWFSTPERNRPLRGRANLVLS	881

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)	GELKGPPQGAHLLSRSLDGALKLTEQPELADKVDVVRI VGGSSVDEEAMNHPGHLKLFVTRVMRGFESDTLFPGID LGKRKLLPEYPGVLSDVREEKGIKYKLEVCGNN	
hDHFR (Amino acid 2-187 of WT; I17V, Y122I)	VGSLNCIVAVSQNMGVGKNGDLPWPPLRNEFRYFQRM TTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVLS RELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWI VGGSSVIKEAMNHPGHLKLFVTRIMQDFESDTFFPEIDL EKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	882
hDHFR (Amino acid 2-187 of WT; Y122I, M140I)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQRMT TTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVLSR ELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWIV GGSSVIKEAMNHPGHLKLFVTRIIQDFESDTFFPEIDLEK YKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	883
hDHFR (Amino acid 2-187 of WT; N127Y, Y122I)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQRMT TTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVLSR ELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWIV GGSSVIKEAMYHPGHLKLFVTRIMQDFESDTFFPEIDLE KYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	884
hDHFR (Amino acid 2-187 of WT; Y122I, H131R, E144G)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQRMT TTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVLSR ELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWIV GGSSVIKEAMNHPGRLKLFVTRIMQDFGSDTFFPEIDLE KYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	885
hDHFR (Amino acid 2-187 of WT; D22S, F32M, R33S, Q36S, N65S)	VGSLNCIVAVSQNMGIGKNGSLPWPPLRNEMSYFSRMT TTSSVEGKQNLVIMGKKTWFSIPEKSRPLKGRINLVLSR ELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWIV GGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFPEIDLE KYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	886
hDHFR (Amino acid 2-187 of WT; E31D, F32M, V116I)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNDMRYFQRM TTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVLS RELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWII GGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFPEIDLE KYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	887
hDHFR (Amino acid 2-187 of WT; E162G, I176F)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQRMT TTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVLSR ELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWIV GGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFPEIDLE KYKLLPGYPGVLSDVQEEKGFKYKFEVYEKND	888
hDHFR (Amino acid 2-187 of WT; K185E)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQRMT TTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVLSR ELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWIV GGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFPEIDLE KYKLLPEYPGVLSDVQEEKGIKYKFEVYEEND	889
hDHFR (Amino acid 2-187 of WT; Y122I, A125F)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQRMT TTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVLSR ELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWIV GGSSVIKEFMNHPGHLKLFVTRIMQDFESDTFFPEIDLEK YKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	890
hDHFR (Amino acid 2-187 of WT; Q36F, N65F, Y122I)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFFRMT TTSSVEGKQNLVIMGKKTWFSIPEKFRPLKGRINLVLSR ELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWIV GGSSVIKEAMNHPGHLKLFVTRIMQDFESDTFFPEIDLE KYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	891
hDHFR (Amino acid 2-187 of WT; N127Y)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQRMT TTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVLSR ELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWIV	892

	GGSSVYKEAMYHPGHLKLFVTRIMQDFESDTFFPEIDLE KYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	
hDHFR (Amino acid 2-187 of WT; H131R, E144G)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQRMT TTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVLSR ELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWIV GGSSVYKEAMNHPGRLKLFVTRIMQDFGSDTFFPEIDLE KYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	893
hDHFR (Amino acid 2-187 of WT; I17V)	VGSLNCIVAVSQNMGVGKNGDLPWPPLRNEFRYFQRM TTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVLS RELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWI VGGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFPEIDL EKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	894
hDHFR (Amino acid 2-187 of WT; Y122I)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQRMT TTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVLSR ELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWIV GGSSVIKEAMNHPGHLKLFVTRIMQDFESDTFFPEIDLE KYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	895
hDHFR (E162G, I176F)	MVGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVL SRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVW IVGGSSVYKEAMNHPGHLKLFVTRIMQDFESDTFFPEID LEKYKLLPGYPGVLSDVQEEKGFKYKFEVYEKND	896
hDHFR (Amino acid 2-187 of WT; Q36K, Y122I)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFKRMT TTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINLVLSR ELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMVWIV GGSSVIKEAMNHPGHLKLFVTRIMQDFESDTFFPEIDLE KYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	981

[00150] 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.

[00151] 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.

[00152] 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.

[00153] 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. Payloads: Immunotherapeutic agents

[00154] 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, an antibody and fragments and variants thereof, a chimeric antigen receptor

(CAR), a chimeric switch receptor, a cytokine, chemokine, a cytokine receptor, a chemokine receptor, a cytokine-cytokine receptor fusion polypeptide, 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.

Antibodies

[00155] In some embodiments, antibodies, fragments and variants thereof are payloads of the present invention.

[00156] 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 each of which are incorporated herein by reference in their entirety.

Antibody fragments and variants

[00157] 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.

[00158] 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.

[00159] 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).

[00160] 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).

[00161] 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).

[00162] 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.

[00163] 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.

[00164] 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.

[00165] 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.

[00166] 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.

[00167] 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.

[00168] 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.

[00169] 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.

[00170] 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.

[00171] 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.

[00172] 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.

[00173] 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.

[00174] 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.

[00175] 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.

[00176] 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).

[00177] 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).

[00178] 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).

[00179] 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.

[00180] 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.

[00181] 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.

[00182] 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).

[00183] 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.

[00184] 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.

[00185] 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

[00186] 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.

[00187] 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.

[00188] 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

[00189] 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.

[00190] 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.

[00191] In one embodiment, the antigen is CD19, a B-cell surface protein expressed throughout B-cell development. CD19 is a well-known B cell surface molecule, which upon B cell receptor activation enhances B-cell antigen receptor induced signaling and expansion of B cell populations. CD19 is broadly expressed in both normal and neoplastic B cells. Malignancies derived from B cells such as chronic lymphocytic leukemia, acute lymphocytic leukemia and many non-Hodgkin lymphomas frequently retain CD19 expression. This near universal expression and specificity for a single cell lineage has made CD19 an attractive target for immunotherapies. Human CD19 has 14 exons wherein exon 1-4 encode the extracellular portion of the CD19, exon 5 encodes the transmembrane portion of CD19 and exons 6-14 encode the cytoplasmic tail.

[00192] In one embodiment, payloads of the present invention may be antibodies, fragments and variants thereof which are specific to CD19 antigen.

[00193] In one embodiment, the payload of the invention may be a FMC63 antibody, antibody fragment of variant. FMC63 is an IgG2a mouse monoclonal antibody clone specific to the CD19 antigen that reacts with CD19 antigen on cells of the B cell lineage. The epitope of CD19 recognized by the FMC63 antibody is in exon 2 (Sotillo et al (2015) Cancer Discov ;5(12):1282-95; the contents of which are incorporated by reference in their entirety). In some embodiments,

the payload of the invention may be other CD19 monoclonal antibody clones including but not limited to 4G7, SJ25C1, CVID3/429, CVID3/155, HIB19, and J3-119.

[00194] In some embodiments, the payloads of the present invention may include variable heavy chain and variable light chain comprising the amino acid sequences selected from those in Table 4.

Table 4: Variable Heavy and Light Chain Sequences

Target	Antibody chain	SEQ ID NO	Source
CD19	VH	49	SEQ ID NO: 28 in WO2016168773A3
CD19	VH	50	SEQ ID NO: 29 in WO2016168773A3
CD19	VH	51	SEQ ID NO: 32 in WO2016168773A3
CD19	VH	52	SEQ ID NO: 33 in WO2016168773A3
CD19	VH	53	SEQ ID NO: 34 in WO2016168773A3
CD19	VH	54	SEQ ID NO: 35 in WO2016168773A3
CD19	VH	55	SEQ ID NO: 51 in WO2016187349A1
CD19	VH	56	SEQ ID NO: 20 in US20160039942
CD19	VH	57	SEQ ID NO. 1 in WO2014184143
CD19	VH	58	SEQ ID NO. 5 in US20160145337A1
CD19	VH	59	SEQ ID NO: 15 in US20160319020
CD19	VH	60	SEQ ID NO: 166 in US20160152723
CD19	VH	61	SEQ ID NO: 167 in US20160152723
CD19	VH	62	SEQ ID NO: 168 in US20160152723
CD19	VH	63	SEQ ID NO: 17 in EP3057991A1
CD19	VH	64	SEQ ID NO: 172 in US20160152723
CD19	VH	65	SEQ ID NO: 176 in US20160152723
CD19	VH	66	SEQ ID NO: 177 in US20160152723
CD19	VH	67	SEQ ID NO: 181 in US20160152723
CD19	VH	68	SEQ ID NO: 183 in US20160152723
CD19	VH	69	SEQ ID NO: 184 in US20160152723
CD19	VH	70	SEQ ID NO: 185 in US20160152723
CD19	VH	71	SEQ ID NO: 62 in US20160152723
CD19	VH	72	SEQ ID NO: 62 in WO2016097231
CD19	VH	73	SEQ ID NO. 12 in WO2016134284
CD19	VH	74	SEQ ID NO: 111 in US20160333114A1
CD19	VH	75	SEQ ID NO: 113 in US20160333114A1
CD19	VH	76	SEQ ID NO: 33 in EP3057994A1
CD19	VH	77	SEQ ID NO: 34 in EP3057994A1
CD19	VH	78	SEQ ID NO: 35 in EP3057994A1
CD19	VH	79	SEQ ID NO. 53 in WO2016120216
CD19	VH	80	SEQ ID NO. 55 in WO2016120216
CD19	VK	81	SEQ ID NO: 13 in US20160319020

CD19	VK	82	SEQ ID NO: 6 in US20160319020
CD19	VL	83	SEQ ID NO: 27 in WO2016168773A3
CD19	VL	84	SEQ ID NO: 31 in WO2016168773A3
CD19	VL	85	SEQ ID NO: 49 in WO2016187349A1
CD19	VL	86	SEQ ID NO. 11 in WO2016134284
CD19	VL	87	SEQ ID NO. 194 in US20140134142A1
CD19	VL	88	SEQ ID NO. 54 in WO2016120216
CD19	VL	89	SEQ ID NO. 56 in WO2016120216
CD19	VL	90	SEQ ID NO: 13 in US20160152723
CD19	VL	91	SEQ ID NO: 14 in US20160152723
CD19	VL	92	SEQ ID NO: 15 in US20160152723
CD19	VL	93	SEQ ID NO: 16 in US20160152723
CD19	VL	94	SEQ ID NO: 17 in US20160152723
CD19	VL	95	SEQ ID NO: 186 in US20160152723
CD19	VL	96	SEQ ID NO: 187 in US20160152723
CD19	VL	97	SEQ ID NO: 188 US20160152723
CD19	VL	98	SEQ ID NO: 189 in US20160152723
CD19	VL	99	SEQ ID NO: 192 in US20160152723
CD19	VL	100	SEQ ID NO: 196 in US20160152723
CD19	VL	101	SEQ ID NO: 197 in US20160152723
CD19	VL	102	SEQ ID NO: 198 in US20160152723
CD19	VL	103	SEQ ID NO: 199 in US20160152723
CD19	VL	104	SEQ ID NO: 200 in US20160152723
CD19	VL	105	SEQ ID NO: 201 in US20160152723
CD19	VL	106	SEQ ID NO: 202 in US20160152723
CD19	VL	107	SEQ ID NO: 203 in US20160152723
CD19	VL	108	SEQ ID NO: 204 in US20160152723
CD19	VL	109	SEQ ID NO: 205 in US20160152723
CD19	VL	110	SEQ ID NO: 22 in US20160039942
CD19	VL	111	SEQ ID NO: 63 in WO2016097231
CD19	VL	112	SEQ ID NO: 64 in US20160152723
CD19	VL	113	SEQ ID NO: 66 in US20160152723
CD19	VL	114	SEQ ID NO: 67 in US20160152723
CD19	VL	115	SEQ ID NO: 68 in US20160152723
CD19	VL	116	SEQ ID NO: 69 in US20160152723
CD19	VL	117	SEQ ID NO: 70 in US20160152723
CD19	VL	118	SEQ ID NO: 71 in US20160152723
CD19	VL	119	SEQ ID NO: 91 in US20160152723
CD19	VL	120	SEQ ID NO. 3 in US20160145337A1
CD19	VL	121	SEQ ID NO: 112 in US20160333114A1
CD19	VL	122	SEQ ID NO: 114 in US20160333114A1

[00195] A tumor specific antigen (TSA) may be a tumor neoantigen. A neoantigen is a mutated antigen 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). In the context of CD19, neoantigens such as a transcript variant of CD19 lacking exon 2 or lacking exon 5-6 or both have been described (see International patent publication No. WO2016061368; the contents of which are incorporated herein by reference in their entirety). Since FMC63 binding epitope is in exon 2, CD19 neoantigen lacking exon 2 is not recognized by FMC63 antibody. Thus, in some embodiments, payloads of the invention may include FMC63-distinct antibodies, or fragments thereof. As used herein "FMC63-distinct" refers, to an antibody or fragment thereof that is immunologically specific and binds to an epitope of the CD19 antigen that is different or unlike the epitope of CD19 antigen that is bound by FMC63. In some instances, antibodies of the invention may include CD19 antibodies, antibody fragments or variants that recognize CD19 neoantigens including the CD19 neoantigen lacking exon2. In one embodiment, the antibody or fragment thereof is immunologically specific to the CD19 encoded by exon 1, 3 and/or 4. In one example, the antibody or fragment thereof is specific to the epitope that bridges the portion of CD19 encoded by exon 1 and the portion of CD19 encoded by exon 3.

[00196] Chimeric antigen receptors (CARs)

[00197] 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.

[00198] 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.

[00199] 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.

[00200] 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).

[00201] 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.

[00202] 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.

[00203] 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 *in vivo*.

[00204] 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*.

[00205] In one embodiment, selection of a CAR with a lower affinity may provide more T cell signaling and less toxicity.

Extracellular targeting domain/moiety

[00206] 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.

[00207] 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.

[00208] In one embodiment, the targeting moiety of the CAR may recognize CD19. CD19 is a well-known B cell surface molecule, which upon B cell receptor activation enhances B-cell antigen receptor induced signaling and expansion of B cell populations. CD19 is broadly expressed in both normal and neoplastic B cells. Malignancies derived from B cells such as chronic lymphocytic leukemia, acute lymphocytic leukemia and many non-Hodgkin lymphomas frequently retain CD19 expression. This near universal expression and specificity for a single cell lineage has made CD19 an attractive target for immunotherapies. Human CD19 has 14 exons wherein exon 1-4 encode the extracellular portion of the CD19, exon 5 encodes the transmembrane portion of CD19 and exons 6-14 encode the cytoplasmic tail. In one embodiment, the targeting moiety may comprise scFvs derived from the variable regions of the FMC63 antibody. FMC63 is an IgG2a mouse monoclonal antibody clone specific to the CD19 antigen that reacts with CD19 antigen on cells of the B lineage. The epitope of CD19 recognized by the FMC63 antibody is in exon 2 (Sotillo et al (2015) Cancer Discov ;5(12):1282-95; the contents of which are incorporated by reference in their entirety). In some embodiments, the targeting moiety of the CAR may be derived from the variable regions of other CD19 monoclonal antibody clones including but not limited to 4G7, SJ25C1, CVID3/429, CVID3/155, HIB19, and J3-119.

[00209] 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). In the context of CD19, TSAs may include a transcript variant of human CD19 lacking exon 2 or lacking exon 5-6 or both (see International patent publication No. WO2016061368; the contents of which are incorporated herein by reference in their entirety). Since FMC63 binding epitope is in exon 2, CD19 lacking exon 2 is not recognized by FMC63 antibody. Thus, in some embodiments, the targeting moiety of the CAR may be an FMC63-distinct scFV. As used herein "FMC63-distinct" refers, to an antibody, scFv or a fragment thereof that is immunologically specific and binds to an epitope of the CD19 antigen that is different or unlike the epitope of CD19 antigen that is bound by FMC63. In some instances, targeting moiety may recognize a CD19 antigen lacking exon2. In one embodiment, the targeting moiety recognizes a fragment of CD19 encoded by exon 1, 3 and/or 4. In one example, the targeting moiety recognizes the epitope that bridges the portion of CD19 encoded by exon 1 and the portion of CD19 encoded by exon 3.

[00210] In some embodiments, the targeting moieties of the present invention may be scFv comprising the amino acid sequences in Table 5.

Table 5: scFv sequences

Target	Description	SEQ ID NO	Source
CD19	scFv	123	SEQ ID NO. 53 in EP3083671A1
CD19	scFv	124	SEQ ID NO. 54 in EP3083671A1
CD19	scFv	125	SEQ ID NO. 1 in WO2015157252
CD19	scFv	126	SEQ ID NO. 10 in WO2015157252
CD19	scFv	127	SEQ ID NO. 10 in WO2016033570
CD19	scFv	128	SEQ ID NO. 11 in WO2015157252
CD19	scFv	129	SEQ ID NO. 12 in WO2015157252
CD19	scFv	130	SEQ ID NO. 2 in WO2015157252
CD19	scFv	131	SEQ ID NO. 2 in WO2016033570
CD19	scFv	132	SEQ ID NO. 206 in WO2016033570
CD19	scFv	133	SEQ ID NO. 207 in WO2016033570
CD19	scFv	134	SEQ ID NO. 208 in WO2016033570
CD19	scFv	135	SEQ ID NO. 209 in WO2016033570
CD19	scFv	136	SEQ ID NO. 210 in WO2016033570
CD19	scFv	137	SEQ ID NO. 211 in WO2016033570
CD19	scFv	138	SEQ ID NO. 213 in WO2016033570
CD19	scFv	139	SEQ ID NO. 214 in WO2016033570
CD19	scFv	140	SEQ ID NO. 215 in WO2016033570
CD19	scFv	141	SEQ ID NO. 216 in WO2016033570
CD19	scFv	142	SEQ ID NO. 217 in WO2016033570
CD19	scFv	143	SEQ ID NO. 218 in WO2016033570
CD19	scFv	144	SEQ ID NO. 219 in WO2016033570
CD19	scFv	145	SEQ ID NO. 220 in WO2016033570
CD19	scFv	146	SEQ ID NO. 221 in WO2016033570
CD19	scFv	147	SEQ ID NO. 222 in WO2016033570
CD19	scFv	148	SEQ ID NO. 223 in WO2016033570
CD19	scFv	149	SEQ ID NO. 224 in WO2016033570
CD19	scFv	150	SEQ ID NO. 225 in WO2016033570
CD19	scFv	151	SEQ ID NO. 3 in WO2015157252
CD19	scFv	152	SEQ ID NO. 4 in WO2015157252

CD19	scFv	153	SEQ ID NO. 4 in WO2016033570
CD19	scFv	154	SEQ ID NO. 45 in WO2016033570
CD19	scFv	155	SEQ ID NO. 47 in WO2016033570
CD19	scFv	156	SEQ ID NO. 49 in WO2016033570
CD19	scFv	157	SEQ ID NO. 5 in WO2015155341A1
CD19	scFv	158	SEQ ID NO. 5 in WO2015157252
CD19	scFv	159	SEQ ID NO. 51 in WO2016033570
CD19	scFv	160	SEQ ID NO. 53 in WO2016033570
CD19	scFv	161	SEQ ID NO. 55 in WO2016033570
CD19	scFv	162	SEQ ID NO. 57 in WO2016033570
CD19	scFv	163	SEQ ID NO. 59 in WO2015157252
CD19	scFv	164	SEQ ID NO. 59 in WO2016033570
CD19	scFv	165	SEQ ID NO. 6 in WO2015157252
CD19	scFv	166	SEQ ID NO. 6 in WO2016033570
CD19	scFv	167	SEQ ID NO. 7 in WO2014184143
CD19	scFv	168	SEQ ID NO. 7 in WO2015157252
CD19	scFv	169	SEQ ID NO. 8 in WO2015157252
CD19	scFv	170	SEQ ID NO. 8 in WO2016033570
CD19	scFv	171	SEQ ID NO. 87 in WO2016033570
CD19	scFv	172	SEQ ID NO. 9 in WO2015157252
CD19	scFv	173	SEQ ID NO.9 in WO2016139487
CD19	scFv	174	SEQ ID NO. 10 in US20160152723
CD19	scFv	175	SEQ ID NO. 2 in US20160152723
CD19	scFv	176	SEQ ID NO. 206 in US20160152723
CD19	scFv	177	SEQ ID NO. 207 in US20160152723
CD19	scFv	178	SEQ ID NO. 208 in US20160152723
CD19	scFv	179	SEQ ID NO. 209 in US20160152723
CD19	scFv	180	SEQ ID NO. 210 in US20160152723
CD19	scFv	181	SEQ ID NO. 211 in US20160152723
CD19	scFv	182	SEQ ID NO. 212 in US20160152723
CD19	scFv	183	SEQ ID NO. 213 in US20160152723
CD19	scFv	184	SEQ ID NO. 214 in US20160152723
CD19	scFv	185	SEQ ID NO. 215 in US20160152723
CD19	scFv	186	SEQ ID NO. 216 in US20160152723
CD19	scFv	187	SEQ ID NO. 217 in US20160152723
CD19	scFv	188	SEQ ID NO. 218 in US20160152723
CD19	scFv	189	SEQ ID NO. 219 in US20160152723
CD19	scFv	190	SEQ ID NO. 220 in US20160152723
CD19	scFv	191	SEQ ID NO. 221 in US20160152723
CD19	scFv	192	SEQ ID NO. 222 in US20160152723
CD19	scFv	193	SEQ ID NO. 223 in US20160152723
CD19	scFv	194	SEQ ID NO. 224 in US20160152723
CD19	scFv	195	SEQ ID NO. 225 in US20160152723
CD19	scFv	196	SEQ ID NO. 32 in EP3083691A2
CD19	scFv	197	SEQ ID NO. 35 in EP3083691A2
CD19	scFv	198	SEQ ID NO. 38 in EP3083691A2
CD19	scFv	199	SEQ ID NO. 4 in US20160152723
CD19	scFv	200	SEQ ID NO. 45 in US20160152723
CD19	scFv	201	SEQ ID NO. 47 in US20160152723
CD19	scFv	202	SEQ ID NO. 49 in US20160152723
CD19	scFv	203	SEQ ID NO. 51 in US20160152723
CD19	scFv	204	SEQ ID NO. 53 in US20160152723
CD19	scFv	205	SEQ ID NO. 55 in US20160152723
CD19	scFv	206	SEQ ID NO. 57 in US20160152723
CD19	scFv	207	SEQ ID NO. 59 in US20160152723
CD19	scFv	208	SEQ ID NO. 6 in US20160152723
CD19	scFv	209	SEQ ID NO. 8 in US20160152723
CD19	scFv	210	SEQ ID NO. 87 in US20160152723

CD19	scFv	211	SEQ ID NO. 89 in US20160152723
CD19	scFv	212	SEQ ID NO. 39 in WO2016109410
CD19	scFv	213	SEQ ID NO. 37 in EP3083671A1
CD19	scFv	214	SEQ ID NO. 174 in WO2016115482
CD19	scFv	215	SEQ ID NO. 20 in WO2012079000
CD19	scFv	216	SEQ ID NO. 32 in WO2015092024
CD19	scFv	217	SEQ ID NO. 33 in WO2015092024A2
CD19	scFv	218	SEQ ID NO. 35 in WO2015092024A2
CD19	scFv	219	SEQ ID NO. 38 in WO2015092024A2
CD19	scFv	220	SEQ ID NO. 40 in WO2016109410
CD19	scFv	221	SEQ ID NO. 41 in WO2016109410
CD19	scFv	222	SEQ ID NO. 42 in WO2016109410
CD19	scFv	223	SEQ ID NO. 43 in WO2016109410
CD19	scFv	224	SEQ ID NO. 44 in WO2016109410
CD19	scFv	225	SEQ ID NO. 45 in WO2016109410
CD19	scFv	226	SEQ ID NO. 46 in WO2016109410
CD19	scFv	227	SEQ ID NO. 47 in WO2016109410
CD19	scFv	228	SEQ ID NO. 48 in WO2016109410
CD19	scFv	229	SEQ ID NO. 49 in WO2016109410
CD19	scFv	230	SEQ ID NO. 5 in WO2015155341A1
CD19	scFv	231	SEQ ID NO. 50 in WO2016109410
CD19	scFv	232	SEQ ID NO. 51 in WO2016109410
CD19	scFv	233	SEQ ID NO. 7 in US20160145337A1
CD19	scFv	234	SEQ ID NO. 9 in US20160145337A1
CD19	scFv	235	SEQ ID NO.20 in US9499629B2
CD19	scFv	236	SEQ ID NO.6 in WO2015155341A1
CD19	scFv	237	SEQ ID NO.73 in WO2016164580
CD19	scFv	238	SEQ ID NO. 10 US20160152723
CD19	scFv	239	SEQ ID NO. 2 in US20160152723
CD19	scFv	240	SEQ ID NO. 206 in US20160152723
CD19	scFv	241	SEQ ID NO. 207 in US20160152723
CD19	scFv	242	SEQ ID NO. 209 in US20160152723
CD19	scFv	243	SEQ ID NO. 210 in US20160152723
CD19	scFv	244	SEQ ID NO. 212 in US20160152723
CD19	scFv	245	SEQ ID NO. 216 in US20160152723
CD19	scFv	246	SEQ ID NO. 218 in US20160152723
CD19	scFv	247	SEQ ID NO. 219 in US20160152723
CD19	scFv	248	SEQ ID NO. 220 in US20160152723
CD19	scFv	249	SEQ ID NO. 221 in US20160152723
CD19	scFv	250	SEQ ID NO. 222 in US20160152723
CD19	scFv	251	SEQ ID NO. 223 in US20160152723
CD19	scFv	252	SEQ ID NO. 224 in US20160152723
CD19	scFv	253	SEQ ID NO. 225 in US20160152723
CD19	scFv	254	SEQ ID NO. 4 in US20160152723
CD19	scFv	255	SEQ ID NO. 45 in US20160152723
CD19	scFv	256	SEQ ID NO. 47 in US20160152723
CD19	scFv	257	SEQ ID NO. 49 in US20160152723
CD19	scFv	258	SEQ ID NO. 51 in US20160152723
CD19	scFv	259	SEQ ID NO. 53 in US20160152723
CD19	scFv	260	SEQ ID NO. 55 in US20160152723
CD19	scFv	261	SEQ ID NO. 57 in US20160152723
CD19	scFv	262	SEQ ID NO. 59 in US20160152723
CD19	scFv	263	SEQ ID NO. 6 in US20160152723
CD19	scFv	264	SEQ ID NO. 8 in US20160152723
CD19	scFv	265	SEQ ID NO. 87 in US20160152723
CD19	scFv	266	SEQ ID NO. 89 in US20160152723
CD19	scFv	267	SEQ ID NO. 5 in WO2016055551

Intracellular signaling domains

[00211] 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).

[00212] 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 as long as it transduces the effector function signal.

[00213] 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.

[00214] 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. NO.: 9, 175, 308; the contents of which are incorporated herein by reference in its entirety.

[00215] 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, IL15Ra, 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.

[00216] 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.

[00217] 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.

[00218] In some embodiments, the intracellular domain of the present invention may comprise amino acid sequences of Table 6.

Table 6: Intracellular signaling and co-stimulatory domains

Description	Amino Acid Sequence	Amino Acid SEQ ID
2B4 co-stimulatory domain	WRRKRKEKQSETSPKEFLTIYEDVKDLKTRRNHEQEQTFP GGGSTIYSMIQSQSSAPTSQEPAYTLYSLIQPSRKSGSRKRN HSPSFNSTIYEVIGKSQPKAQNPARLSRKELENFDVYS	268
CD27 co-stimulatory domain	HQRRKYRSNKGESPVEPAEPCRYSCPREEEGSTIPIQEDYR KPEPACSP	269
CD272 (BTLA1) co-stimulatory domain	RRHQGKQNELSDTAGREINLVDAHLKSEQTEASTRQNSQ VLLSETGIYDNDPDLCFRMQEGSEVYSNPCLEENKPGVYA SLNHSVIGPNSRLARNVKEAPTEYASICVRS	270
CD272 (BTLA1) co-stimulatory domain	CCLRRHQGKQNELSDTAGREINLVDAHLKSEQTEASTRQ NSQVLLSETGIYDNDPDLCFRMQEGSEVYSNPCLEENKPG IVYASLNHSVIGPNSRLARNVKEAPTEYASICVRS	271
CD28 co-stimulatory	FWVLVVVGGVLACYSLLVTVAFIIFWV	272
CD28 co-stimulatory domain	KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGC EL	273
CD28 co-stimulatory domain	FWVRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDF AAYRS	274
CD28 co-stimulatory domain	RSKRSRGGHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAY RS	275
CD28 co-stimulatory domain	RSKRSRGGHSDYIVINMTPRRPGPTRKHYQPYAPPRDFAA YRS	276
CD28 co-stimulatory signaling region	MLRLLLALNLFPSIQVTGNKILVKQSPMLVAYDNAVNLSC KYSYNLFSREFRASLHKGLDSAVEVCVVYGNYSQQLQVY SKTGFNCDGKLGNESVTFYLQNLYVNQTDIYFCKIEVMYP PPYLDNEKSNGTIIHVKGKHLCPSPLFPGPSKPFWVLVVVG GVLACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTPRRPG PTRKHYQPYAPPRDFAAYRS	277
CD30 co-stimulatory domain	RRACRKRIRQKLHLCYPVQTSQPKLELVDSRPRRSSTQLR SGASVTEPVAEERGLMSQPLMETCHSVGAAYLESLPLQD ASPAGGPSSPRDLPEPRVSTEHTNNKIEKIYIMKADTVIVG TVKAELPEGRGLAGPAEPELEEELEADHTPHYPEQETEPPL GSCSDVMLSVEEEGKEDPLPTAASGK	278
CD30 co-stimulatory domain	RRACRKRIRQKLHLCYPVQTSQPKLELVDSRPRRSSTQLR SGASVTEPVAEERGLMSQPLMETCHSVGAAYLESLPLQD ASPAGGPSSPRDLPEPRVSTEHTNNKIEKIYIMKADTVIVG TVKAELPEGRGLAGPAEPELEEELEADHTPHYPEQETEPPL GSCSDVMLSVEEEGKEDPLPTAASGK	279
GITR co-stimulatory domain	HIWQLRSQCMWPRETQLLLEVPPSTEDARSCQFPEEERGE RSAEEKGRLGDLWV	280
HVEM co-stimulatory domain	CVKRRKPRGDVVKVIVSVQRKRQEAEGEATVIEALQAPP DVTTVAVEETIPSFTGRSPNH	281
ICOS co-stimulatory domain	TKKKYSSSVHDPNGEYMFMRAVNTAKKSRLTDVTL	282
ICOS co-stimulatory signaling domain	CWLTKKKYSSSVHDPNGEYMFMRAVNTAKKSRLTDVTL	283
LAG-3 co-stimulatory region	HLWRRQWRPRRFSALEQGIHPPQAQSKIEELEQEPEPEPEP EPEPEPEPEPEQL	284
OX40 co-stimulatory domain	ALYLLRRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLA KI	285
OX40 co-stimulatory domain	RRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKI	286
4-1BB intracellular domain	KRGRKKLLYIFKQPFMRPVQTIQEEDGCSCRFPEEEEGGCE L	287
4-1BB signaling domain	KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGY EL	288

4-1BB-CD3Zeta intracellular domain	TGTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRG LDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYIF KQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSAD APAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGG KPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKG HDGLYQGLSTATKDTYDALHMQALPPR	289
4-1BB-Z endodomain fusion	KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGC ELRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDK RRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIG MKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR	290
CD127 intracellular domain	KRIKPIVWPSLPDHKKTLEHLCKKPRKNLNVSFNPESFLDC QIHRVDDIQARDEVEGFLQDTFPQQLEESEKQRLGGDVQS PNCPSEDVVITPESFGRDSSLTCLAGNVSACDAPILSSSRSL DCRESGKNGPHVYQDLLLSLGTTNSTLPPPFSLQSGILTLN PVAQGQPILTSLGSNQEEAYVTMSSFYQNQ	291
CD137 intracellular domain	RFSVVKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEE EEGGCEL	292
CD148 intracellular domain	RKKRKDAKNNEVSFSQIKPKKSKLIRVENFEAYFKKQQAD SNCGFAEEYEDLKLVGISQPKYAAELAENRGKNRYNNVL PYDISRVKLSVQTHSTDDYINANYMPGYHSKKDFIATQGP LPNTLKDFWRMVWEKNVYAIIMLTKCVEQGRTKCEEYW PSKQAQDYGDITVAMTSEIVLPEWTIRDFTVKNIQTSESHP LRQFHFTSWPDHGVPDTTDLLINFRYLVRDYMKQSPPESPI LVHCSAGVGRTGTFIAIDRLIYQIENENTVDVYGIVYDLR MHRPLMVQTEDQYVFLNQCVLDIVRSQKDSKVDLIYQNT TAMTIYENLAPVTTFGKTNGYIA	293
CD27 intracellular domain	QRRKYRSNKGESPVEPAEPCHYSCPREEEGSTIPIQEDYRK PEPACSP	294
CD28 intracellular domain	FAAYRS	295
CD28 signaling chain	FWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDY MNMTPRRPGPTRKHYQPYAPPRDFAAYRS	296
CD28 signaling domain	RSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAY RS	297
CD28 signaling domain	SKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYR S	298
CD28 signaling domain	IEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPSKPFW VLVVVGGVLACYSLLVTVAFIIFWRSKRSRLLHSDYMNM TPRRPGPTRKHYQPYAPPRDFAAYRS	299
CD28, 4-1BB, and/or CD3ζ signaling domain	RSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAY RSRFSVVKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFP EEEEGGCELRVKFSRSADAPAYQQGQNQLYNELNLGRRE EYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMA EAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHM QALPPR	300
CD28/CD3C	AAAIEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPSK PFWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDY MNMTPRRPGPTRKHYQPYAPPRDFAAYRSRVKFSRSADA PAYQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGK PRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGH DGLYQGLSTATKDTYDALHMQALPPR	301
CD28-0XZ intracellular domain	RSKRSRLLHSDYNMTPRRPGPTRKHYQPYAPPRDFAAYRS RDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKIRVKFS RSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGRDP EMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERR RGKGHDGLYQGLSTATKDTYDALHMQALPPR	302
CD28-4-1BB intracellular domain	MFWVLVVVGGVLACYSLLVTVAFIIFWVKRGRKKLLYIF KQPFMRPVQTTQEEDGCSCRFPEEEEGGCEL	303

CD28-4-1BB intracellular domain	IEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPSKPFW VLVVVGGVLACYSLLVTVAFIIFWVKRGRKKLLYIFKQPF MRPVQTTQEEDGCSCRFPEEEEGGCEL	304
CD28-CD3 Zeta intracellular domain	RSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAY RSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDK RRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIG MKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR	305
CD28-CD3Zeta intracellular domain	KRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFAAYRS RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKR RGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGM KGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR	306
CD3 delta chain intracellular signaling domain	MEHSTFLSGLVLATLLSQVSPFKIPIEELEDRVFVNCNTSIT WVEGTVGTLLSDITRLDLGKRILDPRGIYRCNGTDIYKDK ESTVQVHYRMCQSCVELDPATVAGIIVTDVIATLLLALGV FCFAGHETGRLSGAADTQALLRNDQVYQPLRDRDDAQYS HLGGNWARNK	307
CD3 delta chain intracellular signaling domain	MEHSTFLSGLVLATLLSQVSPFKIPIEELEDRVFVNCNTSIT WVEGTVGTLLSDITRLDLGKRILDPRGIYRCNGTDIYKDK ESTVQVHYRTADTQALLRNDQVYQPLRDRDDAQYSHLG GNWARNK	308
CD3 delta chain intracellular signaling domain	DQVYQPLRDRDDAQYSHLGGN	309
CD3 delta intracellular domain	MEHSTFLSGLVLATLLSQVSPFKIPIEELEDRVFVNCNTSIT WVEGTVGTLLSDITRLDLGKRILDPRGIYRCNGTDIYKDK ESTVQVHYRMCQSCVELDPATVAGIIVTDVIATLLLALGV FCFAGHETGRLSGAADTQALLRNDQVYQPLRDRDDAQYS HLGGNWARNK	310
CD3 delta intracellular domain	MEHSTFLSGLVLATLLSQVSPFKIPIEELEDRVFVNCNTSIT WVEGTVGTLLSDITRLDLGKRILDPRGIYRCNGTDIYKDK ESTVQVHYRTADTQALLRNDQVYQPLRDRDDAQYSHLG GNWARNK	311
CD3 delta intracellular domain	DQVYQPLRDRDDAQYSHLGGN	312
CD3 epsilon intracellular domain	MQSGTHWRVLGLCLLSVGVWGQDGNEEMGGITQTPYKV SISGTTVILTCPQYPGSEILWQHNDKNIGGDEDDKNIGSDE DHLSLKEFSELEQSGYYVCYPRGSKPEDANFYLYLRARVC ENCMEMDVMSVATIVIVDICITGGLLLLVYYWSKNRKAK AKPVTRGAGAGGRQRGQNKERPPPVPNPDYEPIRKGQRD LYSGLNQRRI	313
CD3 epsilon intracellular domain	NPDYEPIRKGQRDLYSGLNQR	314
CD3 gamma intracellular domain	MEQGKGLAVLILAIILLQGTLAQSIKGNHLVKVYDYQEDG SVLLTCDAEAKNITWFKDGKMIGFLTEDKKKWNLGSNAK DPRGMYQCKGSQNKSKPLQVYYRMCQNCIELNAATISGF LFAEIVSIFVLAVGVYFIAGQDGVRQSRASDKQTLLPNDQ LYQPLKDREDDQYSHLQGNQLRRN,	315
CD3 gamma intracellular domain	DQLYQPLKDREDDQYSHLQGN	316
CD3 gamma intracellular domain	DQLYQPLKDREDDQYSHLQGN	317
CD3 gamma intracellular domain	MEQGKGLAVLILAIILLQGTLAQSIKGNHLVKVYDYQEDG SVLLTCDAEAKNITWFKDGKMIGFLTEDKKKWNLGSNAK DPRGMYQCKGSQNKSKPLQVYYRMCQNCIELNAATISGF LFAEIVSIFVLAVGVYFIAGQDGVRQSRASDKQTLLPNDQ LYQPLKDREDDQYSHLQGNQLRRN	318
CD3 zeta intracellular domain	MKWKALFTAAILQAQLPITEAQSFGLLDPKLCYLLDGILFI YGVILTALFLRVKFSRSADAPAYQQGQNQLYNELNLGRR EEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKM	319

	AEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALH MQALPPR	
CD3 zeta intracellular domain	MKWKALFTAAILQAQLPITEAQSFGLLDPKLCYLLDGILFI YGVILTALFLRVKFSRSADAPAYQQGQNQLYNELNLGRR EEYDVLDKRRGRDPEMGGKPQRRKNPQEGLYNELQKDK MAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDAL HMQALPPR	320
CD3 zeta intracellular domain	MKWKALFTAAILQAQLPITEAQSFGLLDPKLCYLLDGILFI YGVILTALFLRVKFSRSADAPAYQQGQNQLYNELNLGRR EEYDVLDKRRGRDPEMGGKPQRRKNPQEGLYNELQKDK MAEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDAL HMQALPPR	321
CD3 zeta intracellular domain	NQLYNELNLGRREEYDVLDKR	322
CD3 zeta domain 2 (NM_000734.3)	RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKR RGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGM KGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR	323
CD3 zeta intracellular domain	DGLYQGLSTATKDTYDALHMQ	324
CD3 zeta intracellular domain	RVKFSRSAEPPAYQQGQNQLYNELNLGRREEYDVLDKRR GRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMK GERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR	325
CD3 zeta intracellular domain	RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKR RGRDPEMGGKPQRRKNPQEGLYNELQKDKMAEAYSEIG MKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR	326
CD3 zeta intracellular domain	RSRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDK RRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIG MKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR	327
CD3 zeta intracellular domain	RVKFSRSADAPAYQQGEYDVLDKRRGRDPEMGGKPRRK NPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLY QGLSTATKDTYDALHMQALPPR	328
CD3 zeta intracellular domain	RVKFSRSADAPAYQQGQNQLYNELNLGRREEVDVLDKR RGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGM KGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR	329
CD3 zeta intracellular domain	MIPAVVLLLLLLVEQAAALGEPQLCYILDAILFLVGIVLTL LVCRLKIQVRKAAITSYEKSRVKFSRSADAPAYQQGQNQL YNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQEGL YNELQKDKMAEAVSEIGMKGERRRGKGHDGLYQGLSTA TKDTYDALHMQALPPR	330
CD3 zeta intracellular domain	LRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKR RGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGM KGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR	331
CD3 zeta intracellular domain	RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKR RGRDPEMGGKPQRRKNPQEGLY	332
CD3 zeta intracellular domain	LRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKR RGRDPEMGGKPQRRKNPQEGLYNELQKDKMAEAYSEIG MKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR	333
CD3 zeta intracellular domain	RRVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDK RRGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIG MKGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR	334
CD3 zeta intracellular domain	NQLYNELNLGRREEYDVLDKR	335
CD3 zeta intracellular domain	EGLYNELQKDKMAEAYSEIGMK	336
CD3 zeta intracellular domain	DGLYQGLSTATKDTYDALHMQ	337
CD3 zeta intracellular domain	RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKR RGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGM KGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR	338

CD3 zeta intracellular domain	RVKFSRSADAPAYKQGQNQLYNELNLGRREEYDVLDKR RGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGM KGERRRGKGHDGLYQGLSTATKDTYDALHMQALPPR	339
CD3 zeta intracellular domain	RVKFSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKR RGRDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGM KGERRRGKGHDGLYQGLSTATKDTYDALHMQALP	340
CD3 zeta intracellular domain	DPKLCYLLDGILFIYGVILTALFLRVKFSRSADAPAYQQGQ NQLYNELNLGRREEYDVLDKRRGRDPEMGGKPQRRKNP QEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQG LSTATKDTYDALHMQALPPR	341
CD3 zeta intracellular domain	MKWKALFTAAILQAQLPITEAQSFGLLDPKLCYLLDGILFI YGVILTALFLRVKFSRSADAPAYQQGQNQLYNELNLGRR EEYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKM AEAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALH MQALPPR	342
CD40 intracellular domain	RSRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKI	343
CD79A intracellular domain	MPGGPGVLQALPATIFLLFLLSAVYLGPGCQALWMHKVP ASLMVSLGEDAHFQCPHNSSNNANVTWWRVLHGNYTWP PEFLGPGEDPNGTLIIQNVNKSHGGIYVCRVQEGNESYQQ SCGTYLRVRQPPPRPFLDMGEGTKNRIITAEGIILLFCAVVP GTLLLFRKRWQNEKLGLDAGDEYEDENLYEGLNLDDCS MYEDISRGLQGTYQDVGSLNIGDVQLEKP	344
CD79A intracellular domain	MPGGPGVLQALPATIFLLFLLSAVYLGPGCQALWMHKVP ASLMVSLGEDAHFQCPHNSSNNANVTWWRVLHGNYTWP PEFLGPGEDPNEPPPRPFLDMGEGTKNRIITAEGIILLFCAV VPGTLLLFRKRWQNEKLGLDAGDEYEDENLYEGLNLDDC SMYEDISRGLQGTYQDVGSLNIGDVQLEKP	345
CD79A intracellular domain	MPGGPGVLQALPATIFLLFLLSAVYLGPGCQALWMHKVP ASLMVSLGEDAHFQCPHNSSNNANVTWWRVLHGNYTWP PEFLGPGEDPNGTLIIQNVNKSHGGIYVCRVQEGNESYQQ SCGTYLRVRQPPPRPFLDMGEGTKNRIITAEGIILLFCAVVP GTLLLFRKRWQNEKLGLDAGDEYEDENLYEGLNLDDCS MYEDISRGLQGTYQDVGSLNIGDVQLEKP	346
CD79A intracellular domain	ENLYEGLNLDDCSMYEDISRG	347
CD8 intracellular domain	FVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAG GAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCNHR NR	348
CD8 intracellular domain	FVPVFLPAKPITTPAPRPPTPAPTIASQPLSLRPEACRPAAG GAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCNHR NR	349
CD8a intracellular domain	PTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGL DFACDI	350
CTLA4 intracellular domain	AVSLSKMLKKRSPLTTGVFVKMAPTEAECEKQFQPYFIPI N	351
CTLA4 intracellular domain	AVSLSKMLKKRSPLTTGVYMNMTPRRPECEKQFQPYAPP RDFAAYRS	352
DAP10 intracellular domain	RPRRSPAQDGKVYINMPGRG	353
DAP12 intracellular domain	MGGLEPCSRLLLLPLLLAVSGLRPVQAQAQSDCSCSTVSP GVLAGIVMGDLVLTVLIALAVYFLGRLVPRGRGAAEAAT RKQRITETESPYQELQGQRSDVYSDLNTQRPYYK	354
DAP12 intracellular domain	MGGLEPCSRLLLLPLLLAVSGLRPVQAQAQSDCSCSTVSP GVLAGIVMGDLVLTVLIALAVYFLGRLVPRGRGAAEATR KQRITETESPYQELQGQRSDVYSDLNTQRPYYK	355
DAP12 intracellular domain	MGGLEPCSRLLLLPLLLAVSDCSCSTVSPGVLAGIVMGDL VLTVLIALAVYFLGRLVPRGRGAAEAATRKQRITETESPY QELQGQRSDVYSDLNTQRPYYK	356

DAP12 intracellular domain	MGGLEPCSRLLLLPLLLAVSDCSCSTVSPGVLAGIVMGDL VLTVLIALAVYFLGRLVPRGRGAAEATRKQRITETESPYQ ELQGQRSDVYSDLNTQRPYYK	357
DAP12 intracellular domain	MGGLEPCSRLLLLPLLLAVSGLRPVQAQAQSDCSCSTVSP GVLAGIVMGDLVLTVLIALAVYFLGRLVPRGRGAAEAAT RKQRITETESPYQELQGQRSDVYSDLNTQRPYYK	358
DAP12 intracellular domain	MGGLEPCSRLLLLPLLLAVSGLRPVQAQAQSDCSCSTVSP GVLAGIVMGDLVLTVLIALAVYFLGRLVPRGRGAAEATR KQRITETESPYQELQGQRSDVYSDLNTQRPYYK;	359
DAP12 intracellular domain	MGGLEPCSRLLLLPLLLAVSDCSCSTVSPGVLAGIVMGDL VLTVLIALAVYFLGRLVPRGRGAAEAATRKQRITETESPY QELQGQRSDVYSDLNTQRPYYK	360
DAP12 intracellular domain	MGGLEPCSRLLLLPLLLAVSDCSCSTVSPGVLAGIVMGDL VLTVLIALAVYFLGRLVPRGRGAAEATRKQRITETESPYQ ELQGQRSDVYSDLNTQRPYYK	361
DAP12 intracellular domain	ESPYQELQGQRSDVYSDLNTQ	362
DAP12 intracellular domain	ESPYQELQGQRSDVYSDLNTQ	363
GITR intracellular domain	RSQCMWPRETQLLLEVPPSTEDARSCQFPEEERGERSAEE KGRLGDLWV	364
ICOS intracellular domain	TKKKYSSSVHDPNGEFMFMRAVNTAKKSRLTDVTL	365
IL15Ra intracellular domain	KSRQTPPLASVEMEAMEALPVTWGTSSRDEDLENCSHHL	366
OX40-CD3 Zeta intracellular domain	RRDQRLPPDAHKPPGGGSFRTPIQEEQADAHSTLAKIRVK FSRSADAPAYQQGQNQLYNELNLGRREEYDVLDKRRGR DPEMGGKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGE RRRGKGHDGLYQGLSTATKDTYDALHMQALPPR	367
ZAP70 intracellular domain	MPDPAAHLPFFYGSISRAEAEEHLKLAGMADGLFLLRQCL RSLGGYVLSLVHDVRFHHFPIERQLNGTYAIAGGKAHCGP AELCEFYSRDPDGLPCNLRKPCNRPSGLEPQPGVFDCLRD AMVRDYVRQTWKLEGEALEQAIISQAPQVEKLIATTAHE RMPWYHSSLTREEAERKLYSGAQTDGKFLLRPRKEQGTY ALSLIYGKTVYHYLISQDKAGKYCIPEGTKFDTLWQLVEY LKLKADGLIYCLKEACPNSSASNASGAAAPTLPAHPSTLT HPQRRIDTLNSDGYTPEPARITSPDKPRPMPMDTSVYESPY SDPEELKDKKLFLKRDNLLIADIELGCGNFGSVRQGVYRM RKKQIDVAIKVLKQGTEKADTEEMMREAQIMHQLDNPYI VRLIGVCQAEALMLVMEMAGGGPLHKFLVGKREEIPVSN VAELLHQVSMGMKYLEEKNFVHRDLAARNVLLVNRHYA KISDFGLSKALGADDSYYTARSAGKWPLKWYAPECINFR KFSSRSDVWSYGVTMWEALSYGQKPYKKMKGPEVMAFI EQGKRMECPPECPPELYALMSDCWIYKWEDRPDFLTVEQ RMRACYYSLASKVEGPPGSTQKAEAACA	368
CD28 intracellular domain	MLRLLLALNLFPSIQVTGNKILVKQSPMLVAYDNAVNLSC KYSYNLFSREFRASLHKGLDSAVEVCVVYGNYSQQLQVY SKTGFNCDGKLGNESVTFYLQNLYVNQTDIYFCKIEVMYP PPYLDNEKSNGTIIHVKGKHLCPSPLFPGPSKPFWVLVVVG GVLACYSLLVTVAFIIFWVR	369
4-1BB intracellular domain	MGNSCYNIVATLLLVLNFERTRSLQDPCSNCPAGTFCDNN RNQICSPCPPNSFSSAGGQRTCDICRQCKGVFRTRKECSST SNAECDCTPGFHCLGAGCSMCEQDCKQGQELTKKGCKD CCFGTFNDQKRGICRPWTNCSLDGKSVLVNGTKERDVVC GPSPADLSPGASSVTPPAPAREPGHSPQIISFFLALTSTALLF LLFFLTLRFSVVKRGRKKLLYIFKQPFMRPVQTTQEEDG	370
Fc epsilon Receptor I gamma chain intracellular domain	MIPAVVLLLLLLVEQAAALGEPQLCYILDAILFLYGIVLTL LYCRLKIQVRKAAITSYEKSDGVYTGLSTRNQETYETLKH EKPPQ	371

Fc epsilon Receptor I gamma chain intracellular domain	DGVYTGLSTRNQETYETLKHE	372
Fc epsilon Receptor I gamma chain intracellular domain	DPKLCYILDAILFLYGIVLTLLYCRLKIQVRKAAITSYEKSD GVYTGLSTRNQETYETLKHEKPPQ	373
Fc epsilon Receptor I gamma chain intracellular domain	DGVYTGLSTRNQETYETLKHE	374

Transmembrane domains

[00219] 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.

[00220] 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.

[00221] 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 Ig_{G4} 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 NO.: WO2014/100385; and a PD-1 transmembrane domain comprising the amino acid sequences of SEQ ID NOs.: 6-8 of International Patent Publication NO.: WO2014100385; the contents of each of which are incorporated herein by reference in their entirety.

[00222] 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 7 provides various transmembrane regions that can be used in the CARs described herein.

Table 7: Transmembrane domains

Transmembrane domain	Amino Acid Sequence	SEQ ID NO.
CD8 Transmembrane domain	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDF ACDI	375
2B4 Transmembrane domain	FLVIIVILSALFLGTLACFCV	424
4-1BB Transmembrane domain	IISFFLALTSTALLFLLFFLTLRFSVVKRGR	376
4-1BB Transmembrane domain	IISFFLALTSTALLFLLFFLTLRFSVV	377
CD134 (OX40) Transmembrane domain	VAAILGLGLVLGLLGPLAILLALYLL	378
CD148 Transmembrane and intracellular domain	AVFGCIFGALVIVTVGGFIFWRKKRKDAKNNEVSFSQIKPK KSKLIRVENFEAYFKKQQADSNCGFAEEYEDLKLVGISQPK YAAELAENRGKNRYNNVLPYDISRVKLSVQTHSTDDYINA NYMPGYHSKKDFIATQGPLPNTLKDFWRMVWEKNVYAII MLTKCVEQGRTKCEEYWPSKQAQDYGDITVAMTSEIVLPE VVTIRDFTVKNIQTSESHPLRQFHFTSWPDHGVPDTTDLLIN FRYLVRDYMKQSPPESPILVHCSAGVGRTGTFIAIDRLIYQI ENENTVDVYGIVYDLRMHRPLMVQTEDQYVFLNQCVLDI VRSQKDSKVDLIYQNTTAMTIYENLAPVTTFGKTNGYIA	379
CD148 Transmembrane domain	AVFGCIFGALVIVTVGGFIFW	380
CD2 Transmembrane domain	KEITNALETWGALGQDINLDIPSFQMSDDIDDIKWEKTSDK KKIAQFRKEKETFKEKDTYKLFKNGTLKIKHLKTDDQDIYK VSIYDTKGKNVLEKIFDLKIQERVSKPKISWTCINTTLTCEV MNGTDPELNLYQDGKHLKLSQRVITHKWTTSLSAKFKCTA GNKVSKESSVEPVSCPEKGLD	381
CD28 Transmembrane and intracellular domain	IEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPSKPFW VLVVVGGVLACYSLLVTVAHIFWVRSKRSRLLHSDYMNM TPRRPGPTRKHYQPYAPPRDFAAYRS	382
CD28 Transmembrane domain	FWVLVVVGGVLACYSLLVTVAFIIFWV	383
CD28 Transmembrane domain	IEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPSKPFWV LVVVGGVLACYSLLVTVAFIIFWV	384

CD28 Transmembrane domain	IFWVLVVVGGVLACYSLLVTVAFIIFWVRSKRR	385
CD28 Transmembrane domain	FWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDYM NMTPRRPGPTRKHYQP YAPPRDFAAYRS	386
CD28 Transmembrane domain	MFWVLVVVGGVLACYSLLVTVAFIIFWV	387
CD28 Transmembrane domain	FWVLVVVGGVLACYSLLVTVAFHFWV	388
CD28 Transmembrane domain	FWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDYM NMTPRRPGPTRKHYQAYAAARDFAAYRS	425
CD28 Transmembrane domain	IEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPSKPFWV LWVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDYMNMTP RRPGPTRKHYQPYAPPRDFAAYRS	897
CD28 Transmembrane domain	MFWVLVVVGGVLACYSGGVTVAFIIFWV	389
CD28 Transmembrane domain	WVLVVVGGVLACYSLLVTVAFIIFWV	390
CD28 Transmembrane domain	FWVLVVVGGVLACYSLLVTVAFIIFWVR	898
CD28 Transmembrane domain	PFWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDY MNMTPRRPGPTRKHYQPYAPPRDFAAYRS	391
CD28 Transmembrane domain and CD28 and CD3 Zeta intracellular domain	FWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDYM NMTPRRPGPTRKHYQPYAPPRDFAAYRSRVKFSRSADAPA YQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRR KNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLY QGLSTATKDTYDALHMQALPPR	392
CD28 Transmembrane domain and CD28, OX40, and CD3 Zeta intracellular domain	FWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDYM NMTPRRPGPTRKHYQPYAPPRDFAAYRSRDQRLPPDAHKP PGGGSFRTPIQEEQADAHSTLAKIRVKFSRSADAPAYQQGQ NQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQE GLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLYQGLST ATKDTYDALHMQALPPR	393
CD28 Transmembrane domain and CD3 Zeta intracellular domain	FWVLVVVGGVLACYSLLVTVAFIIFWVRRVKFSRSADAPA YQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRR KNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLY QGLSTATKDTYDALHMQALPPR	394
CD28 transmembrane-CD3 zeta signaling domain ("28z")	AAAIEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPSKP FWVLVVVGGVLACYSLLVTVAFIIFWVRSKRSRLLHSDYM NMTPRRPGPTRKHYQPYAPPRDFAAYRSRVKFSRSADAPA YQQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGGKPRR KNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGHDGLY QGLSTATKDTYDALHMQALPPR	395
CD3 zeta Transmembrane domain	LCYLLDGILFIYGVILTALFLRV	396
CD3 zeta Transmembrane domain	MKWKALFTAAILQAQLPITEAQSFGLLDPKLCYLLDGILFIY GVILTALFL	397
CD3 zeta Transmembrane domain	LCYLLDGILFIYGVILTALFL	398
CD4 Transmembrane domain	ALIVLGGVAGLLLFIGLGIFFCVRC	399
CD4 Transmembrane domain	MALIVLGGVAGLLLFIGLGIFF	400
CD45 Transmembrane and intracellular domain	ALIAFLAFLIIVTSIALLVVLYKIYDLHKKRSCNLDEQQELV ERDDEKQLMNVEPIHADILLETYKRKIADEGRLFLAEFQSIP RVFSKFPIKEARKPFNQNKNRYVDILPYDYNRVELSEINGD AGSNYINASYIDGFKEPRKYIAAQGPRDETVDDFWRMIWE QKATVIVMVTRCEEGNRNKCAEYWPSMEEGTRAFGDVVV KINQHKRCPDYIIQKLNIVNKKEKATGREVTHIQFTSWPDH GVPEDPHLLLKLRRRVNAFSNFFSGPIWHCSAGVGRTGTYI	401

	GIDAMLEGLEAENKVDVYGYVVKLRRQRCLMVQVEAQYI LIHQALVEYNQFGETEVNLSELHPYLHNMKKRDPPSEPSPL EAEFQRLPSYRSWRTQHIGNQEENKSKNRNSNVIPYDYNR VPLKHELEMSKESEHDSDESSDDDSDSEEPSKYINASFIMSY WKPEVMIAAQGPLKETIGDFWQMIFQRKVKVIVMLTELKH GDQEICAQYWGEGKQTYGDIEVDLKDTDKSSTYTLRVFEL RHSKRKDSRTVYQYQYTNWSVEQLPAEPKELISMIQWKQK LPQKNSSEGNKHHKSTPLLIHCRDGSQQTGIFCALLNLLES AETEEWDIFQWKALRKARPGMVSTFEQYQFLYDVIASTYP AQNGQVKKNNHQEDKIEFDNEVDKVKQDANCVNPLGAPE KLPEAKEQAEGSEPTSGTEGPEHSVNGPASPALNQGS	
CD62L Transmembrane domain	PLFIPVAVMVTAFSGLAFIIWLA	402
CD7 Transmembrane domain	ALPAALAVISFLLGLGLGVACVLA	403
CD8 Transmembrane domain	MALPVTALLLPLALLLHAARP	404
CD8 Transmembrane domain and CD28 signaling domain	AAAFVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEACRPA AGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCN HRNRSKRSRLLHSDYMNMTPRRPGPTRKHYQPYAPPRDFA AYRSRFSVVKRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRF PEEEEGGCELRVKFSRSADAPAYQQGQNQLYNELNLGRRE EYDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKMA EAYSEIGMKGERRRGKGHDGLYQGLSTATKDTYDALHMQ ALPPR	405
CD8 transmembrane domain-CD137 (4-1BB) signaling domain and CD3 zeta signaling domain ("BBz")	AAATTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTR GLDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLYI FKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRSAD APAYKQGQNQLYNELNLGRREEYDVLDKRRGRDPEMGG KPRRKNPQEGLYNELQKDKMAEAYSEIGMKGERRRGKGH DGLYQGLSTATKDTYDALHMQALPPR	406
CD8a Transmembrane domain	FVPVFLPAKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGG AVHTRGLDFACDIYIWAPLAGTCGVLLLSLVITLYCNHRN	407
CD8a Transmembrane domain	IWAPLAGTCGVLLLSLVITLYC	408
CD8a Transmembrane domain	IYIWAPLAGTCGVLLLSLVITLYC	409
CD8a Transmembrane domain	IYIWAPLAGTCGVLLLSLVITLYCR	410
CD8a Transmembrane domain	PTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLD FACDIYIWAPLAGTCGVLLLSLVITLYCN	411
CD8a Transmembrane domain	IYIWAPLAGTCGVLLLSLVITLVCR	412
CD8a Transmembrane domain	IYIWAPLAGTCGVLLLSLVIT	413
CD8a Transmembrane domain	IYIWAPLAGTCGVLLLSLVITLY	414
CD8a Transmembrane domain (NP 001139345.1)	TPAPRPPTPAPTIASQPLSLRPEACRPAAGGAWTRGLDFAC DIYIWAPLAGTCGVLLLSLVITLYCNHRNRRR	899
CD8b Transmembrane domain	LGLLVAGVLVLLVSLGVAIHLCC	900
DAP10 Transmembrane domain	ILLAGLVAADAVASLLIVGAVFLCARR	901
EpoR Transmembrane domain	APVGLVARLADESGHVVLRWLPPPETPMTSHIRYEVDVSA GNGAGSVQRVEILEGRTECVLSNLRGRTRYTFAVRARMAE PSFGGFWSAWSEPVSLLTPSD	415
FcERI a Transmembrane domain	FFIPLLVVILFAVDTGLFISTQQQVTFLLKIKRTRKGFRLLNP HPKPNPKNN	416

FcERI a- Transmembrane domain	MAPAMESPTLLCVALLFFAPDGVLAVPQKPKVSLNPPWNR IFKGENVTLTCNGNNFFEVSSTKWFHNGSLSEETNSSLNIV NAKFEDSGEYKCQHQQVNESEPVYLEVFSDWLLLQASAEV VMEGQPLFLRCHGWRNWDVYKVIYYKDGEALKYWYENH NISITNATVEDSGTYYCTGKVWQLDYESEPLNITVIKAPRE KYWLQFFIPLLVVILFAVDTGLFISTQQQVTFLLKIKRTRKG FRLLNPHPKPNPKNN	417
FcERI b- Transmembrane region	MDTESNRRANLALPQEPSSVPAFEVLEISPQEVSSGRLLKSA SSPPLHTWLTVLKKEQEFLGVTQILTAMICLCFGTVVCSVL DISHIEGDIFSSFKAGYPFWGAIFFSISGMLSIISERRNATYLV RGSLGANTASSIAGGTGITILIINLKKSLAYIHIHSCQKFFETK CFMASFSTEIVVMMLFLTILGLGSAVSLTICGAGEELKGNK VPEDRVYEELNIYSATYSELEDPGEMSPPIDL	418
FcERI g- Transmembrane region	MIPAVVLLLLLLVEQAAALGEPQLCYILDAILFLYGIVLTLL YCRLKIQVRKAAITSYEKSDGVYTGLSTRNQETYETLKHEK PPQ	419
FceRIa Transmembrane domain	DIFIPLLVVILFAVDTGLFISTQQQVTFLLKIKRTRKGFRLLN PHPKPNPKNNR	420
GITR Transmembrane domain	PLGWLTVVLLAVAACVLLLTSAQLGLHIWQL	421
Her2 Transmembrane domain	SIISAVVGILLVVVLGVVFGILI	422
Her2 Transmembrane domain	CHPECQPQNGSVTCFGPEADQCVACAHYKDPPFCVARCPS GVKPDLSYMPIWKFPDEEGACQPCPINCTHSCVDLDDKGC PAEQRASPLTSIISAVVGILLVVVLGVVFGILI	423
ICOS Transmembrane domain	FWLPIGCAAFVVVCILGCILI	902
IgG1Transmembrane domain	EPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIARTPE VTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQY NSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEKTI SKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPSDI AVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRW QQGNVFSCSVMHEALHNHYTQKSLSLSPGKKD	903
LAG-3 Transmembrane domain	LLFLILGVLSLLLLVTGAFGF	904
OX40 Transmembrane domain	VAAILGLGLVLGLLGPLAILL	905
PD-1 Transmembrane domain	VGWGGLLGSLVLLVWVLAVI	906
Transmembrane domain	FWALVVVAGVLFCYGLLVTVALCVIWT	907

[00223] Hinge region sequences useful in the present invention are provided in Table 8A.

Table 8A: Hinge regions

Hinge Domain	**Amino Acid Sequence**	**SEQ ID NO.**
Hinge	DKTHT	426
Hinge	CPPC	427
Hinge	CPEPKSCDTPPPCPR	428
Hinge	ELKTPLGDTTHT	429
Hinge	KSCDKTHTCP	430
Hinge	KCCVDCP	431
Hinge	KYGPPCP	432
C233P Hinge	VEPKSPDKTHTCPPCP	433
C233S Hinge	LDPKSSDKTHTCPPCP	434

CD28 Hinge	IEVMYPPPYLDNEKSNGTIIHVKGKHLCPSPLFPGPSKP	435
CD8a Hinge	GGAVHTRGLDFA	436
CD8a Hinge	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLD FACD	437
CD8a Hinge	AKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTR GLDFACD	438
CD8a Hinge	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLD FACD	439
CD8a Hinge	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLD FACD	440
CD8a Hinge	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLD FACDEPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDT	441
CD8a Hinge	PAKPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHT RGLDFACDIY	442
CD8a Hinge	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLD FACDIYIWAPLAGTCGVLLLSLVITLYC	443
CD8a Hinge	TTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDF ACD	444
CD8a Hinge	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLD FACDIY	445
Delta5 Hinge	LDKTHTCPPCP	446
EpoR Hinge	APVGLVARLADESGHVVLRWLPPPETPMTSHIRYEVDVS AGNGAGSVQRVEILEGRTECVLSNLRGRTRYTFAVRARM AEPSFGGFWSAWSEPVSLLTPSD	447
FCRIIα Hinge	GLAVSTISSFFPPGYQ	448
FcγRIIIα Hinge	GLAVSTISSFFPPGYQ	449
Hinge	RWPESPKAQASSVPTAQPQAEGSLAKATTAPATTRNTGR GGEEKKKEKEKEEQEERETKTPECPSHTQPLGVYLLTPAV QDLWLRDKATFTCFVVGSDLKDAHLTWEVAGKVPTGGV EEGLLERHSNGSQSQHSRLTLPRSLWNAGTSVTCTLNHPS LPPQRLMALREPAAQAPVKLSLNLLASSDPPEAASWLLCE VSGFSPPNILLMWLEDQREVNTSGFAPARPPPQPGSTTFW AWSVLRVPAPPSPQPATYTCVVSHEDSRTLLNASRSLEVS YVTDH	450
Hinge	YVTVSSQDPAEPKSPDKTHTCPPCPAPELLGGPSVFLFPPK PKDTLMISRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVH NAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYKCKV SNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTKNQVS LTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSF FLYSKLTVDKSRWQQGNVFSCSVMHEALHNHYTQKSLSL SPGKKDPK	451
Hinge	KPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRG LDFA	452
Hinge	LEPKSCDKTHTCPPCP	453
Hinge	KPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRG LD	454
Hinge	EPKSCDKTHTCPPCP	455
Hinge	ELKTPLGDTHTCPRCP	456
Hinge	EPKSCDTPPPCPRCP	457
Hinge	ESKYGPPCPSCP	458
Hinge	ERKCCVECPPCP	459
Hinge (CH2-CH3)	ESKYGPPCPPCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNS TYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISK AKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIA	460

	VEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRW QEGNVFSCSVMHEALHNHYTQKSLSLSLGK	
Hinge (CH3)	ESKYGPPCPPCPGQPREPQVYTLPPSQEEMTKNQVSLTCL VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYS RLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGK	461
IgD Hinge	RWPESPKAQASSVPTAQPQAEGSLAKATTAPATTRNTGR GGEEKKKEKEKEEQEERETKTPECPSHTQPLGVYLLTPAV QDLWLRDKATFTCFVVGSDLKDAHLTWEVAGKVPTGGV EEGLLERHSNGSQSQHSRLTLPRSLWNAGTSVTCTLNHPS LPPQRLMALREPAAQAPVKLSLNLLASSDPPEAASWLLCE VSGFSPPNILLMWLEDQREVNTSGFAPARPPPQPGSTTFW AWSVLRVPAPPSPQPATYTCVVSHEDSRTLLNASRSLEVS YVTDH	462
IgD Hinge	RWPESPKAQASSVPTAQPQAEGSLAKATTAPATTRNTGR GGEEKKKEKEKEEQEERETKTPECPSHTQPLGVYLLTPAV QDLWLRDKATFTCFVVGSDLKDAHLTWEVAGKVPTGGV EEGLLERHSNGSQSQHSRLTLPRSLWNAGTSVTCTLHPSL PPQRLMALREPAAQAPVKLSLNLLASSDPPEAASWLLCEV SGFSPPNILLMWLEDQREVNTSGFAPARPPPQPGSTTFWA WSVLRVPAPPSPQPATYTCVVSHEDSRTLLNASRSLEVSY VTDH	463
IgD Hinge	RWPESPKAQASSVPTAQPQAEGSLAKATTAPATTRNTGR GGEEKKKEKEKEEQEERETKTPECPSHTQPLGVYLLTPAV QDLWLRDKATFTCFVVGSDLKDAHLTWEVAGKVPTGGV EEGLLERHSNGSQSQHSRLTLPRSLWNAGTSVTCTLNHPS LPPQRLMALREPAAQAPVKLSLNLLASSDPPEAASWLLCE VSGFSPPNILLMVVLEDQREVNTSGFAPARPPPQPGSTTFW AWSVLRVPAPPSPQPATYTCVVSHEDSRTLLNASRSLEVS YVTDH	464
IgD Hinge	ESPKAQASSVPTAQPQAEGSLAKATTAPATTRNTGRGGEE KKKEKEKEEQEERETKTP	465
IgD Hinge	RWPESPKAQASSVPTAQPQAEGSLAKATTAPATTRNTGR GGEEKKKEKEKEEQEERETKTPECPSHTQPLGVYLLTPAV QDLWLRDKATFTCFVVGSDLKDAHLTWEVAGKVPTGGV EEGLLERHSNGSQSQHSRLTLPRSLWNAGTSVTCTLNHPS LPPQRLMALREPAAQAPVKLSLNLLASSDPPEAASWLLCE VSGFSPPNILLMWLEDQREVNTSGFAPARPPPQPGSTTFW AWSVLRVPAPPSPQPATYTCVVSHEDSRTLLNASRSLEVS YVTDH	466
IgD Hinge	RWPESPKAQASSVPTAQPQAEGSLAKATTAPATTRNTGR GGEEKKKEKEKEEQEERETKTPECPSHTQPLGVYLLTPAV QDLWLRDKATFTCFVVGSDLKDAHLTWEVAGKVPTGGV EEGLLERHSNGSQSQHSRLTLPRSLWNAGTSVTCTLNHPS LPPQRLMALREPAAQAPVKLSLNLLASSDPPEAASWLLCE VSGFSPPNILLMWLEDQREVNTSGFAPARPPPQPGSTTFW AWSVLRVPAPPSPQPATYTCVVSHEDSRTLLNASRSLEVS YVTDH	467
IgD Hinge	RWPESPKAQASSVPTAQPQAEGSLAKATTAPATTRNTGR GGEEKKKEKEKEEQEERETKTPECPSHTQPLGVYLLTPAV QDLWLRDKATFTCFVVGSDLKDAHLTWEVAGKVPTGGV EEGLLERHSNGSQSQHSRLTLPRSLWNAGTSVTCTLNHPS LPPQRLMALREPAAQAPVKLSLNLLASSDPPEAASWLLCE VSGFSPPNILLMWLEDQREVNTSGFAPARPPPQPGSTTFW AWSVLRVPAPPSPQPATYTCVVSHEDSRTLLNASRSLEVS YVTDH	468
IgD Hinge	RWPESPKAQASSVPTAQPQAEGSLAKATTAPATTRNTGR GGEEKKKEKEKEEQEERETKTPECPSHTQPLGVYLLTPAV QDLWLRDKATFTCFVVGSDLKDAHLTWEVAGKVPTGGV EEGLLERHSNGSQSQHSRLTLPRSLWNAGTSVTCTLNHPS LPPQRLMALREPAAQAPVKLSLNLLASSDPPEAASWLLCE	469

	VSGFSPPNILLMWLEDQREVNTSGFAPARPPPQPGSTTFW AWSVLRVPAPPSPQPATYTCVVSHEDSRTLLNASRSLEVS YVTDH	
IgG1 (CH2CH3) Hinge domain	AEPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIART PEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREE QYNSTYRVVSVUTVLHQDWLNGKEYKCKVSNKALPAPIE KTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFY PSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVD KSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKKD	470
IgG1 (CH2CH3) Hinge domain	AEPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIART PEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREE QYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIE KTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFY PSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVD KSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKKD	471
IgG1 Hinge	AEPKSPDKTHTCPPCPKDPK	472
IgG1 Hinge	EPKSCDKTHTCPPCP	473
IgG1 Hinge	EPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIARTP EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ YNSTYRVVSVLTVLHQDWLNGKEVKCKVSNKALPAPIEK TISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS DIAVEWESNGQPENNYKTTPPVLDSDGSFFLVSKLTVDKS RWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKKD	474
IgG1 Hinge	SVFLFPPKPKDTL	475
IgG1 Hinge	EPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIARTP EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEK TISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKS RWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK	476
IgG1 Hinge	EPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIARTP EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEK TISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKS RWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKKDPK	477
IgG1 Hinge	VECPPCPAPPVAGPSVFLFPPKPKDTLMISRTPEVTCVVVD VSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVV SVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISKAKGQP REPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWES NGQPENNYKTTPPVLDSDGSFFLYSKLTVDKSRWQQGNV FSCSVMHEALHNHYTQKSLSLSPGK	478
IgG1 Hinge (CH2CH3 domain)	DPAEPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIA RTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPR EEQYNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPA PIEKTISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKG FYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLT VDKSRWQQGNVFSCSVMHEALHNHYTQKSLSLSPGKK	479
IgG2 Hinge	ERKCCVECPPCP	480
IgG3 Hinge	ELKTPLGDTTHTCPRCP	481
IgG3 Hinge	ELKTPLGDTHTCPRCPEPKSCDTPPPCPRCPEPKSCDTPPPC PRCPEPKSCDTPPPCPRCP	482
IgG4 (CH2 and CH3)	ESKYGPPCPPCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNS TYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISK AKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIA VEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRW QEGNVFSCSVMHEALHNHYTQKSLSLSLGKM	483

IgG4 (CH2 and CH3)	ESKYGPPCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFQS TYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISK AKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIA VEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRW QEGNVFSCSVMHEALHNHYTQKSLSLSLGKM	484
IgG4 Hinge	SPNMVPHAHHAQ	485
IgG4 Hinge	GQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVE WESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRWQE GNVFSCSVMHEALHNHYTQKSLSLSLGK	486
IgG4 Hinge	ESKYGPPCPPCPGGGSSGGGSGGQPREPQVYTLPPSQEEM TKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVL DSDGSFFLYSRLTVDKSRWQEGNVFSCSVMHEALHNHYT QKSLSLSLGK	487
IgG4 Hinge	ESKYGPPCPSCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSQEDPEVQFNWYVDGVEVHQAKTKPREEQFNS TYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISK AKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFVPSDIA VEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRW QEGNVFSCSVMHEALHNHYTQKSLSLSLGK	488
IgG4 Hinge	ESKYGPPCPPCPAPEFEGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSQEDPEVQFNWYVDGVEVHQAKTKPREEQFNS TYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISK AKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFVPSDIA VEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRW QEGNVFSCSVMHEALHNHYTQKSLSLSLGK	489
IgG4 Hinge	ESKYGPPCPPCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNS TYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISK AKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIA VEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRW QEGNVFSCSVMHEALHNHYTQKSLSLSLGKM	490
IgG4 Hinge	GAATCTAAGTACGGACCGCCCTGCCCCCCTTGCCCT	491
IgG4 Hinge	ESKYGPPCPPCP	492
IgG4 Hinge	ESKYGPPCPPCPGQPREPQVYTLPPSQEEMTKNQVSLTCL VKGFYPSDIAVEWESNGQPENNYKTTPPVLDSDGSFFLYS RLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGK	493
IgG4 Hinge	ESKYGPPCPPCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNS TYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISK AKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIA VEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRW QEGNVFSCSVMHEALHNHYTQKSLSLSLGK	494
IgG4 Hinge	ESKYGPPCPPCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNS TYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISK AKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIA VEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDKSRW QEGNVFSCSVMHEALHNHYTQKSLSLSLGK	495
IgG4 Hinge	ESKYGPPCPPCP	496
IgG4 Hinge	YGPPCPPCP	497
IgG4 Hinge	KYGPPCPPCP	498
IgG4 Hinge	EVVKYGPPCPPCP	499
IgG4 Hinge	ESKYGPPCPSCPAPEFLGGPSVFLFPPKPKDTLMISRTPEVT CVVVDVSQEDPEVQFNWYVDGVEVHNAKTKPREEQFNS TYRVVSVLTVLHQDWLNGKEYKCKVSNKGLPSSIEKTISK AKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIA	500

	VEWESNGQPENNYKTTPPVLDSDGSFFLYSRLTVDLSRW QEGNVFSCSVMHEALHNHYTQKSLSLSLGK	
IgG4 Hinge and Linker	ESKYGPPCPPCPGGGSSGGGSG	501
IgG1 Hinge	EPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIARTP EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEK TISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS DIAVEWESNGQPENNYKTIPPVLDSDGSFFLYSKLTVDKS RWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK	502
IgG1 Hinge	EPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIARTP EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEK TISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS DIAVEWESNGQPENNYKTIPPVLDSDGSFFLYSKLTVDKS RWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK	503
IgG1 Hinge	EPKSPDKTHTCPPCPAPPVAGPSVFLFPPKPKDTLMIARTP EVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQ YNSTYRVVSVLTVLHQDWLNGKEYKCKVSNKALPAPIEK TISKAKGQPREPQVYTLPPSRDELTKNQVSLTCLVKGFYPS DIAVEWESNGQPENNYKTTPPVLDSDGSFFLYSKLTVDKS RWQQGNVFSCSVMHEALHNHYTQKSLSLSPGK	504

[00224] Hinge and transmembrane region sequences useful in the present invention are provided in Table 8B.

Table 8B: Hinge and Transmembrane regions

Hinge Domain	**Amino Acid Sequence**	**SEQ ID NO.**
CD8a Transmembrane and Hinge	TTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDF ACDIYIWAPLAGTCGVLLLSLVITLYC	505
CD8a Transmembrane and Hinge	DIQMTQSSSYLSVSLGGRVTITCKASDHINNWLAWYQQK PGNAPRLLISGATSLETGVPSRFSGSGSGKDYTLSITSLQTE DVATYYCQQYWSTPFTFGSGTKLEIKGGGGSGGGGSGGG GSQVQLKESGPGLVAPSQSLSITSTVSGFSLSRYSVHWVR QPPGKGLEWLGMIWGGGSTDYNSALKSRLSISKDNSKSQ VFLKMNSLQTDDTAMYYCARNEGDTTAGTWFAYWGQG TLVTVSS	506
CD8a Transmembrane and Hinge	ALSNSIMYFSHFVPVFLPAKPTTTPAPRPPTPAPTIASQPLS LRPEACRPAAGGAVHTRGLD	507
CD8a Transmembrane and Hinge	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRGLD FACDIYIWAPLAGTCGVLLLSLVITLY	508
CD8a Transmembrane and Hinge	KPTTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRG LDFACDIYIWAPLAGTCGVLLLSLVITLY	509

[00225] 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.

[00226] 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.

[00227] In some embodiments, the CAR sequences may be selected from Table 9.

Table 9: CAR sequences

Description	SEQ ID NO	Source
CD19 CAR	510	SEQ ID NO: 12 in US9499629B2
CD19 CAR	511	SEQ ID NO: 24 in US20160333108A1
CD19 CAR	512	SEQ ID NO: 25 in US20160333108A1
CD19 CAR	513	SEQ ID NO: 26 in US20160333108A1
CD19 CAR	514	SEQ ID NO: 27 in US20160333108A1
CD19 CAR	515	SEQ ID NO: 1 in EP2997134A4
CD19 CAR	516	SEQ ID NO: 19 in EP3071687A1
CD19 CAR	517	SEQ ID NO: 20 in EP3071687A1
CD19 CAR	518	SEQ ID NO: 181 in WO2016168773A3
CD19 CAR	519	SEQ ID NO: 2 in WO2015157399A9
CD19 CAR	520	SEQ ID NO: 56 in WO2016174409A1
CD19 CAR	521	SEQ ID NO: 62 in WO2016174409A1
CD19 CAR	522	SEQ ID NO: 145 in WO2016179319A1
CD19 CAR	523	SEQ ID NO: 293 in US20160311907A1
CD19 CAR	524	SEQ ID NO: 294 in US20160311907A1
CD19 CAR	525	SEQ ID NO: 295 in US20160311907A1
CD19 CAR	526	SEQ ID NO: 296 in US20160311907A1
CD19 CAR	527	SEQ ID NO: 297 in US20160311907A1
CD19 CAR	528	SEQ ID NO: 298 in US20160311907A1
CD19 CAR	529	SEQ ID NO. 73 in WO2013176915A1
CD19 CAR	530	SEQ ID NO. 73 in WO2013176916A1
CD19 CAR	531	SEQ ID NO. 73 in US20130315884A1
CD19 CAR	532	SEQ ID NO. 73 in US20140134142A1
CD19 CAR	533	SEQ ID NO. 73 in US20150017136A1
CD19 CAR	534	SEQ ID NO. 73 in US20150203817A1
CD19 CAR	535	SEQ ID NO. 73 in US20160120905A1
CD19 CAR	536	SEQ ID NO. 73 in US20160120906A1
CD19 CAR	537	SEQ ID NO. 8 in WO2015124715
CD19 CAR	538	SEQ ID NO. 5 in WO2015124715
CD19 CAR	539	SEQ ID NO. 73 in WO2014184744
CD19 CAR	540	SEQ ID NO. 73 in WO2014184741
CD19 CAR	541	SEQ ID NO. 14 in US20160145337A1
CD19 CAR	542	SEQ ID NO. 15 in US20160145337A1
CD19 CAR	543	SEQ ID NO. 14 in WO2014184143
CD19 CAR	544	SEQ ID NO. 15 in WO2014184143
CD19 CAR	545	SEQ ID NO. 15 in WO2015075175
CD19 CAR	546	SEQ ID NO. 16 in WO2015075175
CD19 CAR	547	SEQ ID NO. 16 in US20160145337A1
CD19 CAR	548	SEQ ID NO. 16 in WO2014184143
CD19 CAR	549	SEQ ID NO 12 in WO2012079000
CD19 CAR	550	SEQ ID NO.31 in WO2016164580
CD19 CAR	551	SEQ ID NO.32 in WO2016164580
CD19 CAR	552	SEQ ID NO.33 in WO2016164580
CD19 CAR	553	SEQ ID NO.34 in WO2016164580

CD19 CAR	554	SEQ ID NO.35 in WO2016164580
CD19 CAR	555	SEQ ID NO.36 in WO2016164580
CD19 CAR	556	SEQ ID NO.37 in WO2016164580
CD19 CAR	557	SEQ ID NO.38 in WO2016164580
CD19 CAR	558	SEQ ID NO.39 in WO2016164580
CD19 CAR	559	SEQ ID NO.40 in WO2016164580
CD19 CAR	560	SEQ ID NO.41 in WO2016164580
CD19 CAR	561	SEQ ID NO.42 in WO2016164580
CD19 CAR	562	SEQ ID NO.58 in WO2016164580
CD19 CAR	563	SEQ ID NO: 14 in US20160296563A1
CD19 CAR	564	SEQ ID NO: 15 in US20160296563A1
CD19 CAR	565	SEQ ID NO.31 in WO2015157252
CD19 CAR	566	SEQ ID NO.32 in WO2015157252
CD19 CAR	567	SEQ ID NO.33 in WO2015157252
CD19 CAR	568	SEQ ID NO.34 in WO2015157252
CD19 CAR	569	SEQ ID NO.35 in WO2015157252
CD19 CAR	570	SEQ ID NO.36 in WO2015157252
CD19 CAR	571	SEQ ID NO.37 in WO2015157252
CD19 CAR	572	SEQ ID NO.38 in WO2015157252
CD19 CAR	573	SEQ ID NO.39 in WO2015157252
CD19 CAR	574	SEQ ID NO.40 in WO2015157252
CD19 CAR	575	SEQ ID NO.41 in WO2015157252
CD19 CAR	576	SEQ ID NO.42 in WO2015157252
CD19 CAR	577	SEQ ID NO. 14 in WO2016139487
CD19 CAR	578	SEQ ID NO.15 in WO2016139487
CD19 CAR	579	SEQ ID NO: 53 in US20160250258A1
CD19 CAR	580	SEQ ID NO: 54 in US20160250258A1
CD19 CAR	581	SEQ ID NO: 55 in US20160250258A1
CD19 CAR	582	SEQ ID NO: 56 in US20160250258A1
CD19 CAR	583	SEQ ID NO: 57 in US20160250258A1
CD19 CAR	584	SEQ ID NO: 58 in US20160250258A1
CD19 CAR	585	SEQ ID NO. 1 in WO2015187528
CD19 CAR	586	SEQ ID NO. 2 in WO2015187528
CD19 CAR	587	SEQ ID NO. 3 in WO2015187528
CD19 CAR	588	SEQ ID NO. 4 in WO2015187528
CD19 CAR	589	SEQ ID NO. 5 in WO2015187528
CD19 CAR	590	SEQ ID NO. 6 in WO2015187528
CD19 CAR	591	SEQ ID NO. 7 in WO2015187528
CD19 CAR	592	SEQ ID NO. 8 in WO2015187528
CD19 CAR	593	SEQ ID NO. 9 in WO2015187528
CD19 CAR	594	SEQ ID NO. 10 in WO2015187528
CD19 CAR	595	SEQ ID NO. 11 in WO2015187528
CD19 CAR	596	SEQ ID NO. 12 in WO2015187528
CD19 CAR	597	SEQ ID NO. 13 in WO2015187528
CD19 CAR	598	SEQ ID. NO. 31 in WO2015157252
CD19 CAR	599	SEQ ID. NO. 32 in WO2015157252
CD19 CAR	600	SEQ ID. NO. 33 in WO2015157252
CD19 CAR	601	SEQ ID. NO. 34 in WO2015157252
CD19 CAR	602	SEQ ID. NO. 35 in WO2015157252
CD19 CAR	603	SEQ ID. NO. 36 in WO2015157252
CD19 CAR	604	SEQ ID. NO. 37 in WO2015157252
CD19 CAR	605	SEQ ID. NO. 38 in WO2015157252
CD19 CAR	606	SEQ ID. NO. 39 in WO2015157252
CD19 CAR	607	SEQ ID. NO. 40 in WO2015157252
CD19 CAR	608	SEQ ID. NO. 41 in WO2015157252
CD19 CAR	609	SEQ ID. NO. 42 in WO2015157252
CD19 CAR	610	SEQ ID. NO. 58 in WO2015157252
CD19 CAR	611	SEQ ID NO. 31 in WO2014153270

CD19 CAR	612	SEQ ID NO. 32 in WO2014153270
CD19 CAR	613	SEQ ID NO. 33 in WO2014153270
CD19 CAR	614	SEQ ID NO. 34 in WO2014153270
CD19 CAR	615	SEQ ID NO. 35 in WO2014153270
CD19 CAR	616	SEQ ID NO. 36 in WO2014153270
CD19 CAR	617	SEQ ID NO. 37 in WO2014153270
CD19 CAR	618	SEQ ID NO. 38 in WO2014153270
CD19 CAR	619	SEQ ID NO. 39 in WO2014153270
CD19 CAR	620	SEQ ID NO. 40 in WO2014153270
CD19 CAR	621	SEQ ID NO. 41 in WO2014153270
CD19 CAR	622	SEQ ID NO. 42 in WO2014153270
CD19 CAR (Third generation)	623	SEQ ID NO.13 in WO2016139487

[00228] In one embodiment of the present invention, the payload of the invention is a CD19 specific CAR targeting different B cell. In the context of the invention, an effector module may comprise a hDHFR DD, ecDHFR DD, or FKBP DD operably linked to a CD19 CAR fusion construct. In some instances, the promoter utilized to drive the expression of the effector module in the vector may be a CMV promoter or an EF1a. The efficiency of the promoter in driving the expression of the same construct may be compared. For example, two constructs that differ only by their promoter, CMV (in OT-CD19N-001) or EF1a promoter (in OT-CD19N-017) may be compared. The amino acid sequences of CD19 CAR constructs and its components are presented in Table 10a and Table 10b. The amino acid sequences in Table 10a and/or Table 10b may comprise a stop codon which is denoted in the table with a "*" at the end of the amino acid sequence.

Table 10a: Sequences of components of CD19 CARs

Description	Amino Acid Sequence	Amino Acid SEQ ID NO	Nucleic Acid SEQ ID NO
CD19 scFv	DIQMTQTTSSLSASLGDRVTISCRASQDISKYLNW YQQKPDGTVKLLIYHTSRLHSGVPSRFSGSGSGTD YSLTISNLEQEDIATYFCQQGNTLPYTFGGGTKLEI TGGGGSGGGGSGGGGSEVKLQESGPGLVAPSQSL SVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVIW GSETTYYNSALKSRLTIIKDNSKSQVFLKMNSLQT DDTAIYYCAKHYYYGGSYAMDYWGQGTSVTVS S	624	626, 650-654
CD8α hinge--TM	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVH TRGLDFACDIYIWAPLAGTCGVLLLSLVITLYC	625	627, 655, 982-984
CD8α hinge	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVH TRGLDFACD	440	656-660
CD3 zeta signaling domain	RVKFSRSADAPAYKQGQNQLYNELNLGRREEYD VLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDK MAEAYSEIGMKGERRRGKGHDGLYQGLSTATKD TYDALHMQALPPR	339	661-665, 986
4-1BB (41BB) intracellular signaling domain	KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEE EEGGCEL	273	666-670, 985
CD8a Transmembrane domain	IYIWAPLAGTCGVLLLSLVITLYC	409	990, 992

CD8α leader	MALPVTALLLPLALLLHAARP	628	671-675
p40 signal sequence	MCHQQLVISWFSLVFLASPLVA	719	736-744
p40	IWELKKDVYVVELDWYPDAPGEMVVLTCDTPEE DGITWTLDQSSEVLGSGKTLTIQVKEFGDAGQYT CHKGGEVLSHSLLLLHKKEDGIWSTDILKDQKEP KNKTFLRCEAKNYSGRFTCWWLTTISTDLTFSVK SSRGSSDPQGVTCGAATLSAERVRGDNKEYEYSV ECQEDSACPAAEESLPIEVMVDAVHKLKYENYTS SFFIRDIIKPDPPKNLQLKPLKNSRQVEVSWEYPDT WSTPHSYFSLTFCVQVQGKSKREKKDRVFTDKTS ATVICRKNASISVRAQDRYYSSSWSEWASVPCS	723	632-634, 752-761
p35	RNLPVATPDPGMFPCLHHSQNLLRAVSNMLQKA RQTLEFYPCTSEEIDHEDITKDKTSTVEACLPLELT KNESCLNSRETSFITNGSCLASRKTSFMMALCLSSI YEDLKMYQVEFKTMNAKLLMDPKRQIFLDQNML AVIDELMQALNFNSETVPQKSSLEEPDFYKTKIKL CILLHAFRIRAVTIDRVMSYLNAS	724	762-771, 1012
IL15	NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSC KVTAMKCFLLELQVISLESGDASIHDTVENLIILAN NSLSSNGNVTESGCKECEELEEKNIKEFLQSFVHIV QMFINTS	785	794-797, 1001
IL15Ra	ITCPPPMSVEHADIWVKSYSLYSRERYICNSGFKR KAGTSSLTECVLNKATNVAHWTTPSLKCIRDPAL VHQRPAPPSTVTTAGVTPQPESLSPSGKEPAASSPS SNNTAATTAAIVPGSQLMPSKSPSTGTTEISSHESS HGTPSQTTAKNWELTASASHQPPGVYPQGHSDTT VAISTSTVLLCGLSAVSLLACYLKSRQTPPLASVE MEAMEALPVTWGTSSRDEDLENCSHHL	803	812-813, 1003
mCherry (M1L)	LSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIE GEGEGRPYEGTQTAKLKVTKGGPLPFAWDILSPQ FMYGSKAYVKHPADIPDYLKLSFPEGFKWERVM NFEDGGVVTVTQDSSLQDGEFIYKVKLRGTNFPS DGPVMQKKTMGWEASSERMYPEDGALKGEIKQR LKLKDGGHYDAEVKTTYKAKKPVQLPGAYNVNI KLDITSHNEDYTIVEQYERAEGRHSTGGMDELYK	1029	1030
IRES	-	-	999
Linker (GGSGG)	GGSGG	629	676-680
Linker (SG)	SG	-	AGTGGA
Linker ((G4S)3)	GGGGSGGGGSGGGGS	720	910-915
Linker (GGSG)	GGSG	822	823
Linker	MLLLVTSLLLCELPHPAFLLIP	1031	1032
Linker (SG3-(SG4)3-SG3-SLQ)	SGGGSGGGGSGGGGSGGGGSGGGSLQ	802	811, 916-920, 1002
Modified Furin	ESRRVRRNKRSK	630	681-683
BamHI	-	-	GGATCC
Spacer	-	-	1000
HA Tag	YPYDVPDYA	1024	1025-1027
FKBP (F36V, L106P)	GVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGK KVDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMS VGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVE LLKPE	11	684-686, 987, 989
FKBP (E31G, F36V, R71G, K105E)	GVQVETISPGDGRTFPKRGQTCVVHYTGMLGDG KKVDSSRDRNKPFKFMLGKQEVIRGWEEGVAQM SVGQGAKLTISPDYAYGATGHPGIIPPHATLVFDV ELLELE	12	688-691, 994, 1013, 1028
ecDHFR (Amino acid 2-159 of WT) (R12Y, Y100I)	ISLIAALAVDYVIGMENAMPWNLPADLAWFKRN TLNKPVIMGRHTWESIGRPLPGRKNIILSSQPGTDD RVTWVKSVDEAIAACGDVPEIMVIGGGRVIEQFLP	9	692, 772, 814, 687, 988, 991

	KAQKLYLTHIDAEVEGDTHFPDYEPDDWESVFSE FHDADAQNSHSYCFEILERR		
ecDHFR (Amino acid 2-159 of WT) (R12H, E129K)	ISLIAALAVDHVIGMENAMPWNLPADLAWFKRN TLNKPVIMGRHTWESIGRPLPGRKNIILSSQPGTDD RVTWVKSVDEAIAACGDVPEIMVIGGGRVYEQFL PKAQKLYLTHIDAEVEGDTHFPDYKPDDWESVFS EFHDADAQNSHSYCFEILERR	10	798, 815, 993
hDHFR (Amino acid 2-187 of WT; Y122I)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYF QRMTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLK GRINLVLSRELKEPPQGAHFLSRSLDDALKLTEQP ELANKVDMVWIVGGSSVIKEAMNHPGHLKLFVT RIMQDFESDTFFPEIDLEKYKLLPEYPGVLSDVQE EKGIKYKFEVYEKND	895	694, 995
hDHFR (Amino acid 2-187 of WT; Y122I, A125F)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYF QRMTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLK GRINLVLSRELKEPPQGAHFLSRSLDDALKLTEQP ELANKVDMVWIVGGSSVIKEFMNHPGHLKLFVT RIMQDFESDTFFPEIDLEKYKLLPEYPGVLSDVQE EKGIKYKFEVYEKND	890	696, 973, 974, 996
hDHFR (Amino acid 2-187 of WT; Q36K, Y122I)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYF KRMTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLK GRINLVLSRELKEPPQGAHFLSRSLDDALKLTEQP ELANKVDMVWIVGGSSVIKEAMNHPGHLKLFVT RIMQDFESDTFFPEIDLEKYKLLPEYPGVLSDVQE EKGIKYKFEVYEKND	981	698, 997
hDHFR (Q36F, N65F, Y122I)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYF FRMTTTSSVEGKQNLVIMGKKTWFSIPEKFRPLKG RINLVLSRELKEPPQGAHFLSRSLDDALKLTEQPE LANKVDMVWIVGGSSVIKEAMNHPGHLKLFVTRI MQDFESDTFFPEIDLEKYKLLPEYPGVLSDVQEEK GIKYKFEVYEKND	891	700, 975, 976, 998
Description	**Amino Acid Sequence**	**Amino Acid SEQ ID NO**	**Nucleic Acid SEQ ID NO**
CD19 scFv	DIQMTQTTSSLSASLGDRVTISCRASQDISKYLNW YQQKPDGTVKLLIYHTSRLHSGVPSRFSGSGSGTD YSLTISNLEQEDIATYFCQQGNTLPYTFGGGTKLEI TGGGGSGGGGSGGGGSEVKLQESGPGLVAPSQSL SVTCTVSGVSLPDYGVSWIRQPPRKGLEWLGVIW GSETTYYNSALKSRLTIIKDNSKSQVFLKMNSLQT DDTAIYYCAKHYYYGGSYAMDYWGQGTSVTVS S	624	626, 650-654
CD8α hinge--TM	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVH TRGLDFACDIYIWAPLAGTCGVLLLSLVITLYC	625	627, 655, 982-984
CD8α hinge	TTTPAPRPPTPAPTIASQPLSLRPEACRPAAGGAVH TRGLDFACD	440	656-660
CD3 zeta signaling domain	RVKFSRSADAPAYKQGQNQLYNELNLGRREEYD VLDKRRGRDPEMGGKPRRKNPQEGLYNELQKDK MAEAYSEIGMKGERRRGKGHDGLYQGLSTATKD TYDALHMQALPPR	339	661-665, 986
4-1BB (41BB) intracellular signaling domain	KRGRKKLLYIFKQPFMRPVQTTQEEDGCSCRFPEE EEGGCEL	273	666-670, 985
CD8a Transmembrane domain	IYIWAPLAGTCGVLLLSLVITLYC	409	990, 992
CD8α leader	MALPVTALLLPLALLLHAARP	628	671-675
p40 signal sequence	MCHQQLVISWFSLVFLASPLVA	719	736-744
p40	IWELKKDVYVVELDWYPDAPGEMVVLTCDTPEE DGITWTLDQSSEVLGSGKTLTIQVKEFGDAGQYT CHKGGEVLSHSLLLLHKKEDGIWSTDILKDQKEP	723	632-634, 752-761

	KNKTFLRCEAKNYSGRFTCWWLTTISTDLTFSVK SSRGSSDPQGVTCGAATLSAERVRGDNKEYEYSV ECQEDSACPAAEESLPIEVMVDAVHKLKYENYTS SFFIRDIIKPDPPKNLQLKPLKNSRQVEVSWEYPDT WSTPHSYFSLTFCVQVQGKSKREKKDRVFTDKTS ATVICRKNASISVRAQDRYYSSSWSEWASVPCS		
p35	RNLPVATPDPGMFPCLHHSQNLLRAVSNMLQKA RQTLEFYPCTSEEIDHEDITKDKTSTVEACLPLELT KNESCLNSRETSFITNGSCLASRKTSFMMALCLSSI YEDLKMYQVEFKTMNAKLLMDPKRQIFLDQNML AVIDELMQALNFNSETVPQKSSLEEPDFYKTKIKL CILLHAFRIRAVTIDRVMSYLNAS	724	762-771, 1012
IL15	NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSC KVTAMKCFLLELQVISLESGDASIHDTVENLIILAN NSLSSNGNVTESGCKECEELEEKNIKEFLQSFVHIV QMFINTS	785	794-797, 1001
IL15Ra	ITCPPPMSVEHADIWVKSYSLYSRERYICNSGFKR KAGTSSLTECVLNKATNVAHWTTPSLKCIRDPAL VHQRPAPPSTVTTAGVTPQPESLSPSGKEPAASSPS SNNTAATTAAIVPGSQLMPSKSPSTGTTEISSHESS HGTPSQTTAKNWELTASASHQPPGVYPQGHSDTT VAISTSTVLLCGLSAVSLLACYLKSRQTPPLASVE MEAMEALPVTWGTSSRDEDLENCSHHL	803	812-813, 1003
mCherry (M1L)	LSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIE GEGEGRPYEGTQTAKLKVTKGGPLPFAWDILSPQ FMYGSKAYVKHPADIPDYLKLSFPEGFKWERVM NFEDGGVVTVTQDSSLQDGEFIYKVKLRGTNFPS DGPVMQKKTMGWEASSERMYPEDGALKGEIKQR LKLKDGGHYDAEVKTTYKAKKPVQLPGAYNVNI KLDITSHNEDYTIVEQYERAEGRHSTGGMDELYK	1029	1030
IRES	-	-	999
Linker (GGSGG)	GGSGG	629	676-680
Linker (SG)	SG	-	AGTGGA
Linker ((G4S)3)	GGGGSGGGGSGGGGS	720	910-915
Linker (GGSG)	GGSG	822	823
Linker	MLLLVTSLLLCELPHPAFLLIP	1031	1032
Linker (SG3-(SG4)3-SG3-SLQ)	SGGGSGGGGSGGGGSGGGGSGGGSLQ	802	811, 916-920, 1002
Modified Furin	ESRRVRRNKRSK	630	681-683
BamHI	-	-	GGATCC
Spacer	-	-	1000
HA Tag	YPYDVPDYA	1024	1025-1027
FKBP (F36V, L106P)	GVQVETISPGDGRTFPKRGQTCVVHYTGMLEDGK KVDSSRDRNKPFKFMLGKQEVIRGWEEGVAQMS VGQRAKLTISPDYAYGATGHPGIIPPHATLVFDVE LLKPE	11	684-686, 987, 989
FKBP (E31G, F36V, R71G, K105E)	GVQVETISPGDGRTFPKRGQTCVVHYTGMLGDG KKVDSSRDRNKPFKFMLGKQEVIRGWEEGVAQM SVGQGAKLTISPDYAYGATGHPGIIPPHATLVFDV ELLELE	12	688-691, 994, 1013, 1028
ecDHFR (Amino acid 2-159 of WT) (R12Y, Y100I)	ISLIAALAVDYVIGMENAMPWNLPADLAWFKRN TLNKPVIMGRHTWESIGRPLPGRKNIILSSQPGTDD RVTWVKSVDEAIAACGDVPEIMVIGGGRVIEQFLP KAQKLYLTHIDAEVEGDTHFPDYEPDDWESVFSE FHDADAQNSHSYCFEILERR	9	692, 772, 814, 687, 988, 991
ecDHFR (Amino acid 2-159 of WT) (R12H, E129K)	ISLIAALAVDHVIGMENAMPWNLPADLAWFKRN TLNKPVIMGRHTWESIGRPLPGRKNIILSSQPGTDD RVTWVKSVDEAIAACGDVPEIMVIGGGRVYEQFL	10	798, 815, 993

	PKAQKLYLTHIDAEVEGDTHFPDYKPDDWESVFS EFHDADAQNSHSYCFEILERR		
hDHFR (Amino acid 2-187 of WT; Y122I)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYF QRMTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLK GRINLVLSRELKEPPQGAHFLSRSLDDALKLTEQP ELANKVDMVWIVGGSSVIKEAMNHPGHLKLFVT RIMQDFESDTFFPEIDLEKYKLLPEYPGVLSDVQE EKGIKYKFEVYEKND	895	694, 995
hDHFR (Amino acid 2-187 of WT; Y122I, A125F)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYF QRMTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLK GRINLVLSRELKEPPQGAHFLSRSLDDALKLTEQP ELANKVDMVWIVGGSSVIKEFMNHPGHLKLFVT RIMQDFESDTFFPEIDLEKYKLLPEYPGVLSDVQE EKGIKYKFEVYEKND	890	696, 973, 974, 996
hDHFR (Amino acid 2-187 of WT; Q36K, Y122I)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYF KRMTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLK GRINLVLSRELKEPPQGAHFLSRSLDDALKLTEQP ELANKVDMVWIVGGSSVIKEAMNHPGHLKLFVT RIMQDFESDTFFPEIDLEKYKLLPEYPGVLSDVQE EKGIKYKFEVYEKND	981	698, 997
hDHFR (Q36F, N65F, Y122I)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYF FRMTTTSSVEGKQNLVIMGKKTWFSIPEKFRPLKG RINLVLSRELKEPPQGAHFLSRSLDDALKLTEQPE LANKVDMVWIVGGSSVIKEAMNHPGHLKLFVTRI MQDFESDTFFPEIDLEKYKLLPEYPGVLSDVQEEK GIKYKFEVYEKND	891	700, 975, 976, 998

Table 10b: Sequences of CD19 CARs

Description	Amino Acid Sequence	Amino Acid SEQ ID NO	Nucleic Acid SEQ ID NO
OT-CD19 CAR-001 (OT-CD19c-001) (CD8a leader -CD19 scFV - CD8a-Tm -41BB - CD3zeta - stop)	MALPVTALLLPLALLLHAARPDIQMTQTTSSLSAS LGDRVTISCRASQDISKYLNWYQQKPDGTVKLLI YHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIA TYFCQQGNTLPYTFGGGTKLEITGGGGSGGGGSG GGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPD YGVSWIRQPPRKGLEWLGVIWGSETTYYNSALKS RLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHY YYGGSYAMDYWGQGTSVTVSSTTTPAPRPPTPAP TIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYI WAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQP FMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRS ADAPAYKQGQNQLYNELNLGRREEYDVLDKRRG RDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSE IGMKGERRRGKGHDGLYQGLSTATKDTYDALHM QALPPR*	635	701
OT-CD19 CAR-002 (OT-CD19c-002) (CD8a leader - CD19 scFV - FKBP (F36V, L106P) - CD8a-Tm - 41BB - CD3zeta - stop)	MALPVTALLLPLALLLHAARPDIQMTQTTSSLSAS LGDRVTISCRASQDISKYLNWYQQKPDGTVKLLI YHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIA TYFCQQGNTLPYTFGGGTKLEITGGGGSGGGGSG GGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPD YGVSWIRQPPRKGLEWLGVIWGSETTYYNSALKS RLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHY YYGGSYAMDYWGQGTSVTVSSGVQVETISPGDG RTFPKRGQTCVVHYTGMLEDGKKVDSSRDRNKP FKFMLGKQEVIRGWEEGVAQMSVGQRAKLTISPD YAYGATGHPGIIPPHATLVFDVELLKPETTTPAPRP PTPAPTIASQPLSLRPEACRPAAGGAVHTRGLDFA	636	702

	CDIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLLY IFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELRV KFSRSADAPAYKQGQNQLYNELNLGRREEYDVL DKRRGRDPEMGGKPRRKNPQEGLYNELQKDKM AEAYSEIGMKGERRRGKGHDGLYQGLSTATKDT YDALHMQALPPR*		
OT-CD19 CAR-003 (OT-CD19c-003) (CD8a leader - CD19 scFV - ecDHFR - CD8a-Tm - 41BB - CD3zeta - stop)	MALPVTALLLPLALLLHAARPDIQMTQTTSSLSAS LGDRVTISCRASQDISKYLNWYQQKPDGTVKLLI YHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIA TYFCQQGNTLPYTFGGGTKLEITGGGGSGGGGSG GGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPD YGVSWIRQPPRKGLEWLGVIWGSETTYYNSALKS RLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHY YYGGSYAMDYWGQGTSVTVSSISLIAALAVDYVI GMENAMPWNLPADLAWFKRNTLNKPVIMGRHT WESIGRPLPGRKNIILSSQPGTDDRVTWVKSVDEA IAACGDVPEIMVIGGGRVIEQFLPKAQKLYLTHID AEVEGDTHFPDYEPDDWESVFSEFHDADAQNSHS YCFEILERRTTTPAPRPPTPAPTIASQPLSLRPEACR PAAGGAVHTRGLDFACDIYIWAPLAGTCGVLLLS LVITLYCKRGRKKLLYIFKQPFMRPVQTTQEEDGC SCRFPEEEEGGCELRVKFSRSADAPAYKQGQNQL YNELNLGRREEYDVLDKRRGRDPEMGGKPRRKN PQEGLYNELQKDKMAEAYSEIGMKGERRRGKGH DGLYQGLSTATKDTYDALHMQALPPR*	637	703
OT-CD19 CAR-004 (OT-CD19c-004) (CD8a leader - CD19 scFV - CD8a Hinge - FKBP (F36V, L106P) -CD8a Transmembrane domain-41BB - CD3zeta - stop)	MALPVTALLLPLALLLHAARPDIQMTQTTSSLSAS LGDRVTISCRASQDISKYLNWYQQKPDGTVKLLI YHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIA TYFCQQGNTLPYTFGGGTKLEITGGGGSGGGGSG GGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPD YGVSWIRQPPRKGLEWLGVIWGSETTYYNSALKS RLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHY YYGGSYAMDYWGQGTSVTVSSTTTPAPRPPTPAP TIASQPLSLRPEACRPAAGGAVHTRGLDFACDGV QVETISPGDGRTFPKRGQTCVVHYTGMLEDGKKV DSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVG QRAKLTISPDYAYGATGHPGIIPPHATLVFDVELL KPEIYIWAPLAGTCGVLLLSLVITLYCKRGRKKLL YIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGCELR VKFSRSADAPAYKQGQNQLYNELNLGRREEYDV LDKRRGRDPEMGGKPRRKNPQEGLYNELQKDKM AEAYSEIGMKGERRRGKGHDGLYQGLSTATKDT YDALHMQALPPR*	638	704
OT-CD19 CAR-005 (OT-CD19c-005) (CD8a leader - CD19 scFV - CD8a Hinge - ecDHFR (Amino acid 2-159 of WT) (R12Y, Y100I) - CD8a Transmembrane domain -41BB -CD3zeta - stop)	MALPVTALLLPLALLLHAARPDIQMTQTTSSLSAS LGDRVTISCRASQDISKYLNWYQQKPDGTVKLLI YHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIA TYFCQQGNTLPYTFGGGTKLEITGGGGSGGGGSG GGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPD YGVSWIRQPPRKGLEWLGVIWGSETTYYNSALKS RLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHY YYGGSYAMDYWGQGTSVTVSSTTTPAPRPPTPAP TIASQPLSLRPEACRPAAGGAVHTRGLDFACDISLI AALAVDYVIGMENAMPWNLPADLAWFKRNTLN KPVIMGRHTWESIGRPLPGRKNIILSSQPGTDDRV TWVKSVDEAIAACGDVPEIMVIGGGRVIEQFLPK AQKLYLTHIDAEVEGDTHFPDYEPDDWESVFSEF HDADAQNSHSYCFEILERRIYIWAPLAGTCGVLLL	639	705

	SLVITLYCKRGRKKLLYIFKQPFMRPVQTTQEEDG CSCRFPEEEEGGCELRVKFSRSADAPAYKQGQNQ LYNELNLGRREEYDVLDKRRGRDPEMGGKPRRK NPQEGLYNELQKDKMAEAYSEIGMKGERRRGKG HDGLYQGLSTATKDTYDALHMQALPPR*		
OT-CD19c-006 (CD8a leader-CD19 scFV - CD8a-Tm - 41BB - CD3zeta -linker (GGSGG) - ecDHFR (Amino acid 2-159 of WT) (R12H, E129K) - stop)	MALPVTALLLPLALLLHAARPDIQMTQTTSSLSAS LGDRVTISCRASQDISKYLNWYQQKPDGTVKLLI YHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIA TYFCQQGNTLPYTFGGGTKLEITGGGGSGGGGSG GGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPD YGVSWIRQPPRKGLEWLGVIWGSETTYYNSALKS RLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHY YYGGSYAMDYWGQGTSVTVSSTTTPAPRPPTPAP TIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYI WAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQP FMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRS ADAPAYKQGQNQLYNELNLGRREEYDVLDKRRG RDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSE IGMKGERRRGKGHDGLYQGLSTATKDTYDALHM QALPPRGGSGGISLIAALAVDHVIGMENAMPWNL PADLAWFKRNTLNKPVIMGRHTWESIGRPLPGRK NIILSSQPGTDDRVTWVKSVDEAIAACGDVPEIMV IGGGRVYEQFLPKAQKLYLTHIDAEVEGDTHFPD YKPDDWESVFSEFHDADAQNSHSYCFEILERR*	640	706
OT-CD19c-007 (CD8a leader - CD19 scFV- CD8a-Tm - 41BB - CD3zeta - linker (GGSGG) -FKBP (E31G, F36V, R71G, K105E) - stop)	MALPVTALLLPLALLLHAARPDIQMTQTTSSLSAS LGDRVTISCRASQDISKYLNWYQQKPDGTVKLLI YHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIA TYFCQQGNTLPYTFGGGTKLEITGGGGSGGGGSG GGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPD YGVSWIRQPPRKGLEWLGVIWGSETTYYNSALKS RLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHY YYGGSYAMDYWGQGTSVTVSSTTTPAPRPPTPAP TIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYI WAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQP FMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRS ADAPAYKQGQNQLYNELNLGRREEYDVLDKRRG RDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSE IGMKGERRRGKGHDGLYQGLSTATKDTYDALHM QALPPRGGSGGGVQVETISPGDGRTFPKRGQTCV VHYTGMLGDGKKVDSSRDRNKPFKFMLGKQEVI RGWEEGVAQMSVGQGAKLTISPDYAYGATGHPG IIPPHATLVFDVELLELE*	641	707
OT-CD19c-008 (CD8a leader - CD19 scFV - CD8a-Tm -41BB - CD3zeta - linker (GGSGG) - hDHFR (Amino acid 2-187 of WT; Y122I) - stop)	MALPVTALLLPLALLLHAARPDIQMTQTTSSLSAS LGDRVTISCRASQDISKYLNWYQQKPDGTVKLLI YHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIA TYFCQQGNTLPYTFGGGTKLEITGGGGSGGGGSG GGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPD YGVSWIRQPPRKGLEWLGVIWGSETTYYNSALKS RLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHY YYGGSYAMDYWGQGTSVTVSSTTTPAPRPPTPAP TIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYI WAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQP FMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRS ADAPAYKQGQNQLYNELNLGRREEYDVLDKRRG RDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSE IGMKGERRRGKGHDGLYQGLSTATKDTYDALHM QALPPRGGSGGVGSLNCIVAVSQNMGIGKNGDLP	642	708

	WPPLRNEFRYFQRMTTTSSVEGKQNLVIMGKKT WFSIPEKNRPLKGRINLVLSRELKEPPQGAHFLSRS LDDALKLTEQPELANKVDMVWIVGGSSVIKEAM NHPGHLKLFVTRIMQDFESDTFFPEIDLEKYKLLP EYPGVLSDVQEEKGIKYKFEVYEKND*		
OT-CD19c-009 (CD8a leader - CD19 scFV - CD8a-Tm - 41BB - CD3zeta -linker (GGSGG) - hDHFR (Amino acid 2-187 of WT; Y122I, A125F) - stop)	MALPVTALLLPLALLLHAARPDIQMTQTTSSLSAS LGDRVTISCRASQDISKYLNWYQQKPDGTVKLLI YHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIA TYFCQQGNTLPYTFGGGTKLEITGGGGSGGGGSG GGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPD YGVSWIRQPPRKGLEWLGVIWGSETTYYNSALKS RLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHY YYGGSYAMDYWGQGTSVTVSSTTTPAPRPPTPAP TIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYI WAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQP FMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRS ADAPAYKQGQNQLYNELNLGRREEYDVLDKRRG RDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSE IGMKGERRRGKGHDGLYQGLSTATKDTYDALHM QALPPRGGSGGVGSLNCIVAVSQNMGIGKNGDLP WPPLRNEFRYFQRMTTTSSVEGKQNLVIMGKKT WFSIPEKNRPLKGRINLVLSRELKEPPQGAHFLSRS LDDALKLTEQPELANKVDMVWIVGGSSVIKEFM NHPGHLKLFVTRIMQDFESDTFFPEIDLEKYKLLP EYPGVLSDVQEEKGIKYKFEVYEKND*	643	709
OT-CD19c-010 (CD8a leader - CD19 scFV - CD8a-Tm - 41BB - CD3zeta - linker (GGSGG) -hDHFR (Amino acid 2-187 of WT; Q36K, Y122I) - stop)	MALPVTALLLPLALLLHAARPDIQMTQTTSSLSAS LGDRVTISCRASQDISKYLNWYQQKPDGTVKLLI YHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIA TYFCQQGNTLPYTFGGGTKLEITGGGGSGGGGSG GGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPD YGVSWIRQPPRKGLEWLGVIWGSETTYYNSALKS RLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHY YYGGSYAMDYWGQGTSVTVSSTTTPAPRPPTPAP TIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYI WAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQP FMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRS ADAPAYKQGQNQLYNELNLGRREEYDVLDKRRG RDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSE IGMKGERRRGKGHDGLYQGLSTATKDTYDALHM QALPPRGGSGGVGSLNCIVAVSQNMGIGKNGDLP WPPLRNEFRYFKRMTTTSSVEGKQNLVIMGKKT WFSIPEKNRPLKGRINLVLSRELKEPPQGAHFLSRS LDDALKLTEQPELANKVDMVWIVGGSSVIKEAM NHPGHLKLFVTRIMQDFESDTFFPEIDLEKYKLLP EYPGVLSDVQEEKGIKYKFEVYEKND*	644	710
OT-CD19c-011 (CD8a leader -CD19 scFV - CD8a-Tm - 41BB - CD3zeta -linker (GGSGG) - hDHFR (Amino acid 2-187 of WT; Q36K,N65F, Y122I) -stop)	MALPVTALLLPLALLLHAARPDIQMTQTTSSLSAS LGDRVTISCRASQDISKYLNWYQQKPDGTVKLLI YHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIA TYFCQQGNTLPYTFGGGTKLEITGGGGSGGGGSG GGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPD YGVSWIRQPPRKGLEWLGVIWGSETTYYNSALKS RLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHY YYGGSYAMDYWGQGTSVTVSSTTTPAPRPPTPAP TIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYI WAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQP FMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRS ADAPAYKQGQNQLYNELNLGRREEYDVLDKRRG	645	711

	RDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSE IGMKGERRRGKGHDGLYQGLSTATKDTYDALHM QALPPRGGSGGVGSLNCIVAVSQNMGIGKNGDLP WPPLRNEFRYFFRMTTTSSVEGKQNLVIMGKKTW FSIPEKFRPLKGRINLVLSRELKEPPQGAHFLSRSL DDALKLTEQPELANKVDMVWIVGGSSVIKEAMN HPGHLKLFVTRIMQDFESDTFFPEIDLEKYKLLPE YPGVLSDVQEEKGIKYKFEVYEKND*		
OT-CD19n-012 (CD8a leader - Linker (SG)-FKBP (F36V, L106P) - Furin Site -CD19 scFV - CD8a-Tm - 41BB - CD3zeta - stop)	MALPVTALLLPLALLLHAARPSGGVQVETISPGDG RTFPKRGQTCVVHYTGMLEDGKKVDSSRDRNKP FKFMLGKQEVIRGWEEGVAQMSVGQRAKLTISPD YAYGATGHPGIIPPHATLVFDVELLKPEESRRVRR NKRSKDIQMTQTTSSLSASLGDRVTISCRASQDIS KYLNWYQQKPDGTVKLLIYHTSRLHSGVPSRFSG SGSGTDYSLTISNLEQEDIATYFCQQGNTLPYTFG GGTKLEITGGGGSGGGGSGGGGSEVKLQESGPGL VAPSQSLSVTCTVSGVSLPDYGVSWIRQPPRKGLE WLGVIWGSETTYYNSALKSRLTIIKDNSKSQVFLK MNSLQTDDTAIYYCAKHYYYGGSYAMDYWGQG TSVTVSSTTTPAPRPPTPAPTIASQPLSLRPEACRPA AGGAVHTRGLDFACDIYIWAPLAGTCGVLLLSLV ITLYCKRGRKKLLYIFKQPFMRPVQTTQEEDGCSC RFPEEEEGGCELRVKFSRSADAPAYKQGQNQLYN ELNLGRREEYDVLDKRRGRDPEMGGKPRRKNPQ EGLYNELQKDKMAEAYSEIGMKGERRRGKGHDG LYQGLSTATKDTYDALHMQALPPR*	646	712
OT-CD19n-013 (CD8a leader - Linker (SG)-ecDHFR (Amino acid 2-159 of WT) (R12Y, Y100I) - Furin Site - CD19 scFV -CD8a-Tm - 41BB - CD3zeta - stop)	MALPVTALLLPLALLLHAARPSGISLIAALAVDYVI GMENAMPWNLPADLAWFKRNTLNKPVIMGRHT WESIGRPLPGRKNIILSSQPGTDDRVTWVKSVDEA IAACGDVPEIMVIGGGRVIEQFLPKAQKLYLTHID AEVEGDTHFPDYEPDDWESVFSEFHDADAQNSHS YCFEILERRESRRVRRNKRSKDIQMTQTTSSLSAS LGDRVTISCRASQDISKYLNWYQQKPDGTVKLLI YHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIA TYFCQQGNTLPYTFGGGTKLEITGGGGSGGGGSG GGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPD YGVSWIRQPPRKGLEWLGVIWGSETTYYNSALKS RLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHY YYGGSYAMDYWGQGTSVTVSSTTTPAPRPPTPAP TIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYI WAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQP FMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRS ADAPAYKQGQNQLYNELNLGRREEYDVLDKRRG RDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSE IGMKGERRRGKGHDGLYQGLSTATKDTYDALHM QALPPR*	647	713
OT-CD19n-014 (CD8a leader - Linker (SG)-hDHFR (Amino acid 2-187 of WT, Y122I, A125F) - Furin Site - CD19 scFV - CD8a-Tm - 41BB -CD3zeta - stop)	MALPVTALLLPLALLLHAARPSGVGSLNCIVAVSQ NMGIGKNGDLPWPPLRNEFRYFQRMTTTSSVEGK QNLVIMGKKTWFSIPEKNRPLKGRINLVLSRELKE PPQGAHFLSRSLDDALKLTEQPELANKVDMVWIV GGSSVIKEFMNHPGHLKLFVTRIMQDFESDTFFPEI DLEKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKN DESRRVRRNKRSKDIQMTQTTSSLSASLGDRVTIS CRASQDISKYLNWYQQKPDGTVKLLIYHTSRLHS GVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQG NTLPYTFGGGTKLEITGGGGSGGGGSGGGGSEVK LQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIR	648	714

	QPPRKGLEWLGVIWGSETTYYNSALKSRLTIIKDN SKSQVFLKMNSLQTDDTAIYYCAKHYYYGGSYA MDYWGQGTSVTVSSTTTPAPRPPTPAPTIASQPLS LRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGT CGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQT TQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAY KQGQNQLYNELNLGRREEYDVLDKRRGRDPEMG GKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGE RRRGKGHDGLYQGLSTATKDTYDALHMQALPPR *		
OT-CD19n-015 (CD8a leader - Linker (SG)-hDHFR (Amino acid 2-187 of WT; Q36K, Y122I) -Furin Site - CD19 scFV - CD8a-Tm - 41BB - CD3zeta -stop)	MALPVTALLLPLALLLHAARPSGVGSLNCIVAVSQ NMGIGKNGDLPWPPLRNEFRYFKRMTTTSSVEGK QNLVIMGKKTWFSIPEKNRPLKGRINLVLSRELKE PPQGAHFLSRSLDDALKLTEQPELANKVDMVWIV GGSSVIKEAMNHPGHLKLFVTRIMQDFESDTFFPE IDLEKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKN DESRRVRRNKRSKDIQMTQTTSSLSASLGDRVTIS CRASQDISKYLNWYQQKPDGTVKLLIYHTSRLHS GVPSRFSGSGSGTDYSLTISNLEQEDIATYFCQQG NTLPYTFGGGTKLEITGGGGSGGGGSGGGGSEVK LQESGPGLVAPSQSLSVTCTVSGVSLPDYGVSWIR QPPRKGLEWLGVIWGSETTYYNSALKSRLTIIKDN SKSQVFLKMNSLQTDDTAIYYCAKHYYYGGSYA MDYWGQGTSVTVSSTTTPAPRPPTPAPTIASQPLS LRPEACRPAAGGAVHTRGLDFACDIYIWAPLAGT CGVLLLSLVITLYCKRGRKKLLYIFKQPFMRPVQT TQEEDGCSCRFPEEEEGGCELRVKFSRSADAPAY KQGQNQLYNELNLGRREEYDVLDKRRGRDPEMG GKPRRKNPQEGLYNELQKDKMAEAYSEIGMKGE RRRGKGHDGLYQGLSTATKDTYDALHMQALPPR *	649	715
OT-CD19-056 (CD8a leader - CD19 scFV - CD8a-Tm - 41BB - CD3zeta - linker (GGSGG) - hDHFR (Amino acid 2-187 of WT; Y122I) -stop)	MALPVTALLLPLALLLHAARPDIQMTQTTSSLSAS LGDRVTISCRASQDISKYLNWYQQKPDGTVKLLI YHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIA TYFCQQGNTLPYTFGGGTKLEITGGGGSGGGGSG GGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPD YGVSWIRQPPRKGLEWLGVIWGSETTYYNSALKS RLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHY YYGGSYAMDYWGQGTSVTVSSTTTPAPRPPTPAP TIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYI WAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQP FMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRS ADAPAYKQGQNQLYNELNLGRREEYDVLDKRRG RDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSE IGMKGERRRGKGHDGLYQGLSTATKDTYDALHM QALPPRGGSGGVGSLNCIVAVSQNMGIGKNGDLP WPPLRNEFRYFQRMTTTSSVEGKQNLVIMGKKT WFSIPEKNRPLKGRINLVLSRELKEPPQGAHFLSRS LDDALKLTEQPELANKVDMVWIVGGSSVIKEAM NHPGHLKLFVTRIMQDFESDTFFPEIDLEKYKLLP EYPGVLSDVQEEKGIKYKFEVYEKND*	1005	1022
OT-CD19-057 (CD8a leader -CD19 scFV - CD8a-Tm - 41BB - CD3zeta - BamHI (GS)-stop)	MALPVTALLLPLALLLHAARPDIQMTQTTSSLSAS LGDRVTISCRASQDISKYLNWYQQKPDGTVKLLI YHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIA TYFCQQGNTLPYTFGGGTKLEITGGGGSGGGGSG GGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPD YGVSWIRQPPRKGLEWLGVIWGSETTYYNSALKS	1006	1023

	RLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHY YYGGSYAMDYWGQGTSVTVSSTTTPAPRPPTPAP TIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYI WAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQP FMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRS ADAPAYKQGQNQLYNELNLGRREEYDVLDKRRG RDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSE IGMKGERRRGKGHDGLYQGLSTATKDTYDALHM QALPPRGS*		
OT-CD19-058 (CD8a leader -CD19 scFV - CD8a-Tm - 41BB - CD3zeta - p2A - BamHI (GS)- stop)	MALPVTALLLPLALLLHAARPDIQMTQTTSSLSAS LGDRVTISCRASQDISKYLNWYQQKPDGTVKLLI YHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIA TYFCQQGNTLPYTFGGGTKLEITGGGGSGGGGSG GGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPD YGVSWIRQPPRKGLEWLGVIWGSETTYYNSALKS RLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHY YYGGSYAMDYWGQGTSVTVSSTTTPAPRPPTPAP TIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYI WAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQP FMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRS ADAPAYKQGQNQLYNELNLGRREEYDVLDKRRG RDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSE IGMKGERRRGKGHDGLYQGLSTATKDTYDALHM QALPPRGATNFSLLKQAGDVEENPGPGS*	1007	1033
OT-CD19-059 (CD8a leader - HA Tag - CD19 scFV - CD8a-Tm - 41BB - CD3zeta - BamHI (GS)- stop)	MALPVTALLLPLALLLHAARPYPYDVPDYADIQM TQTTSSLSASLGDRVTISCRASQDISKYLNWYQQK PDGTVKLLIYHTSRLHSGVPSRFSGSGSGTDYSLTI SNLEQEDIATYFCQQGNTLPYTFGGGTKLEITGGG GSGGGGSGGGGSEVKLQESGPGLVAPSQSLSVTC TVSGVSLPDYGVSWIRQPPRKGLEWLGVIWGSET TYYNSALKSRLTIIKDNSKSQVFLKMNSLQTDDTA IYYCAKHYYYGGSYAMDYWGQGTSVTVSSTTTP APRPPTPAPTIASQPLSLRPEACRPAAGGAVHTRG LDFACDIYIWAPLAGTCGVLLLSLVITLYCKRGRK KLLYIFKQPFMRPVQTTQEEDGCSCRFPEEEEGGC ELRVKFSRSADAPAYKQGQNQLYNELNLGRREE YDVLDKRRGRDPEMGGKPRRKNPQEGLYNELQK DKMAEAYSEIGMKGERRRGKGHDGLYQGLSTAT KDTYDALHMQALPPRGS*	1008	1034
OT-CD19-060 (CD8a leader - CD19 scFV- CD8a-Tm- 41BB - CD3zeta - Linker (SG)- Furin - BamHI (GS)- stop)	MALPVTALLLPLALLLHAARPDIQMTQTTSSLSAS LGDRVTISCRASQDISKYLNWYQQKPDGTVKLLI YHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIA TYFCQQGNTLPYTFGGGTKLEITGGGGSGGGGSG GGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPD YGVSWIRQPPRKGLEWLGVIWGSETTYYNSALKS RLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHY YYGGSYAMDYWGQGTSVTVSSTTTPAPRPPTPAP TIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYI WAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQP FMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRS ADAPAYKQGQNQLYNELNLGRREEYDVLDKRRG RDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSE IGMKGERRRGKGHDGLYQGLSTATKDTYDALHM QALPPRSGESRRVRRNKRSKGS*	1009	1035
OT-CD19-063 (CD8a leader - CD19 scFV -	MALPVTALLLPLALLLHAARPDIQMTQTTSSLSAS LGDRVTISCRASQDISKYLNWYQQKPDGTVKLLI	1010	1036

CD8a-Tm - 41BB - CD3zeta - stop)	YHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIA TYFCQQGNTLPYTFGGGTKLEITGGGGSGGGGSG GGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPD YGVSWIRQPPRKGLEWLGVIWGSETTYYNSALKS RLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHY YYGGSYAMDYWGQGTSVTVSSTTTPAPRPPTPAP TIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYI WAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQP FMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRS ADAPAYKQGQNQLYNELNLGRREEYDVLDKRRG RDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSE IGMKGERRRGKGHDGLYQGLSTATKDTYDALHM QALPPR*		
OT-CD19-064 (CD8a leader - CD19 scFV - CD8a-Tm - 41BB - CD3zeta - Linker (TR)-HA Tag - FKBP (E31G, F36V, R71G, K105E) – stop-IRES- mCherry)	MALPVTALLLPLALLLHAARPDIQMTQTTSSLSAS LGDRVTISCRASQDISKYLNWYQQKPDGTVKLLI YHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIA TYFCQQGNTLPYTFGGGTKLEITGGGGSGGGGSG GGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPD YGVSWIRQPPRKGLEWLGVIWGSETTYYNSALKS RLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHY YYGGSYAMDYWGQGTSVTVSSTTTPAPRPPTPAP TIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYI WAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQP FMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRS ADAPAYKQGQNQLYNELNLGRREEYDVLDKRRG RDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSE IGMKGERRRGKGHDGLYQGLSTATKDTYDALHM QALPPRTRYPYDVPDYAGVQVETISPGDGRTFPK RGQTCVVHYTGMLGDGKKVDSSRDRNKPFKFML GKQEVIRGWEEGVAQMSVGQGAKLTISPDYAYG ATGHPGIIPPHATLVFDVELLELE*MHRSAAAAT*I PPPPPLSLPPP*RYWPKPLGIRPVCVCLYVIFHHIAV FWQCEGPETWPCLLDEHS*GSFPSRQRNARSVEC REGSSSSGSFLKTNNVCSDPLQAAEPPTWRQVPLR PKATCIRYTCKGGTTPVPRCELDSCGKSQMALLK RIQQGAEGCPEGTPLYGI*SGASVHMLYMCLVEV KKTSRPPEPRGRGFPLKNTMIIWPQP**ARARRIT WPSSRSSCASRCTWRAP*TATSSRSRARARAAPTR APRPPS*R*PRVAPCPSPGTSCPLSSCTAPRPT*STP PTSPTT*SCPSPRASSGSA**TSRTAAW*P*PRTPPC RTASSSTR*SCAAPTSPPTAP*CRRRPWAGRPPPSG CTPRTAP*RARSSRG*S*RTAATTTLRSRPPTRPRS PCSCPAPTTSTSSWTSPPTTRTTPSWNSTNAPRAA TPPAAWTSCTS*	1215-1231	1037
OT-CD19-066 (CD8a leader - CD19 scFV - CD8a-Tm - 41BB - CD3zeta - Linker (GS)-P2A peptide -mCherry (M1L) - stop)	MALPVTALLLPLALLLHAARPDIQMTQTTSSLSAS LGDRVTISCRASQDISKYLNWYQQKPDGTVKLLI YHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIA TYFCQQGNTLPYTFGGGTKLEITGGGGSGGGGSG GGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPD YGVSWIRQPPRKGLEWLGVIWGSETTYYNSALKS RLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHY YYGGSYAMDYWGQGTSVTVSSTTTPAPRPPTPAP TIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYI WAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQP FMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRS ADAPAYKQGQNQLYNELNLGRREEYDVLDKRRG RDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSE IGMKGERRRGKGHDGLYQGLSTATKDTYDALHM	1015	1039

	QALPPRGSGATNFSLLKQAGDVEENPGPLSKGEE DNMAIIKEFMRFKVHMEGSVNGHEFEIEGEGEGR PYEGTQTAKLKVTKGGPLPFAWDILSPQFMYGSK AYVKHPADIPDYLKLSFPEGFKWERVMNFEDGG VVTVTQDSSLQDGEFIYKVKLRGTNFPSDGPVMQ KKTMGWEASSERMYPEDGALKGEIKQRLKLKDG GHYDAEVKTTYKAKKPVQLPGAYNVNIKLDITSH NEDYTIVEQYERAEGRHSTGGMDELYK*		
OT-CAR19-IL15-001 (CD8a leader - CD19 scFV -CD8a-Tm - 41BB - CD3zeta - Linker (GS)-P2A - IL15 -Linker (SG3-(SG4)3-SG3-SLQ) - IL15Ra - stop)	MALPVTALLLPLALLLHAARPDIQMTQTTSSLSAS LGDRVTISCRASQDISKYLNWYQQKPDGTVKLLI YHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIA TYFCQQGNTLPYTFGGGTKLEITGGGGSGGGGSG GGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPD YGVSWIRQPPRKGLEWLGVIWGSETTYYNSALKS RLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHY YYGGSYAMDYWGQGTSVTVSSTTTPAPRPPTPAP TIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYI WAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQP FMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRS ADAPAYKQGQNQLYNELNLGRREEYDVLDKRRG RDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSE IGMKGERRRGKGHDGLYQGLSTATKDTYDALHM QALPPRGSGATNFSLLKQAGDVEENPGPNWVNVI SDLKKIEDLIQSMHIDATLYTESDVHPSCKVTAMK CFLLELQVISLESGDASIHDTVENLIILANNSLSSNG NVTESGCKECEELEEKNIKEFLQSFVHIVQMFINTS SGGGSGGGGSGGGGSGGGGSGGGSLQITCPPPMS VEHADIWVKSYSLYSRERYICNSGFKRKAGTSSLT ECVLNKATNVAHWTTPSLKCIRDPALVHQRPAPP STVTTAGVTPQPESLSPSGKEPAASSPSSNNTAATT AAIVPGSQLMPSKSPSTGTTEISSHESSHGTPSQTT AKNWELTASASHQPPGVYPQGHSDTTVAISTSTV LLCGLSAVSLLACYLKSRQTPPLASVEMEAMEAL PVTWGTSSRDEDLENCSHHL*	1016	1040
OT-CAR19-IL15-002 (CD8a leader -CD19 scFV -CD8a-Tm -41BB - CD3zeta - Linker (GS)-P2A - Linker (MLLLVTSLLLCELPHP AFLLIP) (SEQ ID NO: 1031) - IL15 - Linker (SG3-(SG4)3-SG3-SLQ) - IL15Ra - stop)	MALPVTALLLPLALLLHAARPDIQMTQTTSSLSAS LGDRVTISCRASQDISKYLNWYQQKPDGTVKLLI YHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIA TYFCQQGNTLPYTFGGGTKLEITGGGGSGGGGSG GGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPD YGVSWIRQPPRKGLEWLGVIWGSETTYYNSALKS RLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHY YYGGSYAMDYWGQGTSVTVSSTTTPAPRPPTPAP TIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYI WAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQP FMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRS ADAPAYKQGQNQLYNELNLGRREEYDVLDKRRG RDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSE IGMKGERRRGKGHDGLYQGLSTATKDTYDALHM QALPPRGSGATNFSLLKQAGDVEENPGPMLLLVT SLLLCELPHPAFLLIPNWVNVISDLKKIEDLIQSMH IDATLYTESDVHPSCKVTAMKCFLLELQVISLESG DASIHDTVENLIILANNSLSSNGNVTESGCKECEEL EEKNIKEFLQSFVHIVQMFINTSSGGGSGGGGSGG GGSGGGGSGGGSLQITCPPPMSVEHADIWVKSYS LYSRERYICNSGFKRKAGTSSLTECVLNKATNVA HWTTPSLKCIRDPALVHQRPAPPSTVTTAGVTPQP ESLSPSGKEPAASSPSSNNTAATTAAIVPGSQLMPS KSPSTGTTEISSHESSHGTPSQTTAKNWELTASAS	1017	1041

	HQPPGVYPQGHSDTTVAISTSTVLLCGLSAVSLLA CYLKSRQTPPLASVEMEAMEALPVTWGTSSRDED LENCSHHL*		
OT-CD19-IL15-006 (CD8a leader -CD19 scFV - CD8a-Tm - 41BB - CD3zeta - Linker (GS)-P2A - IgE Leader - IL15 - Linker (SG3-(SG4)3-SG3-SLQ) - IL15Ra - stop)	MALPVTALLLPLALLLHAARPDIQMTQTTSSLSAS LGDRVTISCRASQDISKYLNWYQQKPDGTVKLLI YHTSRLHSGVPSRFSGSGSGTDYSLTISNLEQEDIA TYFCQQGNTLPYTFGGGTKLEITGGGGSGGGGSG GGGSEVKLQESGPGLVAPSQSLSVTCTVSGVSLPD YGVSWIRQPPRKGLEWLGVIWGSETTYYNSALKS RLTIIKDNSKSQVFLKMNSLQTDDTAIYYCAKHY YYGGSYAMDYWGQGTSVTVSSTTTPAPRPPTPAP TIASQPLSLRPEACRPAAGGAVHTRGLDFACDIYI WAPLAGTCGVLLLSLVITLYCKRGRKKLLYIFKQP FMRPVQTTQEEDGCSCRFPEEEEGGCELRVKFSRS ADAPAYKQGQNQLYNELNLGRREEYDVLDKRRG RDPEMGGKPRRKNPQEGLYNELQKDKMAEAYSE IGMKGERRRGKGHDGLYQGLSTATKDTYDALHM QALPPRGSGATNFSLLKQAGDVEENPGPMDWTW ILFLVAAATRVHSNWVNVISDLKKIEDLIQSMHID ATLYTESDVHPSCKVTAMKCFLLELQVISLESGDA SIHDTVENLIILANNSLSSNGNVTESGCKECEELEE KNIKEFLQSFVHIVQMFINTSSGGGSGGGGSGGGG SGGGGSGGGSLQITCPPPMSVEHADIWVKSYSLYS RERYICNSGFKRKAGTSSLTECVLNKATNVAHWT TPSLKCIRDPALVHQRPAPPSTVTTAGVTPQPESLS PSGKEPAASSPSSNNTAATTAAIVPGSQLMPSKSPS TGTTEISSHESSHGTPSQTTAKNWELTASASHQPP GVYPQGHSDTTVAISTSTVLLCGLSAVSLLACYLK SRQTPPLASVEMEAMEALPVTWGTSSRDEDLENC SHHL*	1018	1042

[00229] Constructs disclosed in Table 10 which are transcriptionally controlled by a CMV promoter, in some instances may be placed under the transcriptional control of a different promoter to test the role of promoters in CD19 CAR expression. In one embodiment, the CMV promoter may be replaced by an EF1a promoter. In one embodiment, the CMV promoter of the, OT-CD19-001 construct, may be replaced to generate OT-CD19N-017 construct, with a EF1a promoter. In another embodiment, the CMV promoter of the CD19 CAR, OT-CD19 CAR-002 construct, may be replaced to generate OT-CD19N-018 construct, with a EF1a promoter. In another embodiment, the CMV promoter of the CD19 CAR, OT-CD19 CAR-003 construct, may be replaced to generate OT-CD19N-019 construct, with a EF1a promoter. In another embodiment, the CMV promoter of the CD19 CAR, OT-CD19 CAR-004 construct, may be replaced to generate OT-CD19N-020 construct, with a EF1a promoter. In another embodiment, the CMV promoter of the CD19 CAR, OT-CD19 CAR-005 construct, may be replaced to generate OT-CD19N-021 construct, with a EF1a promoter. In another embodiment, the CMV promoter of the CD19 CAR, OT-CD19 CAR-006 construct, may be replaced to generate OT-CD19N-022 construct, with a EF1a promoter. In another embodiment, the CMV promoter of the

CD19 CAR, OT-CD19 CAR-007 construct, may be replaced to generate OT-CD19N-023 construct, with a EF1a promoter. In another embodiment, the CMV promoter of the CD19 CAR, OT-CD19 CAR-008 construct, may be replaced to generate OT-CD19N-024 construct, with a EF1a promoter. In another embodiment, the CMV promoter of the CD19 CAR, OT-CD19 CAR-009 construct, may be replaced to generate OT-CD19N-025 construct, with a EF1a promoter.

[00230] In one embodiment, the CAR construct comprises a CD19 scFV (e.g., CAT13.1E10 or FMC63), a CD8α spacer or transmembrane domain, and a 4-1BB and CD3ζ endodomain. These constructs with CAT13.1E10 may have increased proliferation after stimulation *in vitro*, increased cytotoxicity against the CD19+ targets, and increased effector and target interactions as compared to constructs with FMC63.

[00231] In some embodiments, the payloads of the present invention may be tuned using the catalytic domains of the E3 ubiquitin ligases. The catalytic domains of E3 ligases may be fused to an antibody or a fragment of the antibody. The payload is fused to the antigen recognized by the antibody or a fragment of the antibody that is fused to the E3 ligases catalytic domain. The E3 ligases useful in the present invention include, but are not limited to Ring E3 ligase, HECT E3 ligases and RBR E3 ligases. Any of the methods taught by Kanner SA et al. (2017) eLife; 6: e29744 may be useful in the present invention (the contents of which are incorporated by reference in their entirety).

[00232] In some embodiments, the payloads described herein, may be regulated by E3 ubiquitin ligases constructs. The E3 ligases constructs may comprise the catalytic domain of E3 ligases fused to an SRE and an antibody or a fragment of an antibody. The payloads are fused to the antigen recognized by the antibody or a fragment of an antibody, that is appended to the catalytic domain of E3 ligases. In the absence of the stimulus corresponding to the SRE, the E3 ubiquitin ligases constructs are destabilized, which in turn, allows the expression of the payloads fused to the antigen. In the presence of ligand corresponding to the SRE, the E3 ubiquitin ligases constructs are stabilized and available to bind to the antigen fused to the payloads. Binding of the E3 ligases constructs to the antigens, targets the protein for degradation. The E3 ubiquitin ligases constructs may be used to regulate any payload described herein, provided the payload is fused to an antigen recognized by the antibody or the fragment of the antibody in the E3 ubiquitin ligases construct. In some embodiments, the payload is a chimeric antigen receptor. The E3 ubiquitin ligases constructs may be used to design logic gates. In one embodiment, the E3 ubiquitin ligases constructs may be used to generate a NOT gate, wherein one ligand induces the expression of the payload, while another inhibits the expression of the payload. In some embodiments, the NOT gate may be generated using the E3 ubiquitin ligases constructs and by

fusing the payloads-antigen fusion protein to a second a SRE that is distinct from the SRE in the E3 ubiquitin ligase construct.

[00233] 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.

Tandem CAR (TanCAR)

[00234] 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

[00235] 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.

[00236] 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.

[00237] 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

[00238] 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

[00239] 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

[00240] 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).

Cytokines, chemokines and other soluble factors

[00241] In accordance with the present invention, CARs of the present invention may be utilized along with other 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 a number of effector activities. For example, activated T cells produce a variety of cytokines for cytotoxic function to eliminate tumor cells.

[00242] 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. 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.

[00243] In some embodiments, payloads of the present invention may be cytokine receptors, recombinant receptors, variants, analogs and derivatives thereof; or signal components of cytokines.

[00244] 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.

[00245] 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.

[00246] In one embodiment, the payload of the invention may comprise IL12. IL12 is a heterodimeric protein of two subunits (p35, p40) that is secreted by antigen presenting cells, such as macrophages and dendritic cells. IL12 is type 1 cytokine that acts on natural killer (NK) cells, macrophages, $CD8^+$ Cytotoxic T cells, and $CD4^+$ T helper cells through STAT4 pathway to induce IFN-γ production in these effector immune cells (reviewed by Trinchieri G, *Nat Rev Immunol.* 2003; 3(2): 133–146). IL12 can promote the cytotoxic activity of NK cells and $CD8^+$ T cells, therefore has anti-tumor function. Intravenous injection of recombinant IL12 exhibited modest clinical efficacy in a handful of patients with advanced melanoma and renal cell carcinoma (Gollob et al., *Clin. Cancer Res.* 2000; 6(5):1678–1692). IL12 has been used as an adjuvant to enhance cytotoxic immunity using a melanoma antigen vaccine, or using peptide pulsed peripheral blood mononuclear cells; and to promote NK cell activity in breast cancer with trastuzumab treatment. Local delivery of IL12 to the tumor microenvironment promotes tumor regression in several tumor models. These studies all indicate that locally increased IL12 level can promote anti-tumor immunity. One major obstacle of systemic or local administration of

recombinant IL12 protein, or through oncolytic viral vectors is the severe side effects when IL12 is presented at high level. Developing a system that tightly controls IL12 level may provide a safe use of IL12 in cancer treatment.

[00247] 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.

[00248] In one aspect, the effector module of the invention may be a DD-IL12 fusion polypeptide. This regulatable DD-IL12 fusion polypeptide may be directly used as an immunotherapeutic agent or be transduced into an immune effector cell (T cells and TIL cells) to generate modified T cells with greater *in vivo* expansion and survival capabilities for adoptive cell transfer. The need for harsh preconditioning regimens in current adoptive cell therapies may be minimized using regulated IL12 DD-IL12 may be utilized to modify tumor microenvironment and increase persistence in solid tumors that are currently refractory to tumor antigen targeted therapy. In some embodiments, CAR expressing T cells may be armored with DD regulated IL12 to relieve immunosuppression without systemic toxicity.

[00249] In some embodiments, the IL12 may be a Flexi IL12, wherein both p35 and p40 subunits, are encoded by a single cDNA that produces a single chain polypeptide. The single chain polypeptide may be generated by placing p35 subunit at the N terminus or the c terminus of the single chain polypeptide. Similarly, the p40 subunit may be at the N terminus or C terminus of the single chain polypeptide. In some embodiments, the IL12 constructs of the invention may be placed under the transcriptional control of the CMV promoter (SEQ ID NO. 716), an EF1a promoter (SEQ ID NO. 717, SEQ ID NO. 908) or a PGK promoter (SEQ ID NO. 718). Any portion of IL12 that retains one or more functions of full length or mature IL12 may be useful in the present invention. In some aspects, the DD-IL12 comprises the amino acid sequences listed in Table 11. The amino acid sequences in Table 11 may comprise a stop codon which is denoted in the table with a "*" at the end of the amino acid sequence.

Table 11: DD-IL12 constructs

Description	Promoter	Amino acid Sequence	Amino acid SEQ ID NO	Nucleic Acid SEQ ID NO
p40 signal sequence	-	MCHQQLVISWFSLVFLASPLVA	719	736-744

Linker	-	GGSGG	629	679-680
Linker	-	GGGGSGGGGSGGGGS	720	910-915
Linker		GS	-	GGATCC
Spacer		ATNFSLLKQAGDVEENPGP	745	746
Furin cleavage site	-	SARNRQKRS	721	750
Furin cleavage site	-	ARNRQKRS	722	751
Modified Furin	-	ESRRVRRNKRSK	630	681-683
P2A Cleavable Peptide	-	GATNFSLLKQAGDVEENPGP	925	926
p40	-	IWELKKDVYVVELDWYPDAPGEMVVLTCD TPEEDGITWTLDQSSEVLGSGKTLTIQVKEF GDAGQYTCHKGGEVLSHSLLLLHKKEDGI WSTDILKDQKEPKNKTFLRCEAKNYSGRFT CWWLTTISTDLTFSVKSSRGSSDPQGVTCG AATLSAERVRGDNKEYEYSVECQEDSACP AAEESLPIEVMVDAVHKLKYENYTSSFFIRD IIKPDPPKNLQLKPLKNSRQVEVSWEYPDT WSTPHSYFSLTFCVQVQGKSKREKKDRVFT DKTSATVICRKNASISVRAQDRYYSSSWSE WASVPCS	723	752-761, 632-634
p40 (K217N)	-	IWELKKDVYVVELDWYPDAPGEMVVLTCD TPEEDGITWTLDQSSEVLGSGKTLTIQVKEF GDAGQYTCHKGGEVLSHSLLLLHKKEDGI WSTDILKDQKEPKNKTFLRCEAKNYSGRFT CWWLTTISTDLTFSVKSSRGSSDPQGVTCG AATLSAERVRGDNKEYEYSVECQEDSACP AAEESLPIEVMVDAVHKLKYENYTSSFFIRD IIKPDPPNNLQLKPLKNSRQVEVSWEYPDT WSTPHSYFSLTFCVQVQGKSKREKKDRVFT DKTSATVICRKNASISVRAQDRYYSSSWSE WASVPCS	747	748
p35	-	RNLPVATPDPGMFPCLHHSQNLLRAVSNM LQKARQTLEFYPCTSEEIDHEDITKDKTSTV EACLPLELTKNESCLNSRETSFITNGSCLASR KTSFMMALCLSSIYEDLKMYQVEFKTMNA KLLMDPKRQIFLDQNMLAVIDELMQALNF NSETVPQKSSLEEPDFYKTKIKLCILLHAFRI RAVTIDRVMSYLNAS	724	762-771, 1012
ecDHFR (Amino acid 2-159 of WT) (R12Y, Y100I)	-	ISLIAALAVDYVIGMENAMPWNLPADLAW FKRNTLNKPVIMGRHTWESIGRPLPGRKNII LSSQPGTDDRVTWVKSVDEAIAACGDVPEI MVIGGGRVIEQFLPKAQKLYLTHIDAEVEG DTHFPDYEPDDWESVFSEFHDADAQNSHSY CFEILERR	9	692, 772, 814, 687, 988, 991
FKBP (F36V, L106P)	-	GVQVETISPGDGRTFPKRGQTCVVHYTGML EDGKKVDSSRDRNKPFKFMLGKQEVIRGW EEGVAQMSVGQRAKLTISPDYAYGATGHP GIIPPHATLVFDVELLKPE	11	684-686, 987, 989
FKBP (F36V, E31G, R71G, K105E)	-	GVQVETISPGDGRTFPKRGQTCVVHYTGML GDGKKVDSSRDRNKPFKFMLGKQEVIRGW EEGVAQMSVGQGAKLTISPDYAYGATGHP GIIPPHATLVFDVELLELE	12	688-691, 994, 1013, 1028
hDHFR (Amino acid 2-187 of WT; Q36F, Y122I, A125F)	-	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNE FRYFFRMTTTSSVEGKQNLVIMGKKTWFSI PEKNRPLKGRINLVLSRELKEPPQGAHFLSR SLDDALKLTEQPELANKVDMVWIVGGSSVI KEFMNHPGHLKLFVTRIMQDFESDTFFPEID	46	773

		LEKYKLLPEYPGVLSDVQEEKGIKYKFEVY EKND		
hDHFR (Amino acid 2-187 of WT) (I17V)	-	VGSLNCIVAVSQNMGVGKNGDLPWPPLRN EFRYFQRMTTTSSVEGKQNLVIMGKKTWFS IPEKNRPLKGRINLVLSRELKEPPQGAHFLS RSLDDALKLTEQPELANKVDMVWIVGGSS VYKEAMNHPGHLKLFVTRIMQDFESDTFFP EIDLEKYKLLPEYPGVLSDVQEEKGIKYKFE VYEKND	894	979
hDHFR (Amino acid 2-187 of WT) (Y122I)	-	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNE FRYFQRMTTTSSVEGKQNLVIMGKKTWFSI PEKNRPLKGRINLVLSRELKEPPQGAHFLSR SLDDALKLTEQPELANKVDMVWIVGGSSVI KEAMNHPGHLKLFVTRIMQDFESDTFFPEID LEKYKLLPEYPGVLSDVQEEKGIKYKFEVY EKND	895	694, 995
OT-IL12-001 (p40 signal sequence - FKBP (F36V, L106P) - linker (GGSGG) - p40 - linker2 (G4S)3 - p35-stop)	CMV	MCHQQLVISWFSLVFLASPLVAGVQVETISP GDGRTFPKRGQTCVVHYTGMLEDGKKVDS SRDRNKPFKFMLGKQEVIRGWEEGVAQMS VGQRAKLTISPDYAYGATGHPGIIPPHATLV FDVELLKPEGGSGGIWELKKDVYVVELDW YPDAPGEMVVLTCDTPEEDGITWTLDQSSE VLGSGKTLTIQVKEFGDAGQYTCHKGGEVL SHSLLLLHKKEDGIWSTDILKDQKEPKNKTF LRCEAKNYSGRFTCWWLTTISTDLTFSVKS SRGSSDPQGVTCGAATLSAERVRGDNKEYE YSVECQEDSACPAAEESLPIEVMVDAVHKL KYENYTSSFFIRDIIKPDPPKNLQLKPLKNSR QVEVSWEYPDTWSTPHSYFSLTFCVQVQG KSKREKKDRVFTDKTSATVICRKNASISVR AQDRYYSSSWSEWASVPCSGGGGSGGGGS GGGGSRNLPVATPDPGMFPCLHHSQNLLRA VSNMLQKARQTLEFYPCTSEEIDHEDITKDK TSTVEACLPLELTKNESCLNSRETSFITNGSC LASRKTSFMMALCLSSIYEDLKMYQVEFKT MNAKLLMDPKRQIFLDQNMLAVIDELMQA LNFNSETVPQKSSLEEPDFYKTKIKLCILLH AFRIRAVTIDRVMSYLNAS*	727	774
OT-IL12-002 (Met - FKBP (F36V, L106P) - linker (GGSGG) - p40 signal sequence - p40 - linker ((G4S)3) - p35 - stop)	CMV	MGVQVETISPGDGRTFPKRGQTCVVHYTG MLEDGKKVDSSRDRNKPFKFMLGKQEVIR GWEEGVAQMSVGQRAKLTISPDYAYGATG HPGIIPPHATLVFDVELLKPEGGSGGMCHQ QLVISWFSLVFLASPLVAIWELKKDVYVVE LDWYPDAPGEMVVLTCDTPEEDGITWTLD QSSEVLGSGKTLTIQVKEFGDAGQYTCHKG GEVLSHSLLLLHKKEDGIWSTDILKDQKEP KNKTFLRCEAKNYSGRFTCWWLTTISTDLT FSVKSSRGSSDPQGVTCGAATLSAERVRGD NKEYEYSVECQEDSACPAAEESLPIEVMVD AVHKLKYENYTSSFFIRDIIKPDPPKNLQLK PLKNSRQVEVSWEYPDTWSTPHSYFSLTFC VQVQGKSKREKKDRVFTDKTSATVICRKN ASISVRAQDRYYSSSWSEWASVPCSGGGGS GGGGSGGGGSRNLPVATPDPGMFPCLHHS QNLLRAVSNMLQKARQTLEFYPCTSEEIDH EDITKDKTSTVEACLPLELTKNESCLNSRET SFITNGSCLASRKTSFMMALCLSSIYEDLKM YQVEFKTMNAKLLMDPKRQIFLDQNMLAV IDELMQALNFNSETVPQKSSLEEPDFYKTKI KLCILLHAFRIRAVTIDRVMSYLNAS*	728	775

OT-IL12-003 (p40 signal sequence - FKBP (F36V, L106P)- furin (SARNRQKRS) - p40- linker ((G4S)3)- p35 - stop)	CMV	MCHQQLVISWFSLVFLASPLVAGVQVETISP GDGRTFPKRGQTCVVHYTGMLEDGKKVDS SRDRNKPFKFMLGKQEVIRGWEEGVAQMS VGQRAKLTISPDYAYGATGHPGIIPPHATLV FDVELLKPESARNRQKRSIWELKKDVYVVE LDWYPDAPGEMVVLTCDTPEEDGITWTLD QSSEVLGSGKTLTIQVKEFGDAGQYTCHKG GEVLSHSLLLLHKKEDGIWSTDILKDQKEP KNKTFLRCEAKNYSGRFTCWWLTTISTDLT FSVKSSRGSSDPQGVTCGAATLSAERVRGD NKEYEYSVECQEDSACPAAEESLPIEVMVD AVHKLKYENYTSSFFIRDIIKPDPPKNLQLK PLKNSRQVEVSWEYPDTWSTPHSYFSLTFC VQVQGKSKREKKDRVFTDKTSATVICRKN ASISVRAQDRYYSSSWSEWASVPCSGGGGS GGGGSGGGGSRNLPVATPDPGMFPCLHHS QNLLRAVSNMLQKARQTLEFYPCTSEEIDH EDITKDKTSTVEACLPLELTKNESCLNSRET SFITNGSCLASRKTSFMMALCLSSIYEDLKM YQVEFKTMNAKLLMDPKRQIFLDQNMLAV IDELMQALNFNSETVPQKSSLEEPDFYKTKI KLCILLHAFRIRAVTIDRVMSYLNAS*	729	776
OT-IL12-004 (p40 signal sequence - p40 - linker ((G4S)3) - p35 - furin (ARNRQKRS) - FKBP (E31G, F36V, R71G, K105E) -stop)	CMV	MCHQQLVISWFSLVFLASPLVAIWELKKDV YVVELDWYPDAPGEMVVLTCDTPEEDGIT WTLDQSSEVLGSGKTLTIQVKEFGDAGQYT CHKGGEVLSHSLLLLHKKEDGIWSTDILKD QKEPKNKTFLRCEAKNYSGRFTCWWLTTIS TDLTFSVKSSRGSSDPQGVTCGAATLSAER VRGDNKEYEYSVECQEDSACPAAEESLPIE VMVDAVHKLKYENYTSSFFIRDIIKPDPPKN LQLKPLKNSRQVEVSWEYPDTWSTPHSYFS LTFCVQVQGKSKREKKDRVFTDKTSATVIC RKNASISVRAQDRYYSSSWSEWASVPCSGG GGSGGGGSGGGGSRNLPVATPDPGMFPCL HHSQNLLRAVSNMLQKARQTLEFYPCTSEE IDHEDITKDKTSTVEACLPLELTKNESCLNS RETSFITNGSCLASRKTSFMMALCLSSIYED LKMYQVEFKTMNAKLLMDPKRQIFLDQN MLAVIDELMQALNFNSETVPQKSSLEEPDF YKTKIKLCILLHAFRIRAVTIDRVMSYLNAS ARNRQKRSGVQVETISPGDGRTFPKRGQTC VVHYTGMLGDGKKVDSSRDRNKPFKFML GKQEVIRGWEEGVAQMSVGQGAKLTISPD YAYGATGHPGIIPPHATLVFDVELLELE*	730	777
OT-IL12-005 (p40 signal sequence- p40 -- linker- ((G4S)3) - p35 - linker (GGSG) - FKBP (E31G, F36V, R71G, K105E) - stop)	CMV	MCHQQLVISWFSLVFLASPLVAIWELKKDV YVVELDWYPDAPGEMVVLTCDTPEEDGIT WTLDQSSEVLGSGKTLTIQVKEFGDAGQYT CHKGGEVLSHSLLLLHKKEDGIWSTDILKD QKEPKNKTFLRCEAKNYSGRFTCWWLTTIS TDLTFSVKSSRGSSDPQGVTCGAATLSAER VRGDNKEYEYSVECQEDSACPAAEESLPIE VMVDAVHKLKYENYTSSFFIRDIIKPDPPKN LQLKPLKNSRQVEVSWEYPDTWSTPHSYFS LTFCVQVQGKSKREKKDRVFTDKTSATVIC RKNASISVRAQDRYYSSSWSEWASVPCSGG GGSGGGGSGGGGSRNLPVATPDPGMFPCL HHSQNLLRAVSNMLQKARQTLEFYPCTSEE IDHEDITKDKTSTVEACLPLELTKNESCLNS RETSFITNGSCLASRKTSFMMALCLSSIYED LKMYQVEFKTMNAKLLMDPKRQIFLDQN	731	778

		MLAVIDELMQALNFNSETVPQKSSLEEPDF YKTKIKLCILLHAFRIRAVTIDRVMSYLNAS GGSGGVQVETISPGDGRTFPKRGQTCVVHY TGMLGDGKKVDSSRDRNKPFKFMLGKQEV IRGWEEGVAQMSVGQRAKLTISPDYAYGA TGHPGIIPPHATLVFDVELLELE*		
OT-IL12-006 (p40 signal sequence- p40 – linker ((G4S)3)- p35 - stop)	CMV	MCHQQLVISWFSLVFLASPLVAIWELKKDV YVVELDWYPDAPGEMVVLTCDTPEEDGIT WTLDQSSEVLGSGKTLTIQVKEFGDAGQYT CHKGGEVLSHSLLLLHKKEDGIWSTDILKD QKEPKNKTFLRCEAKNYSGRFTCWWLTTIS TDLTFSVKSSRGSSDPQGVTCGAATLSAER VRGDNKEYEYSVECQEDSACPAAEESLPIE VMVDAVHKLKYENYTSSFFIRDIIKPDPPKN LQLKPLKNSRQVEVSWEYPDTWSTPHSYFS LTFCVQVQGKSKREKKDRVFTDKTSATVIC RKNASISVRAQDRYYSSSWSEWASVPCSGG GGSGGGGSGGGGSRNLPVATPDPGMFPCL HHSQNLLRAVSNMLQKARQTLEFYPCTSEE IDHEDITKDKTSTVEACLPLELTKNESCLNS RETSFITNGSCLASRKTSFMMALCLSSIYED LKMYQVEFKTMNAKLLMDPKRQIFLDQN MLAVIDELMQALNFNSETVPQKSSLEEPDF YKTKIKLCILLHAFRIRAVTIDRVMSYLNAS *	732	779
OT-IL12-007 (p40 signal sequence; ecDHFR (Amino acid 2-159 of WT) (R12Y, 100I) - furin site (ESRRVRRNK RSK) - p40 – linker ((G4S)3) - p35)	CMV	MCHQQLVISWFSLVFLASPLVAISLIAALAV DYVIGMENAMPWNLPADLAWFKRNTLNK PVIMGRHTWESIGRPLPGRKNIILSSQPGTD DRVTWVKSVDEAIAACGDVPEIMVIGGGR VIEQFLPKAQKLYLTHIDAEVEGDTHFPDYE PDDWESVFSEFHDADAQNSHSYCFEILERR ESRRVRRNKRSKIWELKKDVYVVELDWYP DAPGEMVVLTCDTPEEDGITWTLDQSSEVL GSGKTLTIQVKEFGDAGQYTCHKGGEVLSH SLLLLHKKEDGIWSTDILKDQKEPKNKTFLR CEAKNYSGRFTCWWLTTISTDLTFSVKSSR GSSDPQGVTCGAATLSAERVRGDNKEYEY SVECQEDSACPAAEESLPIEVMVDAVHKLK YENYTSSFFIRDIIKPDPPKNLQLKPLKNSRQ VEVSWEYPDTWSTPHSYFSLTFCVQVQGKS KREKKDRVFTDKTSATVICRKNASISVRAQ DRYYSSSWSEWASVPCSGGGGSGGGGSGG GGSRNLPVATPDPGMFPCLHHSQNLLRAVS NMLQKARQTLEFYPCTSEEIDHEDITKDKTS TVEACLPLELTKNESCLNSRETSFITNGSCL ASRKTSFMMALCLSSIYEDLKMYQVEFKT MNAKLLMDPKRQIFLDQNMLAVIDELMQA LNFNSETVPQKSSLEEPDFYKTKIKLCILLH AFRIRAVTIDRVMSYLNAS	733	780
OT-IL12-008 (p40 signal sequence; hDHFR (Amino acid 2-187 of WT) (Q36K, Y122I, A125F) - furin site (ESRRVRRNK RSK)- p40 -	CMV	MCHQQLVISWFSLVFLASPLVAVGSLNCIV AVSQNMGIGKNGDLPWPPLRNEFRYFFRM TTTSSVEGKQNLVIMGKKTWFSIPEKNRPL KGRINLVLSRELKEPPQGAHFLSRSLDDALK LTEQPELANKVDMVWIVGGSSVIKEFMNHP GHLKLFVTRIMQDFESDTFFPEIDLEKYKLL PEYPGVLSDVQEEKGIKYKFEVYEKNDESR RVRRNKRSKIWELKKDVYVVELDWYPDAP GEMVVLTCDTPEEDGITWTLDQSSEVLGSG KTLTIQVKEFGDAGQYTCHKGGEVLSHSLL LLHKKEDGIWSTDILKDQKEPKNKTFLRCE	734	781

linker((G4S)3) - p35)		AKNYSGRFTCWWLTTISTDLTFSVKSSRGS SDPQGVTCGAATLSAERVRGDNKEYEYSV ECQEDSACPAAEESLPIEVMVDAVHKLKYE NYTSSFFIRDIIKPDPPKNLQLKPLKNSRQVE VSWEYPDTWSTPHSYFSLTFCVQVQGKSKR EKKDRVFTDKTSATVICRKNASISVRAQDR YYSSSWSEWASVPCSGGGGSGGGGSGGGG SRNLPVATPDPGMFPCLHHSQNLLRAVSNM LQKARQTLEFYPCTSEEIDHEDITKDKTSTV EACLPLELTKNESCLNSRETSFITNGSCLASR KTSFMMALCLSSIYEDLKMYQVEFKTMNA KLLMDPKRQIFLDQNMLAVIDELMQALNF NSETVPQKSSLEEPDFYKTKIKLCILLHAFRI RAVTIDRVMSYLNAS		
OT-IL12-009 (p40 signal sequence- p40 – linker ((G4S)3) - p35 - furin (ESRRVRRNK RSK) - FKBP (E31G, F36V, R71G, K105E)- stop)	CMV	MCHQQLVISWFSLVFLASPLVAIWELKKDV YVVELDWYPDAPGEMVVLTCDTPEEDGIT WTLDQSSEVLGSGKTLTIQVKEFGDAGQYT CHKGGEVLSHSLLLLHKKEDGIWSTDILKD QKEPKNKTFLRCEAKNYSGRFTCWWLTTIS TDLTFSVKSSRGSSDPQGVTCGAATLSAER VRGDNKEYEYSVECQEDSACPAAEESLPIE VMVDAVHKLKYENYTSSFFIRDIIKPDPPKN LQLKPLKNSRQVEVSWEYPDTWSTPHSYFS LTFCVQVQGKSKREKKDRVFTDKTSATVIC RKNASISVRAQDRYYSSSWSEWASVPCSGG GGSGGGGSGGGGSRNLPVATPDPGMFPCL HHSQNLLRAVSNMLQKARQTLEFYPCTSEE IDHEDITKDKTSTVEACLPLELTKNESCLNS RETSFITNGSCLASRKTSFMMALCLSSIYED LKMYQVEFKTMNAKLLMDPKRQIFLDQN MLAVIDELMQALNFNSETVPQKSSLEEPDF YKTKIKLCILLHAFRIRAVTIDRVMSYLNAS ESRRVRRNKRSKGVQVETISPGDGRTFPKR GQTCVVHYTGMLGDGKKVDSSRDRNKPFK FMLGKQEVIRGWEEGVAQMSVGQGAKLTI SPDYAYGATGHPGIIPPHATLVFDVELLELE *	735	782
OT-IL12-019 (p40 signal sequence- p40- linker((G4S)3)- p35-stop)	PGK	MCHQQLVISWFSLVFLASPLVAIWELKKDV YVVELDWYPDAPGEMVVLTCDTPEEDGIT WTLDQSSEVLGSGKTLTIQVKEFGDAGQYT CHKGGEVLSHSLLLLHKKEDGIWSTDILKD QKEPKNKTFLRCEAKNYSGRFTCWWLTTIS TDLTFSVKSSRGSSDPQGVTCGAATLSAER VRGDNKEYEYSVECQEDSACPAAEESLPIE VMVDAVHKLKYENYTSSFFIRDIIKPDPPKN LQLKPLKNSRQVEVSWEYPDTWSTPHSYFS LTFCVQVQGKSKREKKDRVFTDKTSATVIC RKNASISVRAQDRYYSSSWSEWASVPCSGG GGSGGGGSGGGGSRNLPVATPDPGMFPCL HHSQNLLRAVSNMLQKARQTLEFYPCTSEE IDHEDITKDKTSTVEACLPLELTKNESCLNS RETSFITNGSCLASRKTSFMMALCLSSIYED LKMYQVEFKTMNAKLLMDPKRQIFLDQN MLAVIDELMQALNFNSETVPQKSSLEEPDF YKTKIKLCILLHAFRIRAVTIDRVMSYLNAS *	732	779
OT-IL12-020 (p40 signal sequence- p40-	EF1a	MCHQQLVISWFSLVFLASPLVAIWELKKDV YVVELDWYPDAPGEMVVLTCDTPEEDGIT WTLDQSSEVLGSGKTLTIQVKEFGDAGQYT CHKGGEVLSHSLLLLHKKEDGIWSTDILKD	732	779

linker((G4S)3)-p35-stop)		QKEPKNKTFLRCEAKNYSGRFTCWWLTTIS TDLTFSVKSSRGSSDPQGVTCGAATLSAER VRGDNKEYEYSVECQEDSACPAAEESLPIE VMVDAVHKLKYENYTSSFFIRDIIKPDPPKN LQLKPLKNSRQVEVSWEYPDTWSTPHSYFS LTFCVQVQGKSKREKKDRVFTDKTSATVIC RKNASISVRAQDRYYSSSWSEWASVPCSGG GGSGGGGSGGGGSRNLPVATPDPGMFPCL HHSQNLLRAVSNMLQKARQTLEFYPCTSEE IDHEDITKDKTSTVEACLPLELTKNESCLNS RETSFITNGSCLASRKTSFMMALCLSSIYED LKMYQVEFKTMNAKLLMDPKRQIFLDQN MLAVIDELMQALNFNSETVPQKSSLEEPDF YKTKIKLCILLHAFRIRAVTIDRVMSYLNAS *		
OT-IL12-021 (p40 signal sequence- p40-linker((G4S)3)-p35-stop)	No promoter	MCHQQLVISWFSLVFLASPLVAIWELKKDV YVVELDWYPDAPGEMVVLTCDTPEEDGIT WTLDQSSEVLGSGKTLTIQVKEFGDAGQYT CHKGGEVLSHSLLLLHKKEDGIWSTDILKD QKEPKNKTFLRCEAKNYSGRFTCWWLTTIS TDLTFSVKSSRGSSDPQGVTCGAATLSAER VRGDNKEYEYSVECQEDSACPAAEESLPIE VMVDAVHKLKYENYTSSFFIRDIIKPDPPKN LQLKPLKNSRQVEVSWEYPDTWSTPHSYFS LTFCVQVQGKSKREKKDRVFTDKTSATVIC RKNASISVRAQDRYYSSSWSEWASVPCSGG GGSGGGGSGGGGSRNLPVATPDPGMFPCL HHSQNLLRAVSNMLQKARQTLEFYPCTSEE IDHEDITKDKTSTVEACLPLELTKNESCLNS RETSFITNGSCLASRKTSFMMALCLSSIYED LKMYQVEFKTMNAKLLMDPKRQIFLDQN MLAVIDELMQALNFNSETVPQKSSLEEPDF YKTKIKLCILLHAFRIRAVTIDRVMSYLNAS *	732	779
OT-IL12-022 (p40 signal sequence- p40 – linker- ((G4S)3) - p35 - linker (GGSG) - FKBP (E31G, F36V, R71G, K105E) - stop)	PGK	MCHQQLVISWFSLVFLASPLVAIWELKKDV YVVELDWYPDAPGEMVVLTCDTPEEDGIT WTLDQSSEVLGSGKTLTIQVKEFGDAGQYT CHKGGEVLSHSLLLLHKKEDGIWSTDILKD QKEPKNKTFLRCEAKNYSGRFTCWWLTTIS TDLTFSVKSSRGSSDPQGVTCGAATLSAER VRGDNKEYEYSVECQEDSACPAAEESLPIE VMVDAVHKLKYENYTSSFFIRDIIKPDPPKN LQLKPLKNSRQVEVSWEYPDTWSTPHSYFS LTFCVQVQGKSKREKKDRVFTDKTSATVIC RKNASISVRAQDRYYSSSWSEWASVPCSGG GGSGGGGSGGGGSRNLPVATPDPGMFPCL HHSQNLLRAVSNMLQKARQTLEFYPCTSEE IDHEDITKDKTSTVEACLPLELTKNESCLNS RETSFITNGSCLASRKTSFMMALCLSSIYED LKMYQVEFKTMNAKLLMDPKRQIFLDQN MLAVIDELMQALNFNSETVPQKSSLEEPDF YKTKIKLCILLHAFRIRAVTIDRVMSYLNAS GGSGGVQVETISPGDGRTFPKRGQTCVVHY TGMLGDGKKVDSSRDRNKPFKFMLGKQEV IRGWEEGVAQMSVGQGAKLTISPDYAYGA TGHPGIIPPHATLVFDVELLELE*	731	778
OT-IL12-023 (p40 signal sequence- p40 – linker-	EF1a	MCHQQLVISWFSLVFLASPLVAIWELKKDV YVVELDWYPDAPGEMVVLTCDTPEEDGIT WTLDQSSEVLGSGKTLTIQVKEFGDAGQYT CHKGGEVLSHSLLLLHKKEDGIWSTDILKD	731	778

((G4S)3) - p35 - linker (GGSG) - FKBP (E31G, F36V, R71G, K105E) - stop)		QKEPKNKTFLRCEAKNYSGRFTCWWLTTIS TDLTFSVKSSRGSSDPQGVTCGAATLSAER VRGDNKEYEYSVECQEDSACPAAEESLPIE VMVDAVHKLKYENYTSSFFIRDIIKPDPPKN LQLKPLKNSRQVEVSWEYPDTWSTPHSYFS LTFCVQVQGKSKREKKDRVFTDKTSATVIC RKNASISVRAQDRYYSSSWSEWASVPCSGG GGSGGGGSGGGGSRNLPVATPDPGMFPCL HHSQNLLRAVSNMLQKARQTLEFYPCTSEE IDHEDITKDKTSTVEACLPLELTKNESCLNS RETSFITNGSCLASRKTSFMMALCLSSIYED LKMYQVEFKTMNAKLLMDPKRQIFLDQN MLAVIDELMQALNFNSETVPQKSSLEEPDF YKTKIKLCILLHAFRIRAVTIDRVMSYLNAS GGSGGVQVETISPGDGRTFPKRGQTCVVHY TGMLGDGKKVDSSRDRNKPFKFMLGKQEV IRGWEEGVAQMSVGQGAKLTISPDYAYGA TGHPGIIPPHATLVFDVELLELE*		
OT-IL12-024 (p40 signal sequence- p40 – linker- ((G4S)3) - p35 - linker (GGSG) - FKBP (E31G, F36V, R71G, K105E) - stop)	No promoter	MCHQQLVISWFSLVFLASPLVAIWELKKDV YVVELDWYPDAPGEMVVLTCDTPEEDGIT WTLDQSSEVLGSGKTLTIQVKEFGDAGQYT CHKGGEVLSHSLLLLHKKEDGIWSTDILKD QKEPKNKTFLRCEAKNYSGRFTCWWLTTIS TDLTFSVKSSRGSSDPQGVTCGAATLSAER VRGDNKEYEYSVECQEDSACPAAEESLPIE VMVDAVHKLKYENYTSSFFIRDIIKPDPPKN LQLKPLKNSRQVEVSWEYPDTWSTPHSYFS LTFCVQVQGKSKREKKDRVFTDKTSATVIC RKNASISVRAQDRYYSSSWSEWASVPCSGG GGSGGGGSGGGGSRNLPVATPDPGMFPCL HHSQNLLRAVSNMLQKARQTLEFYPCTSEE IDHEDITKDKTSTVEACLPLELTKNESCLNS RETSFITNGSCLASRKTSFMMALCLSSIYED LKMYQVEFKTMNAKLLMDPKRQIFLDQN MLAVIDELMQALNFNSETVPQKSSLEEPDF YKTKIKLCILLHAFRIRAVTIDRVMSYLNAS GGSGGVQVETISPGDGRTFPKRGQTCVVHY TGMLGDGKKVDSSRDRNKPFKFMLGKQEV IRGWEEGVAQMSVGQGAKLTISPDYAYGA TGHPGIIPPHATLVFDVELLELE*	731	778
OT-IL12-025 (p40 signal sequence- p40 – linker- ((G4S)3) - p35 - linker (GGSG) - FKBP (E31G, F36V, R71G, K105E) - stop)	PGK	MCHQQLVISWFSLVFLASPLVAIWELKKDV YVVELDWYPDAPGEMVVLTCDTPEEDGIT WTLDQSSEVLGSGKTLTIQVKEFGDAGQYT CHKGGEVLSHSLLLLHKKEDGIWSTDILKD QKEPKNKTFLRCEAKNYSGRFTCWWLTTIS TDLTFSVKSSRGSSDPQGVTCGAATLSAER VRGDNKEYEYSVECQEDSACPAAEESLPIE VMVDAVHKLKYENYTSSFFIRDIIKPDPPKN LQLKPLKNSRQVEVSWEYPDTWSTPHSYFS LTFCVQVQGKSKREKKDRVFTDKTSATVIC RKNASISVRAQDRYYSSSWSEWASVPCSGG GGSGGGGSGGGGSRNLPVATPDPGMFPCL HHSQNLLRAVSNMLQKARQTLEFYPCTSEE IDHEDITKDKTSTVEACLPLELTKNESCLNS RETSFITNGSCLASRKTSFMMALCLSSIYED LKMYQVEFKTMNAKLLMDPKRQIFLDQN MLAVIDELMQALNFNSETVPQKSSLEEPDF YKTKIKLCILLHAFRIRAVTIDRVMSYLNAS GGSGGVQVETISPGDGRTFPKRGQTCVVHY TGMLGDGKKVDSSRDRNKPFKFMLGKQEV	731	778

		IRGWEEGVAQMSVGQGAKLTISPDYAYGA TGHPGIIPPHATLVFDVELLELE*		
OT-IL12-026 (p40 signal sequence- p40 – linker- ((G4S)3) - p35 - linker (GGSG) - FKBP (E31G, F36V, R71G, K105E) - stop)	EF1a	MCHQQLVISWFSLVFLASPLVAIWELKKDV YVVELDWYPDAPGEMVVLTCDTPEEDGIT WTLDQSSEVLGSGKTLTIQVKEFGDAGQYT CHKGGEVLSHSLLLLHKKEDGIWSTDILKD QKEPKNKTFLRCEAKNYSGRFTCWWLTTIS TDLTFSVKSSRGSSDPQGVTCGAATLSAER VRGDNKEYEYSVECQEDSACPAAEESLPIE VMVDAVHKLKYENYTSSFFIRDIIKPDPPKN LQLKPLKNSRQVEVSWEYPDTWSTPHSYFS LTFCVQVQGKSKREKKDRVFTDKTSATVIC RKNASISVRAQDRYYSSSWSEWASVPCSGG GGSGGGGSGGGGSRNLPVATPDPGMFPCL HHSQNLLRAVSNMLQKARQTLEFYPCTSEE IDHEDITKDKTSTVEACLPLELTKNESCLNS RETSFITNGSCLASRKTSFMMALCLSSIYED LKMYQVEFKTMNAKLLMDPKRQIFLDQN MLAVIDELMQALNFNSETVPQKSSLEEPDF YKTKIKLCILLHAFRIRAVTIDRVMSYLNAS GGSGGVQVETISPGDGRTFPKRGQTCVVHY TGMLGDGKKVDSSRDRNKPFKFMLGKQEV IRGWEEGVAQMSVGQGAKLTISPDYAYGA TGHPGIIPPHATLVFDVELLELE*	731	778
OT-IL12-027 (p40 signal sequence- p40 – linker- ((G4S)3) - p35 - linker (GGSG) - FKBP (E31G, F36V, R71G, K105E) - stop)	No promoter	MCHQQLVISWFSLVFLASPLVAIWELKKDV YVVELDWYPDAPGEMVVLTCDTPEEDGIT WTLDQSSEVLGSGKTLTIQVKEFGDAGQYT CHKGGEVLSHSLLLLHKKEDGIWSTDILKD QKEPKNKTFLRCEAKNYSGRFTCWWLTTIS TDLTFSVKSSRGSSDPQGVTCGAATLSAER VRGDNKEYEYSVECQEDSACPAAEESLPIE VMVDAVHKLKYENYTSSFFIRDIIKPDPPKN LQLKPLKNSRQVEVSWEYPDTWSTPHSYFS LTFCVQVQGKSKREKKDRVFTDKTSATVIC RKNASISVRAQDRYYSSSWSEWASVPCSGG GGSGGGGSGGGGSRNLPVATPDPGMFPCL HHSQNLLRAVSNMLQKARQTLEFYPCTSEE IDHEDITKDKTSTVEACLPLELTKNESCLNS RETSFITNGSCLASRKTSFMMALCLSSIYED LKMYQVEFKTMNAKLLMDPKRQIFLDQN MLAVIDELMQALNFNSETVPQKSSLEEPDF YKTKIKLCILLHAFRIRAVTIDRVMSYLNAS GGSGGVQVETISPGDGRTFPKRGQTCVVHY TGMLGDGKKVDSSRDRNKPFKFMLGKQEV IRGWEEGVAQMSVGQGAKLTISPDYAYGA TGHPGIIPPHATLVFDVELLELE*	731	778
OT-IL12-028 (p40 signal sequence - p40 - linker ((G4S)3) - p35 - furin (ARNRQKRS) - FKBP (E31G, F36V, R71G, K105E) -stop)	PGK	MCHQQLVISWFSLVFLASPLVAIWELKKDV YVVELDWYPDAPGEMVVLTCDTPEEDGIT WTLDQSSEVLGSGKTLTIQVKEFGDAGQYT CHKGGEVLSHSLLLLHKKEDGIWSTDILKD QKEPKNKTFLRCEAKNYSGRFTCWWLTTIS TDLTFSVKSSRGSSDPQGVTCGAATLSAER VRGDNKEYEYSVECQEDSACPAAEESLPIE VMVDAVHKLKYENYTSSFFIRDIIKPDPPKN LQLKPLKNSRQVEVSWEYPDTWSTPHSYFS LTFCVQVQGKSKREKKDRVFTDKTSATVIC RKNASISVRAQDRYYSSSWSEWASVPCSGG GGSGGGGSGGGGSRNLPVATPDPGMFPCL HHSQNLLRAVSNMLQKARQTLEFYPCTSEE IDHEDITKDKTSTVEACLPLELTKNESCLNS	730	777

		RETSFITNGSCLASRKTSFMMALCLSSIYED LKMYQVEFKTMNAKLLMDPKRQIFLDQN MLAVIDELMQALNFNSETVPQKSSLEEPDF YKTKIKLCILLHAFRIRAVTIDRVMSYLNAS ARNRQKRSGVQVETISPGDGRTFPKRGQTC VVHYTGMLGDGKKVDSSRDRNKPFKFML GKQEVIRGWEEGVAQMSVGQGAKLTISPD YAYGATGHPGIIPPHATLVFDVELLELE*		
OT-IL12-029 (p40 signal sequence- p40 – linker ((G4S)3) - p35 - furin (ESRRVRRNK RSK) - FKBP (E31G, F36V, R71G, K105E)-stop)	EF1a	MCHQQLVISWFSLVFLASPLVAIWELKKDV YVVELDWYPDAPGEMVVLTCDTPEEDGIT WTLDQSSEVLGSGKTLTIQVKEFGDAGQYT CHKGGEVLSHSLLLLHKKEDGIWSTDILKD QKEPKNKTFLRCEAKNYSGRFTCWWLTTIS TDLTFSVKSSRGSSDPQGVTCGAATLSAER VRGDNKEYEYSVECQEDSACPAAEESLPIE VMVDAVHKLKYENYTSSFFIRDIIKPDPPKN LQLKPLKNSRQVEVSWEYPDTWSTPHSYFS LTFCVQVQGKSKREKKDRVFTDKTSATVIC RKNASISVRAQDRYYSSSWSEWASVPCSGG GGSGGGGSGGGGSRNLPVATPDPGMFPCL HHSQNLLRAVSNMLQKARQTLEFYPCTSEE IDHEDITKDKTSTVEACLPLELTKNESCLNS RETSFITNGSCLASRKTSFMMALCLSSIYED LKMYQVEFKTMNAKLLMDPKRQIFLDQN MLAVIDELMQALNFNSETVPQKSSLEEPDF YKTKIKLCILLHAFRIRAVTIDRVMSYLNAS ESRRVRRNKRSKGVQVETISPGDGRTFPKR GQTCVVHYTGMLGDGKKVDSSRDRNKPFK FMLGKQEVIRGWEEGVAQMSVGQGAKLTI SPDYAYGATGHPGIIPPHATLVFDVELLELE *	735	782
OT-IL12-030 (p40 signal sequence- p40 – linker ((G4S)3) - p35 - furin (ESRRVRRNK RSK) - FKBP (E31G, F36V, R71G, K105E)-stop)	No promoter	MCHQQLVISWFSLVFLASPLVAIWELKKDV YVVELDWYPDAPGEMVVLTCDTPEEDGIT WTLDQSSEVLGSGKTLTIQVKEFGDAGQYT CHKGGEVLSHSLLLLHKKEDGIWSTDILKD QKEPKNKTFLRCEAKNYSGRFTCWWLTTIS TDLTFSVKSSRGSSDPQGVTCGAATLSAER VRGDNKEYEYSVECQEDSACPAAEESLPIE VMVDAVHKLKYENYTSSFFIRDIIKPDPPKN LQLKPLKNSRQVEVSWEYPDTWSTPHSYFS LTFCVQVQGKSKREKKDRVFTDKTSATVIC RKNASISVRAQDRYYSSSWSEWASVPCSGG GGSGGGGSGGGGSRNLPVATPDPGMFPCL HHSQNLLRAVSNMLQKARQTLEFYPCTSEE IDHEDITKDKTSTVEACLPLELTKNESCLNS RETSFITNGSCLASRKTSFMMALCLSSIYED LKMYQVEFKTMNAKLLMDPKRQIFLDQN MLAVIDELMQALNFNSETVPQKSSLEEPDF YKTKIKLCILLHAFRIRAVTIDRVMSYLNAS ESRRVRRNKRSKGVQVETISPGDGRTFPKR GQTCVVHYTGMLGDGKKVDSSRDRNKPFK FMLGKQEVIRGWEEGVAQMSVGQGAKLTI SPDYAYGATGHPGIIPPHATLVFDVELLELE *	735	782
OT-IL12-046 (p40 signal sequence – FKBP (F36V, L106P) – Linker	EF1a	MCHQQLVISWFSLVFLASPLVAGVQVETISP GDGRTFPKRGQTCVVHYTGMLEDGKKVDS SRDRNKPFKFMLGKQEVIRGWEEGVAQMS VGQRAKLTISPDYAYGATGHPGIIPPHATLV FDVELLKPEGGSGGIWELKKDVYVVELDW YPDAPGEMVVLTCDTPEEDGITWTLDQSSE	727	774

(GGSGG) – p40 – Linker ((G4S)3) – p35 - stop)		VLGSGKTLTIQVKEFGDAGQYTCHKGGEVL SHSLLLLHKKEDGIWSTDILKDQKEPKNKTF LRCEAKNYSGRFTCWWLTTISTDLTFSVKSS RGSSDPQGVTCGAATLSAERVRGDNKEYE YSVECQEDSACPAAEESLPIEVMVDAVHKL KYENYTSSFFIRDIIKPDPPKNLQLKPLKNSR QVEVSWEYPDTWSTPHSYFSLTFCVQVQGK SKREKKDRVFTDKTSATVICRKNASISVRAQ DRYYSSSWSEWASVPCSGGGGSGGGGSGG GGSRNLPVATPDPGMFPCLHHSQNLLRAVS NMLQKARQTLEFYPCTSEEIDHEDITKDKTS TVEACLPLELTKNESCLNSRETSFITNGSCLA SRKTSFMMALCLSSIYEDLKMYQVEFKTMN AKLLMDPKRQIFLDQNMLAVIDELMQALNF NSETVPQKSSLEEPDFYKTKIKLCILLHAFRI RAVTIDRVMSYLNAS*		

[00250] In one embodiment, the payload of the invention may comprise IL15. Interleukin 15 is a potent immune stimulatory cytokine and an essential survival factor for T cells, and Natural Killer cells. Preclinical studies comparing IL2 and IL15, have shown than IL15 is associated with less toxicity than IL2. In some embodiments, the effector module of the invention may be a DD-IL15 fusion polypeptide. IL15 polypeptide may also be modified to increase its binding affinity for the IL15 receptor. For example, the asparagine may be replaced by aspartic acid at position 72 of IL15 (SEQ ID NO. 2 of US patent publication US20140134128A1; the contents of which are incorporated by reference in their entirety). In some embodiments, the IL15 constructs of the invention may be placed under the transcriptional control of the CMV promoter (SEQ ID NO. 716), an EF1a promoter (SEQ ID NO. 717, SEQ ID NO. 908) or a PGK promoter (SEQ ID NO. 718). In some aspects, the DD-IL15 comprises the amino acid sequences listed in Table 12. The amino acid sequences in Table 12 may comprise a stop codon which is denoted in the table with a "*" at the end of the amino acid sequence.

Table 12: DD IL15 constructs

Description/ Construct ID	Promoter	Amino Acid Sequence	Amino Acid SEQ ID NO	Nucleic Acid SEQ ID NO
IL2 signal sequence	-	MYRMQLLSCIALSLALVTNS	783	788-791
IgE Leader	-	MDWTWILFLVAAATRVHS	801	810, 930, 931
Linker	-	EFSTEF	784	792-793
Linker	-	GGSGG	629	676-680
HA Tag	-	YPYDVPDYA	1024	1025-1027
BamHI	-	GS	-	GGATCC
P2A Cleavable Peptide	-	GATNFSLLKQAGDVEENPGP	925	926
mCherry (M1L)	-	LSKGEEDNMAIIKEFMRFKVHMEGSVNG HEFEIEGEGEGRPYEGTQTAKLKVTKGGP LPFAWDILSPQFMYGSKAYVKHPADIPDY LKLSFPEGFKWERVMNFEDGGVVTVTQD SSLQDGEFIYKVKLRGTNFPSDGPVMQKK	1029	1030

		TMGWEASSERMYPEDGALKGEIKQRLKL KDGGHYDAEVKTTYKAKKPVQLPGAYN VNIKLDITSHNEDYTIVEQYERAEGRHSTG GMDELYK		
IL15	-	NWVNVISDLKKIEDLIQSMHIDATLYTESD VHPSCKVTAMKCFLLELQVISLESGDASIH DTVENLIILANNSLSSNGNVTESGCKECEE LEEKNIKEFLQSFVHIVQMFINTS*	785	794-797, 1001
ecDHFR (Amino acid 2-159 of WT) (R12Y, Y100I)	-	ISLIAALAVDYVIGMENAMPWNLPADLA WFKRNTLNKPVIMGRHTWESIGRPLPGRK NIILSSQPGTDDRVTWVKSVDEAIAACGD VPEIMVIGGGRVIEQFLPKAQKLYLTHIDA EVEGDTHFPDYEPDDWESVFSEFHDADA QNSHSYCFEILERR*	9	692, 772, 814, 687, 988, 991
hDHFR (Amino acid 2-187 of WT) (Y122I)	-	VGSLNCIVAVSQNMGIGKNGDLPWPPLR NEFRYFQRMTTTSSVEGKQNLVIMGKKT WFSIPEKNRPLKGRINLVLSRELKEPPQGA HFLSRSLDDALKLTEQPELANKVDMVWI VGGSSVIKEAMNHPGHLKLFVTRIMQDFE SDTFFPEIDLEKYKLLPEYPGVLSDVQEEK GIKYKFEVYEKND	895	694, 995
OT-IL15-001 (IL2 signal sequence-IL15-stop)	CMV	MYRMQLLSCIALSLALVTNSNWVNVISDL KKIEDLIQSMHIDATLYTESDVHPSCKVTA MKCFLLELQVISLESGDASIHDTVENLIILA NNSLSSNGNVTESGCKECEELEEKNIKEFL QSFVHIVQMFINTS*	786	799
OT-IL15-002 (IL2 signal sequence-linker[EFSTEF]-ecDHFR (amino acid 2-159 of WT, R12Y, 100I)- linker [GGSGG]- IL15-stop)	CMV	MYRMQLLSCIALSLALVTNSEFSTEFISLIA ALAVDYVIGMENAMPWNLPADLAWFKR NTLNKPVIMGRHTWESIGRPLPGRKNIILS SQPGTDDRVTWVKSVDEAIAACGDVPEI MVIGGGRVIEQFLPKAQKLYLTHIDAEVE GDTHFPDYEPDDWESVFSEFHDADAQNS HSYCFEILERRGGSGGNWVNVISDLKKIE DLIQSMHIDATLYTESDVHPSCKVTAMKC FLLELQVISLESGDASIHDTVENLIILANNS LSSNGNVTESGCKECEELEEKNIKEFLQSF VHIVQMFINTS*	787	800
OT-IL15-062 (IgE leader – HA Tag – IL15 – BamHI (GS) – stop)	EF1a	MDWTWILFLVAAATRVHSYPYDVPDYA NWVNVISDLKKIEDLIQSMHIDATLYTESD VHPSCKVTAMKCFLLELQVISLESGDASIH DTVENLIILANNSLSSNGNVTESGCKECEE LEEKNIKEFLQSFVHIVQMFINTSGS*	631	749
OT-IL15-132 (IgE leader –IL15 – BamHI (GS) – P2A cleavable peptide – mCherry (M1L) - stop)	EF1a	MDWTWILFLVAAATRVHSNWVNVISDLK KIEDLIQSMHIDATLYTESDVHPSCKVTA MKCFLLELQVISLESGDASIHDTVENLIILA NNSLSSNGNVTESGCKECEELEEKNIKEFL QSFVHIVQMFINTSGSGATNFSLLKQAGD VEENPGPLSKGEEDNMAIIKEFMRFKVHM EGSVNGHEFEIEGEGEGRPYEGTQTAKLK VTKGGPLPFAWDILSPQFMYGSKAYVKH PADIPDYLKLSFPEGFKWERVMNFEDGGV VTVTQDSSLQDGEFIYKVKLRGTNFPSDG PVMQKKTMGWEASSERMYPEDGALKGEI KQRLKLKDGGHYDAEVKTTYKAKKPVQ LPGAYNVNIKLDITSHNEDYTIVEQYERAE GRHSTGGMDELYK*	725	1055
OT-IL15-134 (IgE leader –IL15 – Linker (GS) – hDHFR (WT 2-187, Y122I) –	EF1a	MDWTWILFLVAAATRVHSNWVNVISDLK KIEDLIQSMHIDATLYTESDVHPSCKVTA MKCFLLELQVISLESGDASIHDTVENLIILA NNSLSSNGNVTESGCKECEELEEKNIKEFL	726	1056

BamHI (GS) – P2A cleavable peptide – mCherry (M1L) - stop)		QSFVHIVQMFINTSGSVGSLNCIVAVSQN MGIGKNGDLPWPPLRNEFRYFQRMTTTSS VEGKQNLVIMGKKTWFSIPEKNRPLKGRI NLVLSRELKEPPQGAHFLSRSLDDALKLT EQPELANKVDMVWIVGGSSVIKEAMNHP GHLKLFVTRIMQDFESDTFFPEIDLEKYKL LPEYPGVLSDVQEEKGIKYKFEVYEKNDG SGATNFSLLKQAGDVEENPGPLSKGEEDN MAIIKEFMRFKVHMEGSVNGHEFEIEGEG EGRPYEGTQTAKLKVTKGGPLPFAWDILS PQFMYGSKAYVKHPADIPDYLKLSFPEGF KWERVMNFEDGGVVTVTQDSSLQDGEFI YKVKLRGTNFPSDGPVMQKKTMGWEAS SERMYPEDGALKGEIKQRLKLKDGGHYD AEVKTTYKAKKPVQLPGAYNVNIKLDITS HNEDYTIVEQYERAEGRHSTGGMDELYK *		
Description/ Construct ID	**Promoter**	**Amino Acid Sequence**	**Amino Acid SEQ ID NO**	**Nucleic Acid SEQ ID NO**
IL2 signal sequence	-	MYRMQLLSCIALSLALVTNS	783	788-791
IgE Leader	-	MDWTWILFLVAAATRVHS	801	810, 930, 931
Linker	-	EFSTEF	784	792-793
Linker	-	GGSGG	629	676-680
HA Tag	-	YPYDVPDYA	1024	1025-1027
BamHI	-	GS	-	GGATCC
P2A Cleavable Peptide	-	GATNFSLLKQAGDVEENPGP	925	926
mCherry (M1L)	-	LSKGEEDNMAIIKEFMRFKVHMEGSVNG HEFEIEGEGEGRPYEGTQTAKLKVTKGGP LPFAWDILSPQFMYGSKAYVKHPADIPDY LKLSFPEGFKWERVMNFEDGGVVTVTQD SSLQDGEFIYKVKLRGTNFPSDGPVMQKK TMGWEASSERMYPEDGALKGEIKQRLKL KDGGHYDAEVKTTYKAKKPVQLPGAYN VNIKLDITSHNEDYTIVEQYERAEGRHSTG GMDELYK	1029	1030
IL15	-	NWVNVISDLKKIEDLIQSMHIDATLYTESD VHPSCKVTAMKCFLLELQVISLESGDASIH DTVENLIILANNSLSSNGNVTESGCKECEE LEEKNIKEFLQSFVHIVQMFINTS*	785	794-797, 1001
ecDHFR (Amino acid 2-159 of WT) (R12Y, Y100I)	-	ISLIAALAVDYVIGMENAMPWNLPADLA WFKRNTLNKPVIMGRHTWESIGRPLPGRK NIILSSQPGTDDRVTWVKSVDEAIAACGD VPEIMVIGGGRVIEQFLPKAQKLYLTHIDA EVEGDTHFPDYEPDDWESVFSEFHDADA QNSHSYCFEILERR*	9	692, 772, 814, 687, 988, 991
hDHFR (Amino acid 2-187 of WT) (Y122I)	-	VGSLNCIVAVSQNMGIGKNGDLPWPPLR NEFRYFQRMTTTSSVEGKQNLVIMGKKT WFSIPEKNRPLKGRINLVLSRELKEPPQGA HFLSRSLDDALKLTEQPELANKVDMVWI VGGSSVIKEAMNHPGHLKLFVTRIMQDFE SDTFFPEIDLEKYKLLPEYPGVLSDVQEEK GIKYKFEVYEKND	895	694, 995
OT-IL15-001 (IL2 signal sequence-IL15-stop)	CMV	MYRMQLLSCIALSLALVTNSNWVNVISDL KKIEDLIQSMHIDATLYTESDVHPSCKVTA MKCFLLELQVISLESGDASIHDTVENLIILA NNSLSSNGNVTESGCKECEELEEKNIKEFL QSFVHIVQMFINTS*	786	799

OT-IL15-002 (IL2 signal sequence-linker[EFSTEF]-ecDHFR (amino acid 2-159 of WT, R12Y, 100I)- linker [GGSGG]- IL15-stop)	CMV	MYRMQLLSCIALSLALVTNSEFSTEFISLIA ALAVDYVIGMENAMPWNLPADLAWFKR NTLNKPVIMGRHTWESIGRPLPGRKNIILS SQPGTDDRVTWVKSVDEAIAACGDVPEI MVIGGGRVIEQFLPKAQKLYLTHIDAEVE GDTHFPDYEPDDWESVFSEFHDADAQNS HSYCFEILERRGGSGGNWVNVISDLKKIE DLIQSMHIDATLYTESDVHPSCKVTAMKC FLLELQVISLESGDASIHDTVENLIILANNS LSSNGNVTESGCKECEELEEKNIKEFLQSF VHIVQMFINTS*	787	800
OT-IL15-062 (IgE leader – HA Tag – IL15 – BamHI (GS) – stop)	EF1a	MDWTWILFLVAAATRVHSYPYDVPDYA NWVNVISDLKKIEDLIQSMHIDATLYTESD VHPSCKVTAMKCFLLELQVISLESGDASIH DTVENLIILANNSLSSNGNVTESGCKECEE LEEKNIKEFLQSFVHIVQMFINTSGS*	631	749
OT-IL15-132 (IgE leader –IL15 – BamHI (GS) – P2A cleavable peptide – mCherry (M1L) - stop)	EF1a	MDWTWILFLVAAATRVHSNWVNVISDLK KIEDLIQSMHIDATLYTESDVHPSCKVTA MKCFLLELQVISLESGDASIHDTVENLIILA NNSLSSNGNVTESGCKECEELEEKNIKEFL QSFVHIVQMFINTSGSGATNFSLLKQAGD VEENPGPLSKGEEDNMAIIKEFMRFKVHM EGSVNGHEFEIEGEGEGRPYEGTQTAKLK VTKGGPLPFAWDILSPQFMYGSKAYVKH PADIPDYLKLSFPEGFKWERVMNFEDGGV VTVTQDSSLQDGEFIYKVKLRGTNFPSDG PVMQKKTMGWEASSERMYPEDGALKGEI KQRLKLKDGGHYDAEVKTTYKAKKPVQ LPGAYNVNIKLDITSHNEDYTIVEQYERAE GRHSTGGMDELYK*	725	1055
OT-IL15-134 (IgE leader –IL15 – Linker (GS) – hDHFR (WT 2-187, Y122I) – BamHI (GS) – P2A cleavable peptide – mCherry (M1L) - stop)	EF1a	MDWTWILFLVAAATRVHSNWVNVISDLK KIEDLIQSMHIDATLYTESDVHPSCKVTA MKCFLLELQVISLESGDASIHDTVENLIILA NNSLSSNGNVTESGCKECEELEEKNIKEFL QSFVHIVQMFINTSGSVGSLNCIVAVSQN MGIGKNGDLPWPPLRNEFRYFQRMTTTSS VEGKQNLVIMGKKTWFSIPEKNRPLKGRI NLVLSRELKEPPQGAHFLSRSLDDALKLT EQPELANKVDMVWIVGGSSVIKEAMNHP GHLKLFVTRIMQDFESDTFFPEIDLEKYKL LPEYPGVLSDVQEEKGIKYKFEVYEKNDG SGATNFSLLKQAGDVEENPGPLSKGEEDN MAIIKEFMRFKVHMEGSVNGHEFEIEGEG EGRPYEGTQTAKLKVTKGGPLPFAWDILS PQFMYGSKAYVKHPADIPDYLKLSFPEGF KWERVMNFEDGGVVTVTQDSSLQDGEFI YKVKLRGTNFPSDGPVMQKKTMGWEAS SERMYPEDGALKGEIKQRLKLKDGGHYD AEVKTTYKAKKPVQLPGAYNVNIKLDITS HNEDYTIVEQYERAEGRHSTGGMDELYK *	726	1056

[00251] A unique feature of IL15 mediated activation is the mechanism of trans-presentation in which IL15 is presented as a complex with the alpha subunit of IL15 receptor (IL15Ra) that binds to and activates membrane bound IL15 beta/gamma receptor, either on the same cell or a different cell. The IL15/IL15Ra complex is more effective in activating IL15 signaling, than

IL15 by itself. Thus, in some embodiments, the effector module of the invention may include a DD-IL15/IL15Ra fusion polypeptide. In one embodiment, the payload may be IL15/IL15Ra fusion polypeptide described in US Patent Publication NO.: US20160158285A1 (the contents of which are incorporated herein by reference in their entirety). The IL15 receptor alpha comprises an extracellular domain called the sushi domain which contains most of the structural elements necessary for binding to IL15. Thus, in some embodiments, payload may be the IL15/IL15Ra sushi domain fusion polypeptide described in US Patent Publication NO.: US20090238791A1 (the contents of which are incorporated herein by reference in their entirety).

[00252] Regulated IL15/IL15Ra may be used to promote expansion, survival and potency of $CD8T_{EM}$ cell populations without impacting regulatory T cells, NK cells and TIL cells. In one embodiment, DD-IL15/IL15Ra may be utilized to enhance CD19 directed T cell therapies in B cell leukemia and lymphomas. In one aspect, IL15/IL15Ra may be used as payload of the invention to reduce the need for pre-conditioning regimens in current CAR-T treatment paradigms.

[00253] The effector modules containing DD-IL15, DD-IL15/IL15Ra and/or DD-IL15/IL15Ra sushi domain may be designed to be secreted (using e.g. IL2 signal sequence) or membrane bound (using e.g. IgE or CD8a signal sequence).

[00254] In some aspects, the DD-IL115/IL15Ra comprises the amino acid sequences provided in Table 13a, 13b, and 13c. The amino acid sequences in Tables 13a, 13b and 13c may comprise a stop codon which is denoted in the table with a "*" at the end of the amino acid sequence.

Table 13a: DD-IL15/IL15Ra construct sequences

Description/ Construct ID	Amino Acid Sequence	Amino Acid SEQ ID NO	Nucleic Acid SEQ ID NO
IgE leader	MDWTWILFLVAAATRVHS	801	810, 930, 931
IL15RA Leader	MAPRRARGCRTLGLPALLLLLLLRPPATRG	932	933
Linker (SG3-(SG4)3-SG3-SLQ)	SGGGSGGGGSGGGGSGGGGSGGGSLQ	802	811, 916-920, 1002
Linker (SG3S)	SGGGS	827	828, 844, 909
Linker (SG3(SG4)5SG3S)	SGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGS	921	923
Linker	SGGGSGGGGSGGGGSGGGGS	922	924
Linker	GS	-	GGTTCC
Linker	SG	-	AGCGGC
Linker	GSG	-	GGATCCGGA or GGATCCGGT
Spacer	-	-	927-929, 1000, TCGCGAATG, TCGCA

IL15	NWVNVISDLKKIEDLIQSMHIDATLYTESDVHPSCKVT AMKCFLLELQVISLESGDASIHDTVENLIILANNSLSSN GNVTESGCKECEELEEKNIKEFLQSFVHIVQMFINTS*	785	794-797, 1001
IL15Ra	ITCPPPMSVEHADIWVKSYSLYSRERYICNSGFKRKAG TSSLTECVLNKATNVAHWTTPSLKCIRDPALVHQRPA PPSTVTTAGVTPQPESLSPSGKEPAASSPSSNNTAATT AAIVPGSQLMPSKSPSTGTTEISSHESSHGTPSQTTAKN WELTASASHQPPGVYPQGHSDTTVAISTSTVLLCGLS AVSLLACYLKSRQTPPLASVEMEAMEALPVTWGTSS RDEDLENCSHHL*	803	812-813, 1003
IL15Ra (31-205 of Uniprot ID: Q13261.1)	ITCPPPMSVEHADIWVKSYSLYSRERYICNSGFKRKAG TSSLTECVLNKATNVAHWTTPSLKCIRDPALVHQRPA PPSTVTTAGVTPQPESLSPSGKEPAASSPSSNNTAATTA AIVPGSQLMPSKSPSTGTTEISSHESSHGTPSQTTAKN WELTASASHQPPGVYPQGHSDTT	1057	1058
mCherry	MSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEG EGRPYEGTQTAKLKVTKGGPLPFAWDILSPQFMYGSK AYVKHPADIPDYLKLSFPEGFKWERVMNFEDGGVVT VTQDSSLQDGEFIYKVKLRGTNFPSDGPVMQKKTMG WEASSERMYPEDGALKGEIKQRLKLKDGGHYDAEVK TTYKAKKPVQLPGAYNVNIKLDITSHNEDYTIVEQYE RAEGRHSTGGMDELYK*	1059	1060
mCherry (M1L)	LSKGEEDNMAIIKEFMRFKVHMEGSVNGHEFEIEGEG EGRPYEGTQTAKLKVTKGGPLPFAWDILSPQFMYGSK AYVKHPADIPDYLKLSFPEGFKWERVMNFEDGGVVT VTQDSSLQDGEFIYKVKLRGTNFPSDGPVMQKKTMG WEASSERMYPEDGALKGEIKQRLKLKDGGHYDAEVK TTYKAKKPVQLPGAYNVNIKLDITSHNEDYTIVEQYE RAEGRHSTGGMDELYK	1029	1030
HA Tag	YPYDVPDYA	1024	1025-1027
Flag	DYKDDDDK	1232	-
BamHI	GS	-	GGATCC
P2A Cleavable Peptide	GATNFSLLKQAGDVEENPGP	925	926
ecDHFR (Amino acid 2-159 of WT R12Y, Y100I)	ISLIAALAVDYVIGMENAMPWNLPADLAWFKRNTLN KPVIMGRHTWESIGRPLPGRKNIILSSQPGTDDRVTWV KSVDEAIAACGDVPEIMVIGGGRVIEQFLPKAQKLYLT HIDAEVEGDTHFPDYEPDDWESVFSEFHDADAQNSHS YCFEILERR*	9	692, 772, 814, 687, 988, 991
ecDHFR (Amino acid 2-159 of WT R12H, E129K)	ISLIAALAVDHVIGMENAMPWNLPADLAWFKRNTLN KPVIMGRHTWESIGRPLPGRKNIILSSQPGTDDRVTWV KSVDEAIAACGDVPEIMVIGGGRVYEQFLPKAQKLYL THIDAEVEGDTHFPDYKPDDWESVFSEFHDADAQNS HSYCFEILERR*	10	798, 815, 993
FKBP (E31G, F36V, R71G, K105E)	GVQVETISPGDGRTFPKRGQTCVVHYTGMLGDGKKV DSSRDRNKPFKFMLGKQEVIRGWEEGVAQMSVGQG AKLTISPDYAYGATGHPGIIPPHATLVFDVELLELE*	12	688-691, 994, 1013, 1028
hDHFR (Amino acid 2-187 of WT; Y122I, A125F)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINL VLSRELKEPPQGAHFLSRSLDDALKLTEQPELANKVD MVWIVGGSSVIKEFMNHPGHLKLFVTRIMQDFESDTF FPEIDLEKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKN D*	890	696, 973, 974, 996
hDHFR (Amino acid 2-187 of WT; Q36F, N65F, Y122I)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFFRM TTTSSVEGKQNLVIMGKKTWFSIPEKFRPLKGRINLVL SRELKEPPQGAHFLSRSLDDALKLTEQPELANKVDMV WIVGGSSVIKEAMNHPGHLKLFVTRIMQDFESDTFFPE IDLEKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKND	891	700, 975, 976, 998

hDHFR (Amino acid 2-187 of WT; K185E)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINL VLSRELKEPPQGAHFLSRSLDDALKLTEQPELANKVD MVWIVGGSSVYKEAMNHPGHLKLFVTRIMQDFESDT FFPEIDLEKYKLLPEYPGVLSDVQEEKGIKYKFEVYEE ND*	889	972
hDHFR (Amino acid 2-187 of WT; E162G, I176F)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINL VLSRELKEPPQGAHFLSRSLDDALKLTEQPELANKVD MVWIVGGSSVYKEAMNHPGHLKLFVTRIMQDFESDT FFPEIDLEKYKLLPGYPGVLSDVQEEKGFKYKFEVYE KND*	888	970-972
hDHFR (Amino acid 2-187 of WT; N127Y)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINL VLSRELKEPPQGAHFLSRSLDDALKLTEQPELANKVD MVWIVGGSSVYKEAMYHPGHLKLFVTRIMQDFESDT FFPEIDLEKYKLLPEYPGVLSDVQEEKGIKYKFEVYEK ND	892	977
hDHFR (Amino acid 2-187 of WT; I17V)	VGSLNCIVAVSQNMGVGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINL VLSRELKEPPQGAHFLSRSLDDALKLTEQPELANKVD MVWIVGGSSVYKEAMNHPGHLKLFVTRIMQDFESDT FFPEIDLEKYKLLPEYPGVLSDVQEEKGIKYKFEVYEK ND	894	979
hDHFR (Amino acid 2-187 of WT; I17V, Y122I)	VGSLNCIVAVSQNMGVGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINL VLSRELKEPPQGAHFLSRSLDDALKLTEQPELANKVD MVWIVGGSSVIKEAMNHPGHLKLFVTRIMQDFESDTF FPEIDLEKYKLLPEYPGVLSDVQEEKGIKYKFEVYEKN D	882	969
hDHFR (Amino acid 2-187 of WT; H131R, E144G)	VGSLNCIVAVSQNMGIGKNGDLPWPPLRNEFRYFQR MTTTSSVEGKQNLVIMGKKTWFSIPEKNRPLKGRINL VLSRELKEPPQGAHFLSRSLDDALKLTEQPELANKVD MVWIVGGSSVYKEAMNHPGRLKLFVTRIMQDFGSDT FFPEIDLEKYKLLPEYPGVLSDVQEEKGIKYKFEVYEK ND	893	978

Table 13b: DD-IL15/IL15Ra constructs

Description	Promoter	Amino acid sequences	Amino Acid SEQ ID NO	Nucleic Acid SEQ ID NO
OT-IL15-006 (IgE signal sequence; IL15; linker1 (SG3-(SG4)5-SG3); IL15Ra; linker2 (GGSGG); ecDHFR (R12H, E129K))	EF1a	MDWTWILFLVAAATRVHSNWVNVISDL KKIEDLIQSMHIDATLYTESDVHPSCKVT AMKCFLLELQVISLESGDASIHDTVENLII LANNSLSSNGNVTESGCKECEELEEKNIK EFLQSFVHIVQMFINTSSGGGSGGGGSGG GGSGGGGSGGGSLQITCPPPMSVEHADI WVKSYSLYSRERYICNSGFKRKAGTSSL TECVLNKATNVAHWTTPSLKCIRDPALV HQRPAPPSTVTTAGVTPQPESLSPSGKEP AASSPSSNNTAATTAAIVPGSQLMPSKSP STGTTEISSHESSHGTPSQTTAKNWELTA SASHQPPGVYPQGHSDTTVAISTSTVLLC GLSAVSLLACYLKSRQTPPLASVEMEAM EALPVTWGTSSRDEDLENCSHHLSGISLI AALAVDHVIGMENAMPWNLPADLAWF KRNTLNKPVIMGRHTWESIGRPLPGRKN IILSSQPGTDDRVTWVKSVDEAIAACGD	804	816

		VPEIMVIGGGRVYEQFLPKAQKLYLTHI DAEVEGDTHFPDYKPDDWESVFSEFHD ADAQNSHSYCFEILERR*		
OT-IL15-007 (IgE signal sequence; IL15; linker1 (SG3-(SG4)5-SG3); IL15Ra; linker2 (GGSGG); FKBP (E31G,F36V, R71G, K105E))	EF1a	MDWTWILFLVAAATRVHSNWVNVISDL KKIEDLIQSMHIDATLYTESDVHPSCKVT AMKCFLLELQVISLESGDASIHDTVENLII LANNSLSSNGNVTESGCKECEELEEKNIK EFLQSFVHIVQMFINTSSGGGSGGGGSGG GGSGGGGSGGGSLQITCPPPMSVEHADI WVKSYSLYSRERYICNSGFKRKAGTSSL TECVLNKATNVAHWTTPSLKCIRDPALV HQRPAPPSTVTTAGVTPQPESLSPSGKEP AASSPSSNNTAATTAAIVPGSQLMPSKSP STGTTEISSHESSHGTPSQTTAKNWELTA SASHQPPGVYPQGHSDTTVAISTSTVLLC GLSAVSLLACYLKSRQTPPLASVEMEAM EALPVTWGTSSRDEDLENCSHHLSGGVQ VETISPGDGRTFPKRGQTCVVHYTGMLG DGKKVDSSRDRNKPFKFMLGKQEVIRG WEEGVAQMSVGQGAKLTISPDYAYGAT GHPGIIPPHATLVFDVELLELE*	805	817
OT-IL15-008 (IgE signal sequence- IL15-linker (SG3-(SG4)3-SG3-SLQ)- IL15Ra-stop)	EF1a	MDWTWILFLVAAATRVHSNWVNVISDL KKIEDLIQSMHIDATLYTESDVHPSCKVT AMKCFLLELQVISLESGDASIHDTVENLII LANNSLSSNGNVTESGCKECEELEEKNIK EFLQSFVHIVQMFINTSSGGGSGGGGSGG GGSGGGGSGGGSLQITCPPPMSVEHADI WVKSYSLYSRERYICNSGFKRKAGTSSL TECVLNKATNVAHWTTPSLKCIRDPALV HQRPAPPSTVTTAGVTPQPESLSPSGKEP AASSPSSNNTAATTAAIVPGSQLMPSKSP STGTTEISSHESSHGTPSQTTAKNWELTA SASHQPPGVYPQGHSDTTVAISTSTVLLC GLSAVSLLACYLKSRQTPPLASVEMEAM EALPVTWGTSSRDEDLENCSHHL*	806	818
OT-IL15-009 (IgE signal sequence- IL15-linker (SG3-(SG4)3-SG3-SLQ)- IL15Ra-linker (SG)-ecDHFR (Amino acid 2-159 of WT; R12Y, Y100I)-stop)	EF1a	MDWTWILFLVAAATRVHSNWVNVISDL KKIEDLIQSMHIDATLYTESDVHPSCKVT AMKCFLLELQVISLESGDASIHDTVENLII LANNSLSSNGNVTESGCKECEELEEKNIK EFLQSFVHIVQMFINTSSGGGSGGGGSGG GGSGGGGSGGGSLQITCPPPMSVEHADI WVKSYSLYSRERYICNSGFKRKAGTSSL TECVLNKATNVAHWTTPSLKCIRDPALV HQRPAPPSTVTTAGVTPQPESLSPSGKEP AASSPSSNNTAATTAAIVPGSQLMPSKSP STGTTEISSHESSHGTPSQTTAKNWELTA SASHQPPGVYPQGHSDTTVAISTSTVLLC GLSAVSLLACYLKSRQTPPLASVEMEAM EALPVTWGTSSRDEDLENCSHHLSGISLI AALAVDYVIGMENAMPWNLPADLAWF KRNTLNKPVIMGRHTWESIGRPLPGRKN IILSSQPGTDDRVTWVKSVDEAIAACGD VPEIMVIGGGRVIEQFLPKAQKLYLTHID AEVEGDTHFPDYEPDDWESVFSEFHDAD AQNSHSYCFEILERR*	807	819
OT-IL15-010 (IgE signal sequence- IL15-linker (SG3-(SG4)3-SG3-	EF1a	MDWTWILFLVAAATRVHSNWVNVISDL KKIEDLIQSMHIDATLYTESDVHPSCKVT AMKCFLLELQVISLESGDASIHDTVENLII LANNSLSSNGNVTESGCKECEELEEKNIK EFLQSFVHIVQMFINTSSGGGSGGGGSGG	808	820

SLQ)- IL15Ra-linker (SG)-hDHFR (Y122I, A125F)-stop)		GGSGGGGSGGGSLQITCPPPMSVEHADI WVKSYSLYSRERYICNSGFKRKAGTSSL TECVLNKATNVAHWTTPSLKCIRDPALV HQRPAPPSTVTTAGVTPQPESLSPSGKEP AASSPSSNNTAATTAAIVPGSQLMPSKSP STGTTEISSHESSHGTPSQTTAKNWELTA SASHQPPGVYPQGHSDTTVAISTSTVLLC GLSAVSLLACYLKSRQTPPLASVEMEAM EALPVTWGTSSRDEDLENCSHHLSGVGS LNCIVAVSQNMGIGKNGDLPWPPLRNEF RYFQRMTTTSSVEGKQNLVIMGKKTWF SIPEKNRPLKGRINLVLSRELKEPPQGAH FLSRSLDDALKLTEQPELANKVDMVWIV GGSSVIKEFMNHPGHLKLFVTRIMQDFE SDTFFPEIDLEKYKLLPEYPGVLSDVQEE KGIKYKFEVYEKND*		
OT-IL15-011 (IgE signal sequence- IL15-linker (SG3-(SG4)3-SG3-SLQ)- IL15Ra; linker (SG)-hDHFR (Amino acid 2-187 of WT; Q36F, N65F, Y122I)-stop)	EF1a	MDWTWILFLVAAATRVHSNWVNVISDL KKIEDLIQSMHIDATLYTESDVHPSCKVT AMKCFLLELQVISLESGDASIHDTVENLII LANNSLSSNGNVTESGCKECEELEEKNIK EFLQSFVHIVQMFINTSSGGGSGGGGSGG GGSGGGGSGGGSLQITCPPPMSVEHADI WVKSYSLYSRERYICNSGFKRKAGTSSL TECVLNKATNVAHWTTPSLKCIRDPALV HQRPAPPSTVTTAGVTPQPESLSPSGKEP AASSPSSNNTAATTAAIVPGSQLMPSKSP STGTTEISSHESSHGTPSQTTAKNWELTA SASHQPPGVYPQGHSDTTVAISTSTVLLC GLSAVSLLACYLKSRQTPPLASVEMEAM EALPVTWGTSSRDEDLENCSHHLSGVGS LNCIVAVSQNMGIGKNGDLPWPPLRNEF RYFFRMTTTSSVEGKQNLVIMGKKTWFS IPEKFRPLKGRINLVLSRELKEPPQGAHFL SRSLDDALKLTEQPELANKVDMVWIVG GSSVIKEAMNHPGHLKLFVTRIMQDFES DTFFPEIDLEKYKLLPEYPGVLSDVQEEK GIKYKFEVYEKND*	809	821
OT-IL15-017 (IgE signal sequence- IL15-linker (SG3-(SG4)3-SG3-SLQ)- IL15Ra-linker (SG)-hDHFR (Amino acid 2-187 of WT; K185E)-stop)	EF1a	MDWTWILFLVAAATRVHSNWVNVISDL KKIEDLIQSMHIDATLYTESDVHPSCKVT AMKCFLLELQVISLESGDASIHDTVENLII LANNSLSSNGNVTESGCKECEELEEKNIK EFLQSFVHIVQMFINTSSGGGSGGGGSGG GGSGGGGSGGGSLQITCPPPMSVEHADI WVKSYSLYSRERYICNSGFKRKAGTSSL TECVLNKATNVAHWTTPSLKCIRDPALV HQRPAPPSTVTTAGVTPQPESLSPSGKEP AASSPSSNNTAATTAAIVPGSQLMPSKSP STGTTEISSHESSHGTPSQTTAKNWELTA SASHQPPGVYPQGHSDTTVAISTSTVLLC GLSAVSLLACYLKSRQTPPLASVEMEAM EALPVTWGTSSRDEDLENCSHHLSGVGS LNCIVAVSQNMGIGKNGDLPWPPLRNEF RYFQRMTTTSSVEGKQNLVIMGKKTWF SIPEKNRPLKGRINLVLSRELKEPPQGAH FLSRSLDDALKLTEQPELANKVDMVWIV GGSSVYKEAMNHPGHLKLFVTRIMQDF ESDTFFPEIDLEKYKLLPEYPGVLSDVQE EKGIKYKFEVYEEND*	1061	1086
OT-IL15-018 (IgE signal	EF1a	MDWTWILFLVAAATRVHSNWVNVISDL KKIEDLIQSMHIDATLYTESDVHPSCKVT	1062	1087

sequence- IL15-linker (SG3-(SG4)3-SG3-SLQ)- IL15Ra-linker (SG)-hDHFR (Amino acid 2-187 of WT; E162G, I176F)-stop)		AMKCFLLELQVISLESGDASIHDTVENLII LANNSLSSNGNVTESGCKECEELEEKNIK EFLQSFVHIVQMFINTSSGGGSGGGGSGG GGSGGGGSGGGSLQITCPPPMSVEHADI WVKSYSLYSRERYICNSGFKRKAGTSSL TECVLNKATNVAHWTTPSLKCIRDPALV HQRPAPPSTVTTAGVTPQPESLSPSGKEP AASSPSSNNTAATTAAIVPGSQLMPSKSP STGTTEISSHESSHGTPSQTTAKNWELTA SASHQPPGVYPQGHSDTTVAISTSTVLLC GLSAVSLLACYLKSRQTPPLASVEMEAM EALPVTWGTSSRDEDLENCSHHLSGVGS LNCIVAVSQNMGIGKNGDLPWPPLRNEF RYFQRMTTTSSVEGKQNLVIMGKKTWF SIPEKNRPLKGRINLVLSRELKEPPQGAH FLSRSLDDALKLTEQPELANKVDMVWIV GGSSVYKEAMNHPGHLKLFVTRIMQDF ESDTFFPEIDLEKYKLLPGYPGVLSDVQE EKGFKYKFEVYEKND*		
OT-IL15-038 (IgE leader – IL15 – Linker (SG3-(SG4)3-SG3-SLQ) – IL15Ra – Linker (SG) – hDHFR (Amino acid 2-187 of WT; N127Y)-stop)	EF1a	MDWTWILFLVAAATRVHSNWVNVISDL KKIEDLIQSMHIDATLYTESDVHPSCKVT AMKCFLLELQVISLESGDASIHDTVENLII LANNSLSSNGNVTESGCKECEELEEKNIK EFLQSFVHIVQMFINTSSGGGSGGGGSGG GGSGGGGSGGGSLQITCPPPMSVEHADI WVKSYSLYSRERYICNSGFKRKAGTSSL TECVLNKATNVAHWTTPSLKCIRDPALV HQRPAPPSTVTTAGVTPQPESLSPSGKEP AASSPSSNNTAATTAAIVPGSQLMPSKSP STGTTEISSHESSHGTPSQTTAKNWELTA SASHQPPGVYPQGHSDTTVAISTSTVLLC GLSAVSLLACYLKSRQTPPLASVEMEAM EALPVTWGTSSRDEDLENCSHHLSGVGS LNCIVAVSQNMGIGKNGDLPWPPLRNEF RYFQRMTTTSSVEGKQNLVIMGKKTWF SIPEKNRPLKGRINLVLSRELKEPPQGAH FLSRSLDDALKLTEQPELANKVDMVWIV GGSSVYKEAMYHPGHLKLFVTRIMQDF ESDTFFPEIDLEKYKLLPEYPGVLSDVQE EKGIKYKFEVYEKND*	1063	1088
OT-IL15-051 (IgE leader – IL15 – Linker (SG3-(SG4)3-SG3-SLQ) – HA Tag – IL15Ra – stop)	EF1a	MDWTWILFLVAAATRVHSNWVNVISDL KKIEDLIQSMHIDATLYTESDVHPSCKVT AMKCFLLELQVISLESGDASIHDTVENLII LANNSLSSNGNVTESGCKECEELEEKNIK EFLQSFVHIVQMFINTSSGGGSGGGGSGG GGSGGGGSGGGSLQYPYDVPDYAITCPP PMSVEHADIWVKSYSLYSRERYICNSGF KRKAGTSSLTECVLNKATNVAHWTTPSL KCIRDPALVHQRPAPPSTVTTAGVTPQPE SLSPSGKEPAASSPSSNNTAATTAAIVPG SQLMPSKSPSTGTTEISSHESSHGTPSQTT AKNWELTASASHQPPGVYPQGHSDTTV AISTSTVLLCGLSAVSLLACYLKSRQTPP LASVEMEAMEALPVTWGTSSRDEDLEN CSHHL*	1064	1089
OT-IL15-053 (IgE leader – IL15 – Linker (SG3(SG4)5SG3	EF1a	MDWTWILFLVAAATRVHSNWVNVISDL KKIEDLIQSMHIDATLYTESDVHPSCKVT AMKCFLLELQVISLESGDASIHDTVENLII LANNSLSSNGNVTESGCKECEELEEKNIK EFLQSFVHIVQMFINTSSGGGSGGGGSGG	1066	1091

S) – IL15Ra – Stop)		GGSGGGGSGGGGSGGGGSGGGSITCPPP MSVEHADIWVKSYSLYSRERYICNSGFK RKAGTSSLTECVLNKATNVAHWTTPSLK CIRDPALVHQRPAPPSTVTTAGVTPQPES LSPSGKEPAASSPSSNNTAATTAAIVPGS QLMPSKSPSTGTTEISSHESSHGTPSQTTA KNWELTASASHQPPGVYPQGHSDTTVAI STSTVLLCGLSAVSLLACYLKSRQTPPLA SVEMEAMEALPVTWGTSSRDEDLENCS HHL*		
OT-IL15-054 (IgE leader – IL15 – Linker (SG3(SG4)3S) – HA Tag – Linker (SG3S) – IL15Ra – Stop)	EF1a	MDWTWILFLVAAATRVHSNWVNVISDL KKIEDLIQSMHIDATLYTESDVHPSCKVT AMKCFLLELQVISLESGDASIHDTVENLII LANNSLSSNGNVTESGCKECEELEEKNIK EFLQSFVHIVQMFINTSSGGGSGGGGSGG GGSGGGGSYPYDVPDYASGGGSITCPPP MSVEHADIWVKSYSLYSRERYICNSGFK RKAGTSSLTECVLNKATNVAHWTTPSLK CIRDPALVHQRPAPPSTVTTAGVTPQPES LSPSGKEPAASSPSSNNTAATTAAIVPGS QLMPSKSPSTGTTEISSHESSHGTPSQTTA KNWELTASASHQPPGVYPQGHSDTTVAI STSTVLLCGLSAVSLLACYLKSRQTPPLA SVEMEAMEALPVTWGTSSRDEDLENCS HHL*	1067	1092
OT-IL15-055 (IgE leader – IL15 – Linker (SG) - IL15Ra – Stop)	EF1a	MDWTWILFLVAAATRVHSNWVNVISDL KKIEDLIQSMHIDATLYTESDVHPSCKVT AMKCFLLELQVISLESGDASIHDTVENLII LANNSLSSNGNVTESGCKECEELEEKNIK EFLQSFVHIVQMFINTSSGITCPPPMSVEH ADIWVKSYSLYSRERYICNSGFKRKAGT SSLTECVLNKATNVAHWTTPSLKCIRDP ALVHQRPAPPSTVTTAGVTPQPESLSPSG KEPAASSPSSNNTAATTAAIVPGSQLMPS KSPSTGTTEISSHESSHGTPSQTTAKNWE LTASASHQPPGVYPQGHSDTTVAISTSTV LLCGLSAVSLLACYLKSRQTPPLASVEM EAMEALPVTWGTSSRDEDLENCSHHL*	1068	1093
OT-IL15-060 (IL15Ra signal peptide – IL15 – Linker (SG3-(SG4)3-SG3-SLQ) – IL15Ra – stop)	EF1a	MAPRRARGCRTLGLPALLLLLLLRPPAT RGNWVNVISDLKKIEDLIQSMHIDATLYT ESDVHPSCKVTAMKCFLLELQVISLESG DASIHDTVENLIILANNSLSSNGNVTESG CKECEELEEKNIKEFLQSFVHIVQMFINT SSGGGSGGGGSGGGGSGGGGSGGGSLQI TCPPPMSVEHADIWVKSYSLYSRERYIC NSGFKRKAGTSSLTECVLNKATNVAHW TTPSLKCIRDPALVHQRPAPPSTVTTAGV TPQPESLSPSGKEPAASSPSSNNTAATTA AIVPGSQLMPSKSPSTGTTEISSHESSHGT PSQTTAKNWELTASASHQPPGVYPQGHS DTTVAISTSTVLLCGLSAVSLLACYLKSR QTPPLASVEMEAMEALPVTWGTSSRDE DLENCSHHL*	1069	1094
OT-IL15-063 (IgE leader – IL15 – Linker (SG3-(SG4)3-SG3-SLQ) – IL15Ra –	EF1a	MDWTWILFLVAAATRVHSNWVNVISDL KKIEDLIQSMHIDATLYTESDVHPSCKVT AMKCFLLELQVISLESGDASIHDTVENLII LANNSLSSNGNVTESGCKECEELEEKNIK EFLQSFVHIVQMFINTSSGGGSGGGGSGG GGSGGGGSGGGSLQITCPPPMSVEHADI WVKSYSLYSRERYICNSGFKRKAGTSSL	1070	1095

BamHI (GS) - stop)		TECVLNKATNVAHWTTPSLKCIRDPALV HQRPAPPSTVTTAGVTPQPESLSPSGKEP AASSPSSNNTAATTAAIVPGSQLMPSKSP STGTTEISSHESSHGTPSQTTAKNWELTA SASHQPPGVYPQGHSDTTVAISTSTVLLC GLSAVSLLACYLKSRQTPPLASVEMEAM EALPVTWGTSSRDEDLENCSHHLGS*		
OT-IL15-064 and OT-IL15-071 (IgE leader – IL15 – Linker (SG3-(SG4)3-SG3-SLQ) – IL15Ra – stop)	EF1a	MDWTWILFLVAAATRVHSNWVNVISDL KKIEDLIQSMHIDATLYTESDVHPSCKVT AMKCFLLELQVISLESGDASIHDTVENLII LANNSLSSNGNVTESGCKECEELEEKNIK EFLQSFVHIVQMFINTSSGGGSGGGGSGG GGSGGGGSGGGSLQITCPPPMSVEHADI WVKSYSLYSRERYICNSGFKRKAGTSSL TECVLNKATNVAHWTTPSLKCIRDPALV HQRPAPPSTVTTAGVTPQPESLSPSGKEP AASSPSSNNTAATTAAIVPGSQLMPSKSP STGTTEISSHESSHGTPSQTTAKNWELTA SASHQPPGVYPQGHSDTTVAISTSTVLLC GLSAVSLLACYLKSRQTPPLASVEMEAM EALPVTWGTSSRDEDLENCSHHL*	806	818
OT-IL15-066 (IgE leader – IL15 – Linker (SG3-(SG4)3-SG3-SLQ) – IL15Ra – Linker (SG) ecDHFR (Amino acid 2-159 of WT, R12Y, Y100I) - stop)	EF1a	MDWTWILFLVAAATRVHSNWVNVISDL KKIEDLIQSMHIDATLYTESDVHPSCKVT AMKCFLLELQVISLESGDASIHDTVENLII LANNSLSSNGNVTESGCKECEELEEKNIK EFLQSFVHIVQMFINTSSGGGSGGGGSGG GGSGGGGSGGGSLQITCPPPMSVEHADI WVKSYSLYSRERYICNSGFKRKAGTSSL TECVLNKATNVAHWTTPSLKCIRDPALV HQRPAPPSTVTTAGVTPQPESLSPSGKEP AASSPSSNNTAATTAAIVPGSQLMPSKSP STGTTEISSHESSHGTPSQTTAKNWELTA SASHQPPGVYPQGHSDTTVAISTSTVLLC GLSAVSLLACYLKSRQTPPLASVEMEAM EALPVTWGTSSRDEDLENCSHHLSGISLI AALAVDYVIGMENAMPWNLPADLAWF KRNTLNKPVIMGRHTWESIGRPLPGRKN IILSSQPGTDDRVTWVKSVDEAIAACGD VPEIMVIGGGRVIEQFLPKAQKLYLTHID AEVEGDTHFPDYEPDDWESVFSEFHDAD AQNSHSYCFEILERR*	807	819
OT-IL15-067 (IgE leader – IL15 – Linker (SG3-(SG4)3-SG3-SLQ) – IL15Ra – Linker (SG) - ecDHFR (Amino acid 2-159 of WT, R12Y, Y100I) - stop)	EF1a	MDWTWILFLVAAATRVHSNWVNVISDL KKIEDLIQSMHIDATLYTESDVHPSCKVT AMKCFLLELQVISLESGDASIHDTVENLII LANNSLSSNGNVTESGCKECEELEEKNIK EFLQSFVHIVQMFINTSSGGGSGGGGSGG GGSGGGGSGGGSLQITCPPPMSVEHADI WVKSYSLYSRERYICNSGFKRKAGTSSL TECVLNKATNVAHWTTPSLKCIRDPALV HQRPAPPSTVTTAGVTPQPESLSPSGKEP AASSPSSNNTAATTAAIVPGSQLMPSKSP STGTTEISSHESSHGTPSQTTAKNWELTA SASHQPPGVYPQGHSDTTVAISTSTVLLC GLSAVSLLACYLKSRQTPPLASVEMEAM EALPVTWGTSSRDEDLENCSHHLSGISLI AALAVDYVIGMENAMPWNLPADLAWF KRNTLNKPVIMGRHTWESIGRPLPGRKN IILSSQPGTDDRVTWVKSVDEAIAACGD VPEIMVIGGGRVIEQFLPKAQKLYLTHID	1071	1098

		AEVEGDTHFPDYEPDDWESVFSEFHDAD AQNSHSYCFEILERR*		
OT-IL15-070 (IL15Ra signal peptide – IL15 – Linker (SG3-(SG4)3-SG3-SLQ) – IL15Ra – stop)	EF1a	MAPRRARGCRTLGLPALLLLLLLRPPAT RGNWVNVISDLKKIEDLIQSMHIDATLYT ESDVHPSCKVTAMKCFLLELQVISLESG DASIHDTVENLIILANNSLSSNGNVTESG CKECEELEEKNIKEFLQSFVHIVQMFINT SSGGGSGGGGSGGGGSGGGGSGGGSLQI TCPPPMSVEHADIWVKSYSLYSRERYIC NSGFKRKAGTSSLTECVLNKATNVAHW TTPSLKCIRDPALVHQRPAPPSTVTTAGV TPQPESLSPSGKEPAASSPSSNNTAATTA AIVPGSQLMPSKSPSTGTTEISSHESSHGT PSQTTAKNWELTASASHQPPGVYPQGHS DTTVAISTSTVLLCGLSAVSLLACYLKSR QTPPLASVEMEAMEALPVTWGTSSRDE DLENCSHHL*	1072	1101
OT-IL15-072 (IgE leader – IL15 – Linker (SG3-(SG4)3-SG3-SLQ) – IL15Ra – stop)	EF1a	MDWTWILFLVAAATRVHSNWVNVISDL KKIEDLIQSMHIDATLYTESDVHPSCKVT AMKCFLLELQVISLESGDASIHDTVENLII LANNSLSSNGNVTESGCKECEELEEKNIK EFLQSFVHIVQMFINTSSGGGSGGGGSGG GGSGGGGSGGGSLQITCPPPMSVEHADI WVKSYSLYSRERYICNSGFKRKAGTSSL TECVLNKATNVAHWTTPSLKCIRDPALV HQRPAPPSTVTTAGVTPQPESLSPSGKEP AASSPSSNNTAATTAAIVPGSQLMPSKSP STGTTEISSHESSHGTPSQTTAKNWELTA SASHQPPGVYPQGHSDTTVAISTSTVLLC GLSAVSLLACYLKSRQTPPLASVEMEAM EALPVTWGTSSRDEDLENCSHHL*	806	1096
OT-IL15-089 (IgE leader – FLAG – IL15 – Linker (SG3-(SG4)3-SG3-SLQ) – HA Tag – IL15Ra – linker (GSG) – ecDHFR (Amino acid 2-159 of WT, R12Y, Y100I) - stop)	EF1a	MDWTWILFLVAAATRVHSDYKDDDDK NWVNVISDLKKIEDLIQSMHIDATLYTES DVHPSCKVTAMKCFLLELQVISLESGDA SIHDTVENLIILANNSLSSNGNVTESGCK ECEELEEKNIKEFLQSFVHIVQMFINTSSG GGSGGGGSGGGGSGGGGSGGGSLQYPY DVPDYAITCPPPMSVEHADIWVKSYSLY SRERYICNSGFKRKAGTSSLTECVLNKAT NVAHWTTPSLKCIRDPALVHQRPAPPST VTTAGVTPQPESLSPSGKEPAASSPSSNN TAATTAAIVPGSQLMPSKSPSTGTTEISSH ESSHGTPSQTTAKNWELTASASHQPPGV YPQGHSDTTVAISTSTVLLCGLSAVSLLA CYLKSRQTPPLASVEMEAMEALPVTWG TSSRDEDLENCSHHLGSGISLIAALAVDY VIGMENAMPWNLPADLAWFKRNTLNKP VIMGRHTWESIGRPLPGRKNIILSSQPGT DDRVTWVKSVDEAIAACGDVPEIMVIGG GRVIEQFLPKAQKLYLTHIDAEVEGDTH FPDYEPDDWESVFSEFHDADAQNSHSYC FEILERR*	1074	1102
OT-IL15-109 (IgE leader – IL15 – Linker (SG3-(SG4)3-SG3-SLQ) – IL15Ra – BamHI (GS) - stop)	EF1a	MDWTWILFLVAAATRVHSNWVNVISDL KKIEDLIQSMHIDATLYTESDVHPSCKVT AMKCFLLELQVISLESGDASIHDTVENLII LANNSLSSNGNVTESGCKECEELEEKNIK EFLQSFVHIVQMFINTSSGGGSGGGGSGG GGSGGGGSGGGSLQITCPPPMSVEHADI WVKSYSLYSRERYICNSGFKRKAGTSSL TECVLNKATNVAHWTTPSLKCIRDPALV	1070	1095

		HQRPAPPSTVTTAGVTPQPESLSPSGKEP AASSPSSNNTAATTAAIVPGSQLMPSKSP STGTTEISSHESSHGTPSQTTAKNWELTA SASHQPPGVYPQGHSDTTVAISTSTVLLC GLSAVSLLACYLKSRQTPPLASVEMEAM EALPVTWGTSSRDEDLENCSHHLGS*		
OT-IL15-110 (IgE leader – FLAG – IL15 – Linker (SG3-(SG4)3-SG3-SLQ) – HA Tag – IL15Ra – BamHI (GS) - stop)	EF1a	MDWTWILFLVAAATRVHSDYKDDDDK NWVNVISDLKKIEDLIQSMHIDATLYTES DVHPSCKVTAMKCFLLELQVISLESGDA SIHDTVENLIILANNSLSSNGNVTESGCK ECEELEEKNIKEFLQSFVHIVQMFINTSSG GGSGGGGSGGGGSGGGGSGGGSLQYPY DVPDYAITCPPPMSVEHADIWVKSYSLY SRERYICNSGFKRKAGTSSLTECVLNKAT NVAHWTTPSLKCIRDPALVHQRPAPPST VTTAGVTPQPESLSPSGKEPAASSPSSNN TAATTAAIVPGSQLMPSKSPSTGTTEISSH ESSHGTPSQTTAKNWELTASASHQPPGV YPQGHSDTTVAISTSTVLLCGLSAVSLLA CYLKSRQTPPLASVEMEAMEALPVTWG TSSRDEDLENCSHHLGS*	1075	1103
OT-IL15-114 (IgE leader – FLAG – IL15 – Linker (SG3-(SG4)3-SG3-SLQ) – HA Tag – IL15Ra – Linker (GSG) – hDHFR (Amino acid 2-187 of WT; K185E) - stop)	EF1a	MDWTWILFLVAAATRVHSDYKDDDDK NWVNVISDLKKIEDLIQSMHIDATLYTES DVHPSCKVTAMKCFLLELQVISLESGDA SIHDTVENLIILANNSLSSNGNVTESGCK ECEELEEKNIKEFLQSFVHIVQMFINTSSG GGSGGGGSGGGGSGGGGSGGGSLQYPY DVPDYAITCPPPMSVEHADIWVKSYSLY SRERYICNSGFKRKAGTSSLTECVLNKAT NVAHWTTPSLKCIRDPALVHQRPAPPST VTTAGVTPQPESLSPSGKEPAASSPSSNN TAATTAAIVPGSQLMPSKSPSTGTTEISSH ESSHGTPSQTTAKNWELTASASHQPPGV YPQGHSDTTVAISTSTVLLCGLSAVSLLA CYLKSRQTPPLASVEMEAMEALPVTWG TSSRDEDLENCSHHLGSGVGSLNCIVAV SQNMGIGKNGDLPWPPLRNEFRYFQRM TTTSSVEGKQNLVIMGKKTWFSIPEKNR PLKGRINLVLSRELKEPPQGAHFLSRSLD DALKLTEQPELANKVDMVWIVGGSSVY KEAMNHPGHLKLFVTRIMQDFESDTFFP EIDLEKYKLLPEYPGVLSDVQEEKGIKYK FEVYEEND*	1076	1104
OT-IL15-115 (IgE leader – FLAG – IL15 – Linker (SG3-(SG4)3-SG3-SLQ) – HA Tag – IL15Ra – Linker (GSG) – hDHFR (Amino acid 2-187 of WT; E162G, I176F) - stop)	EF1a	MDWTWILFLVAAATRVHSDYKDDDDK NWVNVISDLKKIEDLIQSMHIDATLYTES DVHPSCKVTAMKCFLLELQVISLESGDA SIHDTVENLIILANNSLSSNGNVTESGCK ECEELEEKNIKEFLQSFVHIVQMFINTSSG GGSGGGGSGGGGSGGGGSGGGSLQYPY DVPDYAITCPPPMSVEHADIWVKSYSLY SRERYICNSGFKRKAGTSSLTECVLNKAT NVAHWTTPSLKCIRDPALVHQRPAPPST VTTAGVTPQPESLSPSGKEPAASSPSSNN TAATTAAIVPGSQLMPSKSPSTGTTEISSH ESSHGTPSQTTAKNWELTASASHQPPGV YPQGHSDTTVAISTSTVLLCGLSAVSLLA CYLKSRQTPPLASVEMEAMEALPVTWG TSSRDEDLENCSHHLGSGVGSLNCIVAV SQNMGIGKNGDLPWPPLRNEFRYFQRM TTTSSVEGKQNLVIMGKKTWFSIPEKNR	1077	1105

		PLKGRINLVLSRELKEPPQGAHFLSRSLD DALKLTEQPELANKVDMVWIVGGSSVY KEAMNHPGHLKLFVTRIMQDFESDTFFP EIDLEKYKLLPGYPGVLSDVQEEKGFKY KFEVYEKND*		
OT-IL15-116 (IgE leader – FLAG – IL15 – Linker (SG3-(SG4)3-SG3-SLQ) – HA Tag – IL15Ra – Linker (GSG) – hDHFR (Amino acid 2-187 of WT; H131R, E144G) - stop)	EF1a	MDWTWILFLVAAATRVHSDYKDDDDK NWVNVISDLKKIEDLIQSMHIDATLYTES DVHPSCKVTAMKCFLLELQVISLESGDA SIHDTVENLIILANNSLSSNGNVTESGCK ECEELEEKNIKEFLQSFVHIVQMFINTSSG GGSGGGGSGGGGSGGGGSGGGSLQYPY DVPDYAITCPPPMSVEHADIWVKSYSLY SRERYICNSGFKRKAGTSSLTECVLNKAT NVAHWTTPSLKCIRDPALVHQRPAPPST VTTAGVTPQPESLSPSGKEPAASSPSSNN TAATTAAIVPGSQLMPSKSPSTGTTEISSH ESSHGTPSQTTAKNWELTASASHQPPGV YPQGHSDTTVAISTSTVLLCGLSAVSLLA CYLKSRQTPPLASVEMEAMEALPVTWG TSSRDEDLENCSHHLGSGVGSLNCIVAV SQNMGIGKNGDLPWPPLRNEFRYFQRM TTTSSVEGKQNLVIMGKKTWFSIPEKNR PLKGRINLVLSRELKEPPQGAHFLSRSLD DALKLTEQPELANKVDMVWIVGGSSVY KEAMNHPGRLKLFVTRIMQDFGSDTFFP EIDLEKYKLLPEYPGVLSDVQEEKGIKYK FEVYEKND*	1078	1106
OT-IL15-117 (IgE leader – FLAG – IL15 – Linker (SG3-(SG4)3-SG3-SLQ) – HA Tag – IL15Ra – Linker (GSG) – hDHFR(Amino acid 2-187 of WT; I17V) - stop)	EF1a	MDWTWILFLVAAATRVHSDYKDDDDK NWVNVISDLKKIEDLIQSMHIDATLYTES DVHPSCKVTAMKCFLLELQVISLESGDA SIHDTVENLIILANNSLSSNGNVTESGCK ECEELEEKNIKEFLQSFVHIVQMFINTSSG GGSGGGGSGGGGSGGGGSGGGSLQYPY DVPDYAITCPPPMSVEHADIWVKSYSLY SRERYICNSGFKRKAGTSSLTECVLNKAT NVAHWTTPSLKCIRDPALVHQRPAPPST VTTAGVTPQPESLSPSGKEPAASSPSSNN TAATTAAIVPGSQLMPSKSPSTGTTEISSH ESSHGTPSQTTAKNWELTASASHQPPGV YPQGHSDTTVAISTSTVLLCGLSAVSLLA CYLKSRQTPPLASVEMEAMEALPVTWG TSSRDEDLENCSHHLGSGVGSLNCIVAV SQNMGVGKNGDLPWPPLRNEFRYFQRM TTTSSVEGKQNLVIMGKKTWFSIPEKNR PLKGRINLVLSRELKEPPQGAHFLSRSLD DALKLTEQPELANKVDMVWIVGGSSVY KEAMNHPGHLKLFVTRIMQDFESDTFFP EIDLEKYKLLPEYPGVLSDVQEEKGIKYK FEVYEKND*	1079	1107
OT-IL15-118 (IgE leader – FLAG – IL15 – Linker (SG3-(SG4)3-SG3-SLQ) – HA Tag – IL15Ra – Linker (GSG) – hDHFR (Amino acid 2-187 of	EF1a	MDWTWILFLVAAATRVHSDYKDDDDK NWVNVISDLKKIEDLIQSMHIDATLYTES DVHPSCKVTAMKCFLLELQVISLESGDA SIHDTVENLIILANNSLSSNGNVTESGCK ECEELEEKNIKEFLQSFVHIVQMFINTSSG GGSGGGGSGGGGSGGGGSGGGSLQYPY DVPDYAITCPPPMSVEHADIWVKSYSLY SRERYICNSGFKRKAGTSSLTECVLNKAT NVAHWTTPSLKCIRDPALVHQRPAPPST VTTAGVTPQPESLSPSGKEPAASSPSSNN TAATTAAIVPGSQLMPSKSPSTGTTEISSH	1080	1108

WT, N127Y) - stop)		ESSHGTPSQTTAKNWELTASASHQPPGV YPQGHSDTTVAISTSTVLLCGLSAVSLLA CYLKSRQTPPLASVEMEAMEALPVTWG TSSRDEDLENCSHHLGSGVGSLNCIVAV SQNMGIGKNGDLPWPPLRNEFRYFQRM TTTSSVEGKQNLVIMGKKTWFSIPEKNR PLKGRINLVLSRELKEPPQGAHFLSRSLD DALKLTEQPELANKVDMVWIVGGSSVY KEAMYHPGHLKLFVTRIMQDFESDTFFP EIDLEKYKLLPEYPGVLSDVQEEKGIKYK FEVYEKND*		
OT-IL15-119 (IgE leader – FLAG – IL15 – Linker (SG3-(SG4)3-SG3-SLQ) – HA Tag – IL15Ra – Linker (GSG) – hDHFR (Amino acid 2-187 of WT, I17V, Y122I) - stop)	EF1a	MDWTWILFLVAAATRVHSDYKDDDDK NWVNVISDLKKIEDLIQSMHIDATLYTES DVHPSCKVTAMKCFLLELQVISLESGDA SIHDTVENLIILANNSLSSNGNVTESGCK ECEELEEKNIKEFLQSFVHIVQMFINTSSG GGSGGGGSGGGGSGGGGSGGGSLQYPY DVPDYAITCPPPMSVEHADIWVKSYSLY SRERYICNSGFKRKAGTSSLTECVLNKAT NVAHWTTPSLKCIRDPALVHQRPAPPST VTTAGVTPQPESLSPSGKEPAASSPSSNN TAATTAAIVPGSQLMPSKSPSTGTTEISSH ESSHGTPSQTTAKNWELTASASHQPPGV YPQGHSDTTVAISTSTVLLCGLSAVSLLA CYLKSRQTPPLASVEMEAMEALPVTWG TSSRDEDLENCSHHLGSGVGSLNCIVAV SQNMGVGKNGDLPWPPLRNEFRYFQRM TTTSSVEGKQNLVIMGKKTWFSIPEKNR PLKGRINLVLSRELKEPPQGAHFLSRSLD DALKLTEQPELANKVDMVWIVGGSSVIK EAMNHPGHLKLFVTRIMQDFESDTFFPEI DLEKYKLLPEYPGVLSDVQEEKGIKYKF EVYEKND*	1081	1109
OT-IL15-128 (IgE leader – IL15 – Linker (SG3-(SG4)3-SG3-SLQ) – IL15Ra– Spacer – Flagx3 - Spacer - BamHI (GS) – P2A cleavable peptide – mCherry (M1L) - stop)	EF1a	MDWTWILFLVAAATRVHSNWVNVISDL KKIEDLIQSMHIDATLYTESDVHPSCKVT AMKCFLLELQVISLESGDASIHDTVENLII LANNSLSSNGNVTESGCKECEELEEKNIK EFLQSFVHIVQMFINTSSGGGSGGGGSGG GGSGGGGSGGGSLQITCPPPMSVEHADI WVKSYSLYSRERYICNSGFKRKAGTSSL TECVLNKATNVAHWTTPSLKCIRDPALV HQRPAPPSTVTTAGVTPQPESLSPSGKEP AASSPSSNNTAATTAAIVPGSQLMPSKSP STGTTEISSHESSHGTPSQTTAKNWELTA SASHQPPGVYPQGHSDTTVAISTSTVLLC GLSAVSLLACYLKSRQTPPLASVEMEAM EALPVTWGTSSRDEDLENCSHHLSRMD YKDDDDKDYKDDDDKDYKDDDDKSRG SGATNFSLLKQAGDVEENPGPLSKGEED NMAIIKEFMRFKVHMEGSVNGHEFEIEG EGEGRPYEGTQTAKLKVTKGGPLPFAW DILSPQFMYGSKAYVKHPADIPDYLKLSF PEGFKWERVMNFEDGGVVTVTQDSSLQ DGEFIYKVKLRGTNFPSDGPVMQKKTM GWEASSERMYPEDGALKGEIKQRLKLK DGGHYDAEVKTTYKAKKPVQLPGAYN VNIKLDITSHNEDYTIVEQYERAEGRHST GGMDELYK*	1082	1110
OT-IL15-129 (IgE leader –	EF1a	MDWTWILFLVAAATRVHSNWVNVISDL KKIEDLIQSMHIDATLYTESDVHPSCKVT	1083	1111

IL15 – Linker (SG3-(SG4)3-SG3-SLQ) – IL15Ra–BamHI (GS) – P2A cleavable peptide – mCherry (M1L)-stop)		AMKCFLLELQVISLESGDASIHDTVENLII LANNSLSSNGNVTESGCKECEELEEKNIK EFLQSFVHIVQMFINTSSGGGSGGGGSGG GGSGGGGSGGGSLQITCPPPMSVEHADI WVKSYSLYSRERYICNSGFKRKAGTSSL TECVLNKATNVAHWTTPSLKCIRDPALV HQRPAPPSTVTTAGVTPQPESLSPSGKEP AASSPSSNNTAATTAAIVPGSQLMPSKSP STGTTEISSHESSHGTPSQTTAKNWELTA SASHQPPGVYPQGHSDTTVAISTSTVLLC GLSAVSLLACYLKSRQTPPLASVEMEAM EALPVTWGTSSRDEDLENCSHHLGSGAT NFSLLKQAGDVEENPGPLSKGEEDNMAI IKEFMRFKVHMEGSVNGHEFEIEGEGEG RPYEGTQTAKLKVTKGGPLPFAWDILSP QFMYGSKAYVKHPADIPDYLKLSFPEGF KWERVMNFEDGGVVTVTQDSSLQDGEF IYKVKLRGTNFPSDGPVMQKKTMGWEA SSERMYPEDGALKGEIKQRLKLKDGGH YDAEVKTTYKAKKPVQLPGAYNVNIKL DITSHNEDYTIVEQYERAEGRHSTGGMD ELYK*		

Table 13c: IL15/IL15Ra constructs

Construct Description	Sequence Description	Promoter	Amino acid sequences	Amino Acid SEQ ID NO	Nucleic Acid SEQ ID NO/ Sequence
OT-IL15-122 (IgE leader – IL15 – linker (GS) – hDHFR (Amino acid 2-187 of WT, Y122I) – stop – spacer – IRES – spacer – mCherry – stop)	Full construct	EF1a	MDWTWILFLVAAATRVHSN WVNVISDLKKIEDLIQSMHID ATLYTESDVHPSCKVTAMKC FLLELQVISLESGDASIHDTVE NLIILANNSLSSNGNVTESGC KECEELEEKNIKEFLQSFVHIV QMFINTSGSVGSLNCIVAVSQ NMGIGKNGDLPWPPLRNEFR YFQRMTTTSSVEGKQNLVIM GKKTWFSIPEKNRPLKGRINL VLSRELKEPPQGAHFLSRSLD DALKLTEQPELANKVDMVWI VGGSSVIKEAMNHPGHLKLF VTRIMQDFESDTFFPEIDLEKY KLLPEYPGVLSDVQEEKGIKY KFEVYEKND*SR*YDSLEIPPL SLPPP*RYWPKPLGIRPVCVC LYVIFHHIAVFWQCEGPETWP CLLDEHS*GSFPSRQRNARSV ECREGSSSSGSFLKTNNVCSD PLQAAEPPTWRQVPLRPKAT CIRYTCKGGTTPVPRCELDSC GKSQMALLKRIQQGAEGCPE GTPLYGI*SGASVHMLYMCL VEVKKTSRPPEPRGRGFPLKN TMIIWPQP**ARARRITWPSSR SSCASRCTWRAP*TATSSRSR ARARAAPTRAPRPPS*R*PRV APCPSPGTSCPLSSCTAPRPT*	1114, 1126-1140	1120

			STPPTSPTT*SCPSPRASSGSA* *TSRTAAW*P*PRTPPCRTASS STR*SCAAPTSPPTAP*CRRRP WAGRPPPSGCTPRTAP*RARS SRG*S*RTAATTTLRSRPPTRP RSPCSCPAPTTSTSSWTSPPTT RTTPSWNSTNAPRAATPPAA WTSCTS		
	IgE leader – IL15 – linker (GS) – hDHFR (Amino acid 2-187 of WT, Y122I) – stop	-	MDWTWILFLVAAATRVHSN WVNVISDLKKIEDLIQSMHID ATLYTESDVHPSCKVTAMKC FLLELQVISLESGDASIHDTVE NLIILANNSLSSNGNVTESGC KECEELEEKNIKEFLQSFVHIV QMFINTSGSVGSLNCIVAVSQ NMGIGKNGDLPWPPLRNEFR YFQRMTTTSSVEGKQNLVIM GKKTWFSIPEKNRPLKGRINL VLSRELKEPPQGAHFLSRSLD DALKLTEQPELANKVDMVWI VGGSSVIKEAMNHPGHLKLF VTRIMQDFESDTFFPEIDLEKY KLLPEYPGVLSDVQEEKGIKY KFEVYEKND*	1115	1121
	mCherry - stop	-	MSKGEEDNMAIIKEFMRFKV HMEGSVNGHEFEIEGEGEGRP YEGTQTAKLKVTKGGPLPFA WDILSPQFMYGSKAYVKHPA DIPDYLKLSFPEGFKWERVM NFEDGGVVTVTQDSSLQDGE FIYKVKLRGTNFPSDGPVMQ KKTMGWEASSERMYPEDGA LKGEIKQRLKLKDGGHYDAE VKTTYKAKKPVQLPGAYNV NIKLDITSHNEDYTIVEQYER AEGRHSTGGMDELYK*	1116	1122
OT-IL15-123 and OT-IL15-127(IgE leader – IL15 – BamHI (GS) – stop – spacer – IRES – spacer – mCherry - stop)	Full construct	EF1a	MDWTWILFLVAAATRVHSN WVNVISDLKKIEDLIQSMHID ATLYTESDVHPSCKVTAMKC FLLELQVISLESGDASIHDTVE NLIILANNSLSSNGNVTESGC KECEELEEKNIKEFLQSFVHIV QMFINTSGS*NLDNTTH*RSR PSPSPPPNVTGRSRLE*GRCAF VYMLFSTILPSFGNVRARKPG PVFLTSIPRGLSPLAKGMQGL LNVVKEAVPLEAS*RQTTSVA TLCRQRNPPPGDRCLCGQKP RV*DTPAKAAQPQCHVVSWI VVERVKWLSSSVFNKGLKDA QKVPHCMGSDLGPRCTCFTC V*SRLKKRLGPPNHGDVVFL* KTR**YGHNHDEQGRGG*HG HHQGVHALQGAHGGLRERP RVRDRGRGRGPPLRGHPDRQ AEGDQGWPPALRLGHPVPSV HVRLQGLREAPRRHPRLLEA VLPRGLQVGARDELRGRRRG DRDPGLLPAGRRVHLQGEAA RHQLPLRRPRNAEEDHGLGG	1117, 1141-1149	1123

			LLRADVPRGRRPEGRDQAEA EAEGRRPLRR*GQDHLQGQE ARAAARRLQRQHQVGHHLP QRGLHHRGTVRTRRGPPLHR RHGRAVQV		
	IgE leader – IL15 – BamHI (GS) – stop	-	MDWTWILFLVAAATRVHSN WVNVISDLKKIEDLIQSMHID ATLYTESDVHPSCKVTAMKC FLLELQVISLESGDASIHDTVE NLIILANNSLSSNGNVTESGC KECEELEEKNIKEFLQSFVHIV QMFINTSGS*	1118	1124
	mCherry - stop	-	MSKGEEDNMAIIKEFMRFKV HMEGSVNGHEFEIEGEGEGRP YEGTQTAKLKVTKGGPLPFA WDILSPQFMYGSKAYVKHPA DIPDYLKLSFPEGFKWERVM NFEDGGVVTVTQDSSLQDGE FIYKVKLRGTNFPSDGPVMQ KKTMGWEASSERMYPEDGA LKGEIKQRLKLKDGGHYDAE VKTTYKAKKPVQLPGAYNV NIKLDITSHNEDYTIVEQYER AEGRHSTGGMDELYK*	1119	1125

[00255] In one embodiment, the payload of the present invention may comprise IL18. IL18 is a proinflammatory and immune regulatory cytokine that promotes IFN-γ production by T and NK cells. IL18 belongs to the IL1 family. Secreted IL18 binds to a heterodimer receptor complex, consisting of IL18Rα and β-chains and initiates signal transduction. IL18 acts in concert with other cytokines to modulate immune system functions, including induction of IFN-γ production, Th1 responses, and NK cell activation in response to pathogen products. IL18 showed anti-cancer effects in several tumors. Administration of recombinant IL18 protein or IL18 transgene induces melanoma or sarcoma regression through the activation of $CD4^+$ T and/or NK cell-mediated responses (reviewed by Srivastava et al., *Curr. Med. Chem.*, 2010, 17: 3353–3357). The combination of IL18 with other cytokines, such as IL12 or co-stimulatory molecules (e.g., CD80) increases IL18 anti-tumor effects. For example, IL18 and IL12A/B or CD80 genes have been integrated successfully in the genome of oncolytic viruses, with the aim to trigger synergistically T cell-mediated anti-tumor immune responses (Choi et al., *Gene Ther.*, 2011, 18: 898-909). IL2/IL18 fusion proteins also display enhanced anti-tumor properties relative to either cytokine alone and low toxicity in preclinical models (Acres et al., *Cancer Res.*, 2005, 65:9536–9546).

[00256] IL18 alone, or in combination of IL12 and IL15, activates NK cells. Preclinical studies have demonstrated that adoptively transferred IL12, IL15 and IL18 pre-activated NK cells display enhanced effector function against established tumors *in vivo* (Ni et al., *J Exp Med.* 2012, 209: 2351–2365; and Romee et al., *Blood.* 2012,120:4751–4760). Human IL12/IL15/IL18

activated NK cells also display memory-like features and secrete more IFN-γ in response to cytokines (e.g., low concentration of IL2). In one embodiment, the effector module of the present invention may be a DD-IL18 fusion polypeptide.

[00257] In one embodiment, the payload of the present invention may comprise IL21. IL21 is another pleiotropic type I cytokine that is produced mainly by T cells and natural killer T (NKT) cells. IL21 has diverse effects on a variety of cell types including but not limited to $CD4^+$ and $CD8^+$ T cells, B cells, macrophages, monocytes, and dendritic cells (DCs). The functional receptor for IL21 is composed of IL21 receptor (IL21R) and the common cytokine receptor gamma chain, which is also a subunit of the receptors for IL2, IL4, IL7, IL9 and IL15. Studies provide compelling evidence that IL21 is a promising immunotherapeutic agent for cancer immunotherapy. IL21 promotes maturation, enhances cytotoxicity, and induces production of IFN-γ and perforin by NK cells. These effector functions inhibit the growth of B16 melanoma (Kasaian et al., *Immunity.* 2002, 16(4):559–569; and Brady et al., *J Immunol.*2004, 172(4):2048–2058). IL21 together with IL15 expands antigen-specific $CD8^+$ T-cell numbers and their effector function, resulting in tumor regression (Zeng et al., *J Exp Med.*2005, 201(1):139–148). IL21 may also be used to rejuvenate multiple immune effector cells in the tumor microenvironment. IL21 may also directly induce apoptosis in certain types of lymphoma such as diffuse large B-cell lymphoma, mantle cell lymphoma, and chronic lymphocytic leukemia cells, via activation of STAT3 or STAT1 signal pathway. IL21, alone or in combination with anti-CD20 mAb (rituximab) can activate NK cell-dependent cytotoxic effects. Interestingly, discovery of the immunosuppressive actions of IL21 suggests that this cytokine is a "double-edged sword"- IL21 stimulation may lead to either the induction or suppression of immune responses. Both stimulatory and suppressive effects of IL21 must be considered when using IL21-related immunotherapeutic agents. The level of IL21 needs to be tightly controlled by regulatory elements. In one aspect, the effector module of the present invention may be a DD-IL21 fusion polypeptide.

[00258] In some embodiments, payloads of the present invention may comprise type I interferons. Type I interferons (IFNs-I) are soluble proteins important for fighting viral infection in humans. IFNs-I include IFN-alpha subtypes (IFN- α1, IFN- α1b, IFN- α1c), IFN-beta, IFN-delta subtypes (IFN-delta 1, IFN-delta 2, IFN-delta 8), IFN-gamma, IFN-kappa, and IFN-epsilon, IFN-lambda, IFN-omega, IFN-tau and IFN-zeta. IFN-α and IFN-β are the main IFN-I subtypes in immune responses. All subtypes of IFN-I signal through a unique heterodimeric receptor, interferon alpha receptor (IFNAR), composed of 2 subunits, IFNAR1 and IFNAR2. IFNR activation regulates the host response to viral infections and in adaptive immunity. Several

signaling cascades can be activated by IFNR, including the Janus activated kinase–signal transducer and activation of transcription (JAK-STAT) pathway, the mitogen activated protein kinase (MAPK) pathway, the phosphoinositide 3-kinase (PI3K) pathway, the v-crk sarcoma virus CT10 oncogene homolog (avian)-like (CRKL) pathway, and NF-κB cascade. It has long been established that type I IFNs directly inhibit the proliferation of tumor cells and virus-infected cells, and increase MHC class I expression, enhancing antigen recognition. IFNs-I have also proven to be involved in immune system regulation. IFNs can either directly, through interferon receptor (IFNR), or indirectly by the induction of chemokines and cytokines, regulate the immune system. Type I IFNs enhance NK cell functions and promote survival of NK cells. Type I IFNs also affect monocytes, supporting the differentiation of monocytes into DC with high capacity for antigen presentation, and stimulate macrophage function and differentiation. Several studies also demonstrate that IFNs-I promote $CD8^+$ T cell survival and functions. In some instances, it may be desirable to tune the expression of Type I IFNs using biocircuits of the present invention to avoid immunosuppression caused by long-term treatment with IFNs.

[00259] New anticancer immunotherapies are being developed that use recombinant type I IFN proteins, type I IFN transgene, type I IFN-encoding vectors and type I IFN-expressing cells. For example, IFN-α has received approval for treatment of several neoplastic diseases, such as melanoma, RCC and multiple myeloma. Though type I IFNs are powerful tools to directly and indirectly modulate the functions of the immune system, side effects of systemic long-term treatments and lack of sufficiently high efficacy have dampened the interest of IFN-α for clinical use in oncology. It is believed that if IFN levels are tightly regulated at the malignant tissues, type I IFNs are likely more efficacious. Approaches for intermittent delivery are proposed according to the observation that intermittency at an optimized pace may help to avoid signaling desensitizing mechanisms (negative feedback mechanisms) induced by IFNs-I (i.e., because of SOCS1 induction) in the responding immune cells. In accordance with the present invention, the effector module may comprise a DD-IFN fusion polypeptide. The DD and its ligand control the expression of IFN to induce an antiviral and antitumor immune responses and in the meantime, to minimize the side effects caused by long-term exposure of IFN.

[00260] In some embodiments, payloads of the present invention may comprise members of tumor necrosis factor (TNF) superfamily. The term "TNF superfamily" as used herein refers to a group of cytokines that can induce apoptosis. Members of TNF family include TNF-alpha, TNF-beta (also known as lymphotoxin-alpha (LT-α)), lymphotoxin-beta (LT-β), CD40L(CD154), CD27L (CD70), CD30L(CD153), FASL(CD178), 4-1BBL (CD137L), OX40L, TRAIL (TNF-related apoptosis inducing ligand), APRIL (a proliferation-inducing ligand), TWEAK,

TRANCE, TALL-1, GITRL, LIGHT and TNFSF1 to TNFSF20 (TNF ligand superfamily member 1 to 20). In one embodiment, the payload of the invention may be TNF-alpha. TNF-alpha can cause cytolysis of tumor cells, and induce cell proliferation differentiation as well. In one aspect, the effector module of the present invention may comprise a DD-TNF alpha fusion polypeptide.

[00261] In some embodiments, payloads of the present invention may comprise inhibitory molecules that block inhibitory cytokines. The inhibitors may be blocking antibodies specific to an inhibitory cytokine, and antagonists against an inhibitory cytokine, or the like.

[00262] In some aspects, payloads of the present invention may comprise an inhibitor of a secondary cytokine IL35. IL35 belongs to the interleukin-12 (IL12) cytokine family, and is a heterodimer composed of the IL27 β chain Ebi3 and the IL12 α chain p35. Secretion of bioactive IL35 has been described only in forkhead box protein 3 $(Foxp3)^+$ regulatory T cells (Tregs) (resting and activated Tregs). Unlike other membranes in the family, IL35 appears to function solely in an anti-inflammatory fashion by inhibiting effector T cell proliferation and perhaps other parameters (Collison et al., *Nature*, 2007, 450(7169): 566–569).

[00263] In some embodiments, payloads of the present invention may comprise inhibitors that block the transforming growth factor beta (TGF-β) subtypes (TGF-β1, TGF-β2 and TGF-β3). TGF-β is secreted by many cell types, including macrophages and is often complexed with two proteins LTBP and LAP. Serum proteinases such as plasmin catalyze the release of active TGF-β from the complex from the activated macrophages. It has been shown that an increase in expression of TGF-β correlates with the malignancy of many cancers. The immunosuppressive activity of TGF-β in the tumor microenvironment contributes to oncogenesis.

[00264] In some embodiments, payloads of the present invention may comprise inhibitors of IDO enzyme.

[00265] In some embodiments, payloads of the present invention may comprise chemokines and chemokine receptors. Chemokines are a family of secreted small cytokines, or signaling proteins that can induce directed chemotaxis in nearby responsive cells. The chemokine may be a SCY (small cytokine) selected from the group consisting of SCYA1-28 (CCL1-28), SCYB1-16 (CXCL1-16), SCYC1-2 (XCL1-2), SCYD-1 and SCYE-1; or a C chemokine selected from XCL1 and XCL2; or a CC chemokine selected from CCL1, CCL2, CCL3, CCL4, CCL5, CCL6, CCL7, CCL8, CCL9, CCL10, CCL11, CCL12, CCL13, CCL14, CCL15, CCL16, CCL17, CCL18, CCL19, CCL20, CCL21, CCL22, CCL23, CCL24, CCL25, CCL26, CCL27 and CCL28; or a CXC chemokine selected from CXCL1, CXCL2, CXCL3, CXCL4, CXCL5, CXCL6, CXCL7, CXCL8, CXCL9, CXCL10, CXCL11, CXCL12, CXCL13, CXCL14,

CXCL15, CXCL16 and CXCL17; or a CX3C chemokine CX3CL1. In some aspects, the chemokine receptor may be a receptor for the C chemokines including XCR1; or a receptor for the CC chemokines including CCR1, CCR2, CCR3, CCR4, CCR5, CCR6, CCR7, CCR8, CCR9 and CCR10; or a receptor for the CXC chemokines including CXCR1, CXCR2, CXCR3, CXCR4 and CXCR5; or a CX3C chemokine receptor CX3CR1.

[00266] In some embodiments, payloads of the present invention may comprise other immunomodulators that play a critical role in immunotherapy, such as GM-CSF (Granulocyte-macrophage colony stimulating factor), erythropoietin (EPO), MIP3a, monocyte chemotactic protein (MCP)-1, intracellular adhesion molecule (ICAM), macrophage colony stimulating factor (M-CSF), Interleukin-1 receptor activating kinase (iRAK-1), lactotransferrin, and granulocyte colony stimulating factor (G-CSF).

[00267] In some embodiments, the payload of the present invention may comprise Amphiregulin. Amphiregulin (AREG) is an EGF-like growth factor which binds to the EGFR receptor and enhances CD4+ regulatory T cells (Tregs) function. AREG promotes immune suppression in the tumor environment. Thus, in some embodiment, the payloads of the present invention may comprise Amhiregulin to dampen immune response during immunotherapy.

[00268] In some embodiments, payloads of the present invention may comprise fusion proteins wherein a cytokine, chemokine and/or other soluble factor may be fused to other biological molecules such as antibodies and or ligands for a receptor. Such fusion molecules may increase the half-life of the cytokines, reduce systemic toxicity, and increase local concentration of the cytokines at the tumor site. Fusion proteins containing two or more cytokines, chemokines and or other soluble factors may be utilized to obtain synergistic therapeutic benefits. In one embodiment, payload may be a GM-CSF/IL2 fusion protein.

3. Additional effector module features

[00269] 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

[00270] 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.

[00271] 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).

[00272] 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.

[00273] 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 comprising amino acid of SEQ ID NO: 783, encoded by the nucleotide of SEQ ID NO: 788-791 and/or p40 signal sequence comprising the amino acid sequence of SEQ ID NO: 719, encoded by the nucleotide of SEQ ID NO: 736-744.

[00274] 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 comprising amino acid SEQ ID NO: 801 and nucleotide sequence of SEQ ID NO: 810, 930, or 931, CD8a signal sequence (also referred to as CD8a leader) comprising amino acid SEQ ID NO: 628 and nucleotide sequence of SEQ ID NO:

671-675, or IL15Ra signal sequence (also referred to as IL15Ra leader) comprising amino acid SEQ ID NO: 932 and nucleotide sequence of SEQ ID NO: 933.

[00275] 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 NO.: 20070141666; and International patent application publication NO.: WO1993018181; the contents of each of which are incorporated herein by reference in their entirety.

[00276] 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. NO.: 8,093,016 to Cervin and Kim), bacterial lipoprotein signal sequences (e.g., PCT application publication NO.: WO199109952 to Lau and Rioux), *E.coli* enterotoxin II signal peptides (e.g., U.S. Pat. NO.: 6,605,697 to Kwon et al.), *E.coli* secretion signal sequence (e.g., U.S. patent publication NO.: US2016090404 to Malley et al.), a lipase signal sequence from a methylotrophic yeast (e.g., U.S. Pat. NO.: 8,975,041), and signal peptides for DNases derived from *Coryneform bacteria* (e.g., U.S. Pat. NO.: 4,965,197); the contents of each of which are incorporated herein by reference in their entirety.

[00277] 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. NO.: 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. NO.: 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).

[00278] In some embodiments, signal sequences of the present invention, include without limitation, any of those taught in Table 6 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 each of which are incorporated herein by reference in their entirety.

Cleavage sites

[00279] 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.

[00280] 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.

[00281] In one embodiment, the cleavage site is a furin cleavage site comprising the amino acid sequence SARNRQKRS (SEQ ID NO: 721), encoded by nucleotide sequence of SEQ ID NO: 750; or a revised furin cleavage site comprising the amino acid sequence ARNRQKRS (SEQ ID NO: 722), encoded by nucleotide sequence of SEQ ID NO: 751; or a modified furin site comprising the amino acid sequence ESRRVRRNKRSK (SEQ ID NO: 630), encoded by nucleotide sequence of SEQ ID NO: 681-683.

[00282] 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 each of which are incorporated herein by reference in their entirety.

Protein tags

[00283] 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.

[00284] 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 NO.: WO2013091661; the contents of each of which are incorporated herein by reference in their entirety.

[00285] 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.

[00286] 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 each of which are incorporated herein by reference in their entirety.

Targeting peptides

[00287] 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.

[00288] 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.

[00289] 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.

[00290] 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.

[00291] 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, 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.

Linkers

[00292] 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.

[00293] 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).

[00294] 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: 822; encoded by the nucleotide sequence SEQ ID NO: 823), GGSGG (SEQ ID NO: 629; encoded by any of the nucleotide sequences SEQ ID NO: 676-680), GGSGGG (SEQ ID NO: 824; encoded by any of the nucleotide sequences SEQ ID NO: 825-826), SGGGS (SEQ ID NO: 827; encoded by the nucleotide sequence SEQ ID NO: 828, 844, 909), GGSGGGSGG (SEQ ID NO: 829; encoded by the nucleotide sequence SEQ ID NO: 830), GGGGG (SEQ ID NO: 831), GGGGS (SEQ ID NO: 832) or (GGGGS)n (n=1 (SEQ ID NO: 832), 2 (SEQ ID NO: 833), 3 (SEQ ID NO: 720, encoded by the nucleotide sequence SEQ ID NO: 910-915), 4 (SEQ ID NO: 834), 5 (SEQ ID NO: 835), or 6 (SEQ ID NO: 836)), SSSSG (SEQ ID NO: 837) or (SSSSG)n (n=1 (SEQ ID NO: 837), 2 (SEQ ID NO: 838), 3 (SEQ ID NO: 839), 4 (SEQ ID NO: 840), 5 (SEQ ID NO: 841), or 6 (SEQ ID NO: 842)), SGGGSGGGGSGGGGSGGGGSGGGSLQ (SEQ ID NO: 802; encoded by the nucleotide sequence SEQ ID NO: 811, 916-920, 1002), EFSTEF (SEQ ID NO: 784; encoded by any of the nucleotide sequences SEQ ID NO: 792-793), GKSSGSGSESKS (SEQ ID NO: 845), GGSTSGSGKSSEGKG (SEQ ID NO: 846), GSTSGSGKSSSEGSGSTKG (SEQ ID NO: 847), GSTSGSGKPGSGEGSTKG (SEQ ID NO: 848), VDYPYDVPDYALD (SEQ ID NO: 849; encoded by nucleotide sequence SEQ ID NO: 850), EGKSSGSGSESKEF (SEQ ID NO: 851), SGGGSGGGGSGGGGSGGGGSGGGGSGGGGSGGGS (SEQ ID NO: 921; encoded by SEQ ID NO: 923 SGGGSGGGGSGGGGSGGGGS (SEQ ID NO: 922; encoded by SEQ ID NO: 924), GS (encoded by GGTTCC), SG (encoded by AGCGGC), GSG (encoded by GGATCCGGA or GGATCCGGT), or MLLLVTSLLLCELPHPAFLLIP (SEQ ID NO: 1031; encoded by SEQ ID NO: 1032).

[00295] 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: 1233), (G)5 (SEQ ID NO: 831), (G)8) (SEQ ID NO: 1234), 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.

[00296] 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.

[00297] 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.

[00298] 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. 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: 1235). 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.

[00299] As a non-limiting example, the P2A cleavable peptide may be GATNFSLLKQAGDVEENPGP (SEQ ID NO: 925; encoded by SEQ ID NO: 926).

[00300] 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.

[00301] 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.

[00302] 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 each of which are incorporated herein by reference in their entirety.

[00303] 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: 927), TATGGCCACAACCATG (SEQ ID NO: 928), AATCTAGATAATACGACTCACTAGAGATCC (SEQ ID NO: 929), GCTTGCCACAACCCACAAGGAGACGACCTTCC (SEQ ID NO: 1000), TCGCGAATG, or TCGCGA.

[00304] 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

[00305] 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.

[00306] 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 each of which are incorporated herein by reference in their entirety.

[00307] 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.

Polynucleotides

[00308] 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.

[00309] 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*.

[00310] 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.

[00311] 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.

[00312] 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.

[00313] 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.

[00314] 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).

[00315] 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.

[00316] 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.)

[00317] 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.

[00318] 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.

[00319] 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.

[00320] 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.

[00321] 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%).

[00322] 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.

[00323] 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 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.

[00324] 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).

[00325] 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.

[00326] 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.

[00327] 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).

[00328] 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.

[00329] 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.

[00330] 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.

[00331] 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.

[00332] In some embodiments, the compositions of the polynucleotides of the invention may 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 are performed prior to Gibson assembly which 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 a method 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).

[00333] 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 NO: 684-686, 688-691, 987-989, 994, 1013, and/or 1028, an ecDHFR mutant encoded by nucleotide sequence of SEQ ID NO: 687, 692, 772, 798, 814-815, 988, 991, and/or 993, hDHFR mutant encoded by nucleotide sequence of SEQ ID NO: 693-700, 773, 852-857 and/or 934-980, and/or 995-998.

[00334] In some embodiments, the polynucleotides of the invention may encode effector modules comprising the CD19 CAR as the payload comprising the nucleotide sequence of SEQ ID NO: 701-715 and/or 1019-1042, or IL12 as the payload comprising the nucleotide sequence of SEQ ID NO. 774-782, or IL15 as the payload comprising the nucleotide sequence of SEQ ID NO: 749, 799-800, and/or 1055-1056, or IL15/IL15Ra fusion polypeptide as the payload comprising the nucleotide sequence of SEQ ID NO: 816-821, 1086-1089, 1091-1095, 1098-1111, 1120, and/or 1123.

Cells

[00335] 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).

[00336] In some embodiments, cells of the invention may be autologous, allogeneic, syngeneic, or xenogeneic in relation to a particular individual subject.

[00337] 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.

[00338] In some embodiments, cells of the invention may include expansion factors as payload to trigger proliferation and expansion of the cells. Exemplary payloads 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.

[00339] 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.

[00340] In some embodiments, immune cells of the invention may be T cells modified to express an antigen-specific T cell receptor (TCR), or an antigen specific chimeric antigen receptor (CAR) taught herein (known as CAR T cells). Accordingly, at least one polynucleotide

encoding a CAR system (or a TCR) described herein, or a vector comprising the polynucleotide is introduced into a T cell. The T cell expressing the CAR or TCR binds to a specific antigen via the extracellular targeting moiety of the CAR or TCR, thereby a signal via the intracellular signaling domain (s) is transmitted into the T cell, and as a result, the T cell is activated. The activated CAR T cell changes its behavior including release of a cytotoxic cytokine (e.g., a tumor necrosis factor, and lymphotoxin, etc.), improvement of a cell proliferation rate, change in a cell surface molecule, or the like. Such changes cause destruction of a target cell expressing the antigen recognized by the CAR or TCR. In addition, release of a cytokine or change in a cell surface molecule stimulates other immune cells, for example, a B cell, a dendritic cell, a NK cell, and a macrophage.

[00341] The CAR introduced into a T cell may be a first-generation CAR including only the intracellular signaling domain from TCR CD3zeta, or a second-generation CAR including the intracellular signaling domain from TCR CD3zeta and a costimulatory signaling domain, or a third-generation CAR including the intracellular signaling domain from TCR CD3zeta and two or more costimulatory signaling domains, or a split CAR system, or an on/off switch CAR system. In one example, the expression of the CAR or TCR is controlled by a destabilizing domain (DD) such as a hDHFR mutant, in the effector module of the invention. The presence or absence of hDHFR binding ligand such as TMP is used to tune the CAR or TCR expression in transduced T cells or NK cells.

[00342] In some embodiments, CAR T cells of the invention may be further modified to express another one, two, three or more immunotherapeutic agents. The immunotherapeutic agents may be another CAR or TCR specific to a different target molecule; a cytokine such as IL2, IL12, IL15 and IL18, or a cytokine receptor such as IL15Ra; a chimeric switch receptor that converts an inhibitory signal to a stimulatory signal; a homing receptor that guides adoptively transferred cells to a target site such as the tumor tissue; an agent that optimizes the metabolism of the immune cell; 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.

[00343] In one embodiment, the CAR T cell (including TCR T cell) of the invention may be an "armed" CAR T cell which is transformed with an effector module comprising a CAR and an effector module comprising a cytokine. The inducible or constitutively secrete active cytokines further armor CAR T cells to improve efficacy and persistence. In this context, such CAR T cell is also referred to as "armored CAR T cell". The "armor" molecule may be selected based on the

tumor microenvironment and other elements of the innate and adaptive immune systems. In some embodiments, the molecule may be a stimulatory factor such as IL2, IL12, IL15, IL18, type I IFN, CD40L and 4-1BBL which have been shown to further enhance CAR T cell efficacy and persistence in the face of a hostile tumor microenvironment via different mechanisms (Yeku et al., *Biochem Soc Trans*., 2016, 44(2): 412-418).

[00344] In some aspects, the armed CAR T cell of the invention is modified to express a CD19 CAR and IL12. Such T cells, after CAR mediated activation in the tumor, release inducible IL12 which augments T-cell activation and attracts and activates innate immune cells to eliminate CD19-negative cancer cells.

[00345] In one embodiment, T cells of the invention may be modified to express an effector module comprising a CAR and an effector module comprising a suicide gene.

[00346] In one embodiment, the CAR T cell (including TCR T cell) of the invention may be transformed with effector modules comprising a cytokine and a safety switch gene (e.g., suicide gene). The suicide gene may be an inducible caspase such as caspase 9 which induces apoptosis, when activated by an extracellular stimulus of a biocircuit system. Such induced apoptosis eliminates transferred cell as required to decrease the risk of direct toxicity and uncontrolled cell proliferation.

[00347] In some embodiments, immune cells of the invention may be NK cells modified to express an antigen-specific T cell receptor (TCR), or an antigen specific chimeric antigen receptor (CAR) taught herein.

[00348] 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.

[00349] 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). Adoptive transfer of NK cells expressing CAR such as DAP12-Based Activating CAR revealed improved eradication of tumor cells (Topfer et al., *J Immunol*. 2015; 194:3201–3212). NK cell engineered to express a CS-1 specific CAR also displayed enhanced cytolysis and interferon-γ (IFN-γ) production in multiple myeloma (Chu et al., *Leukemia*, 2014, 28(4): 917-927).

[00350] 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.

[00351] 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).

[00352] Immune cells expressing effector modules comprising a CAR and/or other immunotherapeutic agents can be used as cancer immunotherapy. The immunotherapy comprises the cells expressing a CAR and/or other immunotherapeutic agents as an active ingredient, and may further comprise a suitable excipient. Examples of the excipient may include the aforementioned pharmaceutically acceptable excipients, including various cell culture media, and isotonic sodium chloride.

[00353] In some embodiments, cells of the present invention may be dendritic cells that are genetically modified to express the compositions of the invention. Such cells may be used as cancer vaccines.

Methods of CD19 antibody development and characterization

[00354] In some embodiments, the present invention provides methods of producing CD19 antibodies, antibody fragments or variants. Such methods may include the steps of: (1) preparing a composition with CD19, (2) contacting a library of antibodies or antibody fragments or variable with the composition, and (3) identifying one or more CD19 antibodies. Also, provided herein are methods for identifying FMC63-distinct CD19 antibodies, antibody fragments or variable.

[00355] In some embodiments, the present invention provides methods of identifying CD19 scFvs. Such methods may involve screening phagemid libraries for CD19 scFvs. Phagemid libraries expressing recombinant scFvs associated with the surface of bacteria or bacteriophages are useful in the present inventions. Phagemid libraries may be generated by PCR implication of the polynucleotides encoding the heavy chain and the kappa light chain of the immunoglobulin IgM and infecting Cre recombinase positive bacteria with the vectors containing the PCR products at a high multiplicity of infection (MOI). The high MOI results in bacteria containing multiple phagemids, each of which encodes a different VH and VL genes, which can be recombined by the Cre recombinase. The resulting library that may be generated by recombination is approximately 10^8 unique scFvs. In some instances, libraries of CD19 scFvs formatted into chimeric antigen receptor constructs may be screened to identify CD19scFvs useful in the present invention.

[00356] In some embodiments, scFvs immunologically specific to CD19 may be identified using cells that ectopically express full length, a fragment or a portion of CD19. Cell lines with low endogenous CD19 expression may be selected for ectopic expression. In some embodiments, the CD19 may be a naturally occurring isoform of human CD19.

[00357] In some embodiments, fusion proteins comprising the extracellular domains of CD19 (i.e. exon 1- exon 4) fused to the Fc region of human IgG1 (CD19sIg) are utilized to identify CD19 specific scFvs. Such fusion proteins have been described by Oliveira et al (2013) Journal of Translational Medicine 11:23; the contents of which are incorporated herein by reference in their entirety.

[00358] Also, provided herein are methods to identify FMC63-distinct scFvs, which include scFvs that are immunologically specific to and bind to an epitope of the CD19 antigen that is different or unlike the epitope of CD19 antigen that is bound by FMC63. In some embodiments, FMC63-distinct scFvs are identified by screening the scFv library with a complex consisting of human CD19 bound to FMC63. The CD19 of Rhesus macaque *(Macaca mulatta)* herein referred to as Rhesus CD19, bears 88% homology to the human CD19. Despite this high degree of homology, the Rhesus CD19 is not recognized by FMC63, indicating that the FMC63 epitope is in the region of human CD19 that is non-homologous to Rhesus CD19. Thus, in some embodiments, Rhesus CD19 may be used to screen scFv libraries for FMC63-distinct scFvs. Mutations in the region of Rhesus CD19 that is non-homologous to the human CD19 have been previously utilized to identify residues of human CD19 that confer binding to FMC63 (Sommermeyer et al. (2017) Leukemia Feb 16. doi: 10.1038/leu.2017.57). In some embodiments, the mutational analysis described by Sommermeyer et al. may be utilized to

design human CD19 mutants that are unable to bind to FMC63. Such mutants may include human CD19 (H218R, A237D, M243V, E244D, P250T) and human CD19 (H218R, A237D) and may be utilized to screen scFv libraries for FMC63-distinct scFvs. Sotillo et al have identified a splice variant of human CD19 lacking exon 2 in cancer patients (Sotillo et al. (2015) Cancer Discov. 2015 Dec;5(12):1282-95). The splice variant lacking exon 2 is not recognized by FMC63 and may also be used to screen scFv libraries for FMC63-distinct scFvs.

[00359] CD19 IgG fusion molecules generated by fusing the Fc region of human IgG1 with the human CD19-complete extracellular domains, i.e., exons 1-4 (CD19sIgG1-4) or extracellular domains lacking exon 2, i.e., exons 1, 3 and 4 (CD19sIgG1,3,4) may also be utilized to screen scFv libraries for FMC63-distinct scFvs.

[00360] CD19 proteins, variants and mutants useful in the invention are provided in Table 14.

Table 14: CD19 proteins, variants and mutants

Description	Sequence	SEQ ID NO
Human CD19, Isoform 1 (NCBI Reference No. NP_001171569.1)	MPPPRLLFFLLFLTPMEVRPEEPLVVKVEEGDNAVLQCLK GTSDGPTQQLTWSRESPLKPFLKLSLGLPGLGIHMRPLAIW LFIFNVSQQMGGFYLCQPGPPSEKAWQPGWTVNVEGSGE LFRWNVSDLGGLGCGLKNRSSEGPSSPSGKLMSPKLYVW AKDRPEIWEGEPPCLPPRDSLNQSLSQDLTMAPGSTLWLS CGVPPDSVSRGPLSWTHVHPKGPKSLLSLELKDDRPARD MWVMETGLLLPRATAQDAGKYYCHRGNLTMSFHLEITA RPVLWHWLLRTGGWKVSAVTLAYLIFCLCSLVGILHLQR ALVLRRKRKRMTDPTRRFFKVTPPPGSGPQNQYGNVLSLP TPTSGLGRAQRWAAGLGGTAPSYGNPSSDVQADGALGSR SPPGVGPEEEEGEGYEEPDSEEDSEFYENDSNLGQDQLSQ DGSGYENPEDEPLGPEDEDSFSNAESYENEDEELTQPVAR TMDFLSPHGSAWDPSREATSLAGSQSYEDMRGILYAAPQ LRSIRGQPGPNHEEDADSYENMDNPDGPDPAWGGGGRM GTWSTR	858
Human CD19, Isoform 2 (NCBI Reference No. NP_001761.3)	MPPPRLLFFLLFLTPMEVRPEEPLVVKVEEGDNAVLQCLK GTSDGPTQQLTWSRESPLKPFLKLSLGLPGLGIHMRPLAIW LFIFNVSQQMGGFYLCQPGPPSEKAWQPGWTVNVEGSGE LFRWNVSDLGGLGCGLKNRSSEGPSSPSGKLMSPKLYVW AKDRPEIWEGEPPCLPPRDSLNQSLSQDLTMAPGSTLWLS CGVPPDSVSRGPLSWTHVHPKGPKSLLSLELKDDRPARD MWVMETGLLLPRATAQDAGKYYCHRGNLTMSFHLEITA RPVLWHWLLRTGGWKVSAVTLAYLIFCLCSLVGILHLQR ALVLRRKRKRMTDPTRRFFKVTPPPGSGPQNQYGNVLSLP TPTSGLGRAQRWAAGLGGTAPSYGNPSSDVQADGALGSR SPPGVGPEEEEGEGYEEPDSEEDSEFYENDSNLGQDQLSQ DGSGYENPEDEPLGPEDEDSFSNAESYENEDEELTQPVAR TMDFLSPHGSAWDPSREATSLGSQSYEDMRGILYAAPQLR SIRGQPGPNHEEDADSYENMDNPDGPDPAWGGGGRMGT WSTR	859
Rhesus CD19 (Uniprot ID: F7F486)	MPPPCLLFFLLFLTPMEVRPQEPLVVKVEEGDNAVLQCLE GTSDGPTQQLVWCRDSPFEPFLNLSLGLPGMGIRMGPLGI WLLIFNVSNQTGGFYLCQPGLPSEKAWQPGWTVSVEGSG ELFRWNVSDLGGLGCGLKNRSSEGPSSPSGKLNSSQLYV WAKDRPEMWEGEPVCGPPRDSLNQSLSQDLTMAPGSTL WLSCGVPPDSVSRGPLSWTHVRPKGPKSSLLSLELKDDRP	860

	DRDMWVVDTGLLLTRATAQDAGKYYCHRGNWTKSFYL EITARPALWHWLLRIGGWKVPAVTLTYLIFCLCSLVGILQ LQRALVLRRKRKRMTDPTRRFFKVTPPPGSGPQNQYGNV LSLPTPTSGLGRAQRWAAGLGGTAPSYGNPSSDVQVDGA VGSRSPPGAGPEEEEGEGYEEPDSEEGSEFYENDSNFGQD QLSQDGSGYENPEDEPLGPEDEDSFSNAESYENEDEELTQ PVARTMDFLSPHGSAWDPSREATSLGSQSYEDMRGLLYA APQLRTIRGQPGPNHEEDADSYENMDNPDGPDPAWGGGG RMGTWSAR	
Human CD19 (H218R, A237D, M243V, E244D, P250T)	MPPPRLLFFLLFLTPMEVRPEEPLVVKVEEGDNAVLQCLK GTSDGPTQQLTWSRESPLKPFLKLSLGLPGLGIHMRPLAIW LFIFNVSQQMGGFYLCQPGPPSEKAWQPGWTVNVEGSGE LFRWNVSDLGGLGCGLKNRSSEGPSSPSGKLMSPKLYVW AKDRPEIWEGEPPCLPPRDSLNQSLSQDLTMAPGSTLWLS CGVPPDSVSRGPLSWTHVRPKGPKSLLSLELKDDRPDRD MWVVDTGLLLTRATAQDAGKYYCHRGNLTMSFHLEITA RPVLWHWLLRTGGWKVSAVTLAYLIFCLCSLVGILHLQR ALVLRRKRKRMTDPTRRFFKVTPPPGSGPQNQYGNVLSLP TPTSGLGRAQRWAAGLGGTAPSYGNPSSDVQADGALGSR SPPGVGPEEEEGEGYEEPDSEEDSEFYENDSNLGQDQLSQ DGSGYENPEDEPLGPEDEDSFSNAESYENEDEELTQPVAR TMDFLSPHGSAWDPSREATSLAGSQSYEDMRGILYAAPQ LRSIRGQPGPNHEEDADSYENMDNPDGPDPAWGGGGRM GTWSTR	861
Human CD19 (H218R, A237D)	MPPPRLLFFLLFLTPMEVRPEEPLVVKVEEGDNAVLQCLK GTSDGPTQQLTWSRESPLKPFLKLSLGLPGLGIHMRPLAIW LFIFNVSQQMGGFYLCQPGPPSEKAWQPGWTVNVEGSGE LFRWNVSDLGGLGCGLKNRSSEGPSSPSGKLMSPKLYVW AKDRPEIWEGEPPCLPPRDSLNQSLSQDLTMAPGSTLWLS CGVPPDSVSRGPLSWTHVRPKGPKSLLSLELKDDRPDRD MWVMETGLLLPRATAQDAGKYYCHRGNLTMSFHLEITA RPVLWHWLLRTGGWKVSAVTLAYLIFCLCSLVGILHLQR ALVLRRKRKRMTDPTRRFFKVTPPPGSGPQNQYGNVLSLP TPTSGLGRAQRWAAGLGGTAPSYGNPSSDVQADGALGSR SPPGVGPEEEEGEGYEEPDSEEDSEFYENDSNLGQDQLSQ DGSGYENPEDEPLGPEDEDSFSNAESYENEDEELTQPVAR TMDFLSPHGSAWDPSREATSLAGSQSYEDMRGILYAAPQ LRSIRGQPGPNHEEDADSYENMDNPDGPDPAWGGGGRM GTWSTR	862
Human CD19 (Delta exon 2)	MPPPRLLFFLLFLTPMEVRPEEPLVVKVEGELFRWNVSDL GGLGCGLKNRSSEGPSSPSGKLMSPKLYVWAKDRPEIWE GEPPCLPPRDSLNQSLSQDLTMAPGSTLWLSCGVPPDSVS RGPLSWTHVHPKGPKSLLSLELKDDRPARDMWVMETGL LLPRATAQDAGKYYCHRGNLTMSFHLEITARPVLWHWLL RTGGWKVSAVTLAYLIFCLCSLVGILHLQRALVLRRKRKR MTDPTRRFFKVTPPPGSGPQNQYGNVLSLPTPTSGLGRAQ RWAAGLGGTAPSYGNPSSDVQADGALGSRSPPGVGPEEE EGEGYEEPDSEEDSEFYENDSNLGQDQLSQDGSGYENPED EPLGPEDEDSFSNAESYENEDEELTQPVARTMDFLSPHGS AWDPSREATSLGSQSYEDMRGILYAAPQLRSIRGQPGPNH EEDADSYENMDNPDGPDPAWGGGGRMGTWSTR	863
Human CD19 (Exon 1-4)	MPPPRLLFFLLFLTPMEVRPEEPLVVKVEEGDNAVLQCLK GTSDGPTQQLTWSRESPLKPFLKLSLGLPGLGIHMRPLAIW LFIFNVSQQMGGFYLCQPGPPSEKAWQPGWTVNVEGSGE LFRWNVSDLGGLGCGLKNRSSEGPSSPSGKLMSPKLYVW AKDRPEIWEGEPPCLPPRDSLNQSLSQDLTMAPGSTLWLS CGVPPDSVSRGPLSWTHVHPKGPKSLLSLELKDDRPARD MWVMETGLLLPRATAQDAGKYYCHRGNLTMSFHLEITA RP	864

Human CD19 (Exon 1,3,4)	MPPPRLLFFLLFLTPMEVRPEEPLVVKVEGELFRWNVSDL GGLGCGLKNRSSEGPSSPSGKLMSPKLYVWAKDRPEIWE GEPPCLPPRDSLNQSLSQDLTMAPGSTLWLSCGVPPDSVS RGPLSWTHVHPKGPKSLLSLELKDDRPARDMWVMETGL LLPRATAQDAGKYYCHRGNLTMSFHLEITARP	865

III. PHARMACEUTICAL COMPOSITIONS AND FORMULATIONS

[00361] 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.

[00362] 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.

[00363] 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.

[00364] 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.

[00365] 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.

[00366] 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.

[00367] 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.

[00368] 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.

[00369] 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.

[00370] 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.

[00371] 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.

IV. APPLICATIONS

[00372] 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).

[00373] 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).

[00374] 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.

1. Adoptive cell transfer (adoptive immunotherapy)

[00375] 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).

[00376] 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 effect CAR therapies, in the manipulation or regulation of TILs, in allogeneic cell therapy, in combination T cell therapy with other treatment lines (e.g. radiation, cytokines), to encode engineered TCRs, or modified TCRs, or to enhance T cells other than TCRs (e.g. by introducing cytokine genes, genes for the checkpoint inhibitors PD1, CTLA4).

[00377] 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.

[00378] 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 IL12 and IL15 as payload using SREs of the present invention to reduce the need for preconditioning (Pengram et al. (2012) Blood 119 (18): 4133-41; the contents of which are incorporated by reference in their entirety).

[00379] 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.

[00380] In some embodiments, cells used for ACT may be T cells engineered to express CARs comprising an antigen-binding domain specific to an antigen on tumor cells of interest. In other embodiments, cells used for ACT may be NK cells engineered to express CARs comprising an antigen-binding domain specific to an antigen on tumor cells of interest. In addition to adoptive transfer of genetically modified T cells (e.g., CAR T cells) for immunotherapy, alternate types of CAR-expressing leukocytes, either alone, or in combination with CAR T cells may be used for adoptive immunotherapy. In one example, a mixture of T cells and NK cells may be used for ACT. The expression level of CARs in T cells and NK cells, according to the present invention, is tuned and controlled by a small molecule that binds to the DD(s) operably linked to the CAR in the effector module.

[00381] In some embodiments, the CARs of the present invention may be placed under the transcriptional control of the T cell receptor alpha constant (TRAC) locus in the T cells to achieve uniform CAR expression while enhancing T cell potency. The TRAC locus may be disrupted using the CRISPR/Cas 9, zinc finger nucleases (ZFNs), TALENs followed by the insertion of the CAR construct. Methods of engineering CAR constructs directed to the TRAC locus are described in Eyquem J. et al (2017) Nature.543(7643):113-117 (the contents of which are incorporated herein by reference in their entirety).

[00382] 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).

[00383] Other examples of genetic modification may include the introduction of chimeric antigen receptors (CARs) and the down-regulation of inhibitory NK cell receptors such as NKG2A.

[00384] 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.

[00385] 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 No.: 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 NO.: US20150152387A1, U.S. Patent NO.: 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.

[00386] In some instances, sub populations of immune cells may be enriched for ACT. Methods for immune cell enrichment are taught in International Patent Publication NO.: 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. NO.: 9,512,401 (the content of each of which are incorporated herein by reference in their entirety).

[00387] 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 NO.: US 20160298081A1; the contents of which are incorporated by reference herein in their entirety.

[00388] 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 NO.: WO2017015427, the content of which are incorporated herein by reference in their entirety.

[00389] 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.

[00390] 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.

[00391] 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.

[00392] 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 IL12 and a CAR construct such as CD19 CAR are linked to the same hDHFR destabilizing domain. The expression of IL12 and CD19 CAR 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 IL12 and a CAR construct such as CD19 CAR are linked to different DDs in two separate effector modules, thereby 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.

[00393] 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.

[00394] 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. The immunotherapeutic agents may be a second CAR or TCR specific to a different target molecule; a cytokine or a cytokine receptor; a chimeric switch receptor that converts an inhibitory signal to a stimulatory signal; a homing receptor that guides adoptively transferred cells to a target site such as the tumor tissue; an agent that optimizes the metabolism of the immune cell; 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.

[00395] 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 and IL15) into immune cells to promote immune cell proliferation and survival. Transduction of cytokine genes (e.g., gamma-cytokines IL2 and IL15) into cells will be able to propagate immune cells without addition of exogenous cytokines and cytokine expressing NK cells have enhanced tumor cytotoxicity.

[00396] 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.

[00397] In some embodiments, the biocircuits, and their components may be utilized to prevent T cell exhaustion in the context of Chimeric Antigen Receptor - T cell therapy (CAR-T). In this context, exhaustion in some instances, may be caused by the oligomerization of the scFvs of the CAR on the cell surface which leads to continuous activation of the intracellular domains of the CAR. As a non-limiting example, CARs of the present invention may include scFvs that are unable to oligomerize. As another non-limiting example, CARs that are rapidly internalized and re-expressed following antigen exposure may also be selected to prevent chronic scFv oligomerization on cell surface. In one embodiment, the framework region of the scFvs may be modified to prevent constitutive CAR signaling (Long et al. 2014. Cancer Research. 74(19) S1; the contents of which are incorporated by reference in their entirety). Tunable biocircuit systems of the present invention may also be used to regulate the surface expression of the CAR on the T cell surface to prevent chronic T cell activation. The CARs of the invention may also be engineered to minimize exhaustion. As a non-limiting example, the 41-BB signaling domain may be incorporated into CAR design to ameliorate T cell exhaustion. In some embodiments, any of the strategies disclosed by Long H A et al. may be utilized to prevent exhaustion (Long A H et al. (2015) Nature Medicine 21, 581–590; the contents of which are incorporated herein by reference in their entirety).

[00398] In some embodiments, the tunable nature of the biocircuits of the present invention may be utilized to reverse human T cell exhaustion observed with tonic CAR signaling. Reversibly silencing the biological activity of adoptively transferred cells using compositions of the present invention may be used to reverse tonic signaling which, in turn, may reinvigorate the T cells. Reversal of exhaustion may be measured by the downregulation of multiple inhibitory receptors associated with exhaustion.

[00399] In some embodiments, T cell metabolic pathways may be modified to diminish the susceptibility of T cells to exhaustion. Metabolic pathways may include, but are not limited to glycolysis, urea cycle, citric acid cycle, beta oxidation, fatty acid biosynthesis, pentose phosphate pathway, nucleotide biosynthesis, and glycogen metabolic pathways. As a non-limiting example, payloads that reduce the rate of glycolysis may be utilized to restrict or prevent T cell exhaustion (Long et al. Journal for Immunotherapy of Cancer 2013, 1(Suppl 1): P21; the contents of which are incorporated by reference in their entirety). In one embodiment, T cells of the present

invention may be used in combination with inhibitors of glycolysis such as 2-deoxyglucose, and rapamycin.

[00400] 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).

[00401] 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.

[00402] In one embodiment, the payload of the invention may be a CD276 CAR (with CD28, 4-IBB, and CD3 zeta intracellular domains), that does not show an upregulation of the markers associated with early T cell exhaustion (see International patent publication No. WO2017044699; the contents of which are incorporated by reference in their entirety).

[00403] 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

[00404] 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.

[00405] 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 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).

[00406] 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 NO.: US20160317633A1, the contents of which are incorporated herein by reference in their entirety.

3. Combination treatments

[00407] 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 for enhancement of immunotherapy.

[00408] 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 or virosomes.

[00409] 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. Dual regulation of CAR and cytokines and ligands to segregate the kinetic control of target-mediated activation from intrinsic cell T cell expansion. Such dual regulation also minimizes the need for pre-conditioning regimens in patients. As a non-limiting example, DD regulated CAR e.g. CD19 CAR may be combined with cytokines e.g. IL12 to enhance the anti-tumor efficacy of the CAR (Pegram H.J., et al. Tumor-targeted T cells modified to secrete IL12 eradicate systemic tumors without need for prior conditioning. Blood.2012;119:4133–41; the contents of each of which are incorporated herein by reference in their entirety). As another non-limiting example, Merchant et al. combined dendritic cell- based vaccinations with recombinant human IL7 to improve outcome in high-risk pediatric sarcomas patients (Merchant, M.S et. al. Adjuvant immunotherapy to Improve Outcome in High-Risk Pediatric Sarcomas. Clin Cancer Res. 2016. 22(13):3182-91; the contents of each of which are incorporated herein by reference in their entirety).

[00410] In some embodiments, immune effector cells modified to express one or more antigen-specific TCRs or CARs may be combined with compositions of the invention comprising immunotherapeutic agents that convert the immunosuppressive tumor microenvironment.

[00411] In one aspect, effector immune cells modified to express CARs specific to different target molecules on the same cell may be combined. In another aspect, different immune cells modified to express the same CAR construct such as NK cells and T cells may be used in combination for a tumor treatment, for instance, a T cell modified to express a CD19 CAR may be combined with a NK cell modified to express the same CD19 CAR to treat B cell malignancy.

[00412] In other embodiments, immune cells modified to express CARs may be combined with checkpoint blockade agents.

[00413] In some embodiments, immune effector cells modified to expressed biocircuits, effector modules, DDs and payloads of the invention may be combined with cancer vaccines of the invention.

[00414] 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.

[00415] 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.

[00416] In one embodiment, an effector module comprising a CD19 CAR may be used in combination with amino pyrimidine derivatives such as the Burkit's tyrosine receptor kinase (BTK) inhibitor using methods taught in International Patent Application NO.: WO2016164580, the contents of which are incorporated herein by reference in their entirety.

[00417] 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.

[00418] 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,ρ'-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.

[00419] 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.

[00420] 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.

[00421] 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.

[00422] 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.

4. Diseases

[00423] 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.

[00424] 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

[00425] 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.

[00426] 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.

[00427] 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.

[00428] 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.

Infectious diseases

[00429] 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.

[00430] "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

[00431] 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, respiratory tract, gastrointestinal tract, and the reproductive tract.

[00432] 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. Commensal Bifidobacterium promotes antitumor immunity and facilitates anti-PD-L1 efficacy. Science 2015; 350:1084–9; the contents of which are incorporated herein by reference in their entirety). In one embodiment, protein, RNA and/or other biomolecules derived from the microbiome may be used as a payload to influence the efficacy of the anti-cancer immunotherapies.

6. Tools and agents for making therapeutics

[00433] 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, type of cells, method of gene transfers, 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

[00434] 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 (e.g. 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), 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

[00435] 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 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).

[00436] 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.*

[00437] In some embodiments, the preferred reporter moiety may be luciferase proteins. In one embodiment, the reporter moiety is the Renilla luciferase (SEQ ID NO. 866, encoded by nucleic acid sequence of SEQ ID NO. 867), or a firefly luciferase (SEQ ID NO. 868, encoded by nucleic acid sequence of SEQ ID NO. 869).

Animal models

[00438] 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, beige mouse, and HLA transgenic mice.

Cellular assays

[00439] 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.

[00440] 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. Markers for T cell exhaustion include PD1, TIM3, BTLA, CD160, 2B4, CD39, and 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

[00441] 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.

[00442] 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.

[00443] 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 Elongation Growth Factor-1. Alpha (EF-1. alpha). Other constitutive promoters may also be used, including, but not limited to simian virus 40 (SV40), mouse mammary tumor virus (MMTV), human immunodeficiency virus (HIV), 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, 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 metallothionine promoter, glucocorticoid promoter, a progesterone promoter, and a tetracycline promoter may be used. In some embodiments, the promoter may be selected from the SEQ ID NO.: 716-718.

[00444] 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.

[00445] 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.

[00446] 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 host cell into which the vector is to be introduced.

1. Lentiviral vectors

[00447] 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).

[00448] 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.

[00449] 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.

[00450] 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.

[00451] Cells that can be 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).

[00452] 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.

[00453] 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.

[00454] 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.

[00455] 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.

[00456] 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 NO.: WO2012079000; the contents of each of which are incorporated herein by reference in their entirety).

2. Retroviral vectors (γ-retroviral vectors)

[00457] 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).

[00458] 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 are able to 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.

[00459] 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.

[00460] 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 NO.: 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).

[00461] 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.

[00462] In some embodiments, polynucleotides encoding the biocircuit, biocircuit components, effector module, 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.

[00463] 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 NO.: 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)

[00464] 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.

[00465] 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.

[00466] 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.

[00467] 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.

[00468] 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.

[00469] 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.

[00470] 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.

[00471] In some embodiments, more than one effector module or SRE (e.g. DD) may be encoded in a viral genome.

4. Oncolytic viral vector

[00472] 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. NO.: 9,226,977).

5. Messenger RNA (mRNA)

[00473] 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.

[00474] 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.

[00475] 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

[00476] 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

[00477] 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.

[00478] 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.

[00479] 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.

[00480] 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

[00481] 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.

[00482] 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.

[00483] 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, IL6, IFN-γ, and TNF-α are elevated.

[00484] In some embodiments, the neurotoxicity may be associated with CAR or TIL therapy. Such neurotoxicity may be associated CD19-CARs. Toxicity may be due to excessive T cell infiltration into the brain. In some embodiments, neurotoxicity may be alleviated by preventing the passage of T cells through the blood brain barrier. This can be achieved by the targeted gene

deletion of the endogenous alpha-4 integrin inhibitors such as tysabri/natalizumab may also be useful in the present invention.

3. Administration

[00485] 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 is described in U.S. Pat. NOs.: 7,435, 596; the contents of which are incorporated by reference herein in its entirety.

[00486] 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 each of which are incorporated herein by reference in their entirety.

[00487] 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.

[00488] 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.

[00489] 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. No.: EP0930892 A1, the contents of which are incorporated herein by reference.

[00490] 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

[00491] 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.

[00492] 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

[00493] 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.

[00494] *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.

[00495] *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).

[00496] *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.

[00497] *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.

[00498] *Antagonist*: the term "antagonist" as used herein refers to any agent that inhibits or reduces the biological activity of the target(s) it binds.

[00499] *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.

[00500] *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).

[00501] *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.

[00502] *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.

[00503] *Barcode*: the term "barcode" as used herein refers to polynucleotide or amino acid sequence that distinguishes one polynucleotide or amino acid from another.

[00504] *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.

[00505] *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

[00506] *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.

[00507] *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.

[00508] *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.

[00509] *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.

[00510] *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.

[00511] *Feature*: As used herein, a "feature" refers to a characteristic, a property, or a distinctive element.

[00512] *Formulation*: As used herein, a "formulation" includes at least a compound and/or composition of the present invention and a delivery agent.

[00513] *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.

[00514] *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.

[00515] *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.

[00516] *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.

[00517] *Immunotherapeutic agent:* the term "immunotherapeutic agent" as used herein, refers to the treatment of disease by the induction or restoration of the reactivity of the immune system towards the disease with a biological, pharmaceutical, or chemical compound.

[00518] *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).

[00519] *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).

[00520] *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.

[00521] *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.

[00522] *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.

[00523] *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.

[00524] *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.

[00525] *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, similar to 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).

[00526] *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.

[00527] *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.

[00528] *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.

[00529] *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.

[00530] *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).

[00531] *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.

[00532] *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.

[00533] *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.

[00534] *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).

[00535] *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.

[00536] *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.

[00537] *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.

[00538] *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.

[00539] *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.

[00540] *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

[00541] 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.

[00542] 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.

[00543] 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.

[00544] 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.

[00545] 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.

[00546] 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

[00547] 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.

[00548] 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.

[00549] 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

[00550] 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.

[00551] 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

[00552] 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.

[00553] 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 IL12 expression

[00554] FKBP (DD)-IL12 and DHFR (DD)-IL12 constructs were packaged into pLVX IRES-Puro lentiviral vectors with CMV, EF1a, or PGK promoters or without a promoter. The IL12 consists of two subunits, p40 and p35 which are separated by a linker. A p40 signal sequence was inserted next to the DD or IL12. In several constructs, a furin protease cleavage site or a modified furin site was included.

[00555] HEK293T cells were transiently transfected with 200ng or 1μg of FKBP-IL12 plasmids (OT-IL12-001 to OT-IL12-005), and subsequently treated with 10μM Shield-1 or vehicle control for 6 hours. Culture media was collected from transfected cells and diluted 1:50 to measure IL12 levels using p40 ELISA. The stabilization ratio was defined as fold change in IL12 expression with ligand treatment compared to treatment with DMSO (i.e. in the absence of ligand) with the same construct. Stabilization ratio greater than 1 is desired. The average IL12 ELISA readings and stabilization ratio are presented in Table 15.

Table 15: Ligand dependent IL12 induction

Construct ID	Vehicle	10μM Shield-1	Stabilization ratio
OT-IL12-001	1289.61	1748.95	1.36
OT-IL12-002	18.01	50.73	2.82
OT-IL12-003	1762.55	2138.25	1.21
OT-IL12-004	385.95	1567.62	4.06
OT-IL12-005	1188.42	2670.80	2.25
HEK293T	-12.921	-22.015	

[00556] OT-IL12-002 and OT- IL12-004 showed low level of IL12 expression in the absence of ligand when compared to IL12 levels in HEK 293T parental cells. Treatment with Shield-1 resulted in an increase in IL12 levels in OT-IL12-002, OT-IL12-004, and OT-IL12-005 constructs and a stabilization ratio between 2 and 4. These data show that OT-IL12-002 and OT-IL12-004 are destabilized in the absence of these constructs are stabilized by Shield-1.

[00557] IL12 expression was measured in cells following stable transduction. 500,000 cells stably transduced with OT-IL12-004 were plated in a 12 well plate and incubated overnight in growth media consisting of Dulbecco's Modified Eagle medium (DMEM) and 10% fetal bovine serum (FBS). The next day, cells were treated with 1μM Shield-1 or vehicle control for 6 or 24 hours. Following treatment with Shield-1, growth media was collected from the cells and diluted 10, 40, 160 or 640 fold and IL12 levels were quantified using IL12-p40 ELISA. The stabilization ratio was defined as fold change in IL12 expression with ligand treatment compared to treatment with DMSO (i.e. in the absence of ligand) with the same construct. Stabilization ratio greater than 1 is desired. The average IL12 ELISA readings and stabilization ratio at 6 hours are presented in Table 16.

Table 16: Ligand dependent IL12 induction (6 hours)

Media dilution (fold)	6 hours		
	Vehicle	Shield-1	Stabilization ratio
10	0.17	0.58	3.35
40	0.10	0.26	2.62
160	0.08	0.12	1.41
640	0.09	0.08	0.93

[00558] IL12 stabilization ratio greater than 1 was observed at 10, 40 and 160-fold dilutions of media, indicating that IL12 is stabilized by Shield-1 treatment at these dilutions at 6 hours.

[00559] The average IL12 ELISA readings and stabilization ratio at 24 hours are presented in Table 17.

Table 17: Ligand dependent IL12 induction (24 hours)

Media dilution (fold)	24 hours		
	Vehicle	Shield-1	Stabilization ratio
10	0.28	1.33	4.69
40	0.12	0.79	6.39
160	0.09	0.30	3.44
640	0.08	0.12	1.46

[00560] IL12 stabilization ratio was greater than 1 at all media dilutions tested and the highest stabilization ratio was observed at 40-fold dilution of media at 24 hours, suggesting ligand dependent stabilization.

[00561] To evaluate Shield-1 dependent FKBP-1L12 induction over time, 2 million cells were plated in growth medium and incubated overnight in the presence of 1μM Shield-1 or vehicle control. Cells were then incubated for with the ligand for 2, 4,6, 8, 24, 48, or 72 hours and growth media was collected for the cells at all time points. Growth media was diluted 400-fold and IL12 levels were measured using IL12 p40 ELISA. The stabilization ratio was defined as fold change in IL12 expression with ligand treatment compared to treatment with DMSO (i.e. in the absence of ligand) with the same construct. Stabilization ratio greater than 1 is desired. Average IL12 ELISA readings and stabilization ratio are presented in Table 18.

Table 18: IL12 induction over time

Time (hrs)	Vehicle	Shield-1	Stabilization ratio
2	0.13	0.18	1.41
4	0.14	0.26	1.89
6	0.13	0.29	2.30
8	0.14	0.28	1.99
24	0.18	0.99	5.47
48	0.23	1.79	7.71
72	0.26	1.63	6.28

[00562] Stabilization ratio increased over time and peaked at 48 hours, suggesting that IL12 is stabilized by Shield-1 with increasing duration of ligand treatment.

[00563] To evaluate the dependence of FKBP-IL12 production on Shield-1 dose levels, OT-IL12-004 transduced HEK293T cells were plated at different densities (40,000 cells, 20,000 cells, 10,000 cells or 5,000 cells per well) onto a 96-well plate. Following overnight incubation, cells were treated with growth medium containing 0 to 10μM Shield-1 for 24 hours. Media was then collected, diluted 400-fold and FKBP-IL12 levels were measured using IL12-p40 ELISA. Average IL12 ELISA readings are presented in Table 19.

Table 19: Dose and cell number dependent IL12 induction

Shield-1 (μM)	40000 cells/well	20000 cells/well	10000 cells/well	5000 cells/well
10	623.77	656.70	214.11	193.62
3.333333	670.64	618.10	273.74	207.55
1.111111	677.27	872.24	322.56	203.71
0.37037	368.17	582.71	250.49	172.50
0.123457	197.29	343.34	156.98	95.92
0.041152	171.50	205.68	63.79	48.89
0.013717	117.25	103.56	13.30	-2.35
0.004572	66.34	60.58	2.11	-8.53
0.001524	100.43	39.55	-13.58	-21.76

0	83.49	7.92	-21.76	-26.97

[00564] A dose dependent IL12 induction was observed at all cell numbers tested. IL12 induction increased with Shield-1 up to a dose of 1μM; following which IL12 induction plateaued. Notably, greater IL12 induction was observed at 2000 and 4000 cells/well.

Example 3. DD regulated recombinant IL12 mediated functions in HEK293T cells

[00565] HEK-Blue sensor cells (InvivoGen, San Diego, CA) were utilized to evaluate whether DD regulated IL12 is capable of regulating signaling downstream of IL12. In these cells, the IL12 receptor, STAT4 and downstream transcriptional elements are linked to a reporter gene such that IL12 signaling can be monitored. One million HEK 293T were transfected with 200ng of OT-IL12-003 plasmid using Lipofectamine 2000 (Thermo Fisher Scientific, Waltham, MA). 48 hours after transfection, cells were treated with growth media containing 10μM Shield-1, incubated for another 24 hours, following which, media was collected. 50,000 HEK 293 Blue sensor cells were plated onto 96 well plates and incubated overnight with media (at different dilutions) from Shield-1 treated OT-IL12-003 expressing HEK293T cells. After overnight incubation, 20 μl media was removed from each well and incubated with 180 μl Quanti-Blue reagent (InvivoGen, San Diego, CA) for 30 minutes at 37°C. Absorption was measured at 620 nm using a spectrophotometer. To generate a standard curve, 180 μl Quanti-Blue reagent was mixed with 20 μl of recombinant IL12 at following concentrations 500, 250, 125, 62.5, 31.25, 15.62, 7.8 and 3.9 pg/ml. Functional IL12 concentrations were determined by comparing the optical density of each sample with IL12 standard curve. Measurable levels of functional IL12 were reached with 640-fold dilutions of IL12 containing growth media and further plateaued at higher concentrations of the media (Figure 19A).

[00566] The dependence of functional IL12 production on the dose of Shield-1 used was measured. 10,000 HEK293T cells stably transduced with OT-IL12-004 were plated onto 96 well plates and treated with growth media containing 10, 3.33, 1.11, 0.37, 0.12, 0.04, 0.01, 0.005, 0.002 or 0 μM Shield-1 for 24 hours. Following Shield-1 treatment, media from cells was diluted 200-fold and 20μL of the diluted media was added to HEK Blue sensor cells. After overnight incubation, 20 μl of media was removed from each well and incubated with 180 μl Quanti-Blue reagent (InvivoGen, San Diego, CA) for 30 minutes at 37°C. Absorption was measured at 620 nm using a spectrophotometer. To generate a standard curve, 180 μl Quanti-Blue reagent was mixed with 20 μl of recombinant IL12 at following concentrations 500, 250, 125, 62.5, 31.25, 15.62, 7.8 and 3.9 pg/ml. Functional IL12 concentrations were determined by comparing the

optical density of each sample with IL12 standard curve. A dose dependent increase in the levels of functional IL12 levels was observed (Figure 19B).

Example 4. DD regulated recombinant IL12 expression *in vivo*

[00567] SKOV3 tumor cells expressing FBP regulated-IL12 (#OT-IL12-009) or parental cells were implanted into SCID Beige mice (Day 0). Mice implanted with FKBP IL12 were dosed intraperitoneally with Shield-1 (10mg/kg) or vehicle control on Day 2 and Day 7, while the parental cells were left untreated. Blood samples were collected at 0, 2, 4, 6, 8 and 24 hours after Shield-1 dosing and plasma human IL12 levels were measured using ELISA. The average adjusted concentration of plasma IL12 is presented in Figure 19C. At Day 2, IL12 levels increased in Shield-1 treatment and the levels were higher than vehicle control at 4, 6, 8, and 24 hours. Maximum IL12 levels were detected in Shield-1 treated mice at 8 hours following treatment. In contrast, at day 7, IL12 levels were very low and almost comparable to the IL12 levels in parental SKOV3 cells.

[00568] The experiment was repeated 28 days following implantation of SKOV3 tumor cells. Mice were split into three groups, with the groups receiving 1, 2 or 3 doses of ligand or vehicle control. Mice received multiple doses with a two-hour interval. Blood samples were collected right before the first dose (0 hours), and 6 hours and 24 hours after the first dosing. Plasma IL12 levels were measured and average IL12 concentrations are shown in Figure 19D. The two dose and three dosing scheme resulted in higher plasma IL12 levels when compared to vehicle treated samples. Peak plasma IL12 levels was detected at 6 hours following shield-1 treatment with all dosing schemes, and the highest IL12 plasma levels were detected with the three-dose regimen. This demonstrates the ligand dependent stabilization of IL12 *in vivo*.

Example 5. DD regulated IL15

[00569] To test ligand dependent IL15 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 at 5% CO2. Cells were then transfected with 100ng of OT-IL15-001(constitutive) or OT-IL15-002 (ecDHFR-IL15) using Lipofectamine 2000 and incubated for 48 hrs. Following the incubation, media was exchanged for growth medium with 10μM Trimethoprim or vehicle control and further incubated for 24 hrs. Media was collected and the undiluted samples or samples diluted 4, 16, 256, 1024, 4096 or 16384-fold were tested using human IL15 ELISA. The stabilization ratio was defined as fold change in IL15 expression with ligand treatment compared to treatment with DMSO (i.e. in the absence of ligand) with the same construct. Stabilization ratio greater than 1 is desired. Average IL15 ELISA readings and stabilization ratio are presented in Table 20.

Table 20: DD-IL15 induction

Media dilution (fold)	Vehicle	10 μM TMP	Stabilization ratio
1	0.396	0.820	2.073
4	0.154	0.287	1.867
16	0.074	0.116	1.567
64	0.056	0.073	1.301
256	0.053	0.057	1.075
1024	0.053	0.048	0.910
4096	0.049	0.049	0.995
16384	0.050	0.049	0.994

[00570] The 16-fold, 4-fold diluted, and undiluted media samples showed stabilization ratio greater than 1.5, suggesting a Trimethoprim dependent stabilization of IL15 at these dilutions.

Example 6. DD regulated expression of IL15-IL15Ra fusion molecule

[00571] A fusion molecule is generated by fusing membrane bound IL15, IL15 Receptor alpha subunit (IL15Ra) and DDs such as ecDHFR (DD), FKBP (DD), or human DHFR (DD). These fusion molecules were cloned into pLVX-EF1a-IRES-Puro vector.

[00572] To test ligand dependent IL15-IL15Ra 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 then transfected with 100ng of constitutive IL15-IL15Ra (OT-IL15-008) or DD linked IL15-IL15Ra (OT-IL15-006, OT-IL15-007, OT-IL15-009, OT-IL15-010, OT-IL15-011) using Lipofectamine 2000 and incubated for 24 hrs. Following the incubation, media is exchanged for growth medium with or without 10μM Trimethoprim or 1μM Shield-1 and further incubated for 24 hrs. Cells were harvested and IL15 levels are analyzed via western blotting using human IL15 antibody (Abcam, Cambridge, UK). OT-IL15-009 showed the strong ligand (Trimethoprim) dependent stabilization of IL15, while OT-IL15-006 and OT-IL15-007 showed modest ligand dependent stabilization of IL15 (Figure 20A).

[00573] Surface expression of membrane bound IL15-IL15Ra constructs (OT-IL15-006, OT-IL15-007, OT-IL15-008, OT-IL15-009, OT-IL15-010, OT-IL15-011) was determined by FACS using anti-IL15 and anti-IL15Ra antibodies. HEK293T cells were transfected with IL15-IL15Ra constructs and then treated with suitable ligand (Shield-1 or Trimethoprim). 48 hours after transfection, cells were analyzed using FACS. As expected, constitutive IL15-IL15Ra construct OT-IL15-008 showed high surface expression of IL15 and IL15Ra both in the presence and absence of ligand. Consistent with the results from the western blot, OT-IL15-009 showed the strong ligand (Trimethoprim) dependent surface expression of IL15 and IL15Ra (Figure 20B, Figure 20C).

[00574] Membrane bound-IL15-IL15Ra constructs (OT-IL15-008 to OT IL15-011) were transduced into human colorectal carcinoma cell line, HCT-116 and stable integrants were selected with 2μg of puromycin. Stably integrated cells were then incubated for 24 hours in the presence or absence of 10μM Trimethoprim or 1μM Methotrexate.

[00575] Surface expression of IL15 -IL15Ra fusion constructs was examined by staining with PE- conjugated IL15Ra antibody (Cat no. 330207, Biolegend, San Diego, CA). The median fluorescence intensity obtained with the different constructs in the presence or absence of the corresponding ligand is presented in Table 21.

Table 21: Surface expression of IL15-IL15Ra fusion constructs

Construct	Median Fluorescence Intensity		
	DMSO	10μM TMP	1μM MTX
HCT-116 cells (control)	273		
OT-IL15-008 (Constitutive)	5315	7019	
OT-IL15-006 (ecDHFR (R12Y, E129K)	764	2978	
OT-IL15-010 (hDHFR (Y122I, A125F))	2657	5775	15864
OT-IL15-011(hDHFR (Q36F, N65F, Y122I))	1560	4010	14509

[00576] The stabilization ratio was calculated as the fold change in GFP intensity in ligand treated samples compared to treatment with DMSO (i.e. in the absence of ligand) with the same construct. The destabilization ratio was calculated as the fold change in GFP intensity in the DD regulated constructs compared to the constitutive construct (OT-IL15-008) in the absence of the ligand. Destabilization ratios less than 1 and stabilization ratios greater than 1 are desired in DDs. The ratios are presented in Table 22.

Table 22: IL15-IL15Ra destabilization and stabilization ratios

Construct	Destabilization ratio	Stabilization ratio	
		TMP	MTX
OT-IL15-008 (Constitutive)	19.47	1.32	
OT-IL15-006 (ecDHFR (R12Y, E129K)	0.14	3.90	-
OT-IL15-010 (hDHFR (Y122I, A125F))	3.48	2.17	5.97
OT-IL15-011(hDHFR (Q36F, N65F, Y122I))	0.59	2.57	9.30

[00577] Destabilization ratios less than one was observed with OT-IL15-006 (ecDHFR (R12H, E129K)) and OT-IL15-011(hDHFR (Q36F, N65F, Y122I)) indicating a strong destabilization in the absence of ligand. Stabilization ratio greater than 1 was observed with all constructs with TMP treatment and with both OT-IL15-010 and 11 with MTX treatment. These data show that OT-IL15-006 and OT-IL15-011 are both strongly destabilized in the absence of ligand and strongly stabilized in the presence of ligand.

[00578] The expression and ligand-dependent stabilization of IL15-IL15Ra constructs (OT-IL15-008 to OT-IL15-011) was measured in HCT-116. Cells were incubated with 10μM Trimethoprim or 1μM Methotrexate or DMSO for 24 hours. Following incubation, cells were harvested and cell extracts were prepared. Cell extracts were run on SDS-PAGE and western blotted with anti-IL15 antibody (Catalog No. 7213, Abcam, Cambridge, UK). As shown in Figure 20D, the IL15/IL15Ra constitutive construct (OT-IL15-008) showed ligand independent IL15 expression while the DD regulated constructs (OT-IL15-009 to OT-IL15-011) showed ligand dependent IL15 expression. The identity of the IL15 bands was also confirmed by immunoblotting with the anti-human DHFR antibody (Catalog No. 117705, Genetex, Irvine, CA). As shown in Figure 20D, both IL15-IL15Ra fusion constructs (OT-IL15-010 and 011) showed ligand dependent expression of DHFR expression.

[00579] To evaluate the dose dependence of ligand induced stabilization, IL15-IL15Ra fusion constructs namely, OT-IL15-009 (ecDHFR (R12Y, Y100I)), OT-IL15-010 (hDHFR (Y122I, A125F)), and OT-IL15-011 (hDHFR (Q36F, N65F, Y122I)) were stably transduced into HCT-116 cells and incubated with increasing concentrations of Trimethoprim for 24 hours. Surface expression of IL15-IL15Ra fusion construct was quantified by FACS using IL15Ra- PE antibody. The median fluorescence intensity with increasing doses of TMP is represented in Table 23.

Table 23: Surface expression of IL15-IL15Ra

Dose (μM)	Median Fluorescence Intensity		
	ecDHFR (R12Y, 100I)	hDHFR (Y122I, A125F)	hDHFR (Q36F, N65F, Y122I)
DMSO	1260	3034	2357
0.01	1384	2791	2291
0.02	1492	2833	2216
0.05	1947	2924	2054
0.14	2741	2740	2150
0.41	3360	2817	2400
1.23	4014	2890	2251
3.7	4265	3117	2494
11.11	4267	3473	2841
33.33	4485	4019	3409
100	4633	5227	4592

[00580] As shown in Table 23, all three constructs showed a dose dependent increase in median fluorescence intensity indicating a dose dependent increase in surface expression of IL15-IL15Ra fusion upon addition of DD stabilizing ligand.

[00581] The time course of ligand dependent stabilization of IL15-IL15Ra fusion constructs was measured in HCT-116 cells. Cells were transduced with OT-IL15-009 (ecDHFR (R12Y, Y100I) construct and incubated with 10μM Trimethoprim for 0, 12, 16, 24, 48 or 72 hours. Following incubations, surface expression of IL15-IL15Ra fusion construct was quantified by FACS using IL15Ra- PE antibody and compared to parental untransfected cells. The median fluorescence intensity (MFI) over time is represented in Table 24.

Table 24: Time course of IL15-IL15Ra surface expression

Time (hours)	MFI
Parental	3054
0	4004
12	7054
16	9390
24	14056
48	28644
72	35303

[00582] As shown in Table 24, OT-IL15-009 (ecDHFR (R12Y, Y100I) showed a time-dependent increase in median fluorescence intensity indicating that the surface expression of IL15-IL15Ra fusion increased with increased duration of treatment with DD stabilizing ligand.

Example 7. DD regulated CD19 CAR expression

[00583] A CD19 CAR fusion polypeptide was linked to either FKBP-DD, ecDHFR -DD or human DHFR- DD and the constructs were cloned into pLVX-IRES-Puro vector.

[00584] FKBP, ecDHFR and hDHFR DDs were positioned either between the CD19 scFv and the CD8αhinge (OT-CD19C-002, OT-CD19C-003), between the CD8αhinge and the transmembrane domain (OT-CD19C-004, OT-CD19C-005) or at the C terminus of the construct (OT-CD19C-007, OT-CD19C-008, OT-CD19C-009, OT-CD19C-010, OT-CD19C-011). In some instances, a furin cleavage site was added between the DD and the CD19 scFv. A constitutively expressed CAR construct, OT-CD19C-001 was used as a positive control.

[00585] To test ligand dependent expression of DD-CD19 CAR constructs, 1 million HEK 293T cells were cultured in growth medium containing DMEM and 10% FBS and transfected with CAR constructs using Lipofectamine 2000. 48 hours after transfection, cells were treated with 1μM or10μM Shield-1, 10μM Trimethoprim, 1μM Methotrexate, or vehicle control and incubated for 24 hours. Cells were harvested, lysed and immunoblotted for CD3 Zeta, a component of the CAR, using anti-CD247 (BD Pharmingen, Franklin Lanes, NJ) and Alexa 555-conjugated-goat-anti mouse antibody (red) (Li-Cor, Lincoln, NE). Lysates were also immunoblotted for Actin with Alexa 488-conjugated secondary antibody (green) to confirm

uniform protein loading in all the samples. Compared to the untreated control, OT-CD19C-002 and OT-CD19C-003 showed increased levels of CD3 Zeta in the presence of ligands Shield-1 and TMP respectively indicating the stabilization of the CD19 CAR (Figure 21A). As shown in Figure 21B, OT-CD19C-008 and OT-CD19C-010 constructs showed strong increase in CD3 Zeta levels in the presence of Methotrexate and low levels in the absence of ligand, indicating a strong ligand-dependent stabilization of CD19 CAR. OT-CD19C-007 and OT-CD19C-009 showed modest increase in CD3 Zeta levels in the presence of Shield-1 and Methotrexate respectively, indicating a modest ligand-dependent stabilization of CD19 CAR. As expected, the constructively expressed, OT-CD19C-001 showed strong expression of CD19 CAR in the absence of ligand treatment.

[00586] Lysates from cells expressing CD19 CAR constructs were also immunoblotted for 4 1-BB, a component of the CAR. As shown in Figure 21C, OT-CD19C-008, OT-CD19C-009, OT-CD19C-010 and OT-CD19C-011 showed low levels of 4-1 BB in the absence of ligand and high levels of 4-1 BB in the presence of the ligand, Methotrexate, indicating a strong ligand dependent stabilization of CD19 CAR using these constructs. OT-CD19C-003, OT-CD19C-006 and OT-CD19C-007 showed modest increase in 4-1BB expression levels with treatment of corresponding ligands- TMP and Shield-1, indicating a modest ligand dependent stabilization of CD19 CAR. Constructs OT-CD19N-014 and OT-CD19N-015, which contain a furin cleavage site, showed an additional, smaller 4 1BB sized protein product upon treatment with MTX. This smaller sized 4-1BB protein band was only seen with the addition of the ligand and its molecular weight is consistent with the size of the CD19 CAR in OT-CD19-001. These data indicate that the furin cleavage occurs only with ligand treatment.

[00587] Surface expression of DD-CD19 CAR constructs in HEK 293T 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). Cells were treated with 1μM Shield-1, 1μM Methotrexate,10μM Trimethoprim or vehicle control for 24 hours and subject to FACS analysis. As shown in Figure 21D, surface expression of OT-CD19C-002 with FKBP-DD was detected only in the presence of Shield-1, while OT-CD19C-003 with ecDHFR-DD showed surface expression only in the presence of Trimethoprim. As expected, constitutively expressed construct OT-C19C-001 showed high expression both in ligand and control vehicle treated cells. Additional constructs were analyzed by FACS with Protein L-Biotin-Strepavidin-Allophycocyanin in a separate experiment. The percentage of GFP positive cells obtained with each construct in the presence or absence of ligand is presented in Table 25. In Table 25, N/A indicates not applicable.

Table 25: Percentage GFP positive cells

Construct	Ligand	Percentage GFP positive cells	
		No Ligand	Ligand
OT-CD19C-001	N/A	46.8	45.4
OT-CD19C-006	TMP	46.6	43.6
OT-CD19C-007	Shield-1	28.9	34
OT-CD19C-008	MTX	15.8	31.3
OT-CD19C-009	MTX	16.5	34.2
OT-CD19C-010	MTX	14.8	33
OT-CD19C-011	MTX	14.5	32.9
OT-CD19C-012	TMP	19.1	18.7
OT-CD19C-013	Shield-1	0.91	0.4
OT-CD19C-014	MTX	4.68	16.2
OT-CD19C-015	MTX	3.04	18.2

[00588] An increase in the percentage GFP positive cells was observed with OT-CD19C-007, OT-CD19C-008, OT-CD19C-009, OT-CD19C-010, OT-CD19C-011, OT-CD19C-014, and OT-CD19C-015. The highest increase in the percentage of GFP positive cells was observed with OT-CD19C-014, and OT-CD19C-015 constructs.

[00589] The mean fluorescence intensities are presented in Table 26. In Table 26, MFI represents mean fluorescence intensity. The stabilization ratio was calculated as the fold change in GFP intensity in ligand treated samples compared to treatment with DMSO (i.e. in the absence of ligand) with the same construct. The destabilization ratio was calculated as the fold change in GFP intensity in the DD regulated constructs compared to the constitutive construct (OT-CD19C-001) in the absence of the ligand. Destabilization ratios less than 1 and stabilization ratios greater than 1 are desired.

Table 26: CD19 CAR surface expression

Construct	Ligand	MFI		Destabilization Ratio	Stabilization Ratio
		No Ligand	Ligand		
OT-CD19C-001	N/A	453	407		0.90
OT-CD19C-006	TMP	425	325	0.94	0.76
OT-CD19C-007	Shield-1	132	123	0.29	0.93
OT-CD19C-008	MTX	89.4	148	0.20	1.66
OT-CD19C-009	MTX	93.3	200	0.21	2.14

OT-CD19C-010	MTX	85.6	160	0.19	1.87
OT-CD19C-011	MTX	83.3	172	0.18	2.06
OT-CD19C-012	TMP	112	105	0.25	0.94
OT-CD19C-013	Shield-1	61	49.5	0.13	0.81
OT-CD19C-014	MTX	78.6	124	0.17	1.58
OT-CD19C-015	MTX	73.3	143	0.16	1.95

[00590] A destabilization ration less than 1 was observed with all constructs indicating that all DD regulated constructs are destabilized in the absence of ligand. A stabilization ratio of greater than 1 was observed with OT-CD19C-008, OT-CD19C-009, OT-CD19C-010, OT-CD19C-011, OT-CD19C-014 and OT-CD19C-015. Notably, these constructs were also destabilized in the absence of ligand and therefore represent suitable CD19-DD constructs.

Example 8. *In vitro* T cell assay development

[00591] The goal of the study was to determine the T cell stimulation regimen and dose of IL12 needed 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 22. 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 9. Measuring human T cell responses *in vitro* and *in vivo*

[00592] 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 23A).

[00593] 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 23B).

[00594] 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 23C, 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.

[00595] 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 23D, 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 10. DD regulated IL12 mediated functions

[00596] DD-IL12 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 50 matrigel

into mice 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 andIL12 as well as the ligand levels are measured.

[00597] To evaluate the ability of IL12 expressing cells to form tumors, HCT-116 cells stably transduced with DD-IL12 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 IL12 levels in ligand treated mice compared to untreated controls is indicative conditional regulation of IL12 *in vivo*.

Example 11. DD regulated recombinant IL12 mediated functions in T cells

[00598] Functional responses to DD-IL12 is evaluated in primary human T cells and in human cell lines/transformed hematopoietic cell lines e.g. Raji cells. Human T cells are purified from peripheral blood mononuclear cells (PBMCs) by negative selection using CD4+ T- cell isolation kit (Miltenyi Biotec, Germany). T cells are treated with growth media from HEK 293T cells expressing DD-IL12 constructs for 5 days. Cells are then activated with beads conjugated with-CD3/CD28 beads (Thermo Fisher Scientific, Waltham, MA) at the ratio of 3 beads per T cell and cultured for 3 days. Functional response to DD-IL12 is determined by measuring Interferon gamma in CD3 positive cells using flow cytometry following treatment with ligand or vehicle control. IL12 promotes the differentiation of naïve T cells into Th1 cells which results in the secretion of IFN gamma from T cells.

[00599] To evaluate IL12 induced phosphorylation of STAT4 (Signal transducer and activator of transcription 4), human T-cells are isolated from PBMCs and activated with phytohemagglutinin (PHA, 2μg/ml) for 3 days followed by treatment with 50 IU/ml of Interleukin 2 (IL2) for 24hrs. Cells are then washed, resuspended in fresh media and rested for 4 hrs in the presence of ligand or vehicle control. Supernatant from DD-IL12 expressing HEK293T cells is added to the primary cells, followed by incubation for 30 minutes. Cells are then harvested and STAT4 phosphorylation is analyzed using STAT4 antibody (Cell Signaling Technology, Danvers, MA).

Example 12. Functional analysis of DD regulated IL15-IL15Ra fusion molecule

[00600] Activation via IL15 can sustain T cell persistence by conferring a survival advantage. In addition, IL15/IL15Ra fusion molecule has been shown to confer a memory phenotype on T cells and increase proliferation of NK cells (Hurton (2016), PNAS, 113: E7788-7797; the contents of which are incorporated herein by reference in their entirety).

[00601] To evaluate signaling by DD regulated IL15-IL15Ra fusion constructs, NK-92 cells are incubated with HCT-116 cells expressing DD regulated IL15-IL15Ra fusion constructs. Trans signaling by IL15/IL15Ra is expected to increase STAT5 phosphorylation in NK92, which is measured by western blotting, and by FACS. Proliferation of NK92 cells is also measured.

[00602] To evaluate the effect of DD regulated IL15-IL15Ra fusion constructs on primary T cells, cells are transduced with the fusion constructs. T cell proliferation in the absence of exogenous IL15 supplementation is measured. The T cell memory phenotype is measured by quantifying CD62L expression by FACS.

[00603] To assess if DD-IL15/IL15R expressing T cells maintain prolonged persistence *in vivo*, DD modified T cells are injected into mice. Constructs are tagged with luciferase reporter to allow *in vivo* tracking in mice. Mice are treated with vehicle control or corresponding ligand, Shield-1, Trimethoprim or Methotrexate depending on the construct utilized and monitored over a period of 40-50 days using bioluminescent imaging (PerkinElmer, Massachusetts). Mice treated with ligand are expected to retain T cells expressing DD-IL15/IL15Ra while T cells in vehicle control treated animals are not expected to persist.

Example 13. DD regulated CD19 CAR expression and function in T cells

[00604] Ligand dependent expression of DD-CD19 CAR constructs is evaluated in primary human T cells and in immortalized/transformed hematopoietic cell lines e.g. Raji cells, Jurkat cells and K562 cells. Human T cells are purified from peripheral blood mononuclear cells (PBMCs) by negative selection using CD4+ T- cell isolation kit (Miltenyi Biotec, Germany). Primary T cells and hematopoietic cell lines are stably transduced with DD-CD19 CAR constructs. Cells are treated with 10μM Shield-1, 10μM Trimethoprim, 1μM Methotrexate or vehicle control and immunoblotted for CD3 Zeta using anti CD247 antibody.

[00605] The production of functional DD-CD19 CAR is analyzed in primary human T cells or human cell lines (NALM6, K562, Jurkat and Raji cells). Cells are incubated with CD19 expressing antigen presenting cells or CD19/Fc fusion protein in the presence of DD stabilizing ligands Shield-1, TMP or MTX. After incubation, cells are stained with fluorescently labelled anti-CD69 antibodies and analyzed by flow cytometry. Cells with high CD69 expression are considered to have a functional DD-CD19 CAR. Functional response to DD-CD19 CAR is also

determined by measuring interferon gamma levels using ELISA. DD-CD19 CAR expressing cells are expected to demonstrate higher Interferon gamma levels in the presence of ligand than untreated cells.

[00606] Cytolytic potential of DD-CD19 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-CD19 CAR and mock transduced cells are coincubated with CD 19 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-CD19 CAR are expected to demonstrate specific cytolysis only in the presence of ligand. Cells with DD-CD19 CAR in the absence of ligand or mock transfected cells are expected to show minimal cytolytic activity.

[00607] The *in vivo* antitumor efficacy of DD-CD19 CAR is also evaluated. Immune compromised mice are injected with luciferase expressing human leukemic cell lines (NALM-6). Subsequently, mice are injected with DD-CD19 CAR T cells via tail vein injections. Mice are subdivided into treatment groups and are treated with a range of ligand doses. Two control groups are also included in the study: a control group that did not receive any ligand and another group that did not receive any T cells. Tumor burden as measured by luciferase activity is monitored over time using bioluminescent imaging. Mice treated with DD-CD19 CAR T cells and ligand are expected to have a reduced tumor burden when compared to control animals.

Example 14. Evaluation of antitumor response of DD regulated payloads in syngeneic mouse models

[00608] 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-IL12, DD-IL15, DD1L15-IL15Ra, and DD-CD19 CAR,. 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 15. Optimizing workflow for discovery of DD-regulated immunotherapeutic agents

[00609] To identify DD-CD19 CAR constructs suitable for immunotherapy, constructs are introduced into cell lines e.g. HEK293T cells and Jurkat cells. The expression of the construct in

the presence or absence of the corresponding ligand is tested. Constructs which show low basal expression in the absence of ligand and robust, ligand-dose responsive expression are selected for further analysis. If no DD-CD19 CAR constructs show ligand-dependent expression, then constructs are redesigned and the experiment is repeated till a regulatable construct is identified. Next, the ligand dependent regulation of the DD-CD19 CAR constructs is tested *in vitro* in primary T cells. If the constructs show low basal expression in the absence of the ligand and ligand dose responsive expression, they are subject to *in vivo* PK/PD proof of concept experiments. Otherwise, the constructs are redesigned and the new constructs are subject to similar analysis. The constitutively expressing CD19 CAR constructs are transduced into T cells and CD19 CAR expression is measured in parallel to the regulated construct. If no expression is detected in vitro, efforts are refocused on testing DD- CD19 CAR constructs *in vitro* in T cells. In contrast, if the constitutive constructs show expression, then the expression of CD19 CAR is measured *in vivo*.

[00610] To test in vivo PK/PD, mice are injected with T cells expressing DD-CD19 CAR constructs and the test group is dosed with the ligand corresponding to the DD, while the control group is dosed with the appropriate vehicle control. Constructs that display ligand-dependent expression of CD19 CAR are selected for in vivo functional proof of concept experiments. Parallel experiments are also conducted using the constitutive CD19 CAR constructs. If constitutive CD19 CAR expression is detected *in vivo*, then the constructs are selected for functional experiments. If no expression is detected *in vivo*, then constructs are redesigned.

[00611] Functional analysis *in vivo* is performed by testing if the constitutive and DD regulated CD19 CAR expressing T cells display anti-tumor activity in a constitutive or ligand dependent manner respectively. If yes, then *in vivo* proof of concept is achieved and constructs suitable for immunotherapy are identified. If none of the DD regulated constructs show anti-tumor activity, then alternate dosing regimens are explored. If the constitutive CD19 CAR constructs do not show anti-tumor activity, then efforts are focused on identifying DD-CD19 CAR constructs that show in vivo expression in T cells.

Example 16. Co-expression of DD regulated payloads

[00612] Toxicity related to systemic administration of interleukins can be circumvented by using CAR-T cells to deliver interleukins to the target tissue. This combinatorial approach also has greater anti-tumor activity than interleukin and CAR therapy alone. Cells are co-transfected with CD19 CAR (constitutive or DD regulated) and DD-Interleukin e.g. DD-IL12, DD-IL15 and DD-IL15/IL15Ra constructs. Transfected cells are treated with stabilizing ligands depending on

the DD utilized. CD19 CAR expression is evaluated by immunoblotting for CD3 zeta. DD-IL12, DD-IL15 and DD-IL15/IL15Ra expression in the media is measured by ELISA.

Example 17. CAR expression and functionality in T cells

[00613] Primary T cells were transduced with CD19 CAR constructs. Surface expression of CD19 CAR construct 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). To determine the percentage of the CD4 and CD8 sub populations of CAR T cells, cells were analyzed by anti CD4, anti CD8 antibodies and Protein L. As shown in Figure 24A, 67.3 % of CAR positive cells obtained were CD4 positive, while only 14.2% cells were CD8 positive.

[00614] To test the ability of CD19 CAR cells to kill target cells, primary T cell populations transduced with OT-CD19N-001 or OT-CD19N-017 were cocultured with K562 cells expressing CD19 (target cells) at a ratio of 5:1. Additional control combinations of T cells and target cells were also set up. These included CAR expressing T cells co cultured with K562 cells, T cells co cultured with K562 cells expressing CD19 and K562 cells expressing CD19 without T cell co culture. K562 cells were fluorescently labelled with NucLight Red and co cultured with T cells for 30 hours. Cell death was monitored by labelling cells with Annexin V and K562 target cell death was measured by evaluating Annexin V staining in NucLight Red positive cells. The ratio of Annexin V staining per target cell area was calculated. As shown in Figure 24B, OT-CD19N-001 or OT-CD19N-017 expressing T cells were effective in killing target K562 cells expressing CD19. A low level of target cell killing was observed when untransduced T cells were cocultured with CD19 expressing target cells. As expected, cell death was minimal in the co-culture of OT-CD19N-001 expressing T cells and K562 cells (without CD19 expression). These data show that CD19 CAR cells are effective in killing their corresponding target cells.

Example 18. Regulated expression of IL15-IL15Ra in T cells

[00615] DD regulated IL15-IL15Ra constructs such as OT-IL15-009 or constitutively expressed constructs such as OT-IL15-008 were transduced into T cells such as primary T cells or SupT1 cells. The transduction was carried out at two different lentivirus concentrations, 5 µl and 20 µl for the DD regulated construct using Lentiboost ™ (Sirion Biotech, Germany). 4 days after transduction, cells were treated with 10µM TMP or DMSO control for 24 and 48 hours. Samples were analyzed with an anti IL15Ra antibody using FACS. Additional controls samples such as cells treated with Lentiboost only, untransduced cells treated with DMSO or TMP, and Isotype

controls were included in the FACS analysis. The FACS results are depicted in Figure 25A for 24 hours of TMP treatment and in Figure 25B for 48 hours of TMP treatment. In both figures, DMSO-A and TMP-A indicate cells treated with 5 µl of lentivirus and DMSO- B and TMP-B indicate cells treated with 20 µl of lentivirus. Treatment of T cells expressing OT-IL15-009 with TMP for 24 hours resulted in an increase in the expression of IL15Ra in T cells with both doses of lentivirus used. Additionally, very low levels of IL15Ra were detected in the DMSO treated samples under the same conditions as well as in untransduced T cells. As expected, the constitutively expressed construct, OT-IL15-008 showed high expression of IL15Ra. TMP dependent expression of OT-IL15-009 was not observed in SupT1 cells (Figure 25A). Similar results were observed for both T cells and SupT1 cells at 48 hours (Figure 25B). These results show that tight regulation of IL15-IL15Ra constructs can be achieved in primary T cells.

[00616] The surface expression of IL15 and IL15Ra was measured for OT-IL15-008 and OT-IL15-009. The percentage of cells expressing IL15, IL15Ra or both on the cell surface is presented in Table 27.

Table 27: Surface expression of IL15 and IL15Ra

	% Positive cells	
	OT-IL15-008	OT-IL15-009
IL15 and IL15Ra positive	2.03	0.51
IL15Ra positive	13.0	15.8
IL15 positive	0.29	0.60

[00617] As shown in Table 27, the percentage of cells with detectable surface expression of IL15 and IL15Ra was less than 5% with both constructs. Further, the percentage of cells with surface expression of IL15Ra was much higher than the percentage of cells with detectable surface expression of IL15.

[00618] The effect of increasing doses of TMP on IL15Ra expression in T cells was measured using the OT-IL15-009 construct. T cells were treated with a range of doses of TMP starting from 0.156 µM to 160 µM for 24 hours. IL15Ra expression was measured using FACS. As shown in Figure 25C, the percentage of IL15Ra expressing T cells with OT-IL15-009 cells was detected even at the lowest concentration of TMP and the percentage of IL15Ra positive cells at the lowest concentration of TMP was higher than the untreated control. The percentage of IL15Ra cells increased with increasing doses of TMP.

Example 19. TMP dose responsive expression of IL15-IL15Ra

[00619] IL15-IL15Ra fusion constructs, OT-IL15-008, OT-IL15-009, and OT-IL15-010 were stably expressed in HCT116 cells treated with increasing doses of TMP ranging from 10μM, 33μM, and 100μM TMP for 24 hours. Cell lysates were immunoblotted with anti IL15Ra antibody. As shown in Figure 26, IL15Ra expression of OT-IL15-009 was virtually undetectable in the absence of TMP, and addition of increasing doses of TMP resulted in an increase in IL15Ra levels. Modest increase in IL15Ra expression was observed with OT-IL15-010 construct with the addition of TMP. As expected, the constitutive construct, OT-IL15-008 showed strong expression of IL15Ra both in the presence and absence of ligand.

Example 20. Effect of IL15-IL15Ra on T cell persistence and T cell memory phenotype

[00620] The effect of constitutively expressed IL15-IL15Ra fusion construct, OT-IL15-008 on T cell persistence was measured in NSG mice. T cells were transduced with OT-IL15-008 and 4 million cells T cells were injected intravenously into NSG mice (number of mice =3). As a control, additional mice were injected with untransduced T cells. Blood samples were obtained from mice at 2, 3, 4, 5 and 6 weeks after injection and analyzed by FACS for the presence of CD8 and/or CD4 positive human T cells expressing IL15 and IL15Ra. The percentage of human T cells in the blood was calculated as the percentage of total T cells i.e. human T cells (measured using anti-human CD45 antibody) and the mouse T cells and endothelial cells (measured using the anti-mouse CD45 antibody). As shown in Figure 27 A, the percentage of T cells in the blood at 2 weeks was greater in mice injected with T cells transduced with OT-IL15-008 compared to control mice that were injected with untransduced T cells. This observed increase in T cells decreased over 3,4, and 5 weeks, and the percentage of T cells was comparable between the two cohorts. At 6 weeks, one of the mice injected with OT-IL15-008 transduced T cells showed a higher percentage of human T cells in the blood. Thus, at 2 weeks, the frequency of human T cells in the blood is increased in the blood of mice injected with OT-IL15-008 transduced T cells.

[00621] The number of T cells in the blood was measured by comparing the number of human T cells in 50 uL of mouse blood using anti-human CD45 antibody as a marker for human T cells and anti-murine CD3 antibody as a marker for murine endothelial cells. As shown in Figure 27B, the number of human T cells in the blood increased at 2 weeks in mice injected with OT-IL15-008 transduced T cells, as compared to mice injected with untransduced T cells. The difference between the two cohorts was diminished at 3 weeks and 4 weeks. At 6 weeks, one of the mice injected with OT-IL15-008 transduced T cells showed a higher number of human T cells in the

blood. Thus, at 2 weeks, the frequency and number of human T cells in the blood is increased in the blood of mice injected with OT-IL15-008 transduced T cells. These data support the role of IL15-IL15Ra fusion proteins in T cell persistence. The increased T cell frequency and number observed at 6 weeks in one of the mice may be due to graft versus host disease.

[00622] The effect of OT-IL15-008 expression on the CD4 and CD8 subset of T cells was measured prior to injecting into mice (Week 0) and 2 weeks after injection. As shown in Figure 27C, the ratio of CD4 and CD8 cells was 1:1 prior to injecting into mice. However, at 2 weeks, the proportion of CD4 positive cells was much higher than the CD8 positive cells in the transduced cells, indicating that OT-IL15-008 causes a preferential expansion of CD4 positive cells. The expression of the OT-IL15-008 construct within the CD4 and CD8 subsets was measured using anti IL15Ra antibody. As shown in Figure 27D, prior to injections, 25 % of the OT-IL15-008 transduced CD4 T cells and CD8 T cells expressed IL15Ra. At week 2, the percentage of IL15Ra positive CD4 and CD8 T cells increased to 80% indicating a preferential expansion of T cells transduced with OT-IL15-008. As expected, untransduced control T cells were negative for IL15Ra expression.

Example 21. IL12 dependent, re-stimulation independent Th1 markers

[00623] T cells require T cell receptor restimulation *in vivo* or *in vitro* stimulation with CD3/CD28 to produce IFNgamma. To study the effect of IL12 activity on T cells in the absence of restimulation, several T cell markers were explored. T cells were expanded using one of the following 4 expansions strategies (i) Day 10 cytokine switch from IL2 to IL12, CD3/CD28 stimulation from day 0 to day10 with no restimulation (ii) Day 10 cytokine switch from IL2 to IL12, CD3/CD28 stimulation from day 0 to day 10 and restimulation at with CD3/CD28 from day 12 to day 14 (iii) Day 10 cytokine switch from IL2 to IL12, CD3/CD28 stimulation from day 0 to day3 with no restimulation (iv) Day 10 cytokine switch from IL2 to IL12, CD3/CD28 stimulation from day 0 to day 3 and restimulation at with CD3/CD28 from day 12 to day 14. Markers tested include CD69, IFNg, Perforin, CXCR3, Granzyme B, CCR5, CXCR6, Ki-67 and T-bet. IFNg appears to be the most robust and consistent marker for IL12 activity on human T cells, but requires re-stimulation of T cells to induce production. Th1 markers which increase in response to IL12 in the absence of re-stimulation and IL2 (likely in vivo conditions) include Ki-67, T-bet, Perforin, CXCR3, and CCR5.

Example 22. Effect of cytokines on NK cell proliferation and activation

[00624] Immune cells such as Natural Killer cells depend on cytokines such as IL15 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.

[00625] 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 IL12 (10 ng/ml) or varying concentrations of IL15 (100 ng/ml, 20 ng/ml, 4 ng/ml, 0.8 ng/ml, 0.16 ng/ml, 0.032 ng/ml, 0.0064 ng/ml and 0.00128 ng/ml). NK-92 cell activation in response to IL15 and IL12 treatment was evaluated by FACS analysis using a panel of markers whose increased expression is associated with NK cell activation. These markers include NKG2D, CD71, CD69; chemokine receptors such as CCR5, CXCR4, and CXCR3, Perforin, Granzyme B and Interferon gamma (IFNg). Prior to FACS analysis for IFNg, cells were cultured for 4 hours with Brefeldin A. 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, signaling 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 IL15 treatment are presented in Table 28.

Table 28: IL15 induced markers

IL15 dose (ng/ml)	CD69	CXCR4	Perforin	Granzyme B	pSTAT5 (15 mins)	pSTAT5 (60 mins)	IFNgamma
100	1.91	3.87	1.67	1.48	1.98	2.34	9.00
20	2.10	3.62	1.57	1.40	1.96	2.35	5.55
4	1.59	3.03	1.28	1.16	1.81	2.35	2.76
0.8	1.18	2.10	1.16	1.09	1.76	2.17	1.92
0.16	0.99	1.41	1.04	1.03	1.44	2.08	2.41
0.032	1.05	1.14	0.92	0.92	1.23	1.67	0.95
0.0064	1.11	1.19	0.79	0.78	1.03	1.26	0.85
0.00128	1.07	1.15	0.85	0.88	0.99	1.04	1.19

[00626] Treatment with IL15 resulted in an increase in the expression of CD69, CXCR4, Perforin, Granzyme B, and IFNg. The effect of IL15 on these markers was dose dependent with a higher dose of IL15 resulting in a corresponding upregulation of markers. Phosphorylation of

STAT5 was increased both at 15 and 60 minutes after the addition of IL2 or IL15. Taken together, these results show that cytokines can activate NK cells.

[00627] The fold change in activation markers observed with IL12 treatment are shown in Table 29.

Table 29: IL12 induced markers

Marker	Fold change
CCR5	1.60
Perforin	1.67
Granzyme	1.87
IFNg	1.74
IFNg (supernatant)	666.15

[00628] Treatment with IL12 resulted in an increase in the expression of markers CD69, CCR5, Perforin, Granzyme B, and IFNgamma. Further, IFNg levels secreted by NK-92 cells into the media higher than untreated controls upon treatment with IL12. Treatment with IL2 resulted in an increase in the expression of CXCR4, Perforin, Granzyme B, and IFNg. Further, IFNg levels secreted by NK-92 cells into the supernatant was higher than untreated controls upon treatment with IL2.

Example 23. Effect of cytokines on T cell expansion and activation

[00629] To test the requirement of IL2 and IL12 for T cells expansion and activation, T cells were stimulated with soluble CD3/CD28, CD3/CD28 Dynabeads or left unstimulated for two days. Each of these groups was further split into two sub groups. One sub group was treated with IL2 and 100 ng/ml of IL12 while the second sub group was treated with IL2 only for the duration of the stimulation. For the soluble CD3/CD28 stimulated cells, a third subgroup that was only treated with 100 ng/ml was also included. T cell expansion over the course of 14 days was measured and the fold change in T cells expansion is shown in Figure 28A. CD3/CD28 dynabeads plus IL2 with or without IL12 had the most profound impact on T cell expansion followed by the T cells treated with soluble CD3/CD28 plus IL2 with or without IL12. Unstimulated cells and cell treated with soluble CD3/CD28 cells that did receive IL2 treatment were unable to expand over the course of the experiment. These results show that IL2 is required for T cell expansion, but IL12 may be dispensable.

[00630] The effect of IL12 on T cell activation was measured by determining the frequency of IFNgamma positive CD4+ and CD8 + T cells. IFNg is produced by activated T cells. Three different stimulation protocols were used. In the first protocol, cells were stimulated with

CD3/CD28 dynabeads for 2 days, following which the beads were washed off and the cells were treated with varying concentrations of IL12 for 7 days (from day 2 to day 9). At day 9, cells were restimulated with soluble CD3/CD28 and the frequency of IFNgamma positive cells was determined by FACS. The results are presented in Figure 28B as the percentage of cells. In the second protocol, following 2 day CD3/CD28 dynabeads stimulation, T cells were maintained in culture for a longer duration of 14 days i.e. from day 2 to day 16. At day 16 cells, were restimulated with soluble CD3/CD28. At day 16, the frequency of IFNgamma positive cells was measured. The results are presented in Figure 28C as the percentage of cells. In the third protocol, T cells were initially stimulated for 2 days with CD3/CD28 dynabeads and IL2, followed by treatment with IL2 only for 9 days (i.e. from day 2 to day 11), followed by IL12 treatment for 2 to 5 days. In the last two days of the experiment, cells were also restimulated with soluble CD3/CD28. IFNgamma positive CD4 and CD8 cells were measured using FACS. The third protocol mimics the environment that is presented to T cells in adoptive cell therapy, both during in vitro transduction and T cells expansion as well as the in vivo. The results are presented in Figure 28D as the percentage of cells. In both 7-day treatment with IL12 as well as 14-day treatment with IL12, shown in Figure 28B and Figure 28C respectively, restimulation with CD3/CD28 cells at the end of the experiment increased the percentage of IFNgamma positive cells. A half maximum effective concentration (EC50) of IL12 observed with the first protocol for CD8 cells was 50 pg/ml. The EC50 of IL12 observed with the second protocol was 12 pg/ml for CD4 cells and 65 pg/ml for CD8 cells. Long-term culture with CD3/CD28 further increased the dependence on re-stimulation and IL12 for IFNg production.

[00631] The results obtained with the third stimulation protocol are presented in Figure 28D. Immune cells treated with IL12 for the final 5 days of the experiment combined with CD3/CD28 restimulation showed the highest percentage of IFN gamma positive cells (EC50 = 24 pg/ml for CD4 cells and 40 pg/ml for CD8 cells), followed by cells that received IL12 for 2 days. Thus, T cells expanded in vitro can later differentiate in response to IL12, but restimulation may be required for IFNg production.

[00632] Taken together these results indicate that IL12 can stimulate IFN production in T cells when restimulated with CD3/CD28.

Example 24. Promoter selection for expression of SREs in T cells

[00633] The expression of SREs in a vector can be driven by either 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. 870) 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 29, both the CMV promoter and the EF1a can drive the expression of GFP in SupT1 cells and T cells. The percentage of GFP positive T cells was higher when 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 when compared to the CMV promoter. Thus, the optimum promoter suitable for expression differs based on the cell type.

Example 25. Effect of ligand on T cell proliferation

[00634] 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 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 26. Effect of DD regulated CD19 CAR on tonic signaling

[00635] Chronic antigen activation can result in T cell exhaustion. To test if DD regulated CD19 CAR constructs induce tonic signaling, irradiated K562 cells expressing CD19 are plated into culture plates 12 hours before the addition of T cells expressing DD regulated CD19 CAR constructs with Interleukin 2. Cells are counted every two days and media is replaced. For repeated stimulations, cells are transferred to a new plate with K562-CD19 cells after 24 hours (for two stimulations) or every 12 hours (for four stimulations). For each condition, T cells are counted and analyzed by FACS for CAR, phenotypic and exhaustion markers every 12 hours. DD regulated constructs were analyzed in the presence or absence of ligand. Markers analyzed include CD25 and CD69 for activation status; CD62 and CD45RA for memory status; and exhaustion markers PD1, TIM3 and LAG3. DD regulated CD19 CAR constructs are expected to induce a lower percentage of cells that are positive for all three exhaustion markers- i.e.PD1,

TIM3 and LAG3 and a higher percentage of cells that are CD45A+/CD62L+ indicating less differentiated T cells. Constitutively expressed CD19CAR constructs may induce the expression of all three exhaustion markers and may have a more differentiated phenotype with a higher proportion of CD45-/CD62L- and CD45+/CD62L- cells.

Example 27. Functional analysis of DD regulated CD19 CAR

[00636] To test the ability of DD regulated CD19 CAR cells to kill target cells, primary T cell populations transduced with DD regulated CD19 CAR constructs are co cultured with K562 cells expressing CD19 (target cells) at a ratio of 5:1 in the presence or absence of the ligand specific to the DD e.g. Shield-1, TMP or MTX. Additional control combinations of T cells and target cells are also set up. These include DD regulated CAR expressing T cells co cultured with K562 cells (in the presence or absence of the ligand), T cells co cultured with K562 cells expressing CD19 and K562 cells expressing CD19 without T cell co culture. The K562 cells are fluorescently labelled with NucLight Red and co cultured with T cells for 30 hours. Cell death is monitored by labelling cells with Annexin V and the cell death in target K562 cells is monitored by evaluating cells that are positive for both Annexin V and NucLight Red. The ratio of Annexin V staining per target cell area is calculated. DD-CD19CAR expressing T cells are expected to be effective in killing target K562 cells expressing CD19 only in the presence of the ligand specific to the DD. Minimal target cell death is expected to occur when untransduced T cells are cocultured with CD19 expressing target cells; and with DD-CAR T cells (with or without ligand treatment) plus K562 cells (without CD19 expression).

Example 28. Generation of CD19 scFvs using the large phage antibody libraries

Construction of Primary Phagemid Library

[00637] Total RNA is prepared from 40 different samples of human peripheral blood lymphocytes and cDNA is synthesized using random primers. IgM variable regions are amplified using an IgM 3' primer and 5'VH primers. Pooled primers are also used to amplify the Vk and VL. An additional PCR step is added to include restriction sites as well as to introduce a region of overlap containing an scFv loxP linker. scFvs are obtained by mixing equimolar amounts of VH and VL genes and performing assembly. The scFvs are then cloned into pDAN5 vectors to obtain a primary library of approximately 10^8.

Recombination and secondary of the secondary library

[00638] To induce recombination, bacterial strain BS1365 (which express Cre-recombinases constitutively) are infected with primary phagemid library at an MOI of 20:1. This results in

bacteria containing multiple phagemids, each of which encodes different VH and VL genes, which can be recombined by the Cre recombinase. Since the phagemid arise from bacteria containing many different scFv, the phenotype and genotype are not coupled. Phagemids derived from the bacteria are used to infect bacteria that do not express Cre (e.g. DH5a) at a low MOI of ≤ 0.1 to couple genotype to phenotype.

CD9 expression constructs and cell lines

[00639] Human CD19 isoforms described in Table 14 are cloned into appropriate vectors and transfected into cell lines with low endogenous CD19 expression such as K562 and 3T3 cell lines. The CD19 expressing lines i.e. K562- CD19 or 3T3 -CD19 cells, are used for positive selection of scFvs in the phage display library. The parental K562 and 3T3 are used for the negative selection.

Selection of antibodies recognizing CD19 on cell surface

[00640] The secondary phage display library is pre-cleared by screening with parental cells to remove non-specific binding phages. The precleared phages are incubated with K562-CD19 or 3T3-CD19 cells, and bound phages are recovered and amplified for next round of selection. Three rounds of selection are performed to enrich for CD19 binders. FMC63-distinct scFvs i.e. scFvs that bind to epitopes distinct from FMC63, are selected in a parallel selection process by blocking the FMC63 epitope with an excess of FMC63 antibody.

[00641] The affinity of the scFv to CD19 is a critical aspect that determines the performance of the antibody in pharmacokinetic and immune response assays. Affinity measurements for 96 scFv clones are made using techniques such as ELISA and surface plasma resonance which provide on-rate (Ka), off-rate (Kd), and affinity constant (KD).

[00642] scFv clones with desired off rates are subjected to Sanger sequencing to identify unique clones that bind to CD19 expressing cells but not parental cells. Identified clones are then subject to epitope binning, using a competitive immunoassay that is used to characterize and then sort a library of scFvs against a target protein, e.g. CD19. scFvs against CD19 are tested against all other CD19 scFvs identified from the library, in a pairwise fashion to identify scFvs that prevent the binding of other scFvs to an epitope of CD19 antigen. After a profile is created for each CD19 scFv, a competitive blocking profile is created for each scFv relative to the others. Closely related binning profiles indicate that the antibodies have the same or a closely related epitope are binned together.

[00643] scFvs obtained at each of step of the selection process are subject to deep sequencing methods such as Ion Torrent/MiSeq. Heavy chain CDR3 sequences, including those that do not

bind to FMC63 are identified using the Abmining ToolBox (D'Angelo S et al. (2014) MAbs. 6(1): 160–172) and top ranking HCDR3s are identified. HCDR3 specific primers designed from the DNA sequence of the top ranked sequences are then used to amplify scFv clones by inverse PCR and the PCR product is cloned into expression vectors.

Example 29. CD19 scFv affinity

[00644] The affinity of the scFv to the CD19 antigen is a critical aspect that determines the performance of the antibody in pharmacokinetic and immune response assays. Affinity measurements are made using techniques such as ELISA and surface plasma resonance which provide on-rate (Ka), off-rate (Kd), and affinity constant (KD).

[00645] Antibodies with varying affinities are identified using cells that have high or low ectopic expression of CD19. K562-CD19 cells and parental K562 cells with low CD19 expression are sorted by FACS using CD19 antibodies e.g. FMC63 to determine surface expression of CD19. Cells are sorted into bottom 5% (i.e. low CD19 expressing cells), top 5% (high CD19 expressing cells) and the rest of the population of cells is categorized as median CD19 expressing cells.

Example 30. Screening strategy for identifying FMC63-distinct CD19 scFvs

CD19/Fc fusion proteins

[00646] FMC63 binds to human CD19 in the region encoded by exon 2. To identify FMC63-distinct CD19 scFvs, human CD19 (Exon 1-4) or human CD19 (Exon 1,3,4) are fused with IgG to generate CD19-IgG fusion proteins, CD19sIgG1-4 and CD19sIgG1,3,4 respectively. CD19-IgG fusion proteins are used for antibody screening. 96-well plates are coated with capture antibodies and incubated with CD19sIgG1-4 or CD19sIgG1,3,4 fusion protein. The plates are washed and incubated with candidate CD19 scFvs identified in Example 28. The plates are washed again and incubated again with reporter (e.g. Alkaline phosphatase) conjugated detection antibodies and detected using reporter compatible detection methods. Capture antibodies may be antihuman IgG Fc antibodies, or the FMC63 antibody (as a control). The detection antibody may be anti-human IgM antibody. FMC63-distinct CD19 scFvs are expected to bind to (CD19sIgG1,3,4) and (CD19sIgG1-4). In contrast, candidate CD19 scFvs that bind to epitopes that are identical or overlap with FMC63's epitope are expected to only bind to (CD19sIgG1-4).

Competition assay

[00647] CD19 expressing K562 cells are incubated with nano molar concentrations of tagged candidate CD19 scFvs e.g. identified in Example 28 and fixed concentration of tagged FMC63

scFv for competition binding assays. Cells are washed and stained with the secondary antibody corresponding to the tag used in the candidate CD19 scFv. Mean fluorescence intensity is measured using flow cytometry. As a negative control, CD19 K562 expressing cells are incubated with varying concentrations of tagged candidate CD19 scFv alone or FMC63 alone. For FMC63-distinct CD19 scFvs, it is expected that there will be no competition for binding to CD19 between the candidate CD19 scFvs and FMC63. Thus, the mean fluorescence intensity of the tagged candidate CD19 scFv is expected to increase with increasing concentrations of the candidate CD19 scFv, while the mean fluorescence intensity of tagged FMC63 antibody is not expected to decrease with increasing concentrations of the candidate CD19 scFv. This would indicate that the FMC63 is not displaced from its epitope by the addition of the candidate CD19 scFv, suggesting distinct binding epitopes. For candidate CD19 scFv that bind to the same epitope as FMC63, a decrease in the fluorescence intensity of FMC63 with increasing concentrations of the candidate CD19 scFv is expected.

Example 31. Functional analysis of FMC63-distinct CD19 CAR constructs

[00648] FMC63-distinct CD19 scFvs engineered to generate FMC63-distinct CD19 CAR constructs with destabilizing domains, linkers, transmembrane and intracellular domains described in Table 1, and Tables 6, 7, 8A or 8B. The ability of FMC63-distinct CD19 CAR to induce cell activation, cytotoxicity and proliferation is compared to the FMC63-CD19 based CAR constructs in Jurkat cells. Constructs are also analyzed for their ability to induce the upregulation of exhaustion markers, PD1, TIM3 and LAG3, and constructs that are positive for multiple exhaustion markers are excluded from the analysis. Constructs that can induce Jurkat cell activation and cytotoxicity but not exhaustion markers are transduced into T cells and their efficacy is compared with the constitutively expressed FMC63-based CD19 CAR construct. It is expected that DD regulated FMC63-distinct CD19 CAR constructs will demonstrate superior cytotoxic capabilities with minimal tonic signaling as compared to FMC63 CD19 CAR constructs.

Example 32. Ligand dependent target cell death induced by DD regulated CD19 CAR

[00649] To test the antigen specificity of cell killing by T cells engineered to express constitutive or DD-containing CAR construct, CD19 was ectopically expressed in the antigen negative K562 cell line. CD19 expression was measured using anti-CD19 antibody conjugated to Phycoerythrin (PE). Figure 30A shows the expression of CD19 in parental K562 cells and K562-CD19 cells, wherein CD19 is ectopically expressed.

[00650] To test the ability of DD regulated CD19 CAR cells to kill target cells, primary T cell populations were transduced with DD regulated CD19 CAR constructs, OT-CD19-024 with human DHFR DD and an EF1a promoter. Transduced T cells were co cultured with K562 cells expressing CD19 (target cells) at a ratio of 5:1 in the presence or absence of TMP (100μM). Additional control combinations of T cells and target cells were also set up. These included DD regulated CAR expressing T cells co cultured with antigen-negative K562 cells (in the presence or absence of the ligand), untransduced T cells co cultured with K562 cells expressing CD19 and K562 cells expressing CD19 without T cell co culture. The T cells utilized for this experiment were transduced with the OT-CD19-024 construct (or untransduced) and expanded for 11 days using protocols described in previous examples, frozen, thawed and co-cultured with target cells. Target cells were treated with Mitomycin C to prevent their proliferation. The K562 or K562-CD19 target cells stably expressing the fluorescent protein NucLight Red were co cultured with T cells for 300 hours. Cell death was monitored by labelling cells with Annexin V and the cell death in target K562 and K562-CD19 cells was monitored by evaluating cells that were positive for both Annexin V and NucLight Red using the IncuCyte® Live Cell Analysis System (Essen Biosciences, Ann Arbor, MI). The results are presented in Figure 30B, where the killed target cells represented on the y axis are based on target cells that are positive for both NucLight Red and Annexin V. Figure 30C, shows the killed target size as measured in (μM/well) at day 5. Target cell killing was observed with the OT-CD19-024 construct only in TMP treated co-cultures of T cells and K562 target cells ectopically expressing CD19. No cell killing was observed in untreated controls of the same co-culture set up and when T cells were co cultured with parental K562 cells that do not express CD19 in the presence or absence of ligand. These data show that regulated CARs display ligand- and target-dependent cell killing with minimal basal off-state.

Example 33. *In vitro* CAR-T cell functional analysis

[00651] The efficacy of T cells expressing DD regulated CD19 CAR constructs in functionally interacting with target cells is evaluated. To interact with the CD19CAR T cells, the chosen target cells express CD19 naturally or ectopically. In this context, target cells which have high endogenous expression of CD19 such as Nalm6, Raji, Reh, Sem, Kopn8, and Daudi cells. Alternatively, target cell lines may be engineered by ectopic expression of CD19 in cell lines that have low endogenous expression of CD19 such as K562. Multiple assays are used to measure functionality. Prior to co culture, the target cells are optionally cultured in the presence of 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 CD19 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 CD19 CAR expressing T cells are co cultured with CD19 expressing target cells in the presence of ligand. Cytotoxicity is not expected in vehicle control cells or when the target cells do not express CD19 are utilized.

[00652] Engagement of the CD19 CAR with CD19 antigen results in the activation of T cells which 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 CD19 CAR expressing T cells are co cultured with CD19 expressing target cells in the presence of ligand. Both parameters are not expected in vehicle control cells or when the target cells do not express CD19 are utilized.

[00653] 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.

Example 34. Ligand dependent target cell death induced by DD regulated CD19 CAR

[00654] To test the ability of DD regulated CD19 CAR cells to kill target cells, primary T cell populations are transduced with DD regulated CD19 CAR constructs are co cultured with K562 cells expressing CD19 (target cells) at a ratio of 5:1 in the presence or absence of the ligand specific to the DD e.g. Shield-1 (1μM), TMP (100μM) or MTX. Constructs with FKBP, ecDHFR or human DHFR DDs may be utilized. Constructs with either CMV, EF1a or PGK promoters may also be used. Multiple combinations of T cells and target cells are set up. These included DD regulated CAR expressing T cells co cultured with K562 cells (in the presence or absence of the ligand), T cells co cultured with K562 cells expressing CD19 and K562 cells expressing CD19 without T cell co culture. Additional controls include target cells only; untransduced T cells; T cells transduced with empty vector. The T cells utilized for this

experiment are expanded for 11 days using protocols described in previous examples, frozen, thawed and transduced with the CD19 CAR constructs. Target cells are treated with Mitomycin C to prevent their proliferation. The K562 cells are fluorescently labelled with NucLight Red and co cultured with T cells for 300 hours. Cell death is monitored by labelling cells with Annexin V and the cell death in target K562 cells is monitored by evaluating cells that are positive for both Annexin V and NucLight Red using the IncuCyte® Live Cell Analysis System (Essen Biosciences, Ann Arbor, MI). Target cell killing is expected with the DD regulated CAR constructs only in the presence of ligand and when K562 target cells ectopically expressing CD19 are utilized. No cell killing is expected in untreated controls of the same co-culture set up and when T cells are co cultured with parental K562 cells that do not express CD19 in the presence or absence of ligand. Constitutive constructs are predicted to show cell killing both in the presence of ligand. Cell killing is also not expected in cocultures with untransduced T cells, T cells transduced with empty vector; and cultures of target cells only.

Example 35. Effect of Ligand on Cytokine Expression

[00655] To study the effect of ligand on the expression of cytokines in regulated CD19 CAR constructs, T cell populations were transduced with empty vector, OT-CD19-017, OT-CD19-023, OT-CD19-024, or OT-CD19-025. $5x10^4$ transduced T cells were co cultured for 48 hours at an E:T (effector to target cell) ratio of 5:1 in the presence or absence of TMP or Shield-1. Target cells were treated with 50 ug/ml of Mitomycin C to prevent their proliferation. The cytokine concentration of IFNγ and IL2 in the media supernatant were determined for each construct using MSD V-PLEX Proinflammatory Panel 1 Human Kit. The readout was obtained using a MESO QuickPlex SQ120. As shown in Figure 31A, a 6 fold increase in IFNγ concentration was seen with the addition of ligand for OT-CD19-024, and a 2 fold increase in IFNγ concentration was seen with the addition of ligand for OT-CD19-025. As shown in Figure 31B, a 6 fold increase in IL2 was seen for OT-CD19-024 with the addition of ligand and a 9 fold increase was seen for OT-CD19-025 with TMP.

Example 36. *In vivo* time course study of IL12 levels in mice

[00656] HCT116 parental cells or cells transduced with IL12 constructs (OT-IL12-020, OT-IL12-026, or OT-IL12-029) were injected into immune compromised CD1 nude mice (n=4 per group) according to the study design in Table 30 below.

Table 30. Study Design

HCT116 cells	Day 15 Dose	Concentration	Route of Day 15 Dose
OT-IL12-026	Vehicle	n/a	Intraperitoneal
	Shield-1 (1x)	10 mg/kg	Intraperitoneal
	Shield-1 (3x, 2h apart)	10 mg/kg	Intraperitoneal
OT-IL12-029	Vehicle	n/a	Intraperitoneal
	Shield-1 (1x)	10 mg/kg	Intraperitoneal
	Shield-1 (3x, 2h apart)	10 mg/kg	Intraperitoneal
OT-IL12-020	Vehicle	n/a	Intraperitoneal
	Shield-1 (1x)	10 mg/kg	Intraperitoneal
Parental	Vehicle	n/a	Intraperitoneal
	Shield-1 (1x)	10 mg/kg	Intraperitoneal

[00657] The mice were bled (blood harvested for plasma PK and IL12 MSD) at day 14 after subcutaneous injection of $5x10^6$ cells (day 0), and 6, 10, and 24 hours post the day 15 dosing. At the end of the study, tumor and kidneys were minced with the razor in 500 ul PBS, spun down, and supernatant isolated for IL12 Meso Scale Diagnostic(MSD) assay.

[00658] As shown in Figure 32A, the basal plasma IL12 levels of the DD constructs were high, but the OT-IL12-026 and OT-IL12-029 constructs were still 100-fold lower than the constitutive (OT-IL12-020) construct. When Figure 32A is shown as fold change from pre-dose plasma, OT-IL12-026 shows regulation at 6 and 10 hours. Figures 32B and 32C show that IL12 is detectable in kidney (Figure 32B) and tumor (Figure 32C) and the levels coordinate with plasma levels.

Example 37. *In vivo* time course study of IL12 levels in mice

[00659] HCT116 parental cells or cells transduced with IL12 constructs (OT-IL12-020, OT-IL12-026) were injected subcutaneously into Matrigel plus in female NSG mice (implant 200 ul matrigel plug with $1x10^7$ cells) (n=4) according to the study design in Table 31 below.

Table 31. Study Design

HCT116 cells	Dose	Harvest Plug	Coverage above EC50
OT-IL12-026	Vehicle (1x)	8 hours after 1st dose	-
	Vehicle (1x)	24 hours after 1st dose	-
	AquaShield-100 mg/kg (1x)	8 hours after 1st dose	4 hours
	AquaShield-100 mg/kg (2x, 4 hours between doses)	8 hours after 1st dose	8 hours
	AquaShield-100 mg/kg (1x)	24 hours after 1st dose	4 hours
	AquaShield-100 mg/kg (2x, 4 hours between doses)	24 hours after 1st dose	8 hours
	AquaShield-100 mg/kg (3x, 4 hours between doses)	24 hours after 1st dose	12 hours
OT-IL12-020	Vehicle (1x)	24 hours after 1st dose	-
Parental	Vehicle (1x)	24 hours after 1st dose	-

[00660] Terminal collection of plasma (for IL12 MSD), plug supernatants and kidneys were collected. As shown in Figure 33A, regulation of IL12 was achieved *in vivo* with high dose Aquashield. There was less regulation observed in the plasma (Figure 33B) and there was some flexi-IL12 detected in the kidneys (Figure 33C).

Example 38. Shield-1 Can Induce ~40-50x Increases in IL12 Production by Primary Human T Cells Transduced with the IL12-026 Construct

[00661] On Day 0, primary human T cells were stimulated with Dynabeads (T-expander CD3/CD28) at a 3:1 bead:cell ratio. The next day, lentiviruses (empty vector (pLVX-EF1a-IRES-Puro), OT-IL12-020 (constitutive), or OT-IL12-026 (regulated)) were added at a multiplicity of infection (MOI) of 10 in the presence of LentiBOOST and 5% FBS. On day 2, the cells were washed to remove the LentiBOOST and the bead:cell ratio was reduced to 1:3, and fresh 10% media and IL2 were added. On days 6, 9, and 13 the cells were counted for equal cell number plating, media replaced, ligand was added, and cells were either left unstimulated or restimulated with soluble ImmunoCult™ Human CD3/CD28 T Cell Activator (StemCell Technologies). After overnight incubation (on days 7, 10, and 14), the supernatants were collected for IL12p40 and p70 MSD assay, and transduction efficiency was analyzed by FACS. OT-IL12-026 T cells were found to be 7% transduced, and OT-IL12-020 (constitutive) T cells were 13% transduced on day 7. Restimulation was shown to increase the expression of IL12 (Figure 34A). Ligand increased production of IL12 by 10-day expanded OT-IL12-026 expressing T cells by 40-50 fold (Figure 34B and Figure 34C).

Example 39. Dose Response of Shield-1 on Transduced T Cells

[00662] Human T cells were activated with CD3/CD28 Dynabeads (Life Technologies) for 1 day prior to transduction with lentiviruses (OT-IL12-026 or vector control), followed by 12-13 days of expansion in culture. T cells that had been transduced with different amounts of virus (4-40 MOI) were exposed to either a dose response of Shield-1 for 24h (left panel). T cells that had been transduced at an MOI of 14 were treated with 1uM Shield-1 or vehicle control for increasing amounts of time (right panel). The levels of IL12 that had accumulated in the supernatants (from 100,000 cells per 200uL media) were measured using human IL12p40 MSD V-plex assay kits (Meso Scale Discovery).

[00663] From the analysis, it was shown that the increase in IL12 production by T cells expressing OT-IL12-026 is dose responsive to the ligand, Shield-1 Figure 35A, and accumulates over time Figure 35B.

Example 40. *In Vivo* Dose Response, and Repeat Dosing of AquaShield in NSG Mice with Transferred T Cells Expressing OT-IL12-026

[00664] Primary human T cells were stimulated with Dynabeads (T-expander CD3/CD28) at a 3:1 bead:cell ratio. The next day, lentiviruses (OT-IL12-020 (constitutive), OT-IL12-026 (regulated), or vector control) were added at a multiplicity of infection (MOI) of 10 in the presence of LentiBOOST and 5% FBS. The following day, T cells were washed to remove the LentiBOOST and the bead:cell ratio was reduced to 1:3, and fresh 10% media and IL2 were added. The T cells were expanded for a total of 10 days, and then $25x10^6$ vector control or OT-IL12-026 transduced T cells or $10x10^6$ constitutive OT-IL12-020 transduced T cells were transferred into NSG mice (study day 0). Three days after cell transfer, the animals were dosed with either vehicle or AquaShield (10, 50 or 100mg/kg). Blood was sampled for plasma analysis of IL12p70 by MSD assay at 0, 4, 8, and 24h post dosing (Figure 36A). Clear dose responsive increases in plasma IL12 was observed.

[00665] On day 5 post T cell transfer, animals were dosed a second time with AquaShield (Figure 36B). A second increase in plasma IL12 was observed upon repeat dosing with AquaShield.

Example 41. *In Vivo* Regulation of DD-12 Expressed in T Cells

[00666] To determine whether ligand can stabilize DD-IL12 *in vivo* upon sequential dosing of AquaShield, T cells are transduced with DD-IL12-expressing constructs (OT-IL12-020 or OT-IL12-026) and implanted into mice (n=4 per group) (day 0) as outlined in the study design below.

Table 32. Study Design

Group	Description
A	Empty Vector, Day 3-6: oral vehicle daily for 4 days
B	OT-IL12-020, Day 3-6: oral vehicle daily for 4 days
C	OT-IL12-026: Day 3-6: oral vehicle daily for 4 days
D	OT-IL12-026, Day 3-6: Aquashield 50 mg/kg orally daily for 4 days
E	OT-IL12-026, Day 4 and 6: Aquashield 50 mg/kg orally
F	Empty Vector, Day 5 and 10: oral vehicle
G	OT-IL12-020, Day 5 and 10: oral vehicle
H	OT-IL12-026, Day 5 and 10: oral vehicle
I	OT-IL12-026, Day 5 and 10: Aquashield 50 mg/kg orally
J	OT-IL12-026, Day 10: Aquashield 50 mg/kg orally

[00667] For each group, a pre-bleed sample is collected as well as samples at 4 hours and 24 hours after each dose. At the end of the study, tissue and organ samples are collected. FACS analysis is conducted to determine cell numbers and Th1 markers.

[00668] On Day 0, primary human T cells were stimulated with Dynabeads (T-expander CD3/CD28) at a 3:1 bead:cell ratio. The next day, lentiviruses (empty vector (pLVX-EF1a-IRES-Puro), OT-IL12-020 (constitutive), or OT-IL12-026 (regulated)) were added at a multiplicity of infection (MOI) of 10 in the presence of LentiBOOST and 5% FBS. On day 2, the cells were washed to remove the LentiBOOST and the bead:cell ratio was reduced to 1:3, and fresh 10% media and IL2 were added.

[00669] In vitro evaluation of these cells is shown under Figure 37A-37C.

[00670] After 10 days of expansion, T cells were injected into NSG mice (12×10^6 cells injected, cells were 15% (constitutive) and 7.5% (regulated) IL12 positive by FACS). For each group, a pre-bleed sample was collected as well as plasma samples at 4 hours and 24 hours after each dose. At the end of the study, tissue and organ samples are collected. FACS analysis was conducted to determine T cell numbers in the blood and to assess Th1 phenotypic markers.

[00671] As shown in Figure 37A, IL12 expression in response to sequential pulsed doses of ligand (50 mg/kg Aquashield administered orally on day 4 and 6 (50 mpk Aquashield q48hr)) was elevated in the plasma of mice with T cells expressing OT-IL12-026 as compared to the vehicle treated controls. T cells expressing the empty vector control did not produce IL12. T cells transduced with OT-IL12-020 (IL12-020), the constitutive control, produced IL12 throughout the time course.

[00672] In Figure 37B, elevated plasma IL12 expression in response to sequential pulsed doses of ligand (50 mg/kg Aquashield administered orally for 4 days (day 3-6) (50 mpk Aquashield QDx4)) was seen in mice bearing OT-IL12-026 expressing T cells as compared to the vehicle treated controls. Cells transduced with OT-IL12-020 (IL12-020), the constitutive control, produced IL12 throughout the time course.

[00673] Figure 37C shows the IL12 expression over 11 days for the constitutive construct OT-IL12-020 (IL12-020). Ligand-regulated expression of IL12 from T cells expressing DD-IL12 from the construct OT-IL12-026 was seen in mice treated with 50 mg/kg Aquashield administered orally on day 5 and 10 (50 mpk Aquashield d5/10). T cells expressing the empty vector control did not produce IL12.

[00674] Figure 37D shows ligand-induced regulation of plasma IL12 expression from T cells expressing DD-IL12 from the construct OT-IL12-026 when mice were treated orally with 50 mg/kg Aquashield on day 10 (50 mpk Aquashield d10). The single ligand pulse increased plasma IL12 levels over those detected in vehicle-treated control mice harboring OT-IL12-026 expressing T cells.

[00675] Regulation of IL12 for all constructs shown in Figures 37A-37D did not impact IFNγ levels, instead the levels of IFNγ gradually rose over time. This is likely due to the exposure of the T cells to IL12 in culture during the *in vitro* expansion phase. However, ligand-induced regulation of IL12 increased granzyme B (GrB) (Figure 37E) and perforin expression (Figure 29F) by CD8+ T cells *in vivo* at day 7 post *in vivo* T cell transfer.

Example 42. Effect of PGK Promoter and N-terminal FKBP

[00676] HEK293T cells were transiently transfected with Lipofectamine 3000 and 2ug plasmid DNA each of: OT-IL12-019 (PGK promoter), OT-IL12-020 (EF1alpha promoter), OT-IL12-025 (PGK promoter, C-terminal FKBP domain), OT-IL12-026 (EF1alpha promoter, C-terminal FKBP domain), OT-IL12-046 (N-terminal FKBP). Ligand (1uM Shield-1) was added one day after transfection, and the cells were further cultured for 2 more days. IL12 secretion into the supernatants was quantitated by IL12p40 MSD assay. Genomic DNA (gDNA) and messenger RNA (mRNA) was purified from the cells. The levels of construct DNA integration into the cellular genome and levels of IL12 mRNA expression were quantitated by qPCR using primers specific to the WPRE element and IL12 within the respective constructs.

[00677] The gDNA qPCR analysis demonstrated that the FKBP DD-containing constructs had integrated to similar levels within the cellular genomes, and that the PGK promoter, as expected, generated less IL12 mRNA expression than the EF1alpha promoter (Figure 38A).

[00678] Due to the lower levels of mRNA transcription induced by the PGK promoter, the IL12p40 MSD assay also demonstrated that the PGK promoter reduced both basal and peak IL12 levels of secretion as compared to the construct using the EF1alpha promoter. The lower basal levels of IL12 production downstream of the PGK promoter resulted in ~2 fold improved ligand-induced IL12 regulation as compared with the construct with the EF1alpha promoter (Figure 38B). More specifically, the ligand-induced regulation of IL12 expression increased from 6-fold to 13-fold with the change from the EF1alpha to the PGK promoter, respectively.

[00679] Constructs containing FKBP either at the N-terminus or at the C-terminus of IL12 were integrated similarly into the cellular genome and generated similar levels of mRNA (Figure 38A). However, while C-terminal containing FKBP constructs regulate IL12 expression, the N-terminal-containing FKBP construct failed to regulate IL12 expression (Figure 38B).

Example 43. Kinetics of ligand-dependent stabilization of DD-IL15- IL15Ra

[00680] The on/off kinetics of ligand-dependent stabilization of DD-IL15-IL15Ra was measured in CD4 positive T cells. T cells were activated with CD3/CD28 beads at 3:1 bead to T cell ratio in 24-well plates for 24 hrs. Lentivirus was added to wells in the presence of

LentiBoost reagent, and cells were incubated for another 24 hrs and washed. Cells were resuspended in fresh media, and media was added every 2-3 days to expand and maintain cells at 0.5-1x10^6/ml. After 7 days of expansion, T cells transduced with the ecDHFR DD-IL15-IL15Ra fusion construct (OT-IL15-009) were treated with 100μM ecDHFR ligand Trimethoprim (TMP) or vehicle control, DMSO. At multiple time points (i.e., 1, 2, 4, 6, 8, 15, 22 and 24 hrs) after TMP treatment, the transduced T cells were collected and analyzed for IL15Ra surface expression using anti-IL15Ra antibodies by flow cytometry. Untransduced T cells were used as a negative control. The T cells were sorted into CD4 positive and CD8 positive populations and the percentage of IL15Ra positive CD4 positive T cells was analyzed. Figure 39 shows the kinetics of surface expression of IL15Ra on CD4 T cells after TMP treatment. Among the CD4 positive T cells transduced with the OT-IL15-009 construct, the proportion of cells with surface expression of IL15Ra remained similar for both TMP treated and DMSO treated cells until 2 hrs after TMP treatment, and was comparable to that of untransduced cells. However, from 4 hrs after TMP treatment, the cells transduced with the OT-IL15-009 construct and treated with TMP exhibited an increased proportion of cells with surface expression of IL15Ra. This trend was observed until 22 hours after treatment with TMP. The CD4 positive T cells with surface-expressed IL15Ra cells constituted ~1% of untransduced cells, indicating that the proportion of cells that expressed endogenous IL15Ra is low.

Example 44. Ligand-dependent stabilization of DD-IL15-IL15Ra fusion molecules *in vivo*

[00681] To examine whether ligand treatment induces stabilization of the DD-IL15-IL15Ra fusion molecules *in vivo*, HCT116 cells transduced with the OT-IL15-009 construct were implanted subcutaneously in BALB/c nude mice and treated with TMP. TMP was orally administered to mice at a dose of 100 mg/kg, twice a day for 11 days after implantation, followed by administration of TMP at the dose of 300 mg/kg, twice a day for 6 days. As a negative control, separate mice implanted with HCT116 cells transduced with the OT-IL15-009 construct were treated with the vehicle twice a day for 17 days. At 4 hrs after the last dosing of TMP or the vehicle control, tumors were harvested from the mice and analyzed for the levels of IL15-IL15Ra fusion molecules by western blotting. As shown in Figure 40, HCT116 tumors harvested from mice treated with TMP exhibited elevated levels of IL15-IL15Ra expression, compared to tumors treated with the vehicle. The GAPDH level was analyzed as a loading control. These data show that administration of ligand enabled stabilization of the DD-IL15-IL15Ra fusion molecule *in vivo*.

[00682] Consistent with the efficacy of TMP-dependent IL15-IL15Ra stabilization *in vivo*, elevated levels of TMP (399.38 ng/g tumor) were observed in HCT116 tumors harvested from

mice treated with TMP for 17 days. The levels of TMP associated with HCT116 tumors were considerably higher than those observed in mouse plasma at day 3 (15.67 ng/ml plasma) and at day 17 (99.5 ng/ml plasma), indicating that the orally administered TMP was successfully delivered to and accumulated in HCT116 tumors implanted in mice.

Example 45. Shedding resistant IL15-IL15Ra constructs

[00683] To maintain the efficiency of the trans-presentation of IL15 via the IL15-IL15Ra fusion molecule, the IL15-IL15Ra shedding needs to be prevented. For this purpose, new DD-IL15-IL15Ra and constitutive IL15-IL15Ra constructs are designed through a variety of modifications on the IL15-IL15Ra fusion molecule. For example, the IL15 molecule or the IL15Ra molecule is truncated or mutated to remove presumable cleavage sites. IL15Ra has a cleavage site (PQGHSDTT from the position 168 to 175 of SEQ ID NO. 803) in the extracellular domain immediately distal to the transmembrane domain of the receptor, as described by Bergamaschi C *et al.* (2008). J Biol Chem ;283(7):4189-99; Anthony SM *et al.* (2015). PLoS One. 10(3): e0120274), and International Patent Application Publication Nos. WO2014066527 and WO2009002562 (the contents of each of which are incorporated herein by reference in their entirety). Tumor necrosis factor-alpha-converting enzyme (TACE/ADAM17) has been implicated as a protease that cleaves between glycine (at the position 170 of SEQ ID NO. 803) and histidine (at the position 171 of SEQ ID NO. 803) and generates a naturally occurring soluble form of IL15Ra. The same mechanism can be responsible for the IL15-IL15Ra shedding. Hence, the cleavage site of IL15Ra is mutated such that cleavage by an endogenous protease is prevented. The mutation of the cleavage site is introduced by substitution, insertion or deletion of amino acid residues. The IL15-IL15Ra fusion molecule is also modified such that the full-length or truncated IL15-IL15Ra fusion molecule is fused to heterologous hinge domains and/or heterologous transmembrane domains. As non-limiting examples, variants of IL15Ra can be utilized. Additionally, the length and sequence of the linkers that connect IL15 and IL15Ra are modified.

[00684] To confirm that the modifications on the IL15-IL15Ra fusion molecule prevent shedding, the new DD-IL15-IL15Ra or constitutive IL15-IL15Ra constructs are introduced into HCT-116 cells. Surface expression of IL15 and IL15Ra on the HCT-116 cells is examined by flow cytometry using anti-IL15 and IL15Ra antibodies to assess surface IL15-IL15Ra shedding. The presence or absence of IL15 in the cell culture supernatant is also analyzed by MSD assay. As a functional assay based on the sensitivity of NK cell activation by shed IL15 in tumor supernatant, the transwell assay is conducted using HCT-116 cells transduced with new DD-IL15-IL15Ra or constitutive IL15-IL15Ra expressing constructs and NK cells. The new DD-

IL15-IL15Ra-expressing constructs that do not induce activation of NK cells in the presence of ligand and the new constitutive IL15-IL15Ra-expressing constructs that do not induce activation of NK cells are chosen for use in future experiments.

Example 46. Regulated expression of IL15-IL15Ra fusion molecule with C-terminal DD

[00685] A fusion molecule is generated by fusing membrane bound IL15, IL15 Receptor alpha subunit (IL15Ra) and a human DHFR (DD). These fusion molecules were cloned into pLVX-EF1a-IRES-Puro vector.

[00686] To test ligand dependent IL15-IL15Ra 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 then transfected with 100ng of constitutive IL15-IL15Ra (OT-IL15-008) or DD linked IL15-IL15Ra (OT-IL15-037 or OT-IL15-040) using Lipofectamine 2000 and incubated for 24 hrs. Following the incubation, media is exchanged for growth medium with or without 50μM Trimethoprim (TMP) and further incubated for 48 hrs. Cells were harvested and IL15 levels are analyzed via western blotting using human IL15 antibody (Abcam, Cambridge, UK). The molecular weight of IL15Ra in OT-IL15-037 and OT-IL15-040 appeared to be the same as OT-IL15-008.

[00687] To test if IL15 is shed into the media, supernatant from HEK293 cells expressing IL15-IL15Ra fusion constructs was subject to immunoassays such as MSD (Rockville, Maryland). 48 hours after transfection, cells were analyzed and, as expected, constitutive IL15-IL15Ra construct OT-IL15-008 showed high surface expression of IL15 in the presence and absence of ligand. OT-IL15-037 and OT-IL15-040 showed the ligand (Trimethoprim) dependent surface expression of IL15 and IL15Ra (Figure 41). The detection of membrane bound IL15-IL15Ra fusion constructs in the supernatant suggests that IL15 constructs are likely shed from the cell surface.

Example 47. Effect TMP exposure to TMP *in vitro* on membrane bound IL15 expression

[00688] In order to determine if the dose and time of exposure to TMP *in vitro* influenced membrane bound IL15 expression, an *in vitro* dose response study was conducted with T cells expressing OT-IL15-073.For this purpose, T cells were activated with CD3/CD28 beads at 3:1 bead to T cell ratio in 24-well plates for 24 hrs. Lentivirus was added to wells. After 24 hrs, fresh media was added every 2-3 days to expand cells while maintaining cells at 0.5-1x10^6/ml. On day 11 of expansion. T cells treated with TMP starting at 100 uM, 10x dilutions and 9 points were analyzed after 2 hours in culture (washed 3x after TMP addition, fresh media added without TMP for 22 hours), 6 hours in culture, or 24 hours in culture and the results are shown in Figure

42A. As shown in Figure 42B and Table 33, this study showed that TMP ligand regulates membrane bound IL15 expression and the dose and time of exposure to TMP *in vitro* influences membrane bound IL15 expression.

Table 33. Membrane Bound IL15 Expression

TMP Treatment	EC50, uM (%IL15+IL15Ra+)	EC90, uM (%IL15+IL15Ra+)	EC50, uM (total IL15 MFI)	EC90, uM (total IL15 MFI)
24 hour	0.035	0.255	0.063	0.75
24 hour (wash at 2 hours)	11.5	59.6	11.1	66.9
6 hours	0.021	0.81	0.030	0.88

Example 48. Regulated membrane bound IL15 expression *in vivo*

[00689] To evaluate regulation of membrane bound IL15 *in vivo*, 2 constructs were selected for evaluation *in vivo*. Four group of T cells were used for this study and are outlined in Table 34. In Table 31, "N" represents the number of mice in each group.

Table 34. T Cell Groups

Group	N	T Cells	Treatment
1	4	Untransduced	-
2	4	OT-IL15-071 (pELNS vector, EF1a promoter, membrane bound IL15 sequence from OT-IL15-008)	-
3	4	OT-IL15-073 (pELNS vector, EF1a promoter, membrane bound IL15 sequence from OT-IL15-009)	Vehicle
4	4	OT-IL15-073 (pELNS vector, EF1a promoter, membrane bound IL15 sequence from OT-IL15-009)	TMP

[00690] The T cells which were to be used as part of the *in vivo* study were evaluated 6 days post transduction, day of implant (day 9 post transduction) and 13 days post transduction and the cells in Groups 2-4 showed expression of the constructs.

[00691] T cells outlined in Table 31 were administered to mice by intravenous administration (3.9×10^6 cells per mouse implanted). On day 3 the mice were dosed with 500 mg/kg of TMP 3 times (4 hours between doses) and bled 2 hours after each dose. The mice were again bled on day 4, 24 hours after the first TMP dose.

[00692] Figures 43A-43C show the expression of membrane bound IL15, 2, 6, 10, and 24 hours after the first TMP dose, using IL15 staining (Figure 43A), IL15Ra staining (Figure 43B), and IL15/IL15Ra double ++ staining (Figure 43C). Figure 43D are FACS plots for each mouse 10 hours after the first TMP dose. Figure 43E shows the expression of membrane bound IL15 in blood 2, 6, 10, and 24 hours after the first TMP dose and Figure 43F shows the plasma TMP levels 2, 6, 10, and 24 hours after the first TMP dose.

Example 49. Effect of long term intraperitoneal (IP) or oral (PO) TMP dosing on T cell function

[00693] In this study, T cells transduced with OT-IL15-071 or OT-IL15-073 (no lentiBoost) were administered intravenously to mice (15×10^6 per mouse). 6 study groups were evaluated for this study: (1) untransduced, (2) OT-IL15-071 T cells, (3) OT-IL15-073 PO vehicle, (4) OT-IL15-073 PO TMP 500 mg/kg), (5) OT-IL15-073 IP vehicle, and (6) OT-IL15-073 IP TMP 300 mg/kg. The study design is shown in Table 35. PO dosing is 500 mg/kg TMP in 0.1M citrate and IP dosing is 300 mg/kg TMP lactate in water.

Table 35. Study Design

Timepoint	Dose	Sample Collection
Day -3	Inject T cells in mice by IV administration	-
Day 0	PO 1x or IP 1x	-
4 hours	PO 1x	-
6 hours	-	Bleed (survival)
24 hours	PO 2x or IP 1x	Bleed (survival)
Day 2	PO 1x or IP 1x	-
Day 3	PO 1x or IP 1x	-
Day 4	PO 2x or IP 1x	-
120 hours	PO 2x or IP 1x	Bleed (survival)
126 hours	-	Bleed (survival)
Day 6	PO 2x or IP 1x	-
Day 7	PO 2x or IP 1x	-
Day 8	-	Bleed (survival)
Day 19	-	Bleed (survival)
Day 25	-	Bleed (terminal)

[00694] The regulated expression in blood was analyzed 6 hours and 24 hours after the first dose, and 6 hours after the 5th dose.

[00695] OT-IL15-071 showed expression of membrane bound IL15 and the untransduced control did not show any expression.

[00696] Regulation of membrane bound IL15 was seen with repeat PO and IP dosing. As seen in Figure 44, regulated expression of membrane bound IL15 was detected 6 hours after the first dose on day 0, and 6 hours after dosing on day 5 (126 hrs) with both PO and IP dosing. There was no increase in expression in mice treated with vehicle.

[00697] 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.

[00698] 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.

2. The composition of claim 1, wherein said at least one immunotherapeutic agent is selected from a chimeric antigen receptor (CAR) and an antibody.

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

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

5. The composition of claim 4, 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, and

(g) NQO2 comprising the amino acid sequence of SEQ ID NO. 7.

6. The composition of claim 5, wherein the parent protein is hDHFR and the DD comprises a mutant protein having:

(a) a single mutation selected from hDHFR (I17V), hDHFR (F59S), hDHFR (N65D), hDHFR (K81R), hDHFR (A107V), hDHFR (Y122I), hDHFR (N127Y), hDHFR (M140I), hDHFR (K185E), hDHFR (N186D), hDHFR (M140I), hDHFR (Amino acid 2-187 of WT; N127Y), hDHFR (Amino acid 2-187 of WT; I17V), hDHFR (Amino acid 2-187 of WT; Y122I), and hDHFR (Amino acid 2-187 of WT; K185E);

(b) a double mutation selected from hDHFR (C7R, Y163C), hDHFR (A10V, H88Y), hDHFR (Q36K, Y122I), hDHFR (M53T, R138I), hDHFR (T57A, I72A), hDHFR (E63G,

I176F), hDHFR (G21T, Y122I), hDHFR (L74N, Y122I), hDHFR (V75F, Y122I), hDHFR (L94A, T147A), DHFR (V121A, Y22I), hDHFR (Y122I, A125F), hDHFR (H131R, E144G), hDHFR (T137R, F143L), hDHFR (Y178H, E18IG), hDHFR (Y183H, K185E), hDHFR (E162G, I176F) hDHFR (Amino acid 2-187 of WT; I17V, Y122I), hDHFR (Amino acid 2-187 of WT; Y122I, M140I), hDHFR (Amino acid 2-187 of WT; N127Y, Y122I), hDHFR (Amino acid 2-187 of WT; E162G, I176F), and hDHFR (Amino acid 2-187 of WT; H131R, E144G), and hDHFR (Amino acid 2-187 of WT; Y122I, A125F);

(c) a triple mutation selected from hDHFR (V9A, S93R, P150L), hDHFR (I8V, K133E, Y163C), hDHFR (L23S, V121A, Y157C), hDHFR (K19E, F89L, E181G), hDHFR (Q36F, N65F, Y122I), hDHFR (G54R, M140V, S168C), hDHFR (V110A, V136M, K177R), hDHFR (Q36F, Y122I, A125F), hDHFR (N49D, F59S, D153G), hDHFR (G21E, I72V, I176T), hDHFR (Amino acid 2-187 of WT; Q36F, Y122I, A125F), hDHFR (Amino acid 2-187 of WT; Y122I, H131R, E144G), hDHFR (Amino acid 2-187 of WT; E31D, F32M, V116I), and hDHFR (Amino acid 2-187 of WT; Q36F, N65F, Y122I); or

(d) a quadruple or higher mutation selected from hDHFR (V2A, R33G, Q36R, L100P, K185R), hDHFR (Amino acid 2-187 of WT; D22S, F32M, R33S, Q36S, N65S), hDHFR (I17N, L98S, K99R, M112T, E151G, E162G, E172G), hDHFR (G16S, I17V, F89L, D96G, K123E, M140V, D146G, K156R), hDHFR (K81R, K99R, L100P, E102G, N108D, K123R, H128R, D142G, F180L, K185E), hDHFR (R138G, D142G, F143S, K156R, K158E, E162G, V166A, K177E, Y178C, K185E, N186S), hDHFR (N14S, P24S, F35L, M53T, K56E, R92G, S93G, N127S, H128Y, F135L, F143S, L159P, L160P, E173A, F180L), hDHFR (F35L, R37G, N65A, L68S, K69E, R71G, L80P, K99G, G117D, L132P, I139V, M140I, D142G, D146G, E173G, D187G), hDHFR (L28P, N30H, M38V, V44A, L68S, N73G, R78G, A97T, K99R, A107T, K109R, D111N, L134P, F135V, T147A, I152V, K158R, E172G, V182A, E184R), 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), 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), and 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).

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

8. The composition of claim 2, wherein the immunotherapeutic agent is a chimeric antigen receptor (CAR).

9. The composition of claim 8, wherein the chimeric antigen receptor (CAR) comprises

(a) an extracellular target moiety;

(b) a transmembrane domain;

(c) an intracellular signaling domain; and

(d) optionally, one or more co-stimulatory domains.

10. The composition of claim 9, wherein the CAR is 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.

11. The composition of claim 9, wherein the extracellular target moiety recognizes a target molecule on the surface of a cancer cell, wherein said target molecule on the surface of the cancer cell is selected from a cancer antigen, a plasma membrane lipid, a receptor and a membrane bound glycoprotein.

12. The composition of any of claims 9-11, 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.

13. The composition of claim 12, wherein the extracellular target moiety is a scFv derived from an antibody that specifically binds a CD19 antigen.

14. The composition of claim 13, wherein the scFv is a CD19 scFv is selected from one that comprises:

(a) a heavy chain variable region having an amino acid sequence independently selected from the group consisting of SEQ ID NO: 49-80, and a light chain variable region having an amino acid sequence independently selected from the group consisting of any of SEQ ID NOs: 81-122; or

(b) an amino acid sequence selected from the group consisting of any of SEQ ID NOs: 123-267 and 624.

15. The composition of claim 9, 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.

16. The composition of claim 15, wherein the intracellular signaling domain of the CAR is a T cell receptor CD3zeta signaling domain comprising the amino acid sequence of SEQ ID NO: 339.

17. The composition of claim 9, wherein the intracellular signaling domain of the CAR is a T cell receptor CD3zeta signaling domain comprising the amino acid sequence of SEQ ID NO: 626 and the co-stimulatory domain is present, said co-stimulatory domain being selected from amino acid sequence of any of SEQ ID NOs: 268-374.

18. The composition of claim 9, wherein the transmembrane domain is derived from any of the members of the group consisting of:

(a) a transmembrane region of an alpha, beta or zeta chain of a T-cell receptor;

(b) the CD3 epsilon chain of a T-cell receptor;

(c) a molecule selected from CD4, CD5, CD8, CD8α, CD9, CD16, CD22, CD33, CD28, CD37, CD45, CD64, CD80, CD86, CD148, DAP 10, EpoRI, GITR, LAG3, ICOS, Her2, OX40 (CD134), 4-1BB (CD137), CD152, CD154, PD-1, or CTLA-4; and

(d) an immunoglobulin selected from IgG1, IgD, IgG4, and an IgG_4 Fc region.

19. The composition of claim 9, wherein the transmembrane domain comprises an amino acid sequence selected from the group consisting of any of SEQ ID NOs: 375-425 and 897-907.

20. The composition of claim 9, wherein the CAR further comprises

(e) a hinge region near the transmembrane domain, said hinge region comprising an amino acid sequence selected from the group consisting of any of SEQ ID NOs: 426-504.

21. The composition of claim 2, wherein the immunotherapeutic agent is an antibody that is specifically immunoreactive to an antigen selected from a tumor specific antigen (TSA), a tumor associated antigen (TAA), or an antigenic epitope.

22. The composition of claim 21, wherein the antigen is an antigenic epitope and said antigenic epitope is CD19.

23. The composition of claim 22, wherein the antibody is selected from one that comprises

(a) a heavy chain variable region having an amino acid sequence independently selected from the group consisting of any of SEQ ID NOs: 49-80 and a light chain variable region having an amino acid sequence independently selected from the group consisting of any of SEQ ID NOs: 81-122; or
(b) an amino acid sequence selected from the group consisting of any of SEQ ID NOs: 123-267.

24. The composition of claim 1 wherein said first effector module comprises the amino acid sequence of any of SEQ ID NO: 635-649, 1005-1010, 1015-1018, and 1215-1231.

25. The composition of claim 24, wherein said first SRE of the effector module stabilizes the immunotherapeutic agent by a stabilization ratio of 1 or more, wherein the stabilization ratio comprises the ratio of expression, function or level of the immunotherapeutic agent in the presence of the stimulus to the expression, function or level of the immunotherapeutic agent in the absence of the stimulus.

26. The composition of any of claims 24-25, wherein the SRE destabilizes the immunotherapeutic agent by a destabilization ratio between 0, and 0.09, wherein the destabilization ratio comprises the ratio of expression, function or level of the immunotherapeutic agent in the absence of the stimulus specific to the SRE to the expression, function or level of the immunotherapeutic agent that is expressed constitutively, and in the absence of the stimulus specific to the SRE.

27. A polynucleotide encoding any of the compositions of claims 1-26.

28. The polynucleotide of claim 27, wherein the polynucleotide is a DNA molecule, or a RNA molecule.

29. The polynucleotide of claim 28, wherein the polynucleotide is an RNA molecule and said RNA molecule is a messenger RNA.

30. The polynucleotide of claim 29, which is chemically modified.

31. The polynucleotide of claim 28, which comprises spatiotemporally selected codons.

32. The polynucleotide of claim 29, further encoding a promoter, a linker, a signal peptide, a tag, a cleavage site and/or a targeting peptide.

33. A vector comprising a polynucleotide of any of claims 27-32.

34. The vector of claim 33, wherein the vector is a viral vector, or a plasmid.

35. The vector of claim 34, which is a viral vector and wherein the 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.

36. An immune cell for adoptive cell transfer (ACT), which expresses any of the compositions of any of claims 1-26, the polynucleotides of any of claims 27-32, and/or is infected or transfected with the vector of any of claims 33-35.

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 immune cell 49which further expresses a composition comprising a second effector module, said second effector module comprising a second SRE linked to a second immunotherapeutic agent wherein the second immunotherapeutic agent is selected from a cytokine, and a cytokine- cytokine receptor fusion.

39. The immune cell of claim 38, wherein the second immunotherapeutic agent is a cytokine.

40. The immune cell of claim 39, wherein the cytokine is IL12 or IL15.

41. The immune cell of claim 38, wherein the second immunotherapeutic agent is a cytokine-cytokine receptor fusion polypeptide.

42. The immune cell of claim 41, wherein the cytokine-cytokine receptor fusion polypeptide is selected from a IL12-IL12 receptor fusion polypeptide, a IL15-IL15 receptor fusion polypeptide, and a IL15-IL15 receptor sushi domain fusion polypeptide.

43. The immune cell of claim 36 or 37, wherein the immune cell is autologous, allogeneic, syngeneic, or xenogeneic in relation to a particular individual subject.

44. A method of reducing a tumor volume or burden in a subject, comprising contacting said subject with a composition of any of claims 1-26, the polynucleotides of any of claims 27-32, the vectors of any of claims 33-35 or the immune cells of any of claims 36-43, wherein the SRE responds to a stimulus and regulates the expression and function of the immunotherapeutic agent.

45. 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 1-26, the polynucleotides of any of claims 27-32, the vectors of any of claims 33-35 or the immune cells of any of claims 36-43.

46. A method of identifying a domain of a CD19 antigen which will not bind the FMC63 antibody (FMC63-distinct CD19 binding domain), said method comprising:

(a) preparing a composition comprising a CD19 antigen,

(b) contacting the composition in (a) with saturating levels of FMC63 antibody,

(c) contact the composition of step (b) with one or more selected members of a library of potential CD19 binders; and

(d) identifying a binding domain on the CD19 antigen based on the differential binding of the selected members of the library of CD19 binders compared to the binding of FMC63.

47. The method of claim 46, wherein said binding domains of the library are generated using phage display techniques with the CD19 antigen as the seed sequence.

48. The method of claim 47, wherein the binding domain is selected from 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 unibody, a nanobody, or an antigen binding region of an antibody, and an antibody fragment.

49. The method of claim 48, wherein the CD19 antigen is selected from a whole or a portion of a human CD19 antigen, and a whole or a portion of a Rhesus CD19 antigen.

50. A chimeric antigen receptor comprising the FMC63-distinct CD19 binding domain obtained according to the method of any of claims 46-49.

51. An effector module comprising a stimulus response element (SRE) operably lined to the chimeric antigen receptor of claim 50.

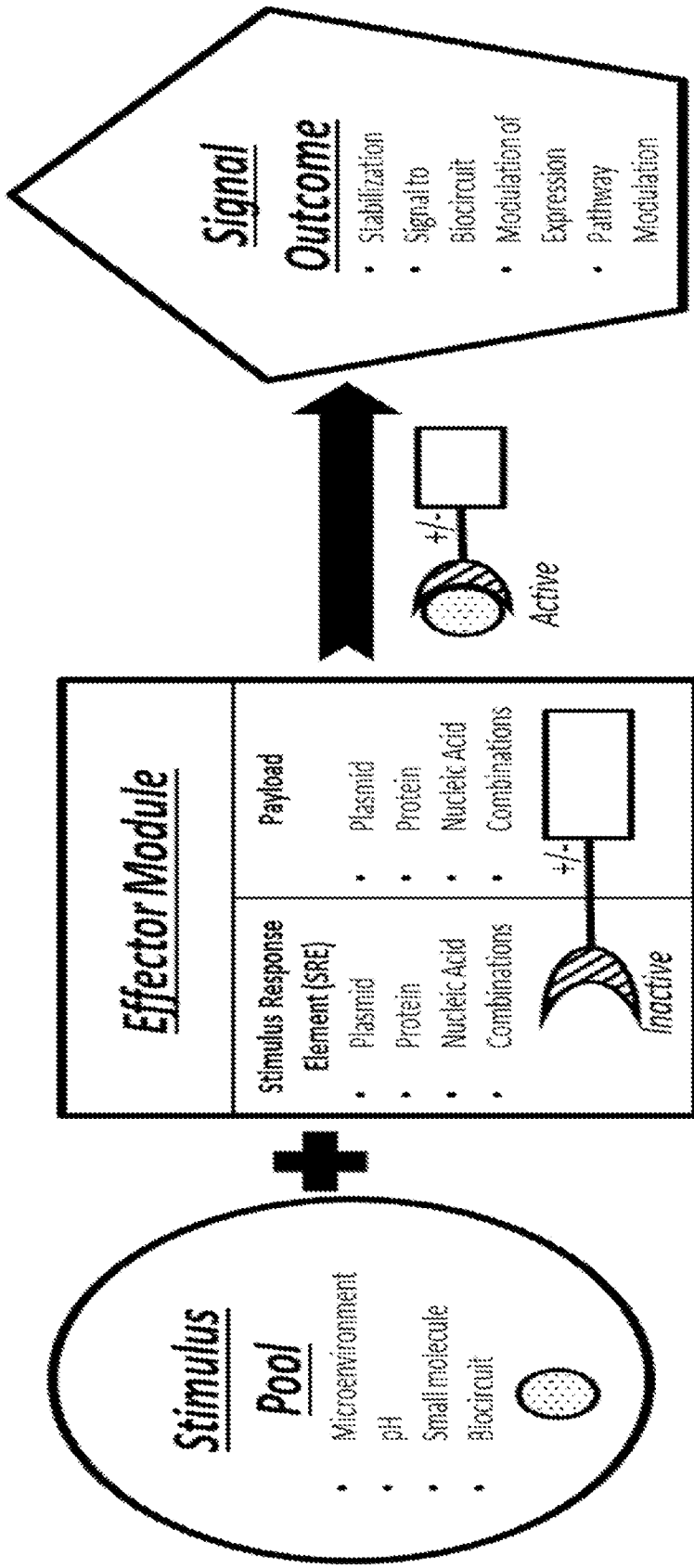

Signal Outcome
• Stabilization
• Signal to Biocircuit
• Modulation of Expression
• Pathway Modulation
+/-
Active
Effector Module
Stimulus Response Element (SRE)
• Plasmid
• Protein
• Nucleic Acid
• Combinations
Payload
• Plasmid
• Protein
• Nucleic Acid
• Combinations
+/-
Inactive
Stimulus Pool
• Microenvironment
• pH
• Small molecule
• Biocircuit

Figure 1

Figure 2

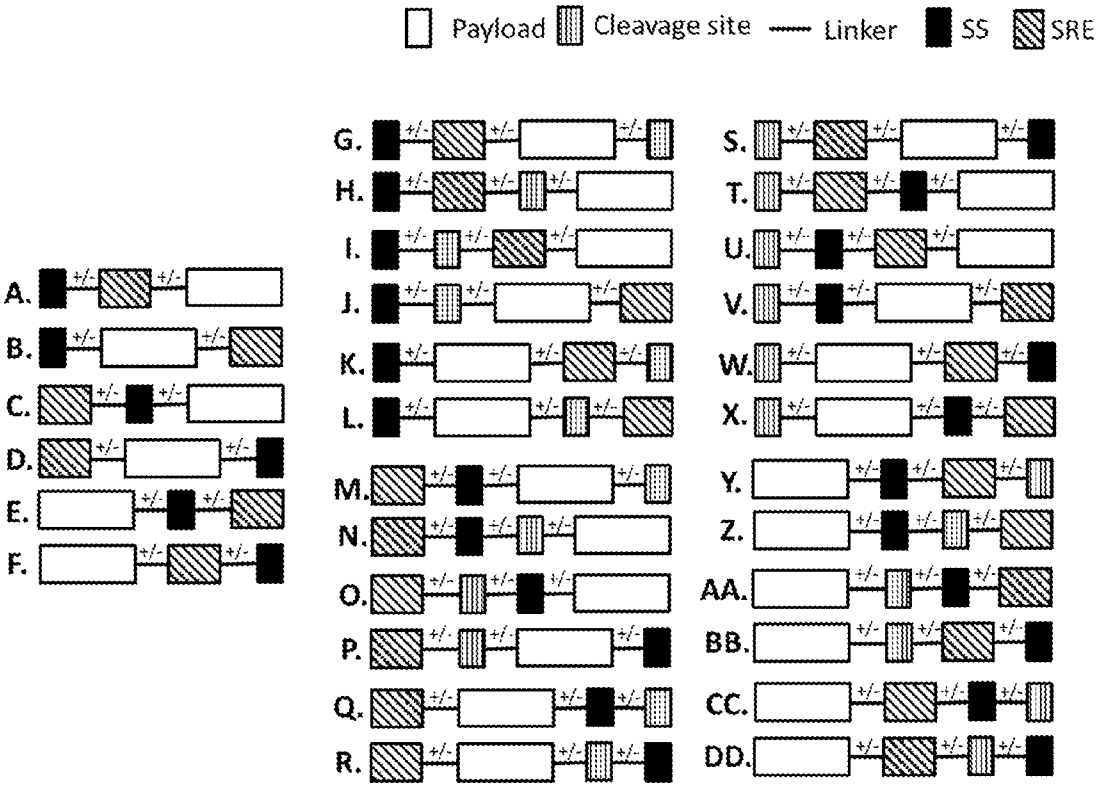


Figure 3

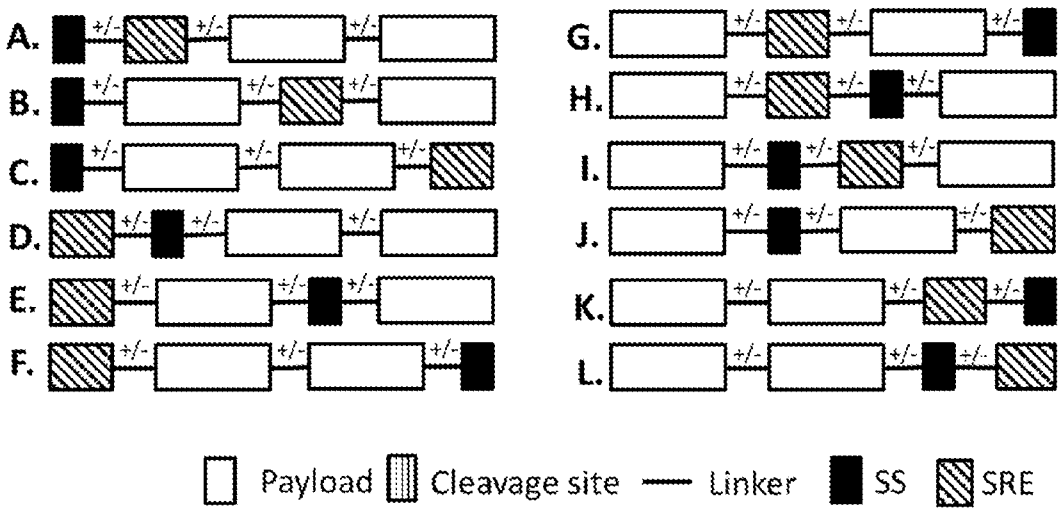


Figure 4

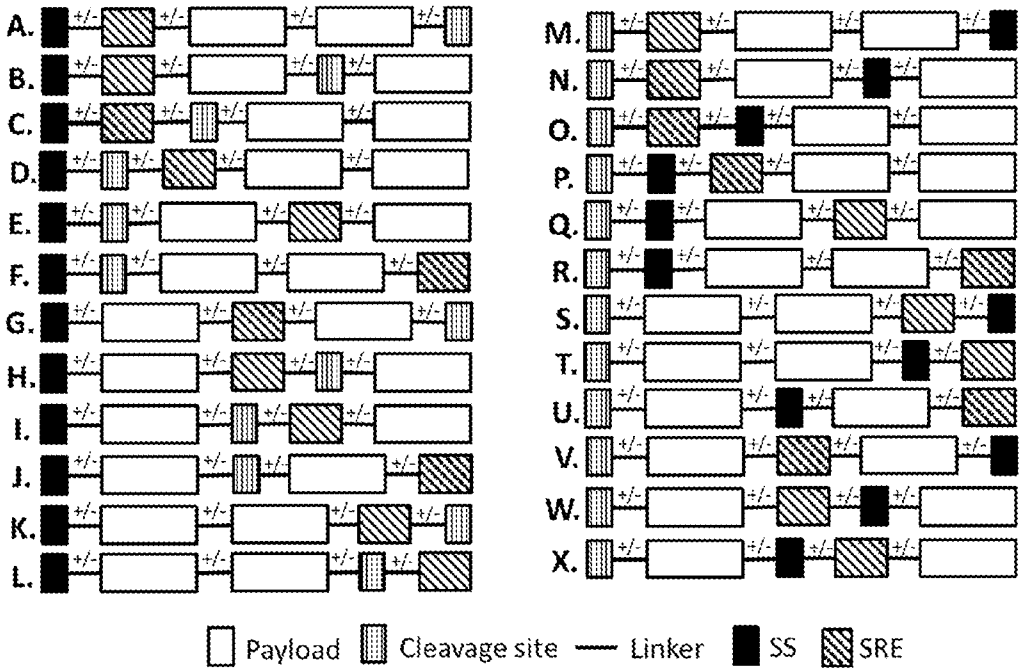


Figure 5

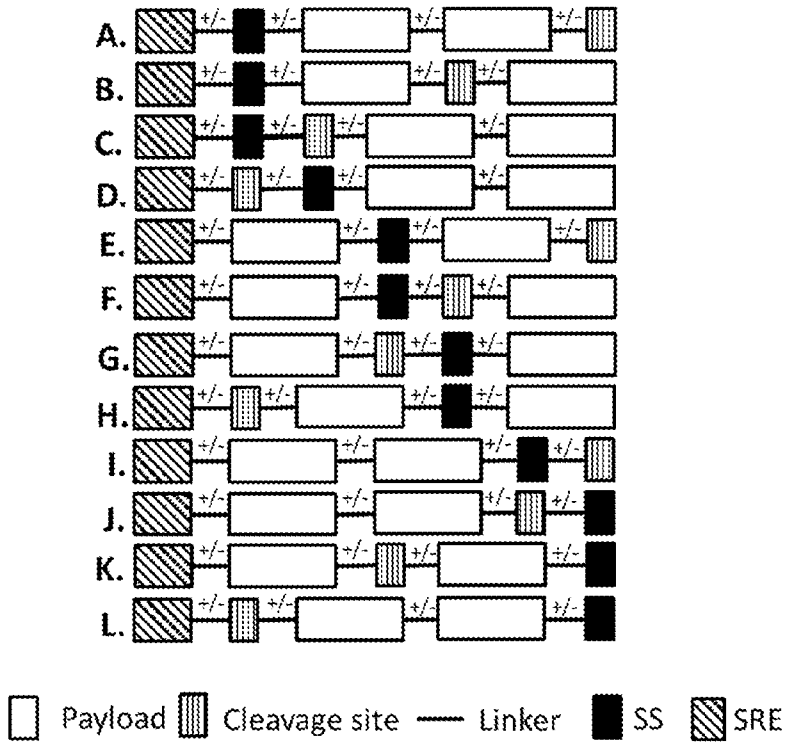


Figure 6

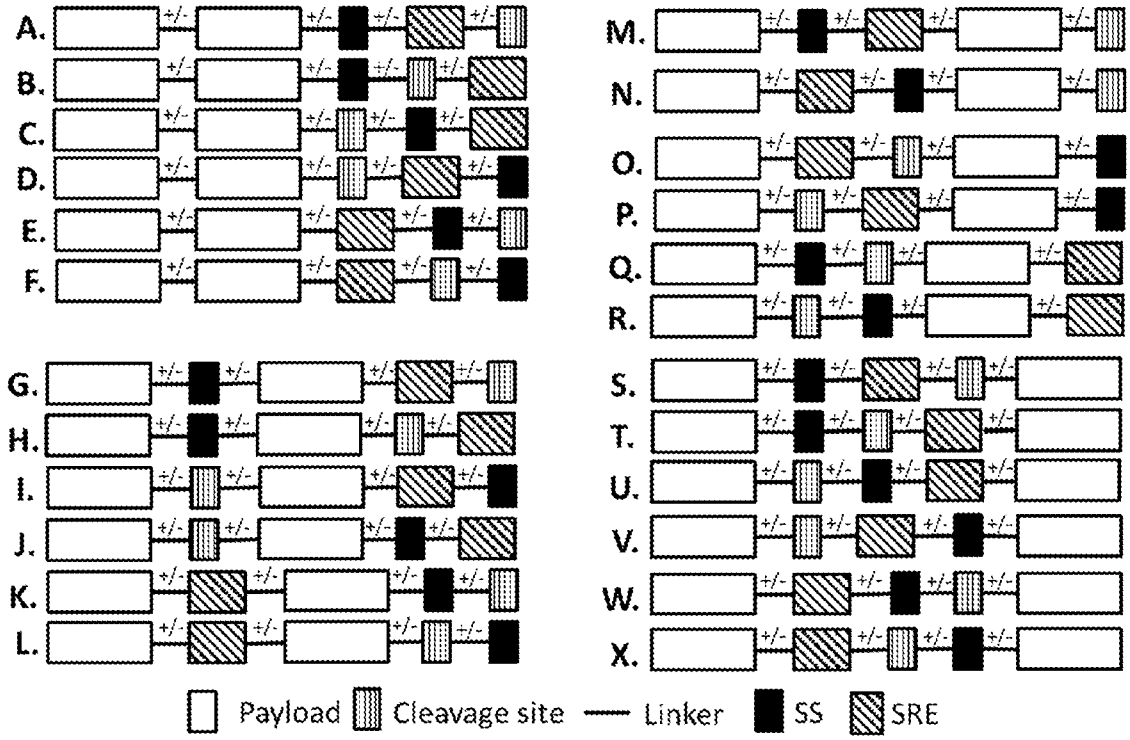


Figure 7

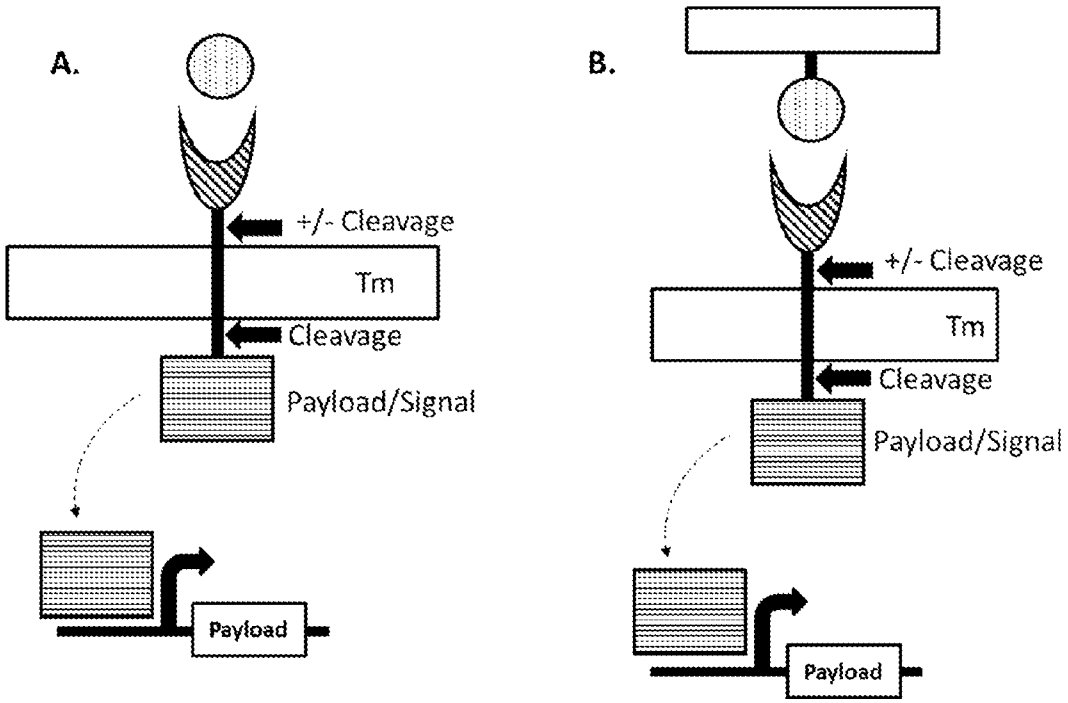


Figure 8

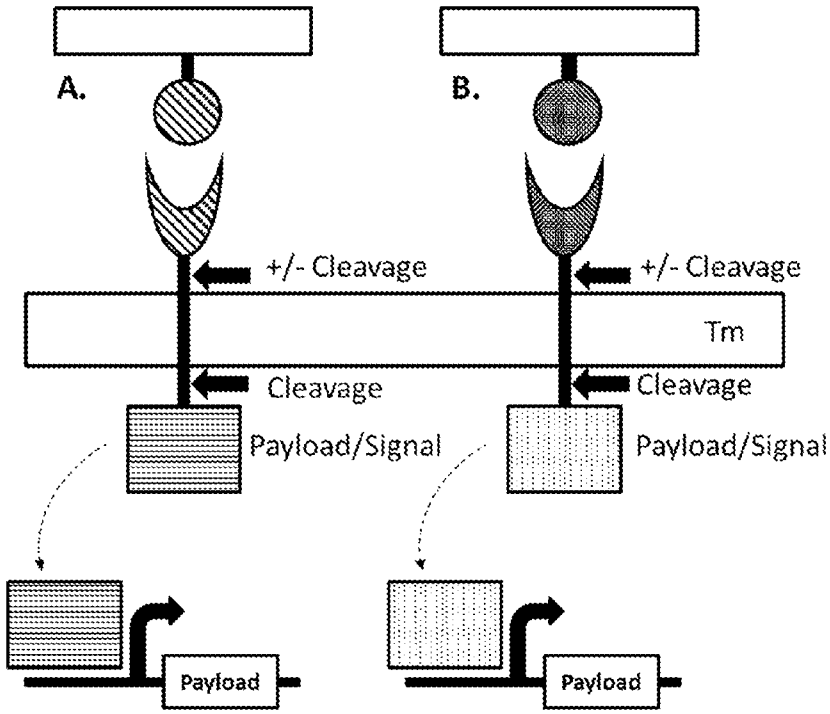


Figure 9

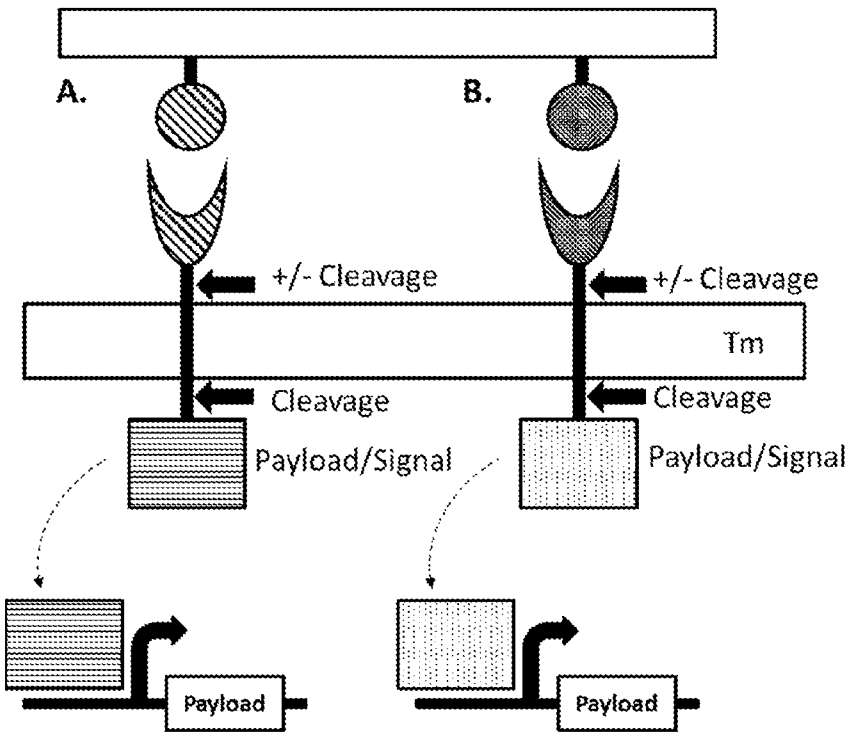


Figure 10

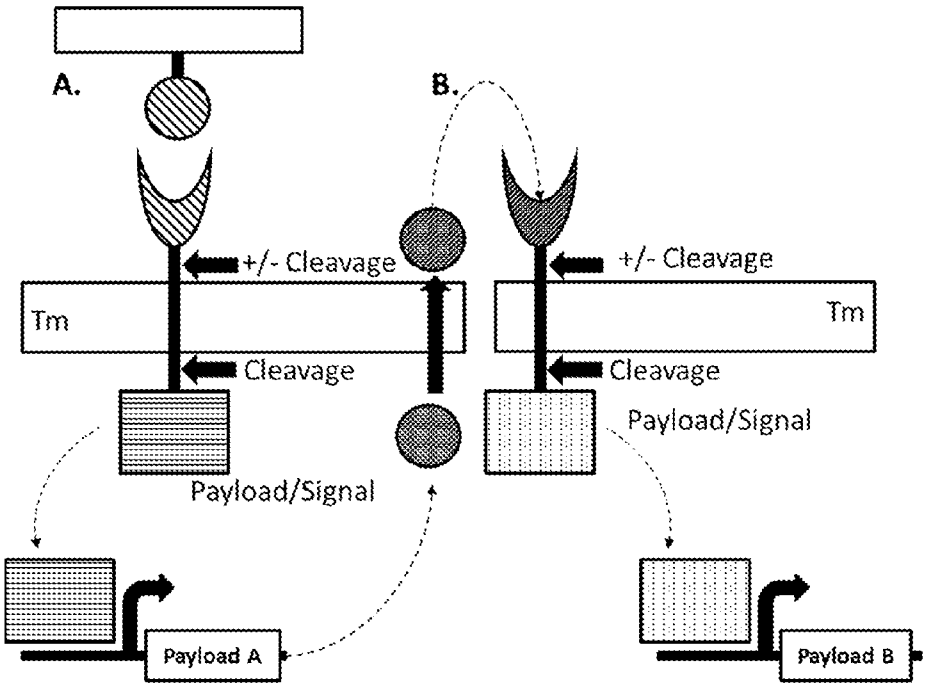


Figure 11

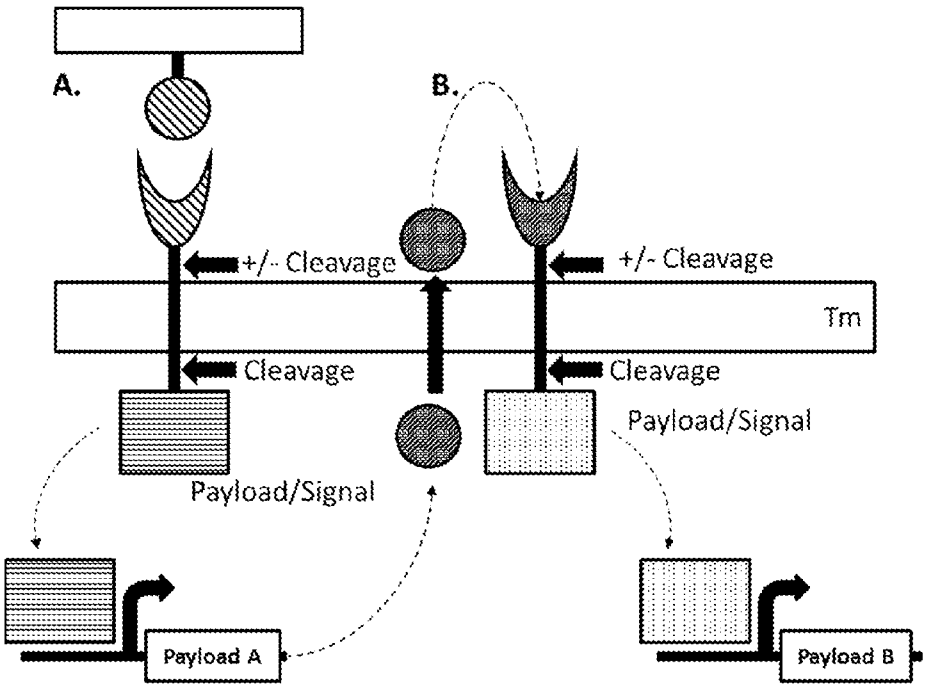


Figure 12

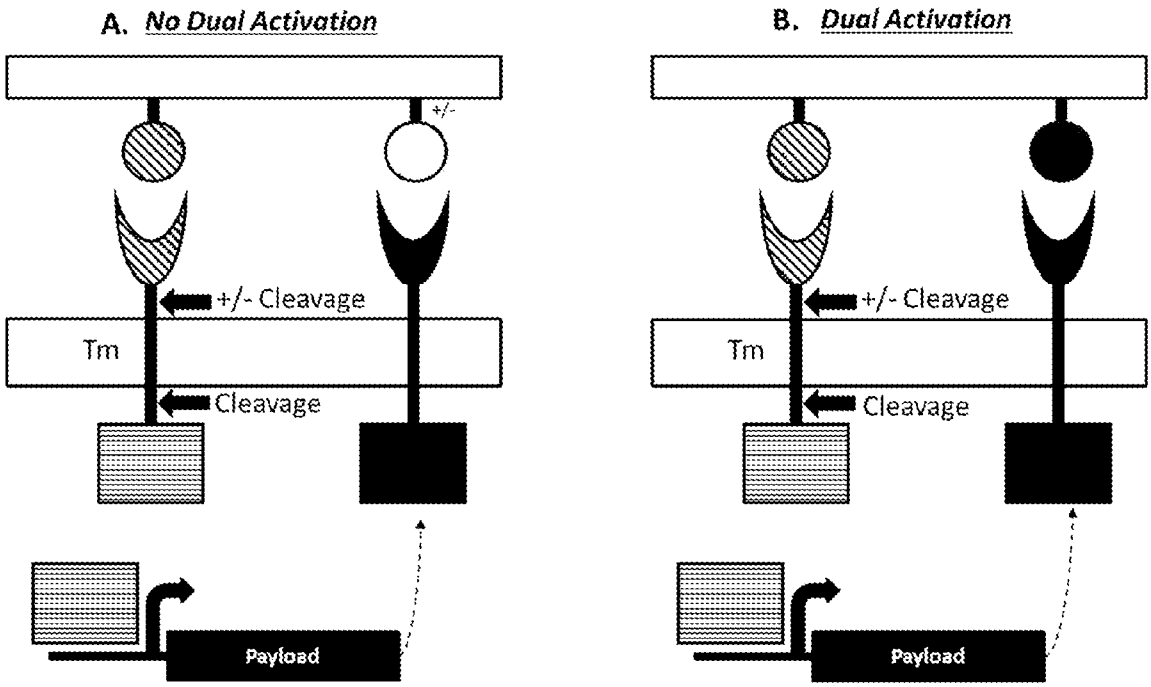


Figure 13

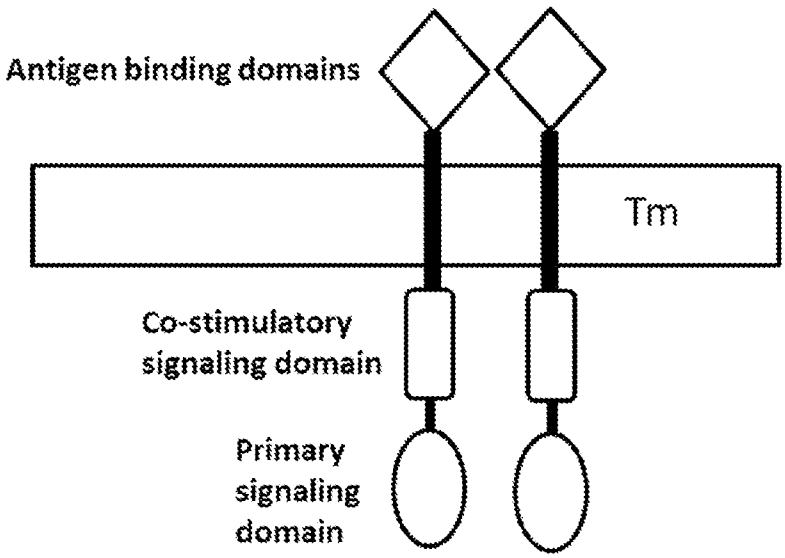


Figure 14

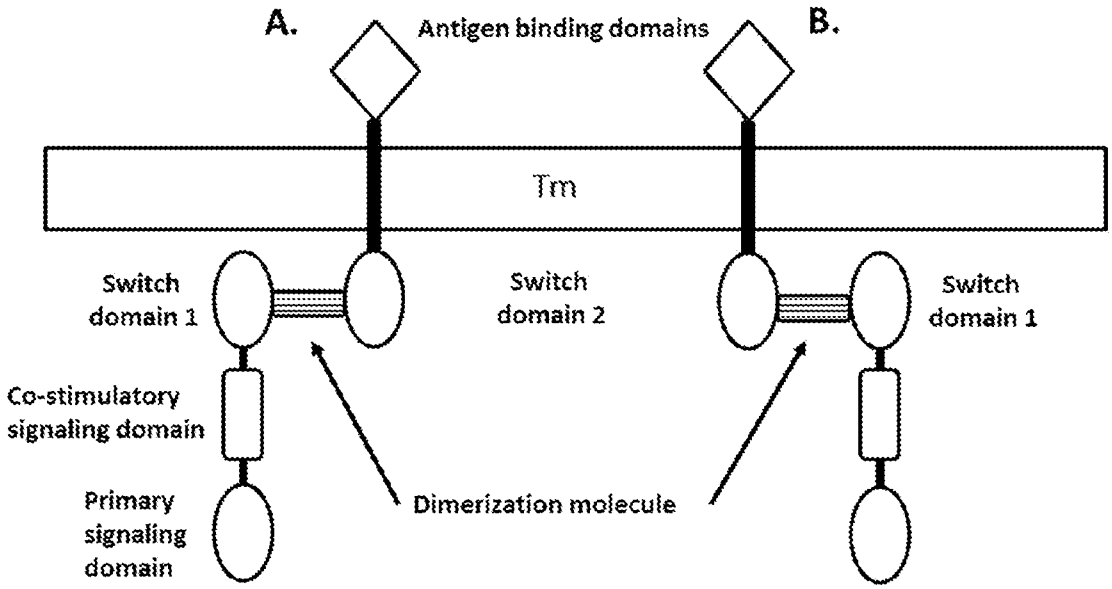


Figure 15

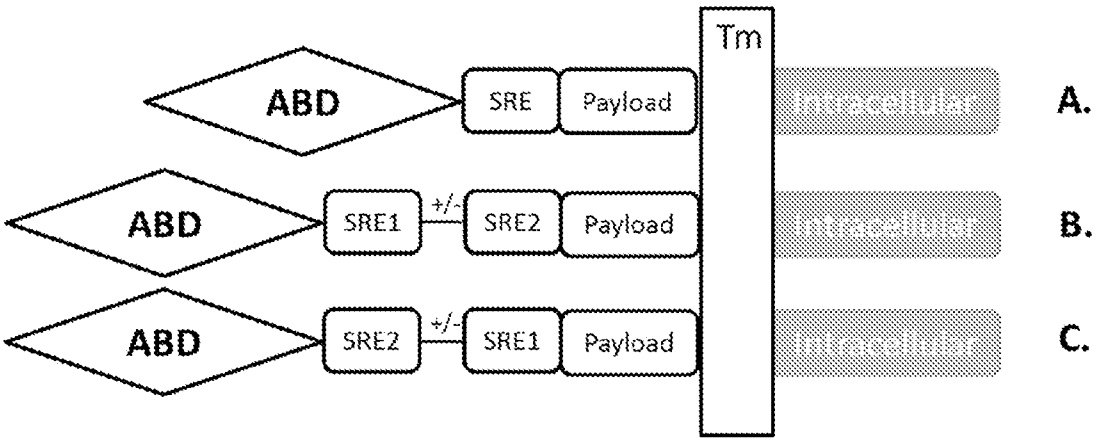


Figure 16

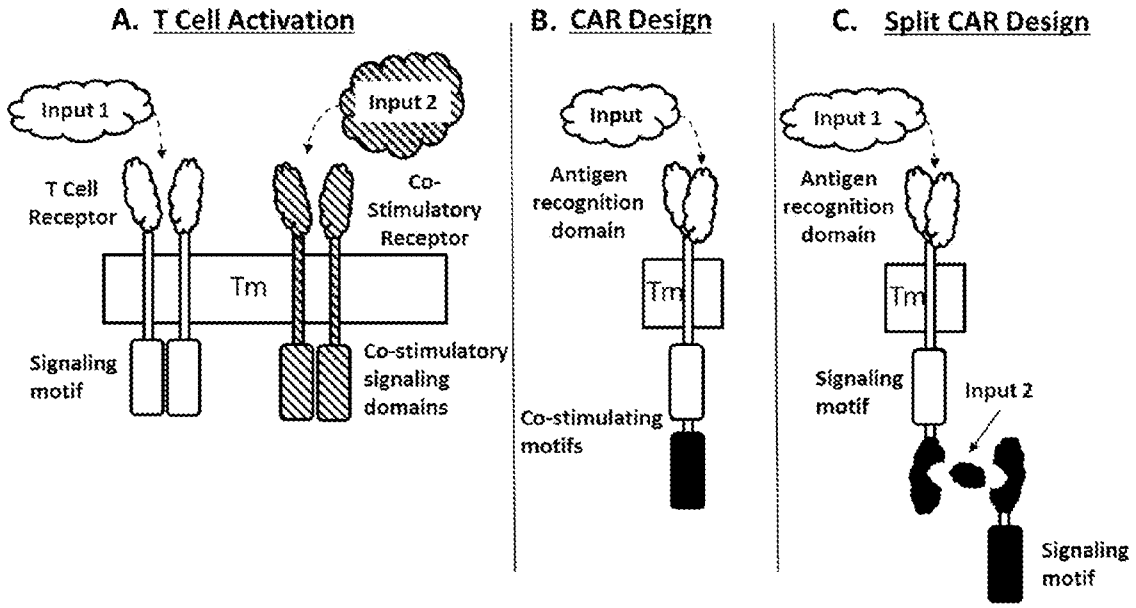


Figure 17

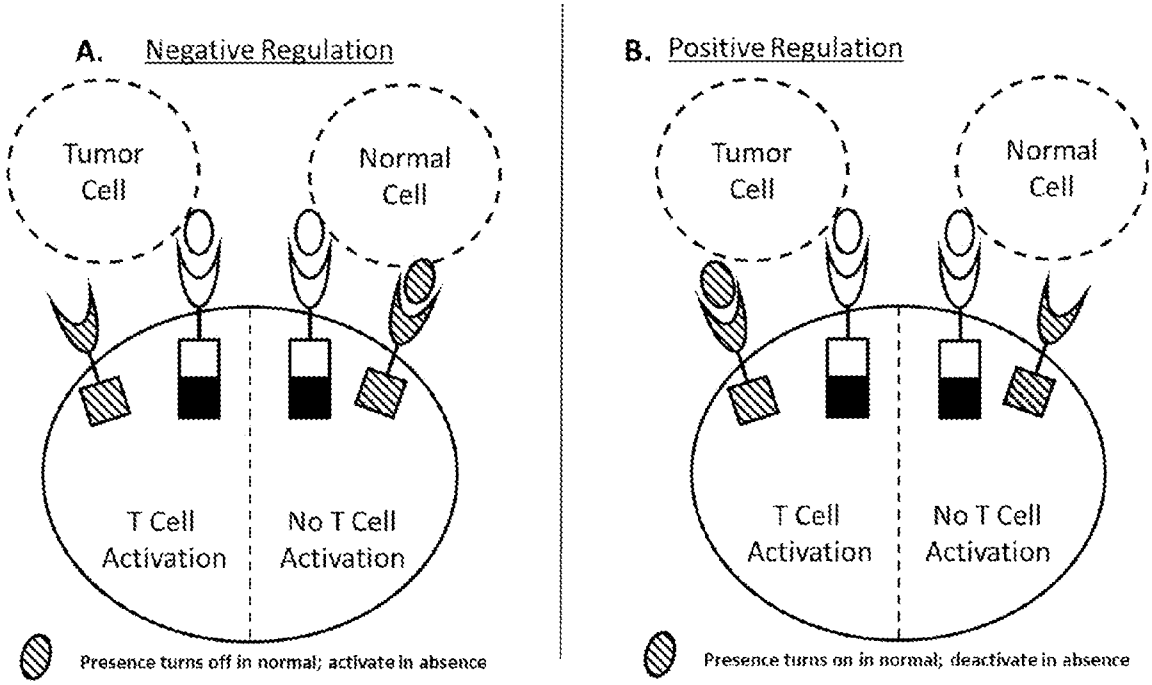


Figure 18

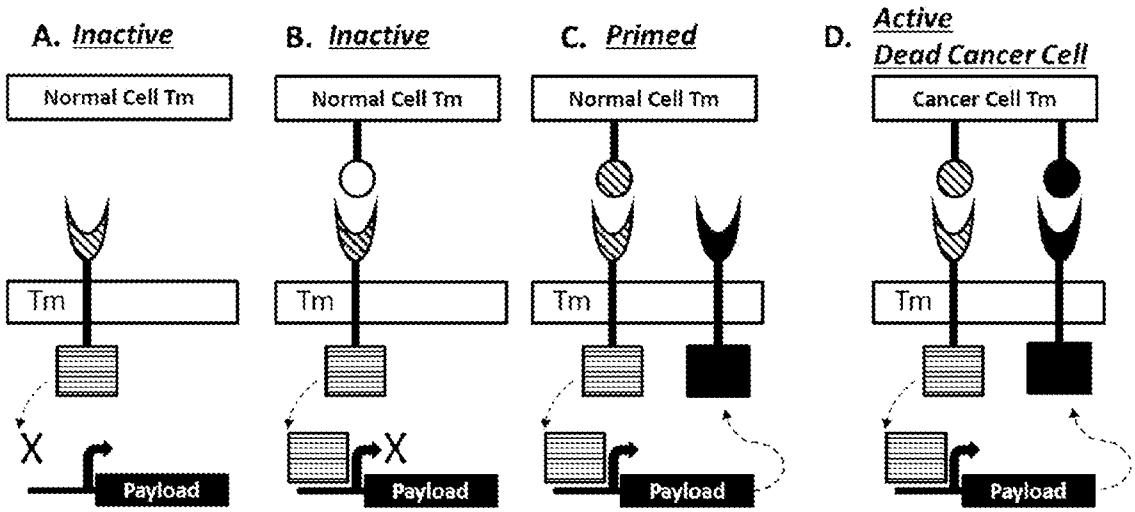


Figure 19A

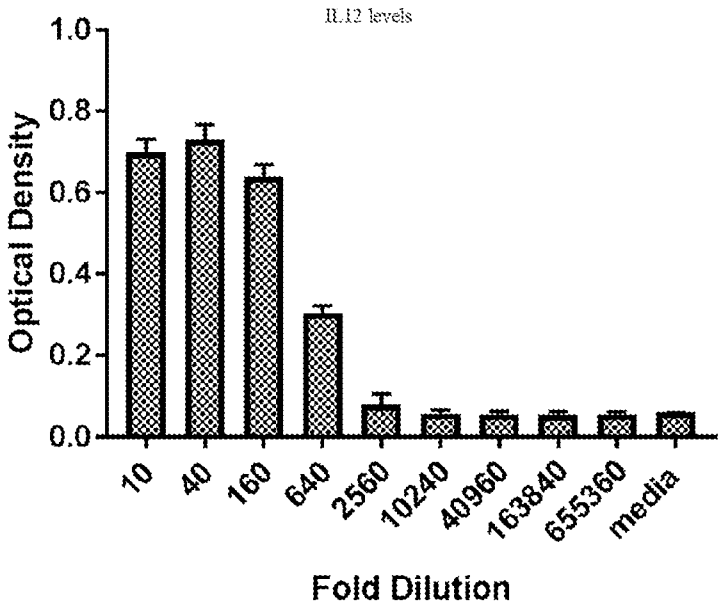


Figure 19B

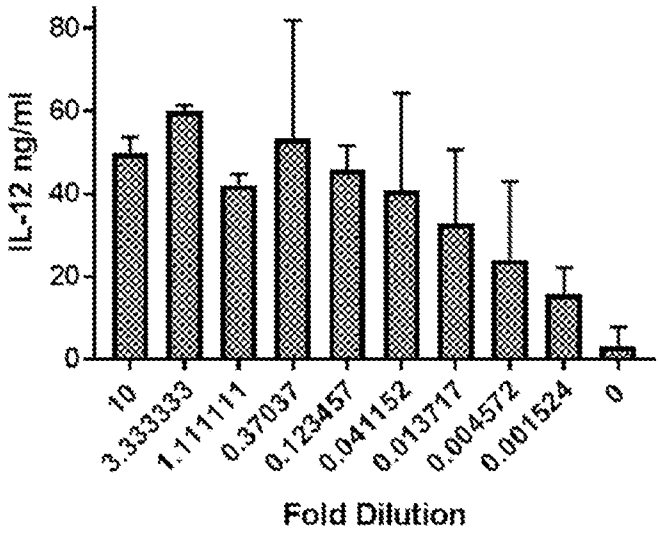
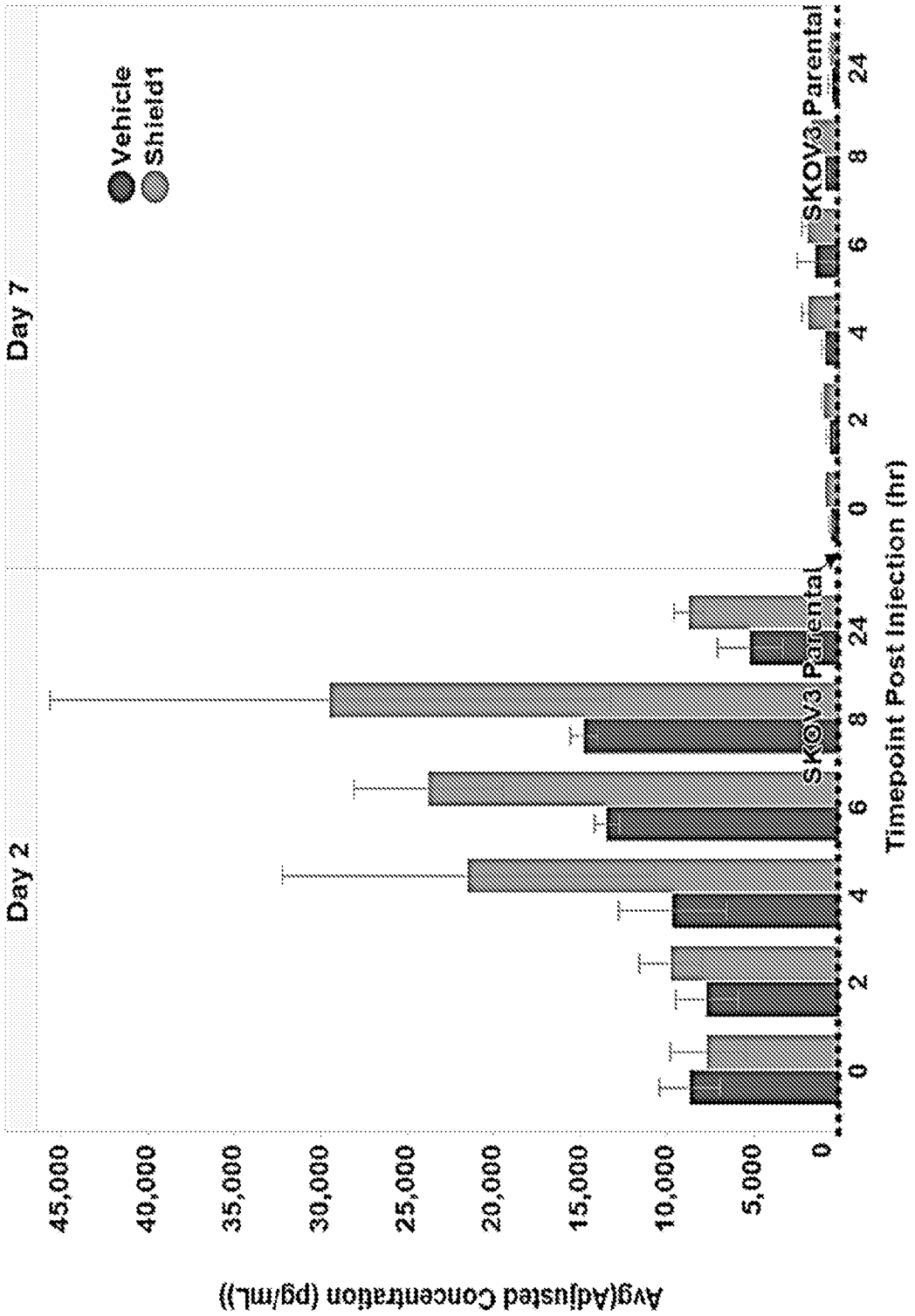

IL-12 ng/ml
80
60
40
20
0
10
3.333333
1.111111
0.37037
0.123457
0.041152
0.013717
0.004572
0.001524
0
Fold Dilution

Figure 19C


Day 2
Day 7
Vehicle
Shield1
SKOV3 Parental
SKOV3 Parental
Timepoint Post Injection (hr)
Avg(Adjusted Concentration (pg/mL))
45,000
40,000
35,000
30,000
25,000
20,000
15,000
10,000
5,000
0
0
2
4
6
8
24
0
2
4
6
8
24

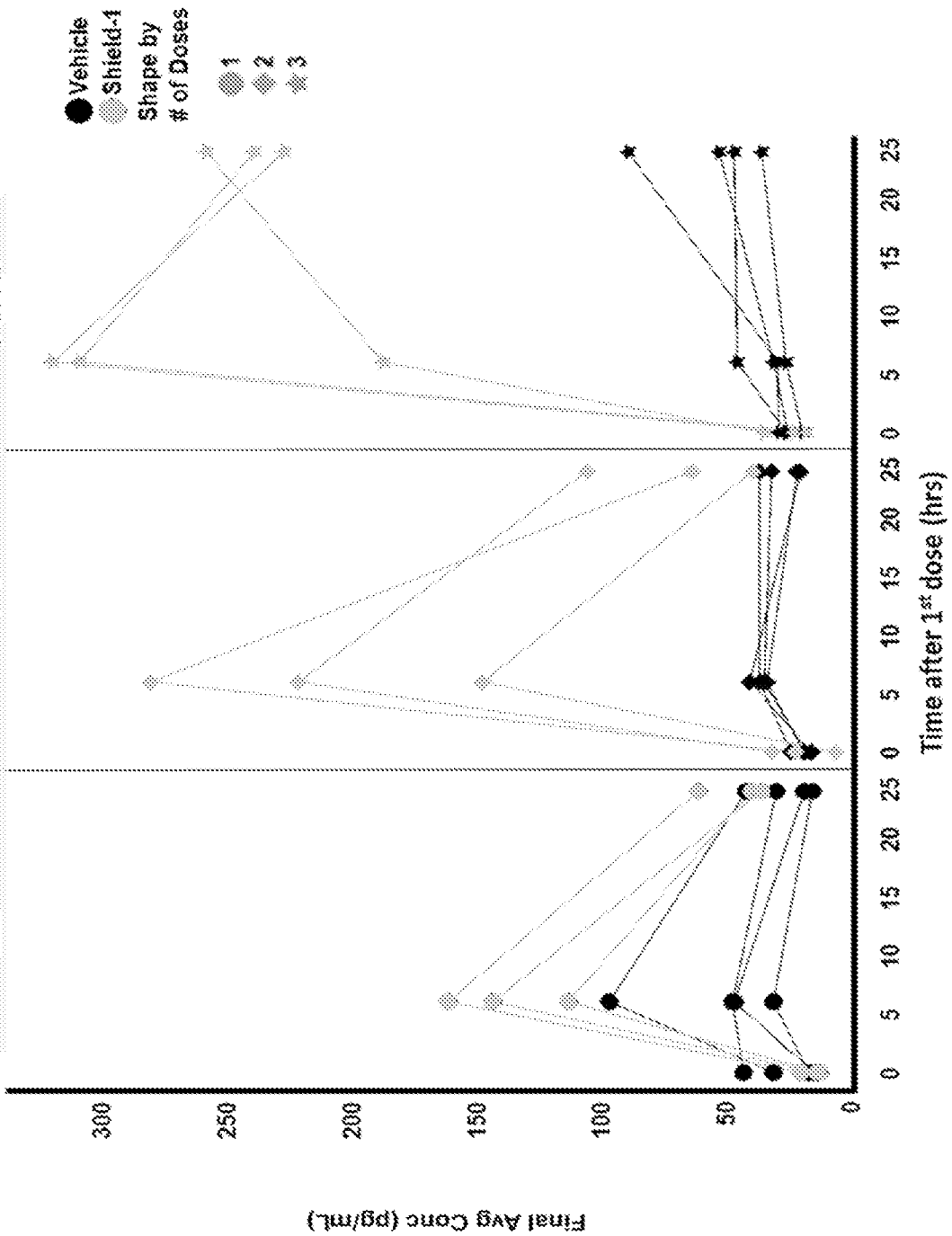

Vehicle
Shield-1
Shape by
# of Doses
1
2
3
1 dose
2 doses
3 doses
Final Avg Conc (pg/mL)
Time after 1st dose (hrs)

Figure 19D

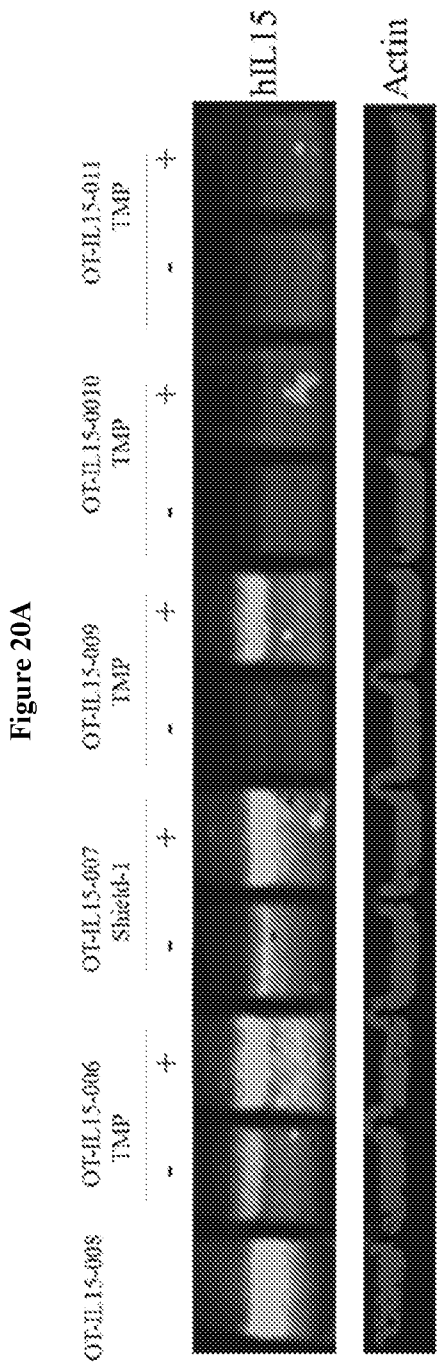

hIL15
Actin
OT-IL15-008
OT-IL15-006 TMP
OT-IL15-007 Shield-1
OT-IL15-009 TMP
OT-IL15-0010 TMP
OT-IL15-011 TMP
- + - + - + - +

Figure 20A

Figure 20B

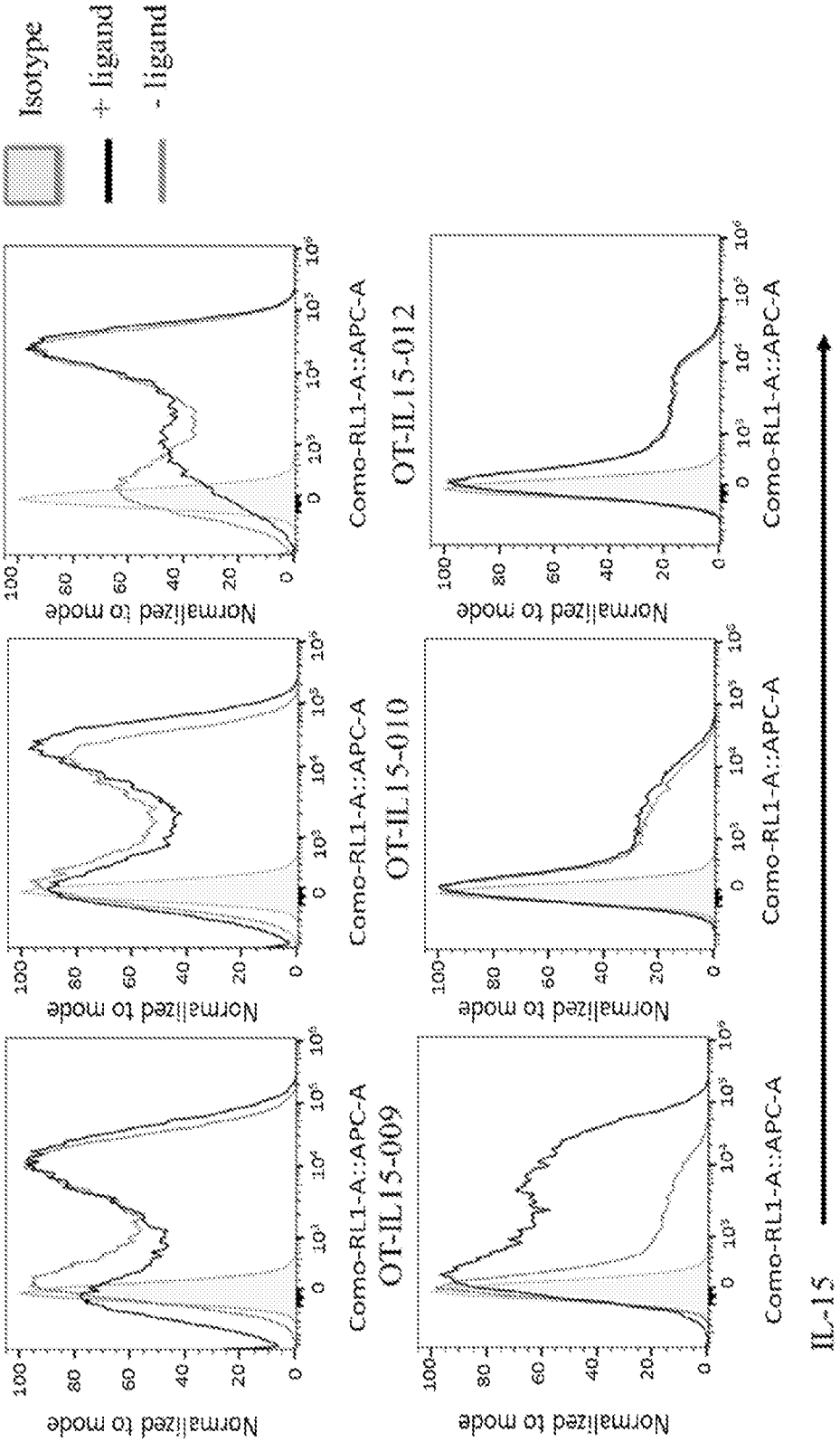

Isotype
+ ligand
- ligand
OT-IL15-006
OT-IL15-007
OT-IL15-008
OT-IL15-009
OT-IL15-010
OT-IL15-012
Como-RL1-A::APC-A
Normalized to mode
IL-15

Figure 20C

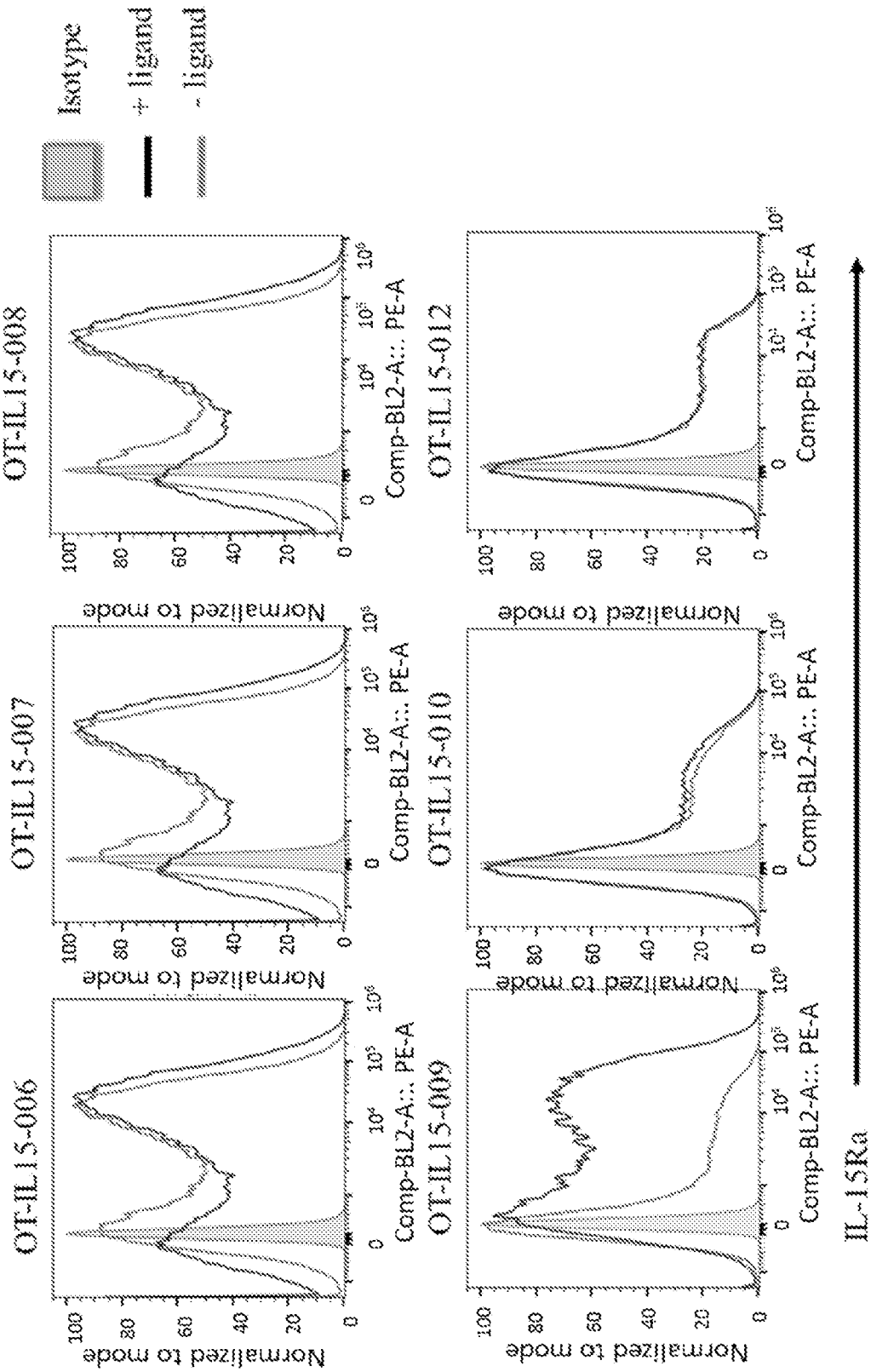

Isotype
+ ligand
- ligand
OT-IL15-006
OT-IL15-007
OT-IL15-008
OT-IL15-009
OT-IL15-010
OT-IL15-012
Normalized to mode
Comp-BL2-A::: PE-A
IL-15Rα

Figure 20D

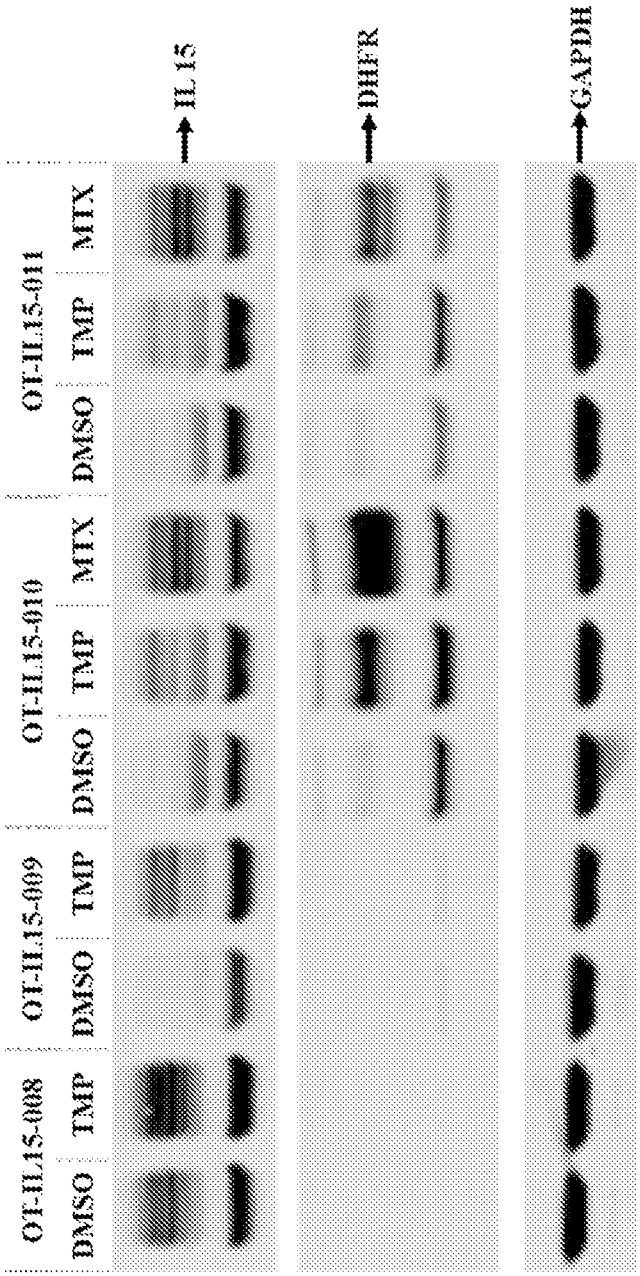

IL 15
DHFR
GAPDH
OT-IL15-008
DMSO
TMP
OT-IL15-009
DMSO
TMP
OT-IL15-010
DMSO
TMP
MTX
OT-IL15-011
DMSO
TMP
MTX

Figure 21A

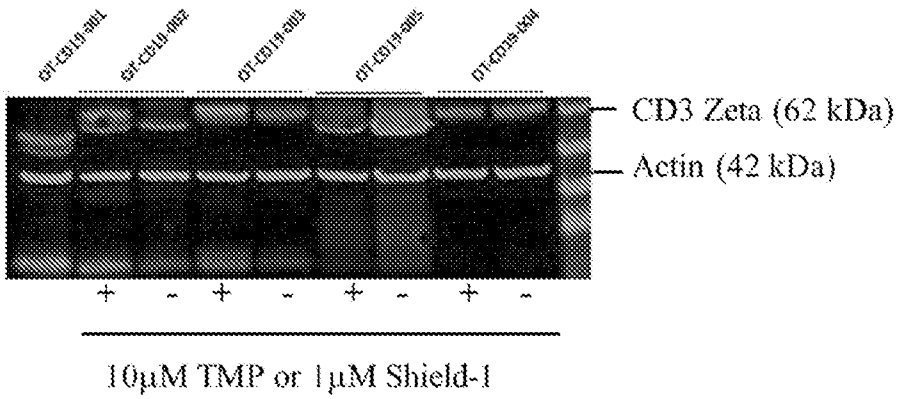


Figure 21B

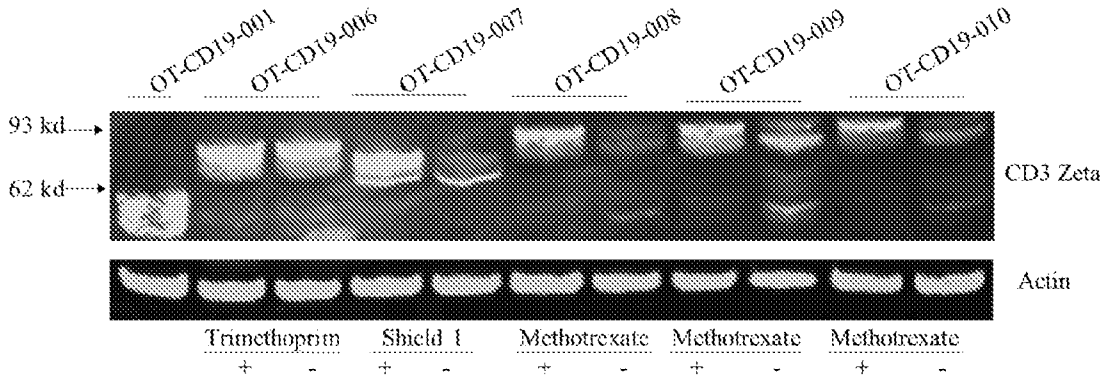


Figure 21C

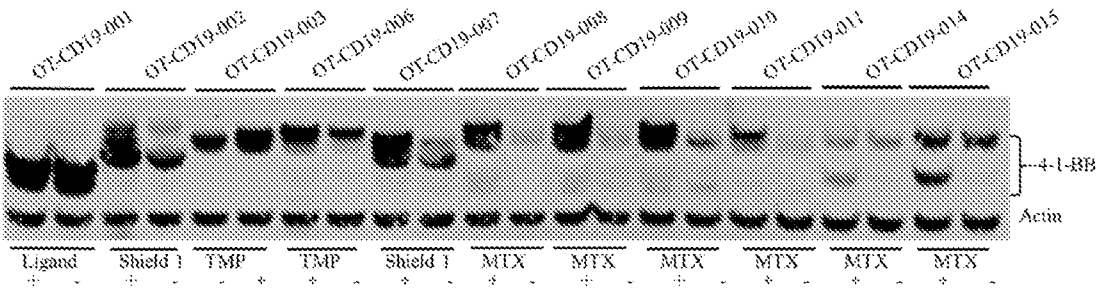


Figure 21D

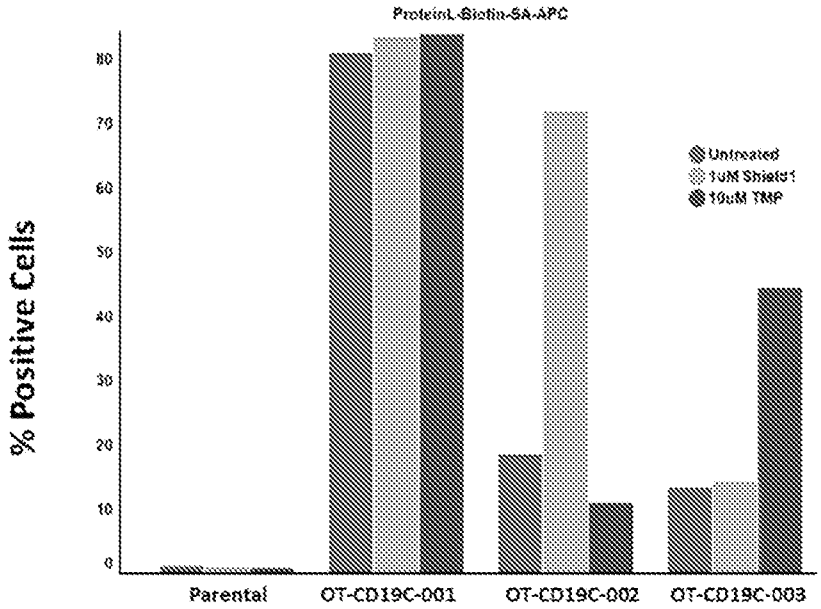


Figure 22

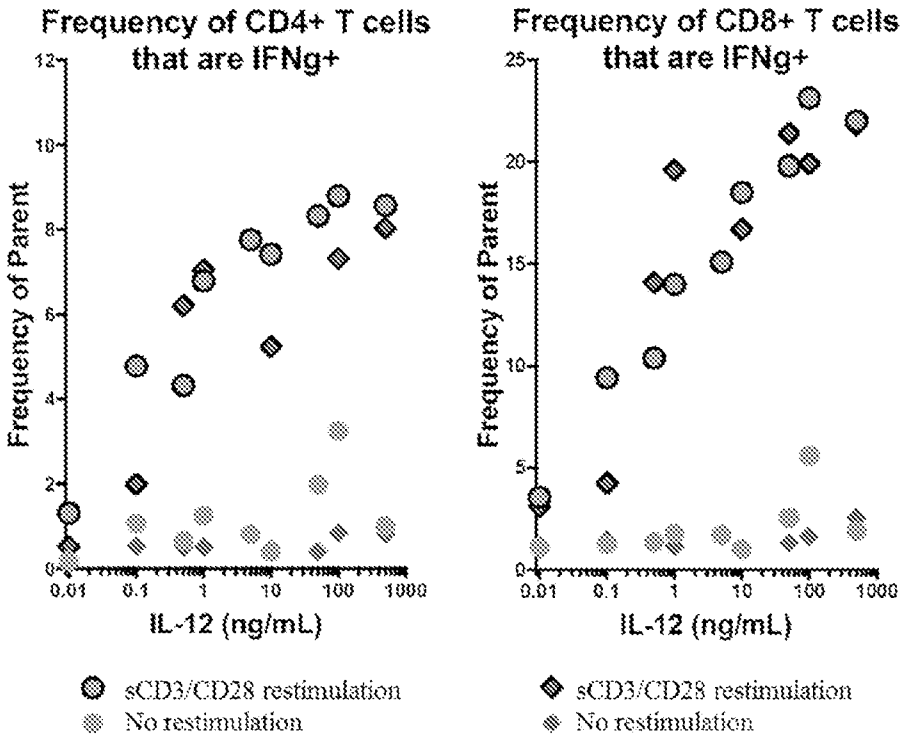


Figure 23A

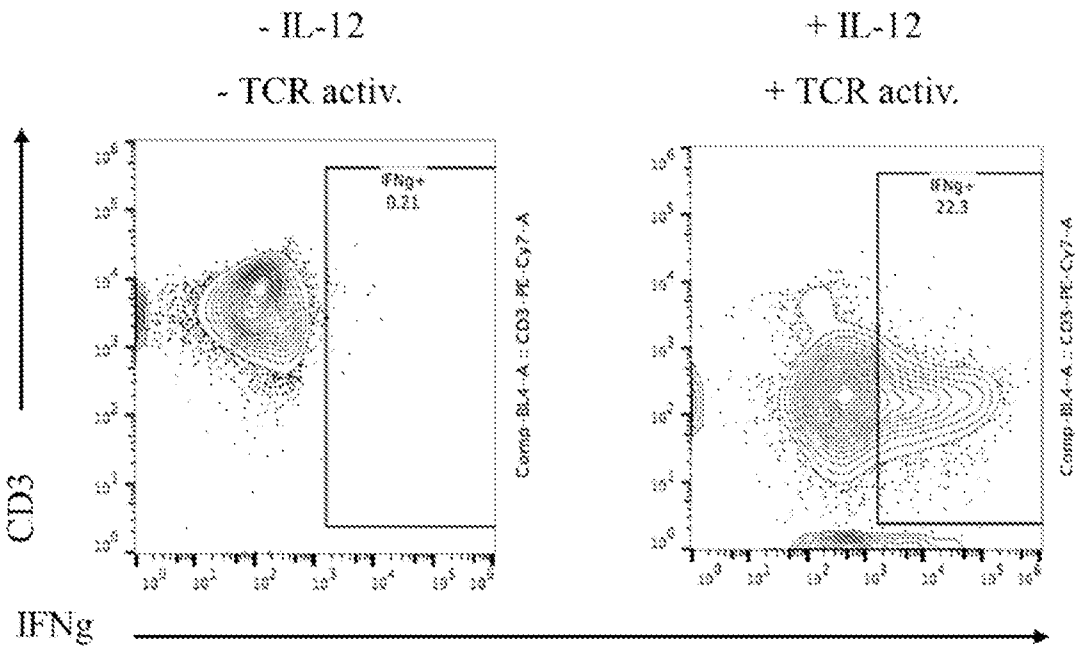


Figure 23B

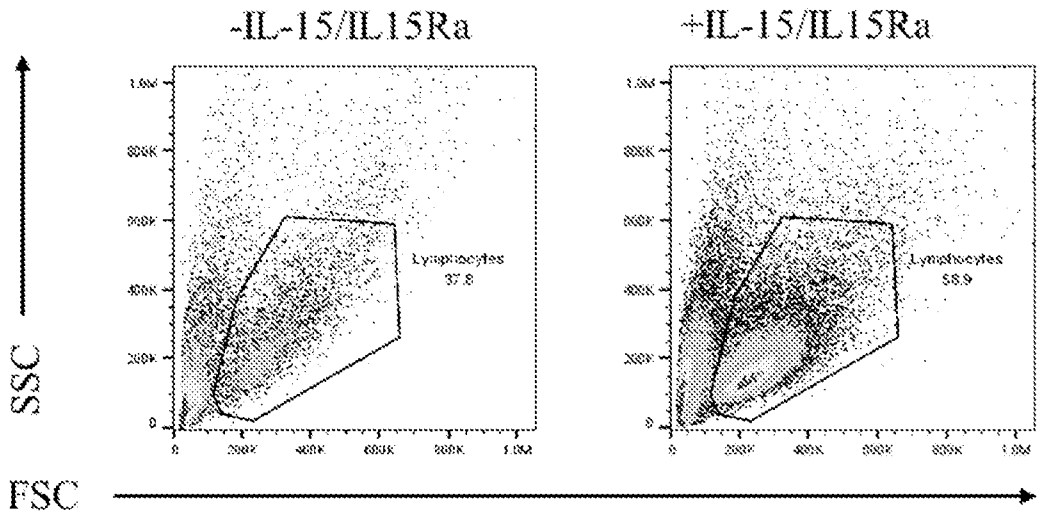


Figure 23C

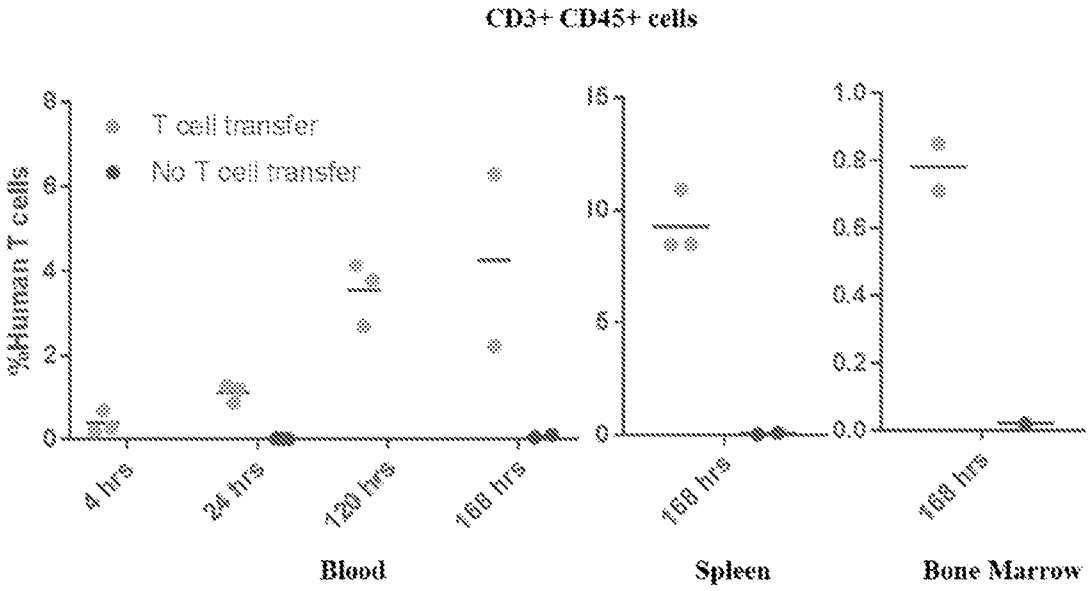


Figure 23D

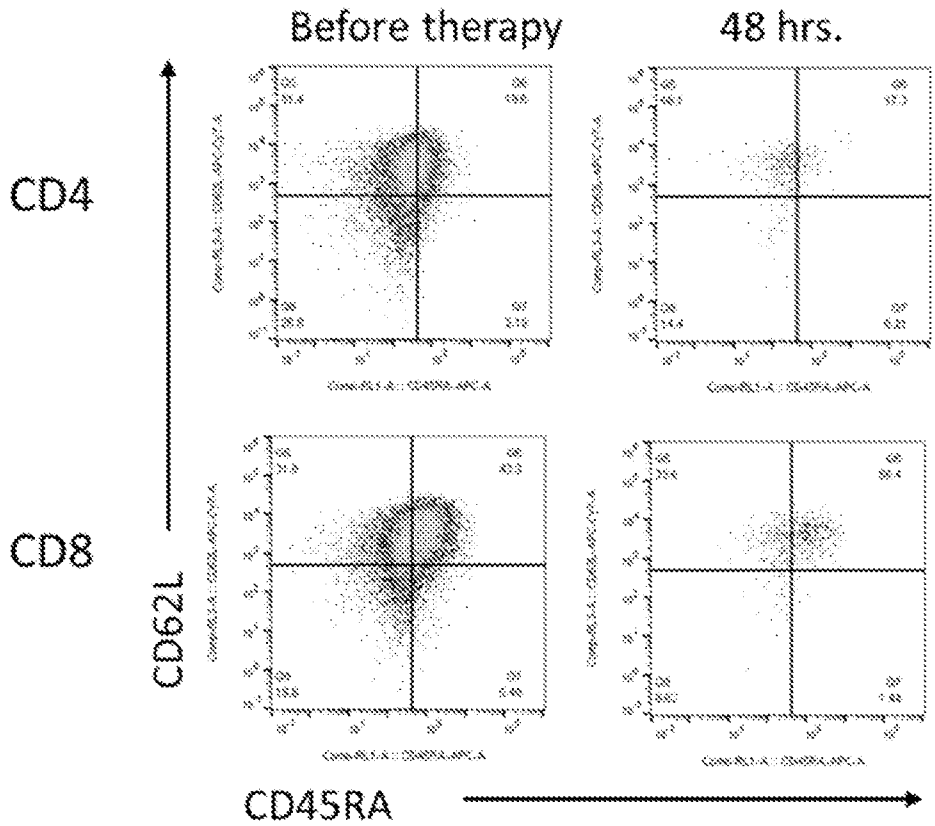


Figure 24A

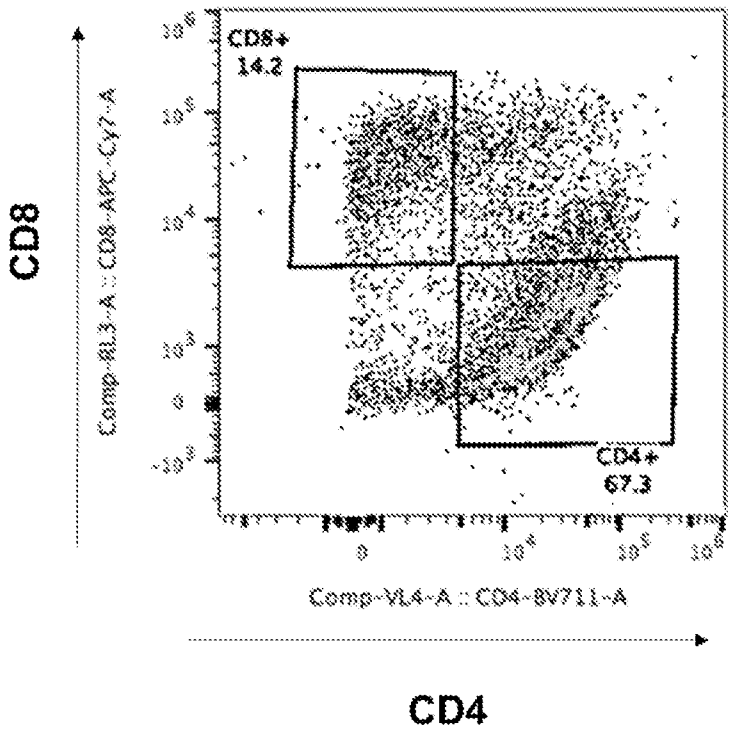


Figure 24B

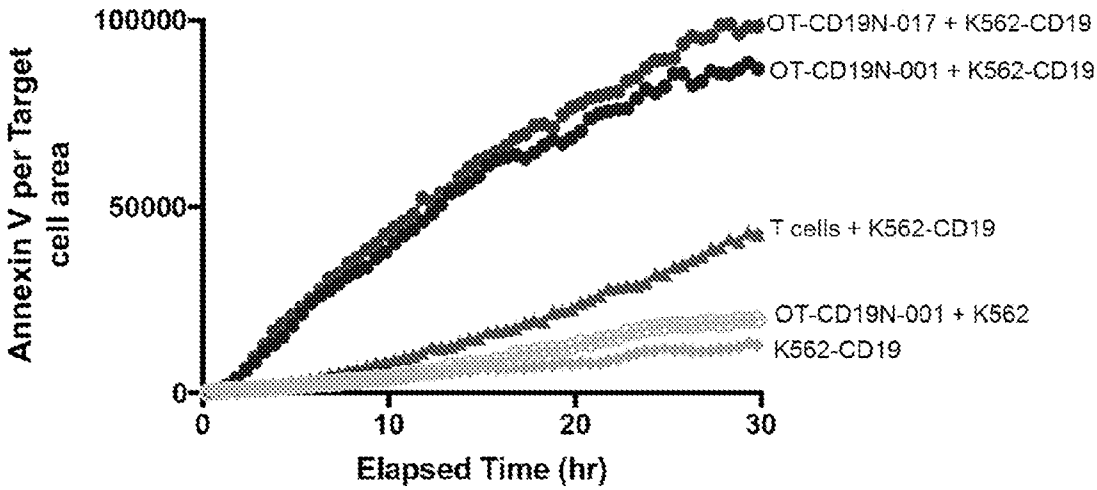


Figure 25A

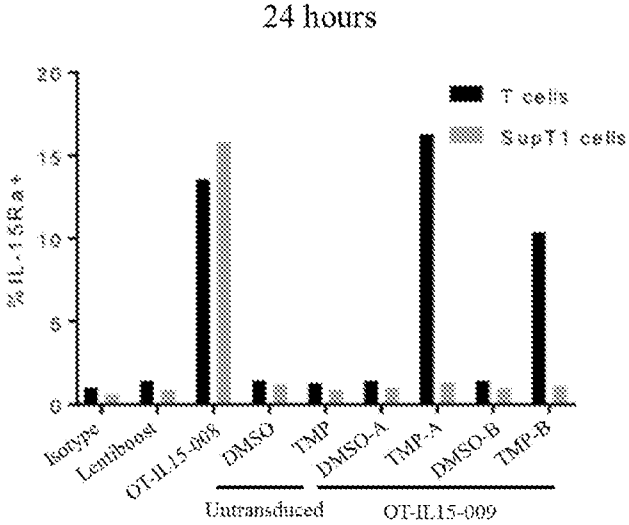


Figure 25B

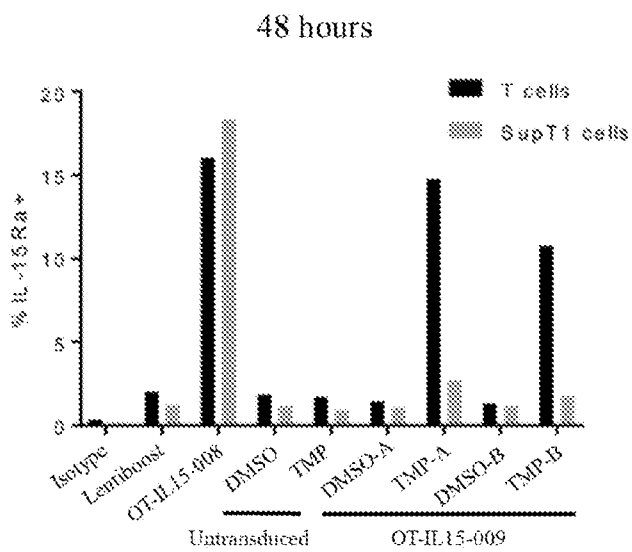


Figure 25C

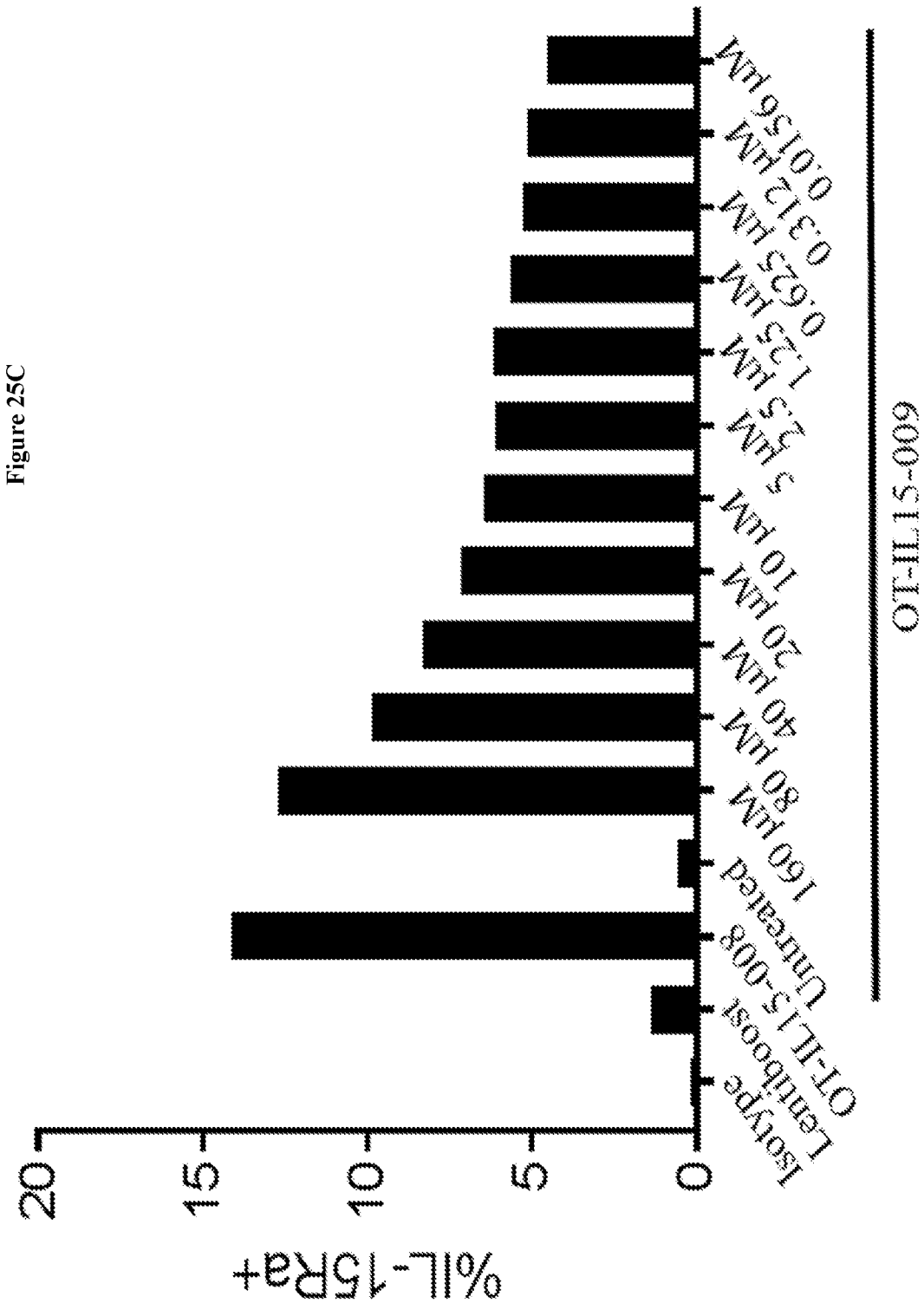


Figure 25C
%IL-15Ra+
20
15
10
5
0
Isotype
Lenzilumab
OT-IL15-008
Unmeated
160 µM
80 µM
40 µM
20 µM
10 µM
5 µM
2.5 µM
1.25 µM
0.625 µM
0.312 µM
0.0156µM
OT-IL15-009

Figure 26

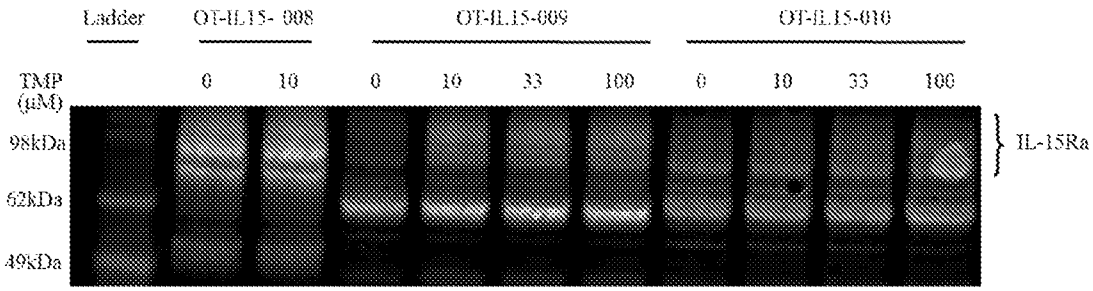

Ladder
OT-IL15- 008
OT-IL15-009
OT-IL15-010
TMP (μM)
0
10
0
10
33
100
0
10
33
100
98kDa
62kDa
49kDa
IL-15Ra

Figure 27A

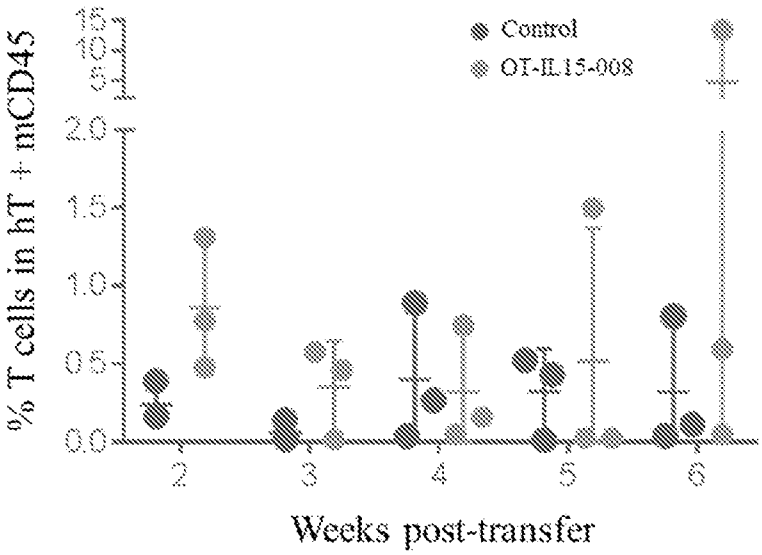


Figure 27B

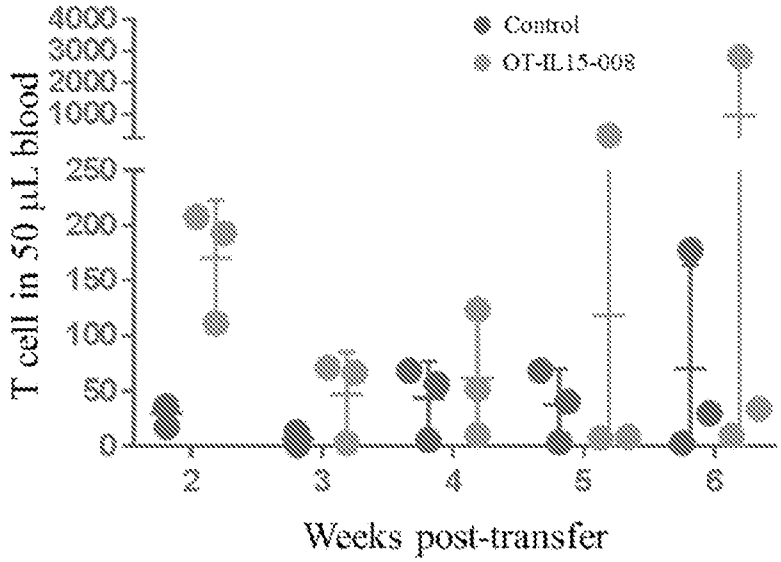


Figure 27C

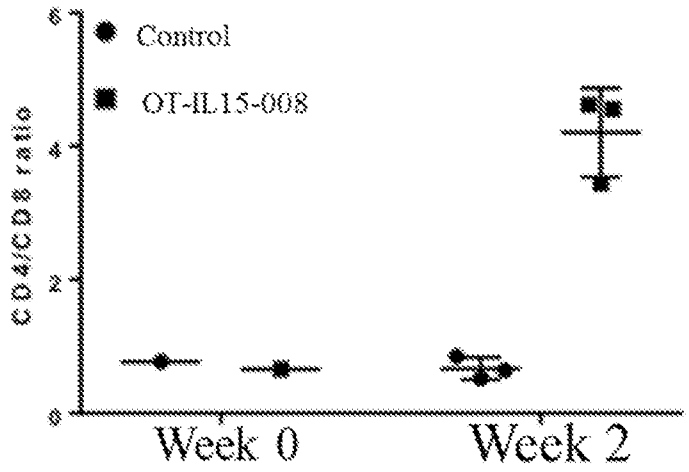


Figure 27D

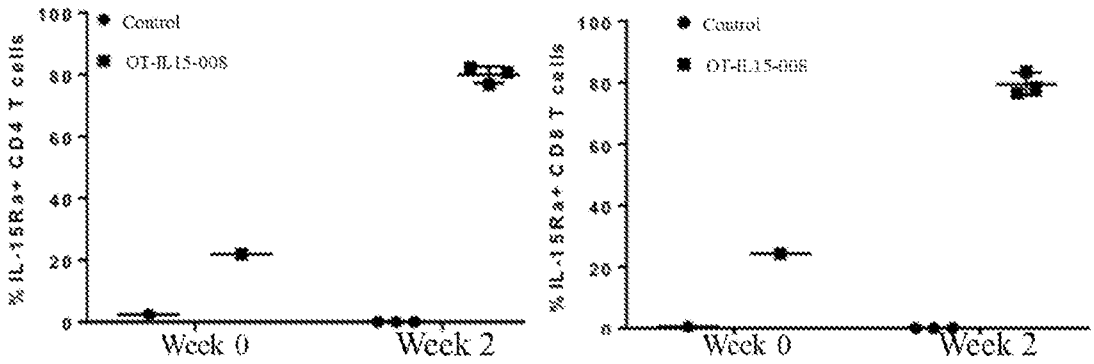


Figure 28A

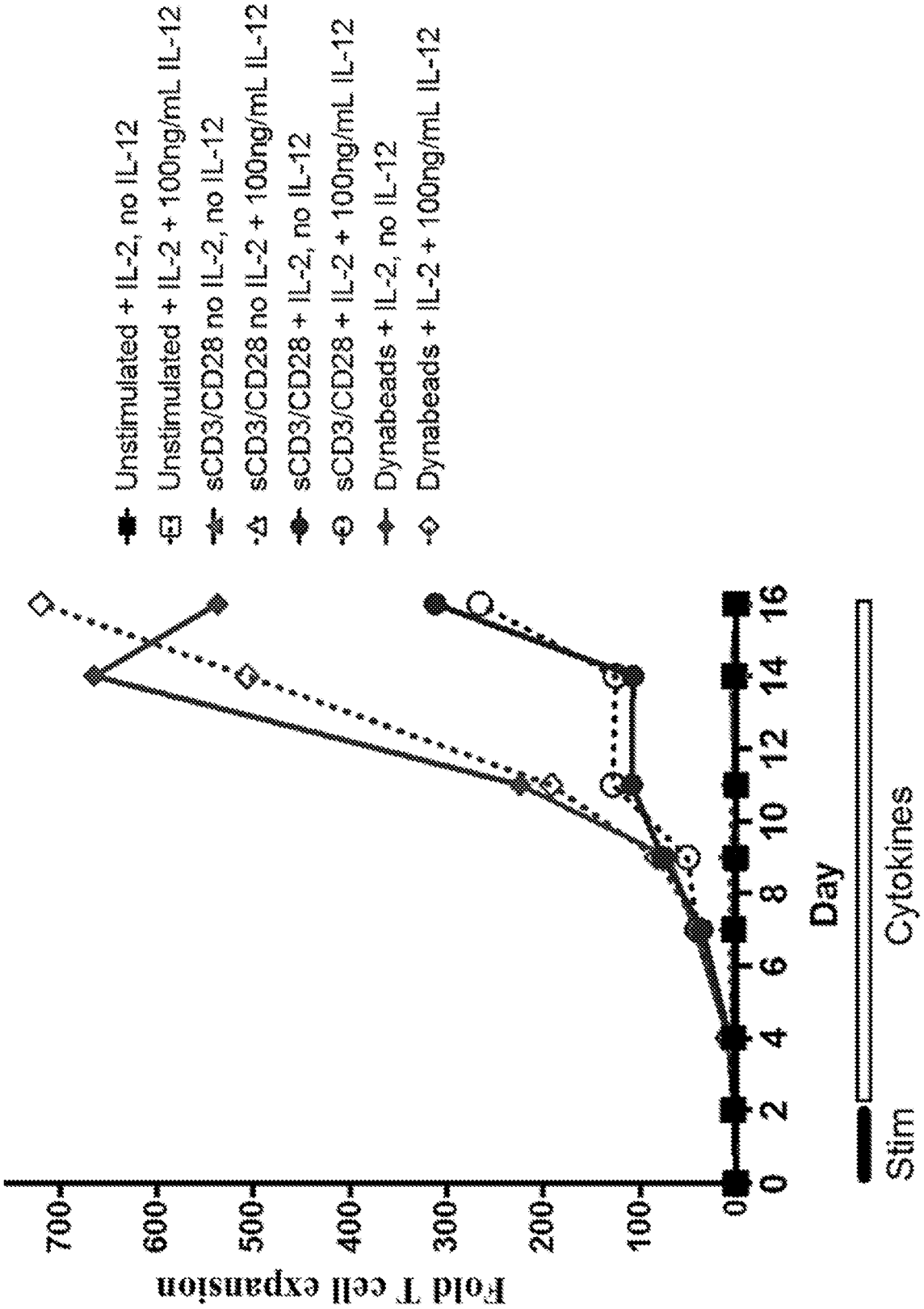

Unstimulated + IL-2, no IL-12
Unstimulated + IL-2 + 100ng/mL IL-12
sCD3/CD28 no IL-2, no IL-12
sCD3/CD28 no IL-2 + 100ng/mL IL-12
sCD3/CD28 + IL-2, no IL-12
sCD3/CD28 + IL-2 + 100ng/mL IL-12
Dynabeads + IL-2, no IL-12
Dynabeads + IL-2 + 100ng/mL IL-12
Fold T cell expansion
700
600
500
400
300
200
100
0
0
2
4
6
8
10
12
14
16
Day
Stim
Cytokines

Figure 28B

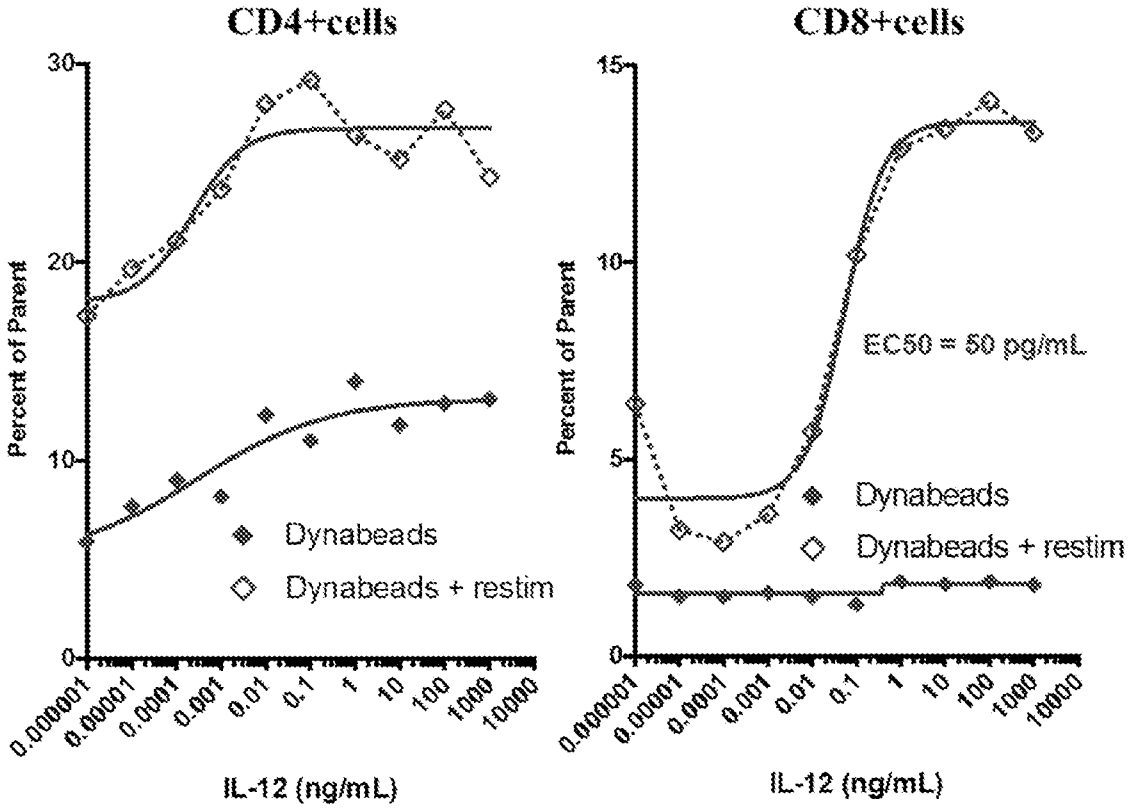

CD4+cells
Percent of Parent
30
20
10
0
Dynabeads
Dynabeads + restim
0.000001
0.00001
0.0001
0.001
0.01
0.1
1
10
100
1000
10000
IL-12 (ng/mL)
CD8+cells
Percent of Parent
15
10
5
0
EC50 = 50 pg/mL
Dynabeads
Dynabeads + restim
0.000001
0.00001
0.0001
0.001
0.01
0.1
1
10
100
1000
10000
IL-12 (ng/mL)

Figure 28C

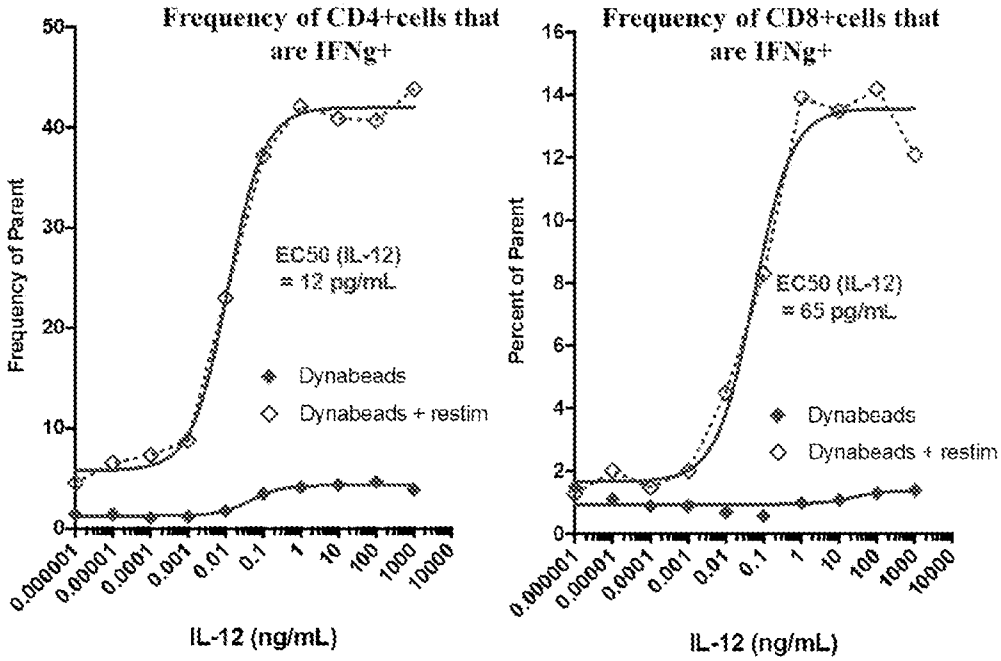


Figure 28D

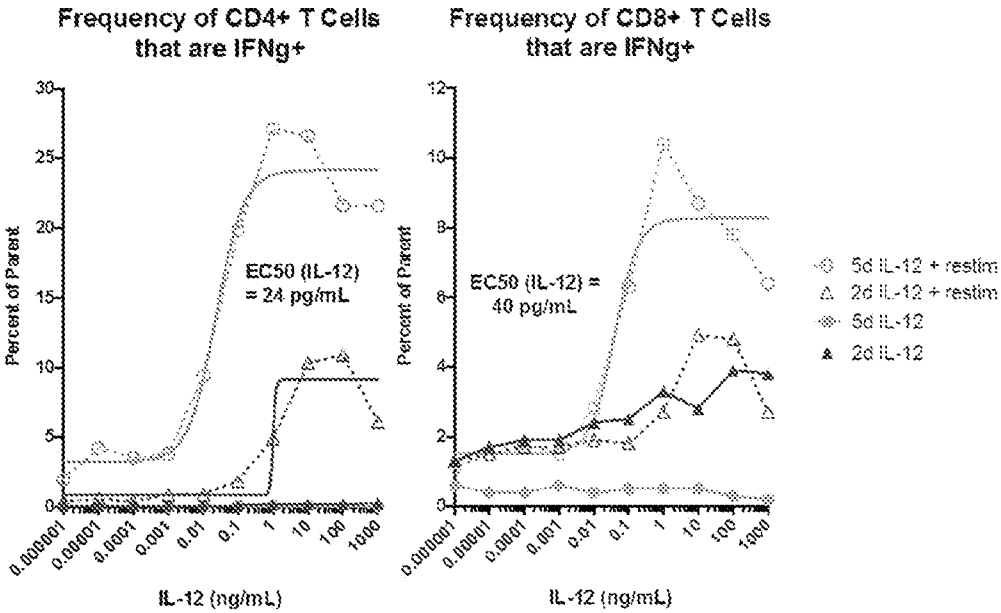


Figure 29

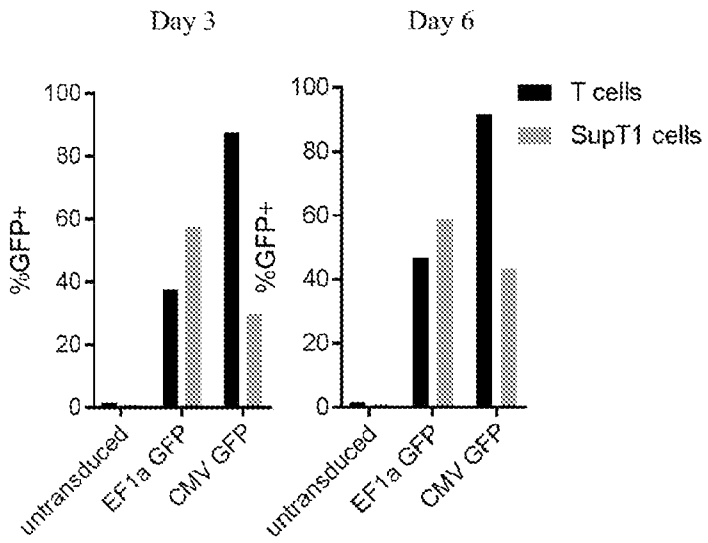

Day 3
Day 6
T cells
SupT1 cells
%GFP+
%GFP+
100
80
60
40
20
0
untransduced
EF1a GFP
CMV GFP

Figure 30A

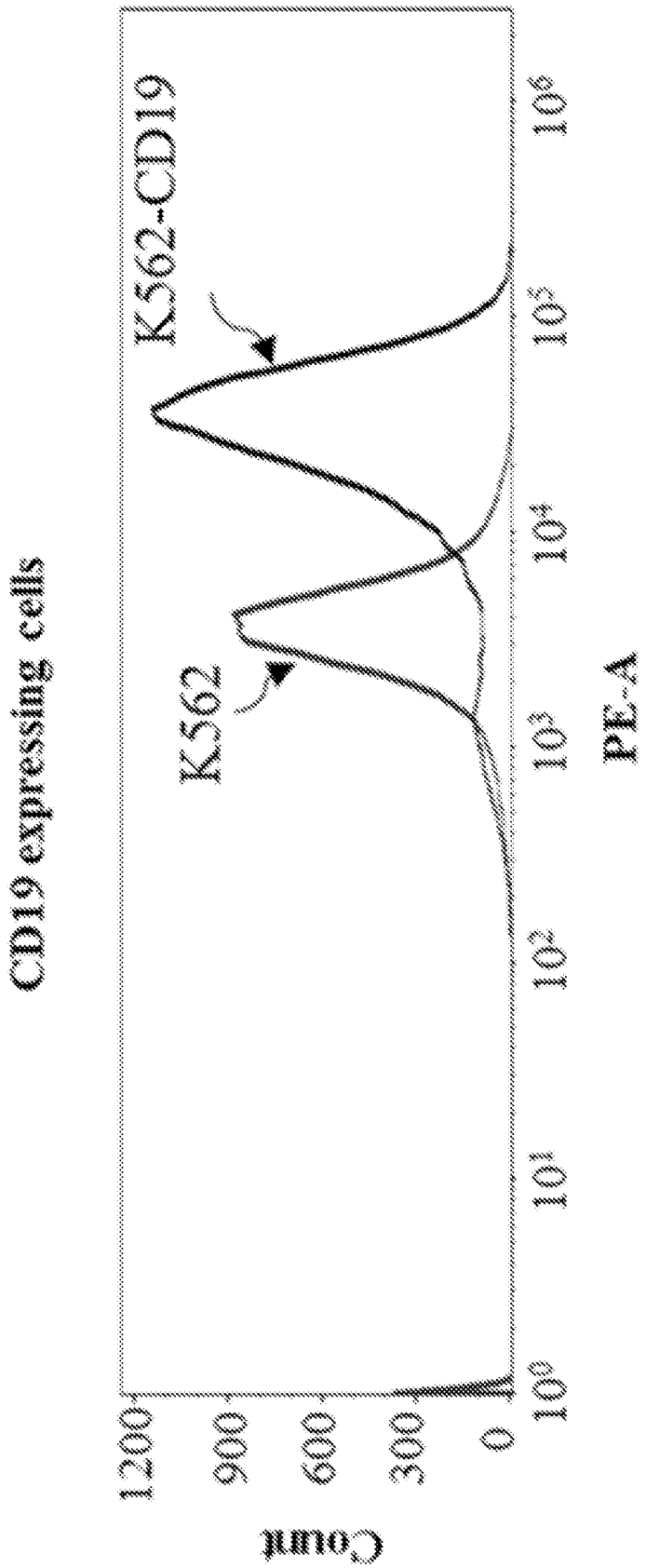

CD19 expressing cells
K562-CD19
K562
Count
PE-A
1200
900
600
300
0
10^0
10^1
10^2
10^3
10^4
10^5
10^6

Figure 30B

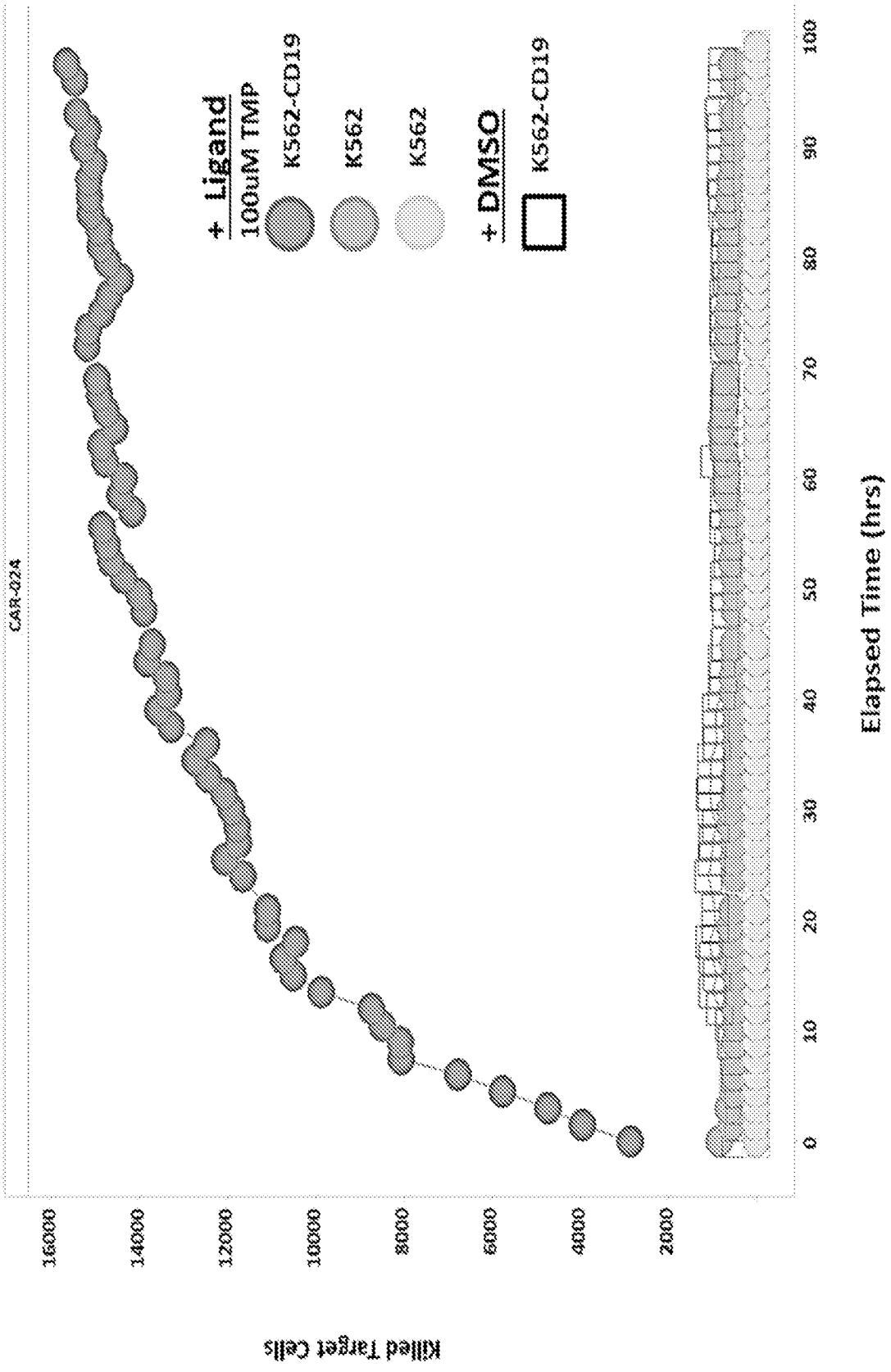

CAR-024
+ Ligand
100uM TMP
K562-CD19
K562
K562
+ DMSO
K562-CD19
Killed Target cells
16000
14000
12000
10000
8000
6000
4000
2000
0
10
20
30
40
50
60
70
80
90
100
Elapsed Time (hrs)

Figure 30C

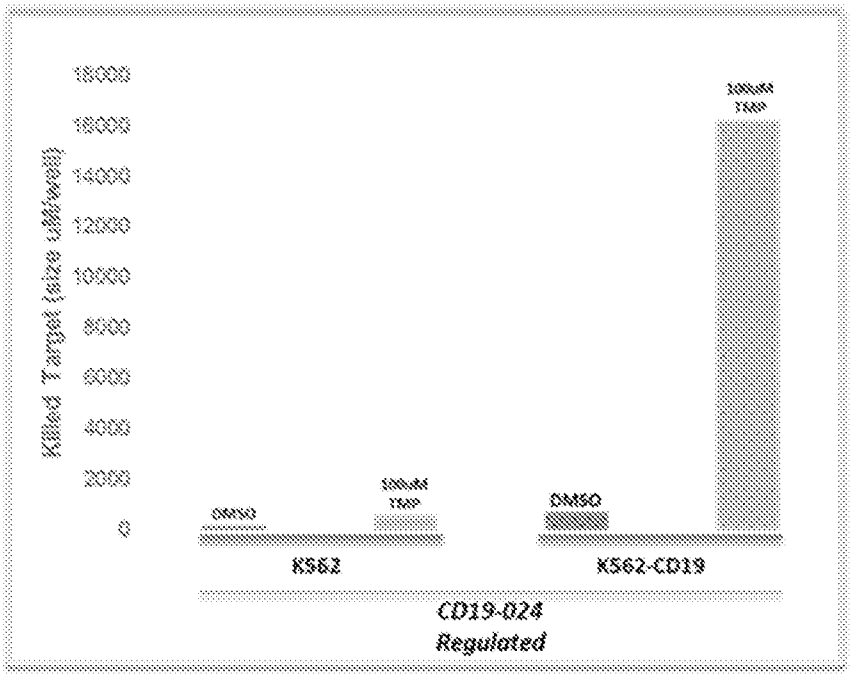

18000
16000
14000
12000
10000
8000
6000
4000
2000
0
K562 Target (size adjusted)
DMSO
100uM TMP
DMSO
100uM TMP
K562
K562-CD19
CD19-024
Regulated

Figure 31A

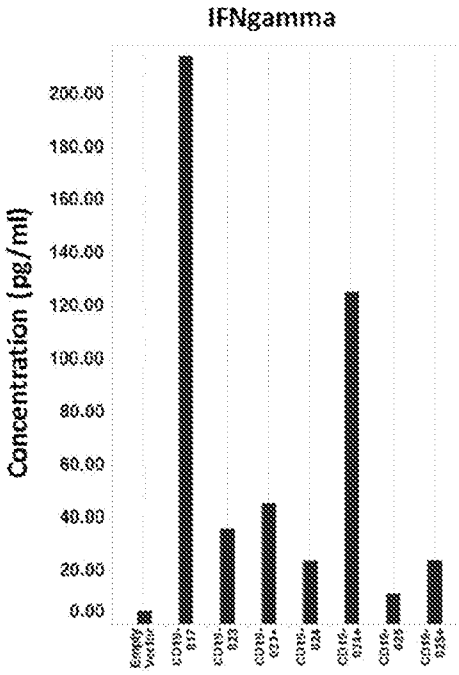


Figure 31B

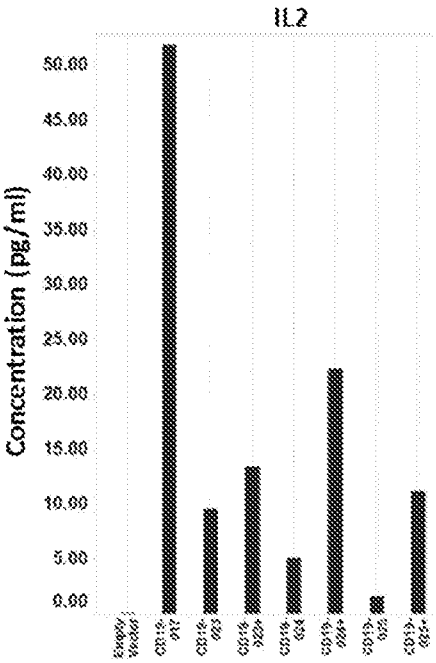


Figure 32A

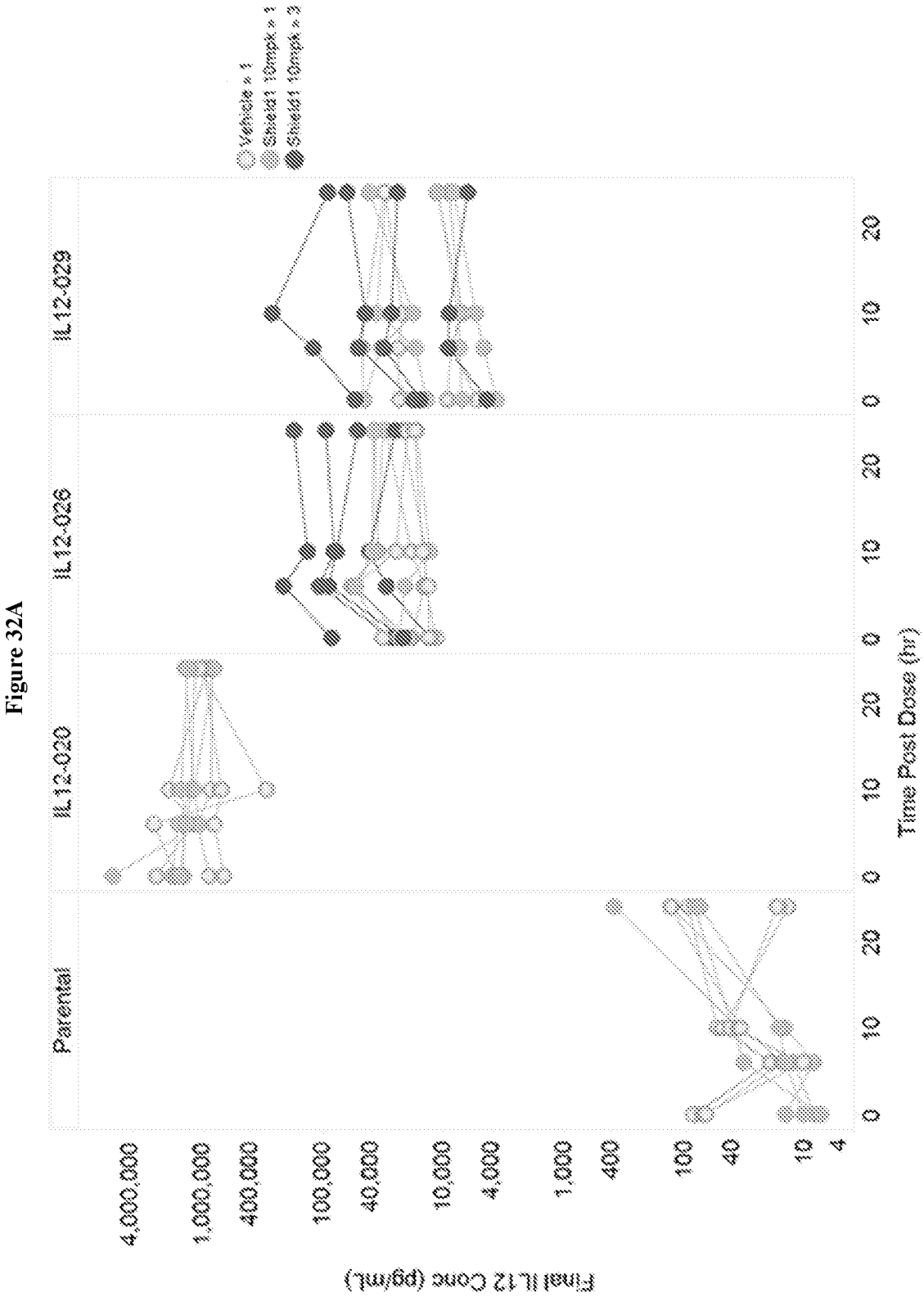

Vehicle × 1
Sheld1 10mpk × 1
Sheld1 10mpk × 3
Parental
IL12-020
IL12-026
IL12-029
Final IL12 Conc (pg/mL)
4,000,000
1,000,000
400,000
100,000
40,000
10,000
4,000
1,000
400
100
40
10
4
0
10
20
Time Post Dose (hr)

Figure 32B

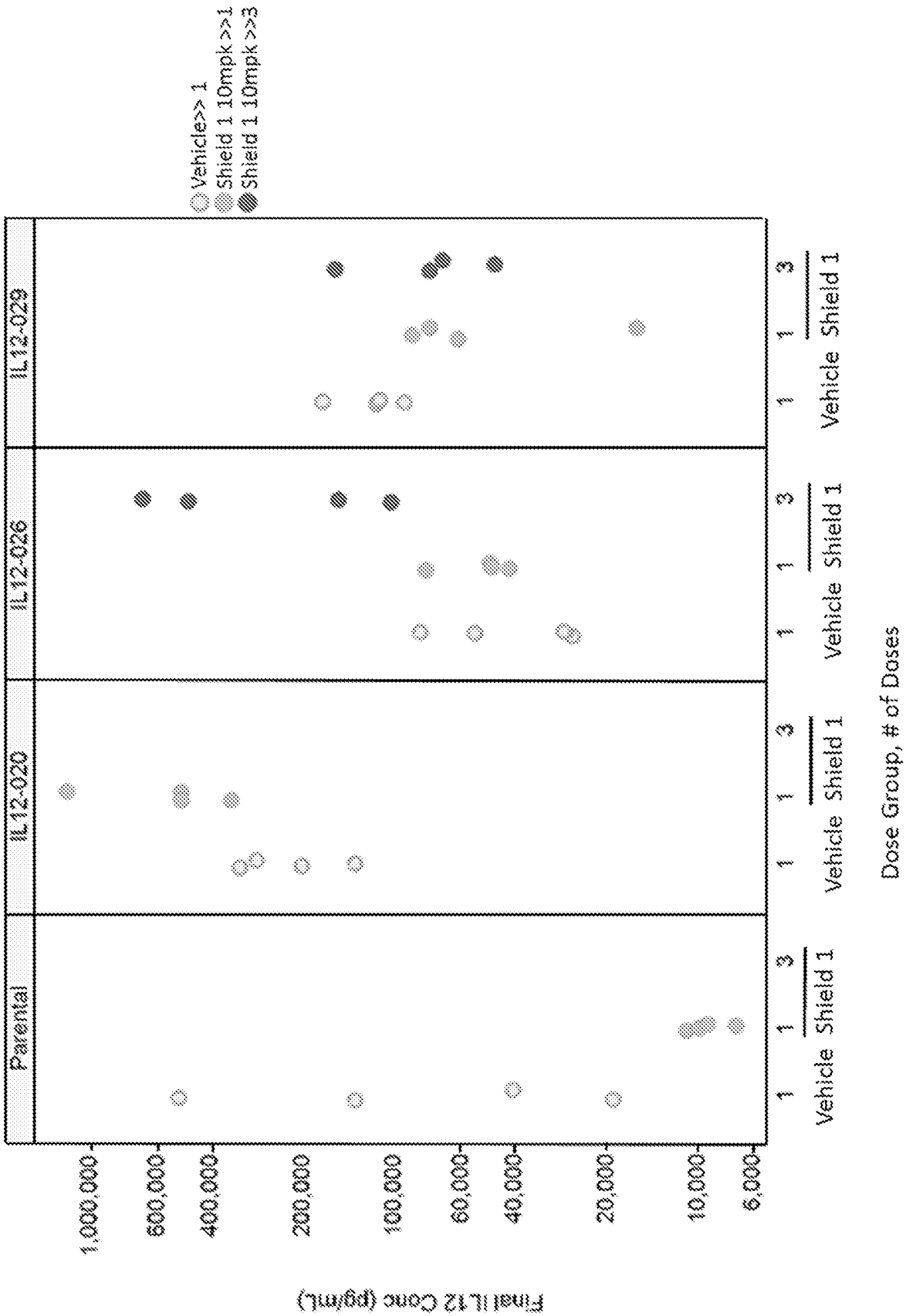
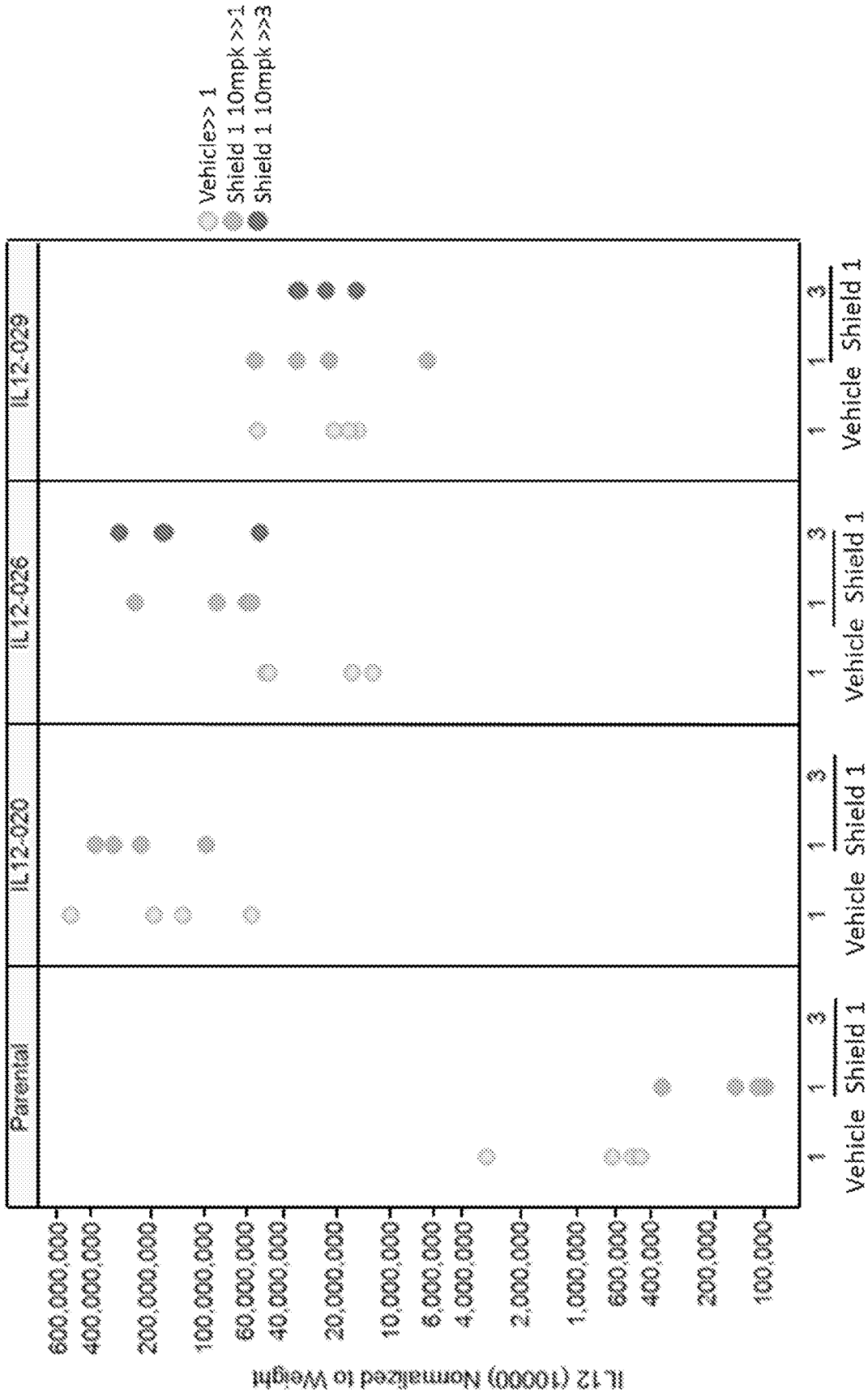

Vehicle>> 1
Shield 1 10mpk >>1
Shield 1 10mpk >>3
Parental
IL12-020
IL12-026
IL12-029
Final IL12 Conc (pg/mL)
1,000,000
600,000
400,000
200,000
100,000
60,000
40,000
20,000
10,000
6,000
1 1 3
Vehicle Shield 1
Dose Group, # of Doses

Figure 32C


Vehicle>> 1
Shield 1 10mpk >>1
Shield 1 10mpk >>3
Parental
IL12-020
IL12-026
IL12-029
IL-12 (10000) Normalized to Weight
600,000,000
400,000,000
200,000,000
100,000,000
60,000,000
40,000,000
20,000,000
10,000,000
6,000,000
4,000,000
2,000,000
1,000,000
600,000
400,000
200,000
100,000
1 1 3
Vehicle Shield 1
Dose Group, # of Doses

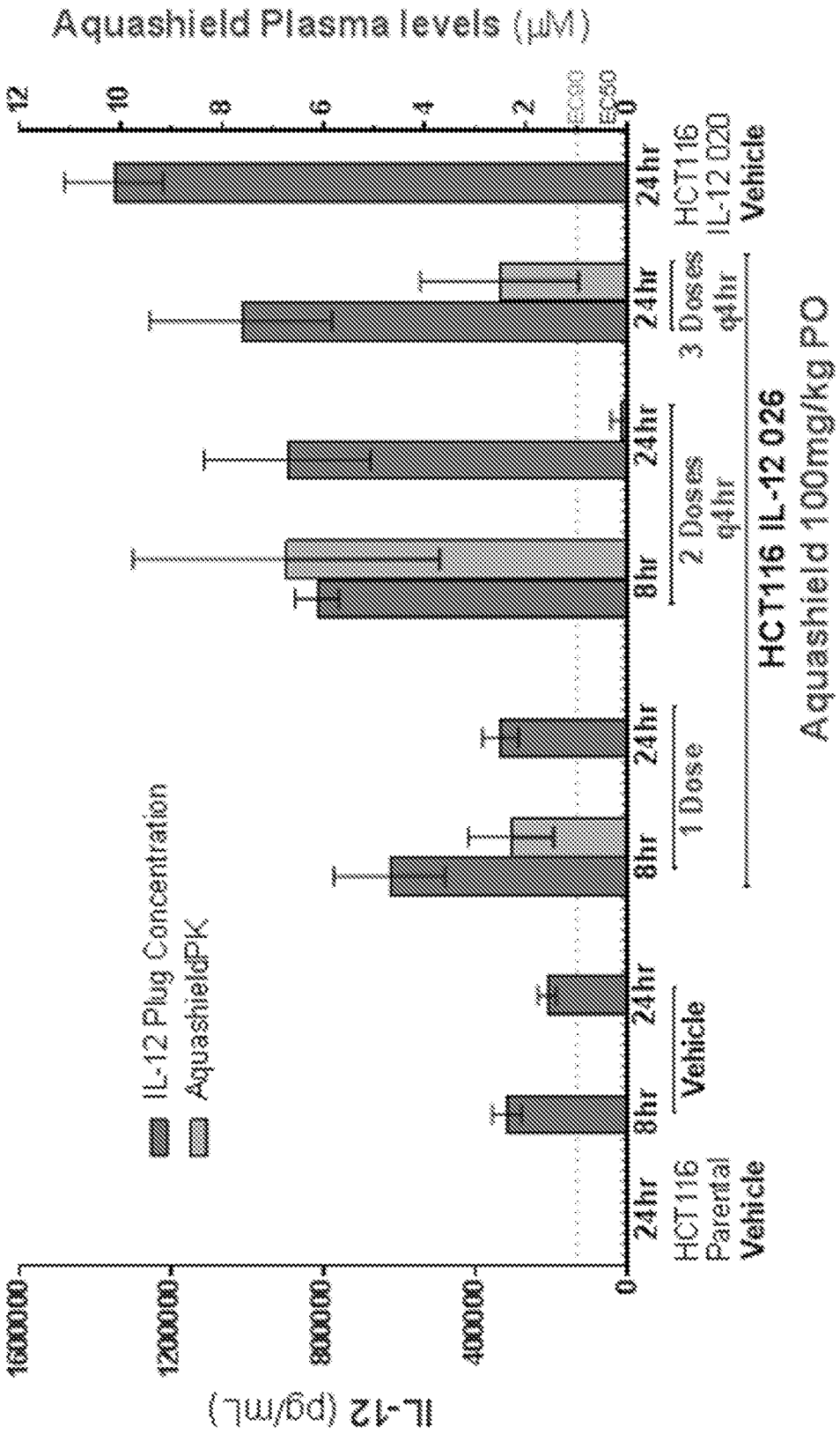

Aquashield Plasma levels (μM)
12
10
8
6
4
2
0
EC90
EC50
IL-12 Plug Concentration
AquashieldPK
IL-12 (pg/mL)
1600000
1200000
800000
400000
0
24hr
8hr
24hr
HCT116 Parental
Vehicle
1 Dose
Vehicle
2 Doses
q4hr
3 Doses
q4hr
HCT116 IL-12 020
Vehicle
HCT116 IL-12 026
Aquashield 100mg/kg PO

Figure 33A

Figure 33B

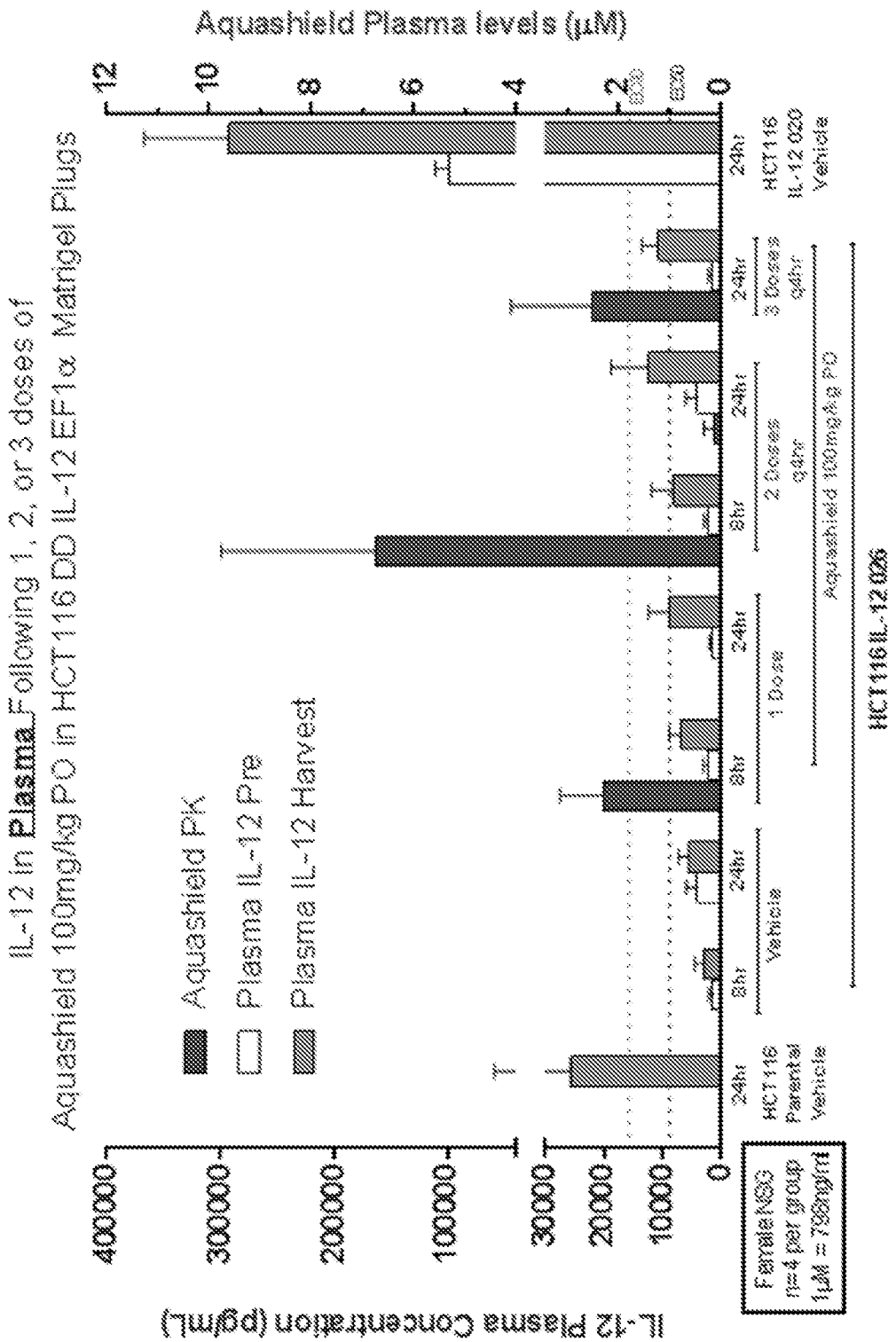

IL-12 in Plasma Following 1, 2, or 3 doses of
Aquashield 100mg/kg PO in HCT116 DD IL-12 EF1α Matrigel Plugs
Aquashield PK
Plasma IL-12 Pre
Plasma IL-12 Harvest
Aquashield Plasma levels (µM)
IL-12 Plasma Concentration (pg/mL)
Female NSG
n=4 per group
1µM = 786ng/ml
HCT116 Parental Vehicle
HCT116 IL-12 020 Vehicle
Vehicle
1 Dose
2 Doses q4hr
3 Doses q4hr
Aquashield 100mg/kg PO
HCT116 IL-12 026
8hr
24hr

Figure 33C

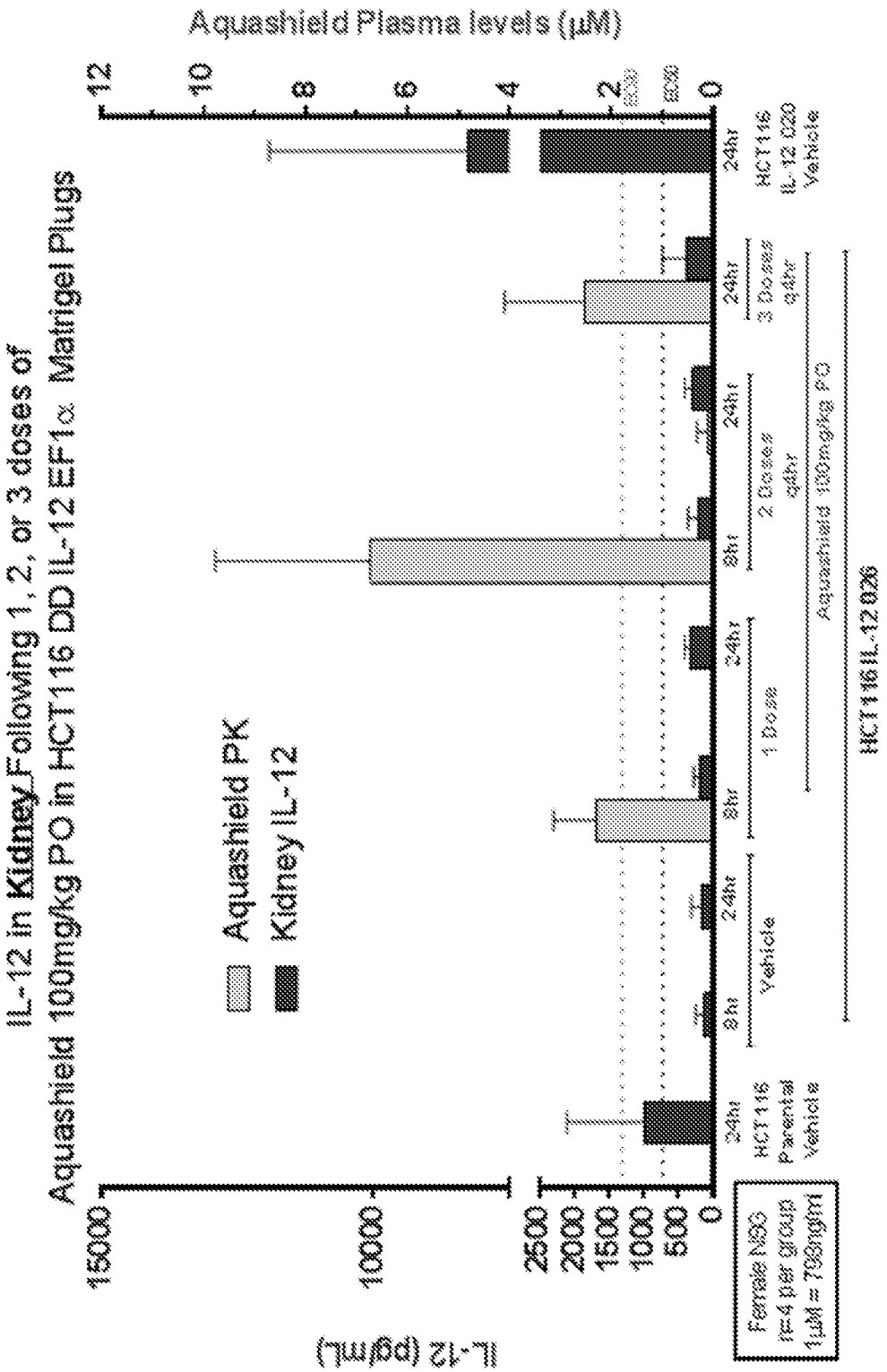

IL-12 in Kidney Following 1, 2, or 3 doses of
Aquashield 100mg/kg PO in HCT116 DD IL-12 EF1α Matrigel Plugs
Aquashield Plasma levels (μM)
IL-12 (pg/mL)
Aquashield PK
Kidney IL-12
Female NSG
n=4 per group
1μM = 798ng/ml
HCT116 Parental Vehicle
HCT116 IL-12 026
Vehicle
1 Dose
2 Doses
3 Doses
q8hr
Aquashield 100mg/kg PO
HCT116 IL-12 026
Vehicle

Figure 34A

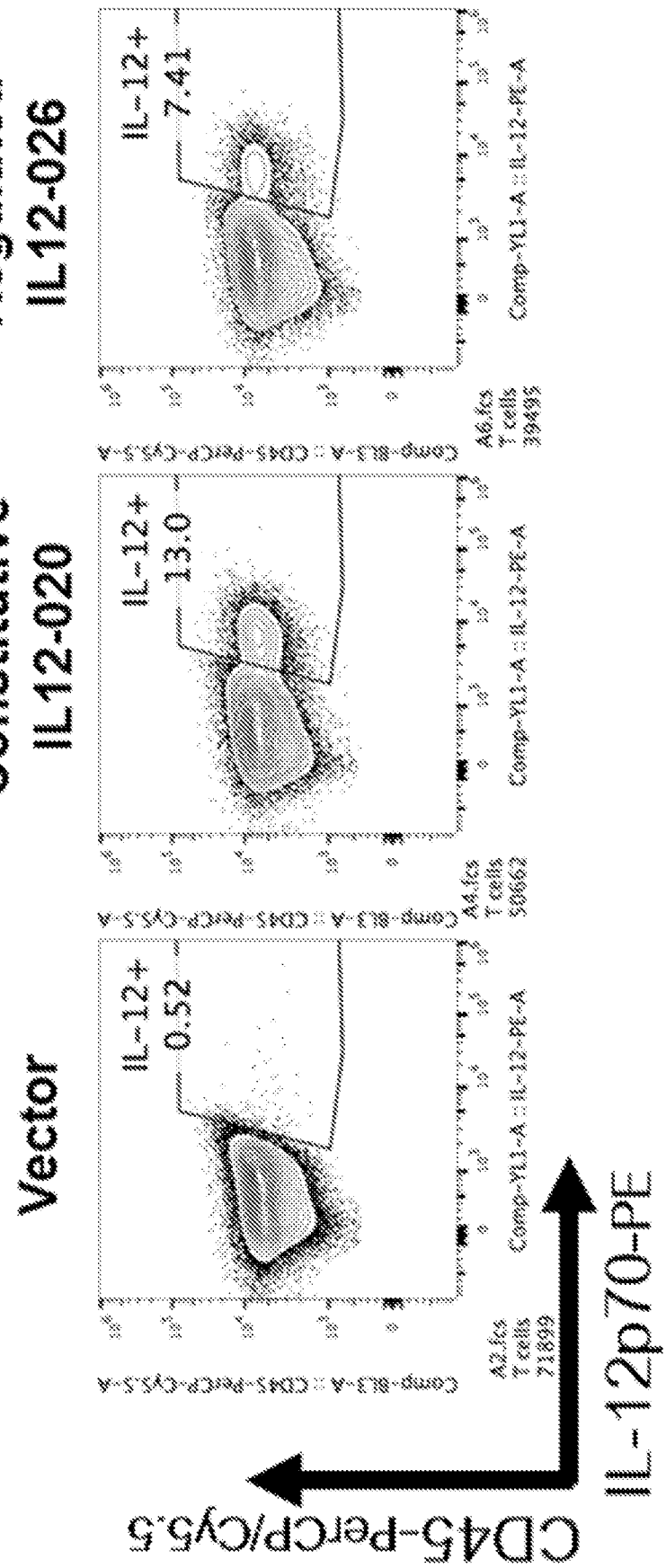

Vector
Constitutive IL12-020
Regulated IL12-026
IL-12+ 0.52
IL-12+ 13.0
IL-12+ 7.41
A2.fcs T cells 21899
A4.fcs T cells 50662
A6.fcs T cells 39495
CD45-PerCP/Cy5.5
IL-12p70-PE

Figure 34B

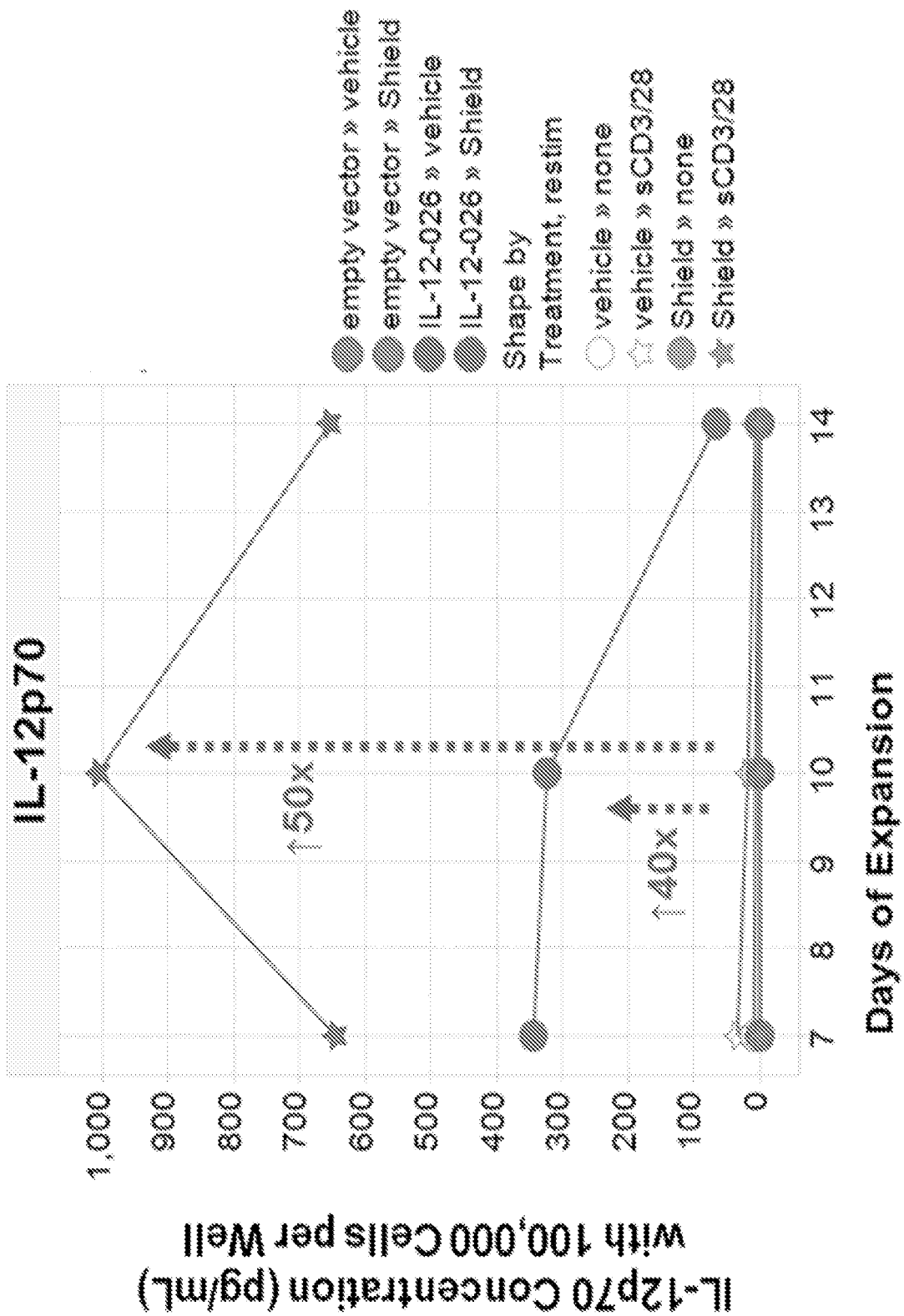

IL-12p70
IL-12p70 Concentration (pg/mL) with 100,000 Cells per Well
1,000
900
800
700
600
500
400
300
200
100
0
7
8
9
10
11
12
13
14
Days of Expansion
↑50x
↑40x
empty vector » vehicle
empty vector » Shield
IL-12-026 » vehicle
IL-12-026 » Shield
Shape by
Treatment, restim
vehicle » none
vehicle » sCD3/28
Shield » none
Shield » sCD3/28

Figure 34C

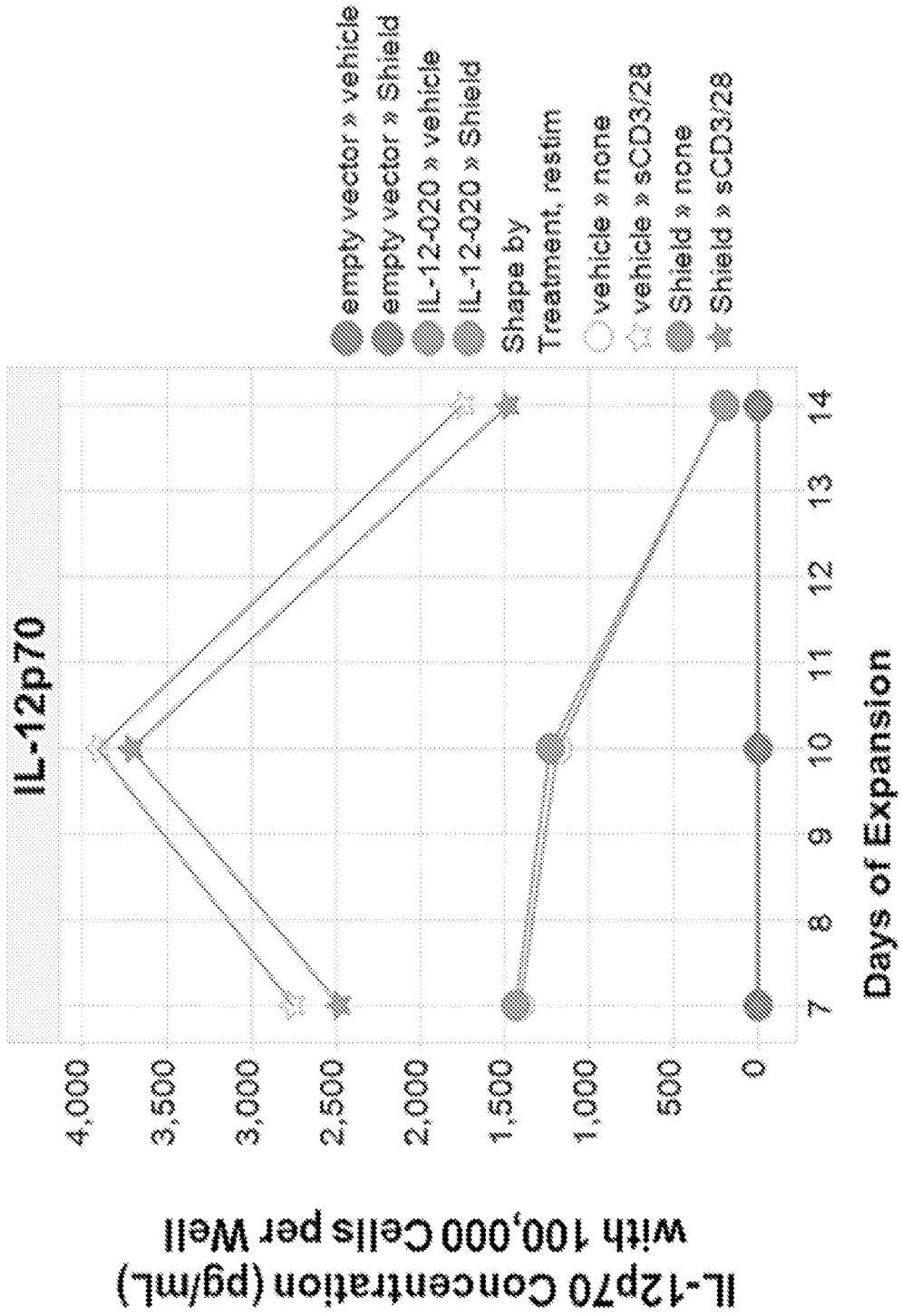

IL-12p70
IL-12p70 Concentration (pg/mL) with 100,000 Cells per Well
4,000
3,500
3,000
2,500
2,000
1,500
1,000
500
0
7
8
9
10
11
12
13
14
Days of Expansion
empty vector » vehicle
empty vector » Shield
IL-12-020 » vehicle
IL-12-020 » Shield
Shape by
Treatment, restim
vehicle » none
vehicle » sCD3/28
Shield » none
Shield » sCD3/28

Figure 35A

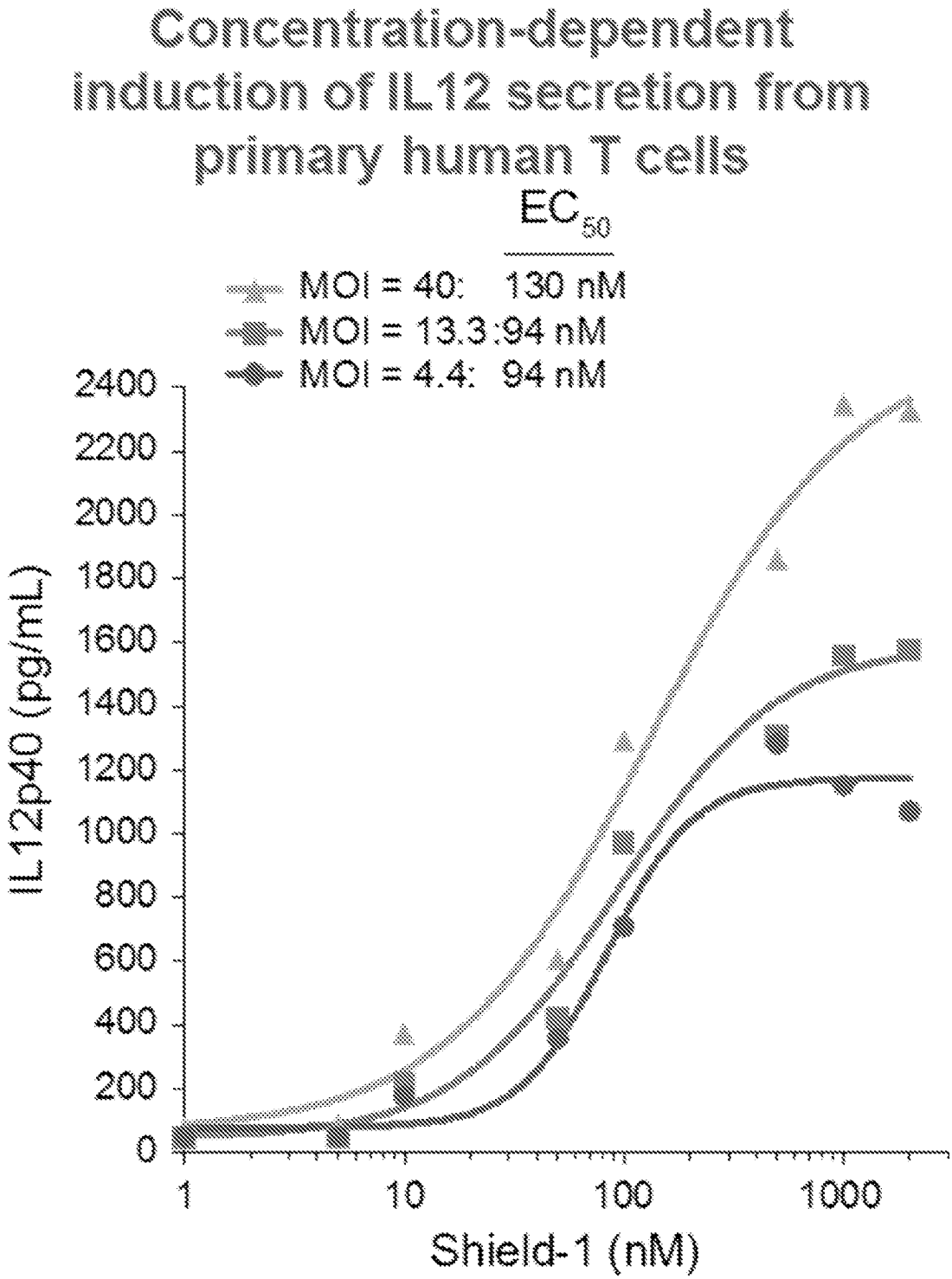

Concentration-dependent induction of IL12 secretion from primary human T cells
EC50
MOI = 40: 130 nM
MOI = 13.3 :94 nM
MOI = 4.4: 94 nM
IL12p40 (pg/mL)
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
200
0
1
10
100
1000
Shield-1 (nM)

Figure 35B

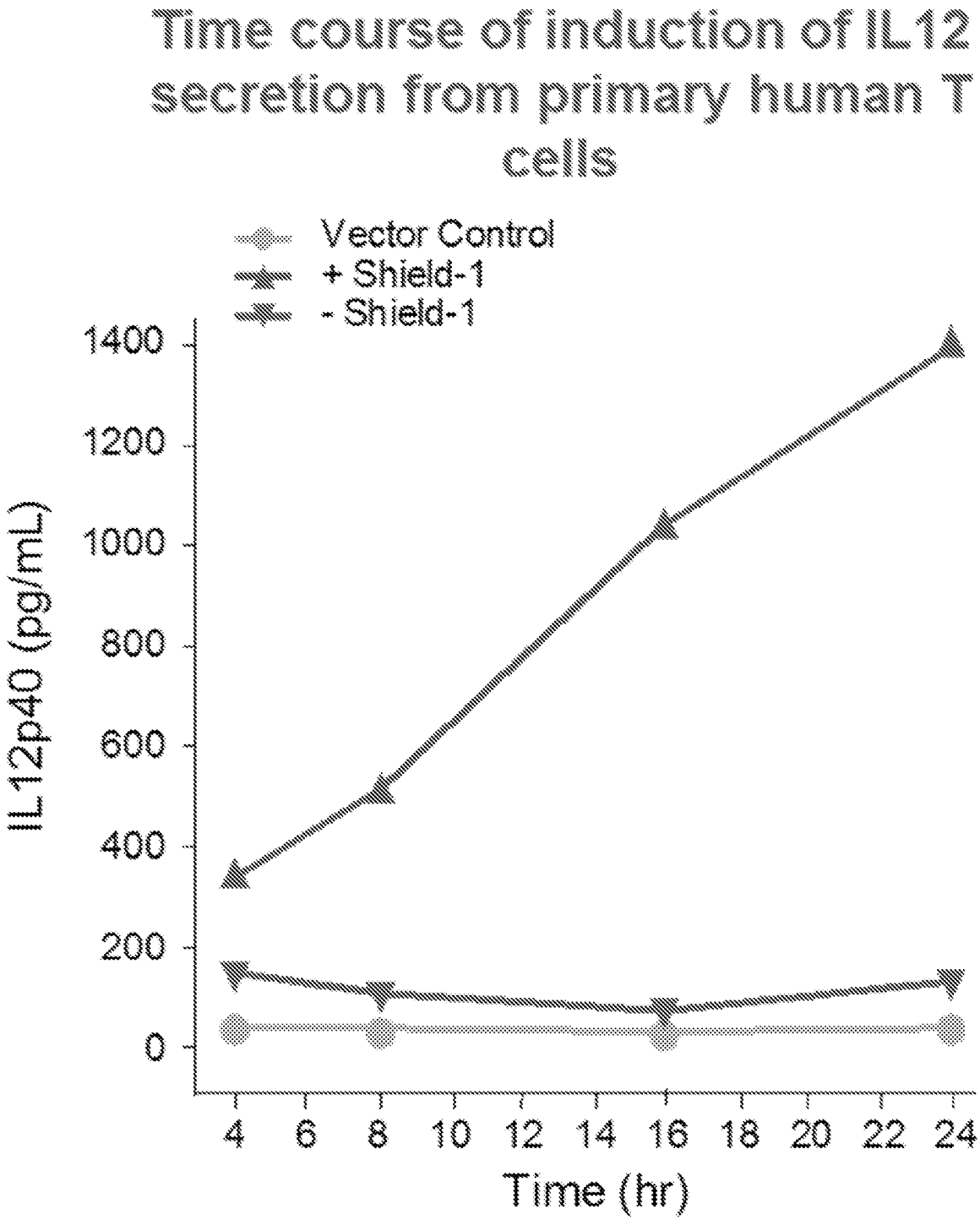

Time course of induction of IL12 secretion from primary human T cells
Vector Control
+ Shield-1
- Shield-1
IL12p40 (pg/mL)
1400
1200
1000
800
600
400
200
0
4
6
8
10
12
14
16
18
20
22
24
Time (hr)

Figure 36A

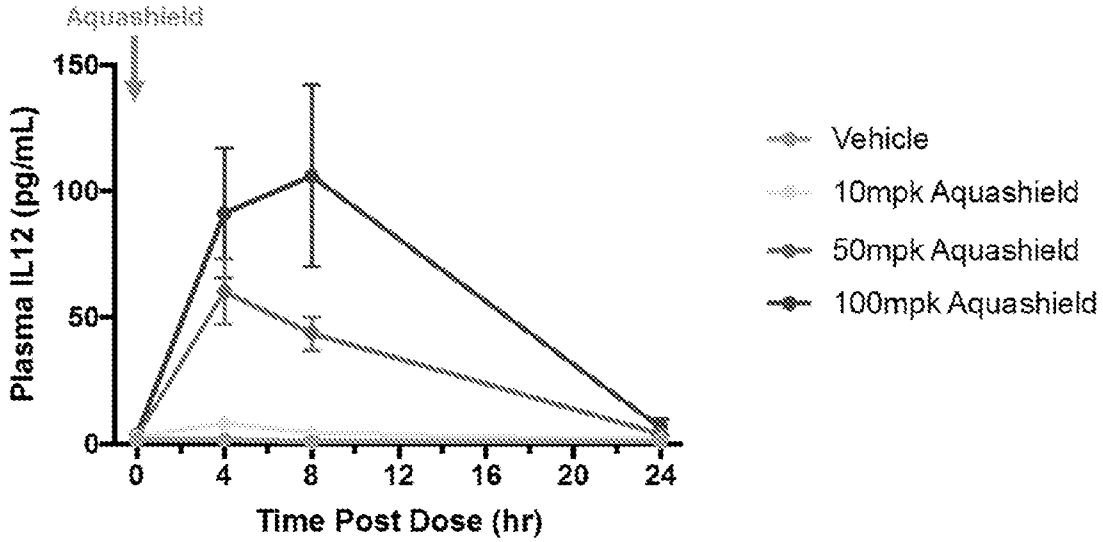


Figure 36B

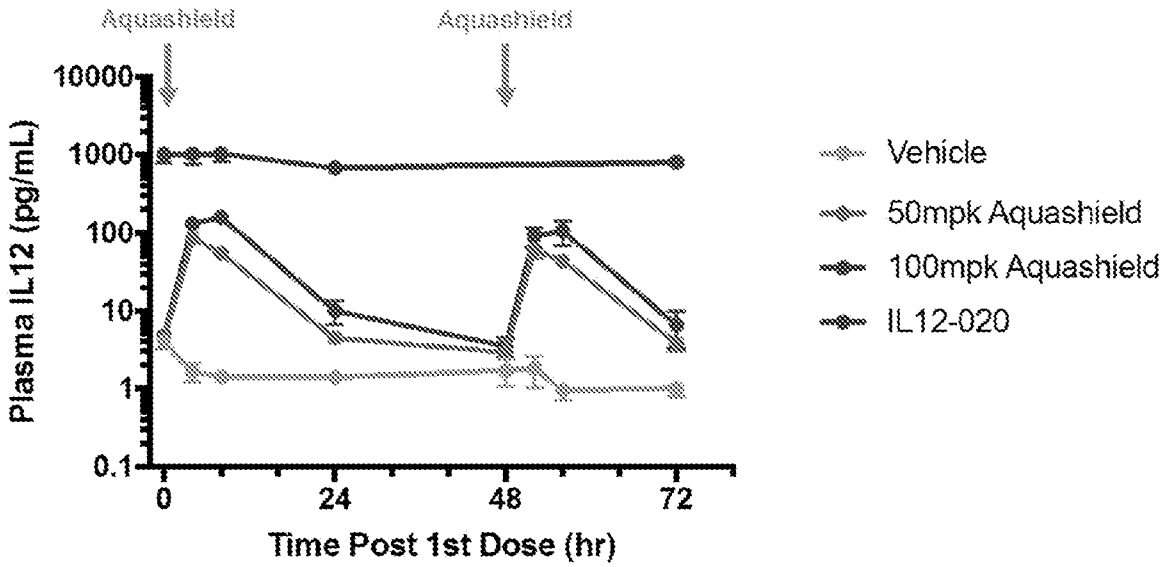


Figure 37A

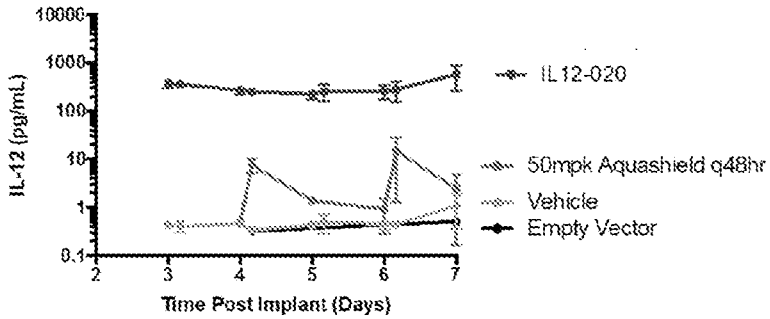


Figure 37B

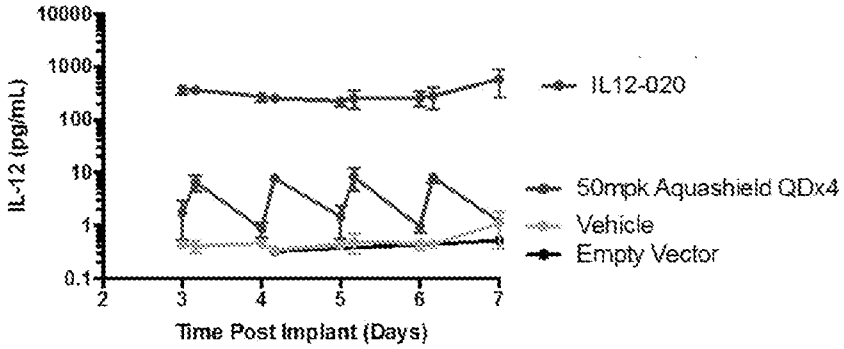


Figure 37C

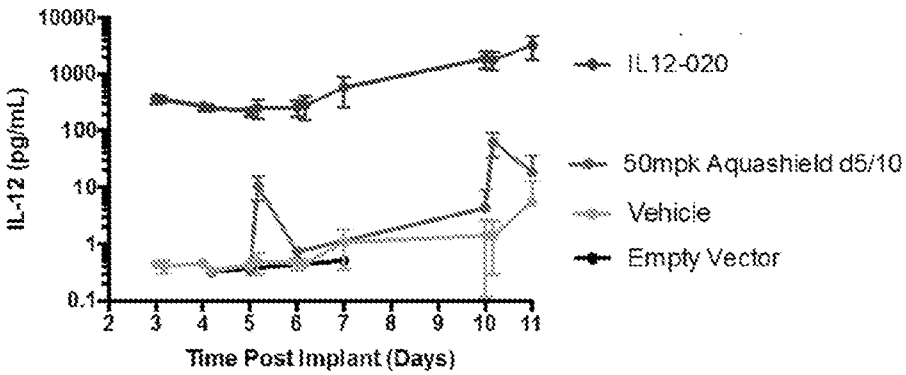


Figure 37D

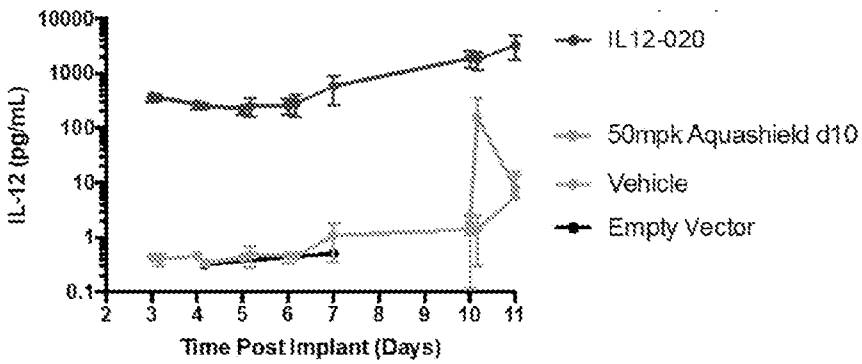


Figure 37E

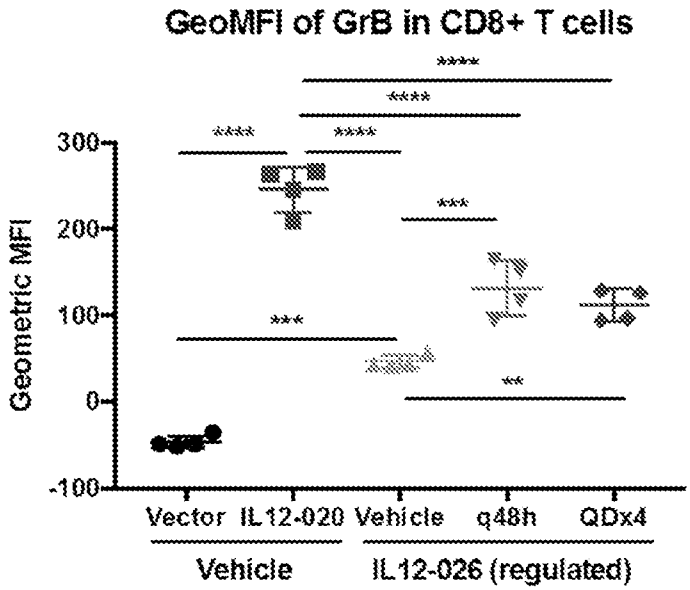


Figure 37F

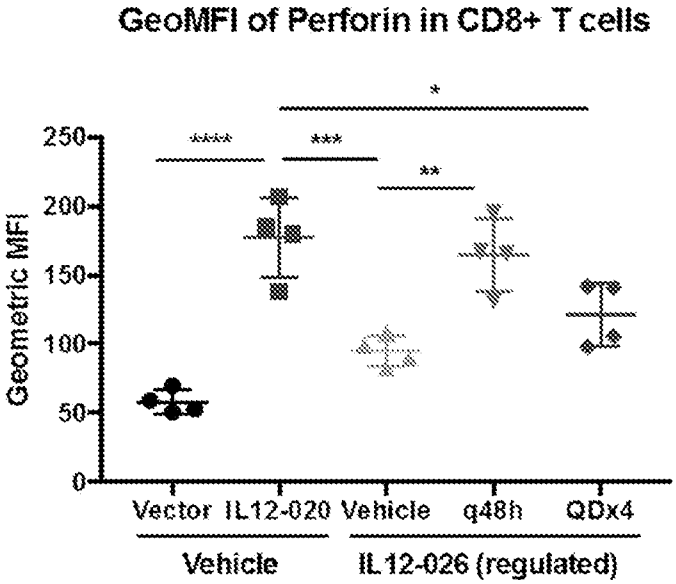


Figure 38A

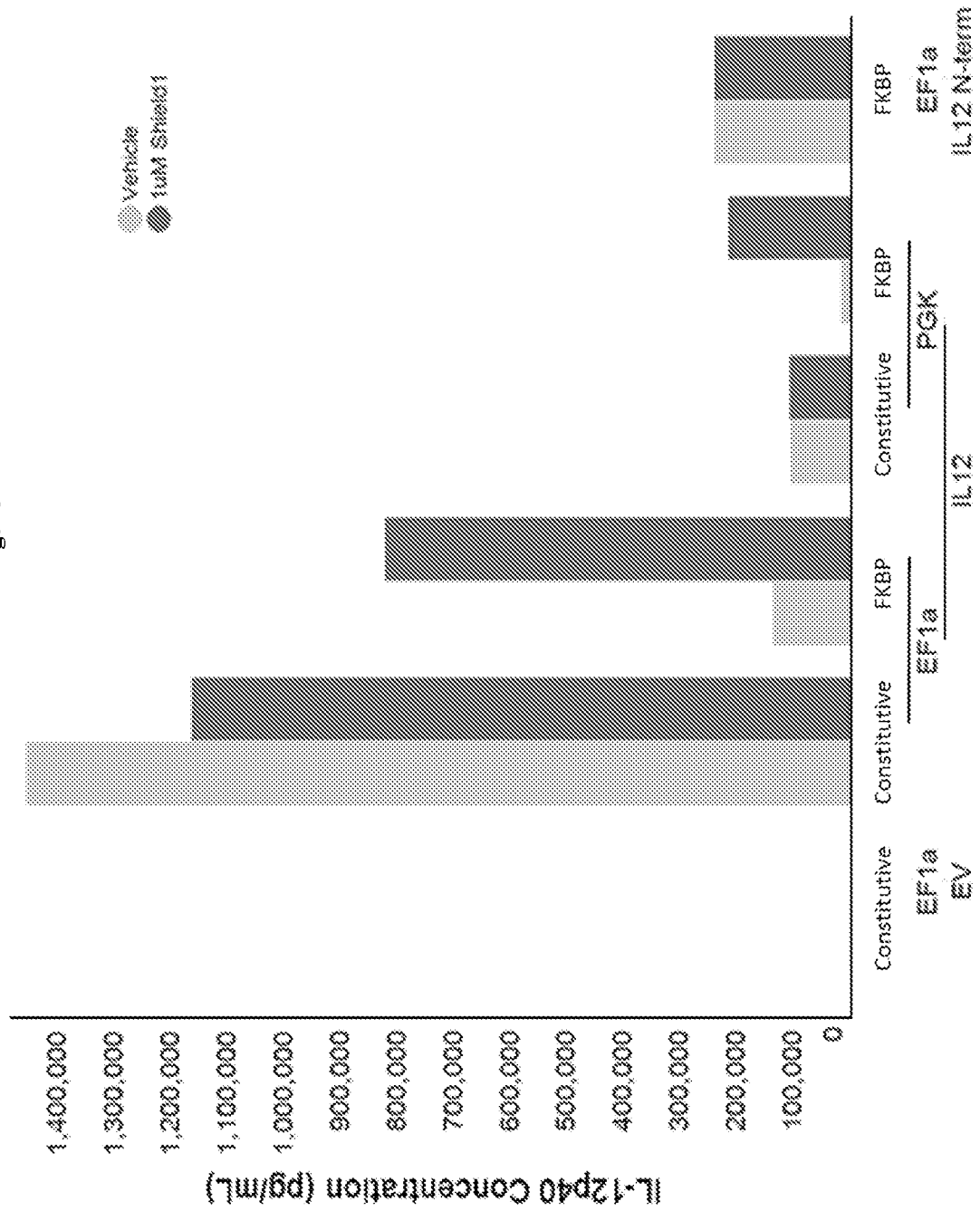

Vehicle
1uM Shield1
IL-12p40 Concentration (pg/mL)
1,400,000
1,300,000
1,200,000
1,100,000
1,000,000
900,000
800,000
700,000
600,000
500,000
400,000
300,000
200,000
100,000
0
Constitutive
EF1a
EV
Constitutive
FKBP
EF1a
IL12
Constitutive
FKBP
PGK
FKBP
EF1a
IL12 N-term

Figure 38B

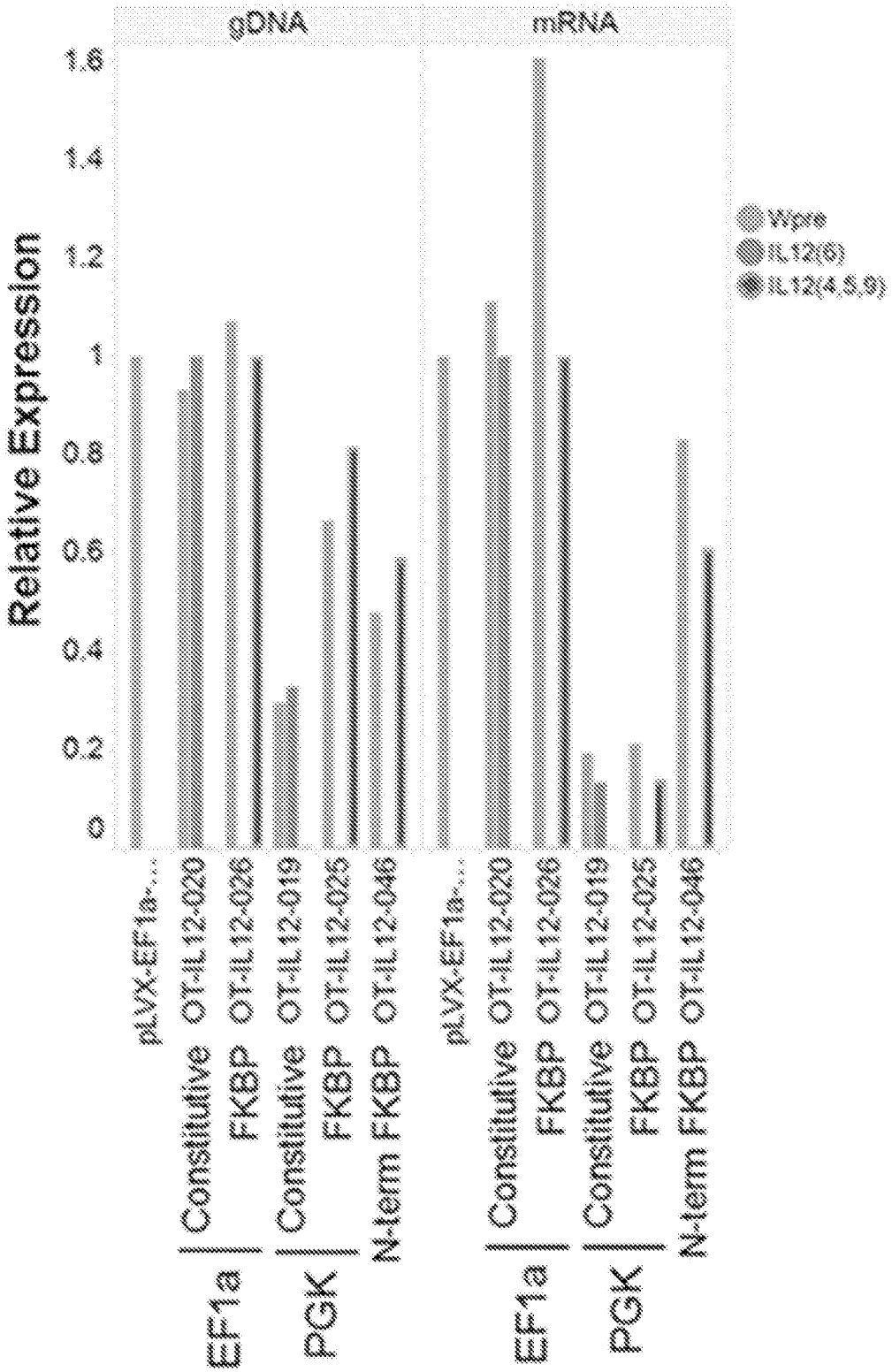

gDNA
mRNA
Relative Expression
0
0.2
0.4
0.6
0.8
1
1.2
1.4
1.6
Wpre
IL12(p)
IL12(4.5.9)
pLVX-EF1a-...
OT-IL12-020
OT-IL12-026
OT-IL12-019
OT-IL12-025
OT-IL12-046
EF1a
PGK
Constitutive
FKBP
N-term FKBP

Figure 39

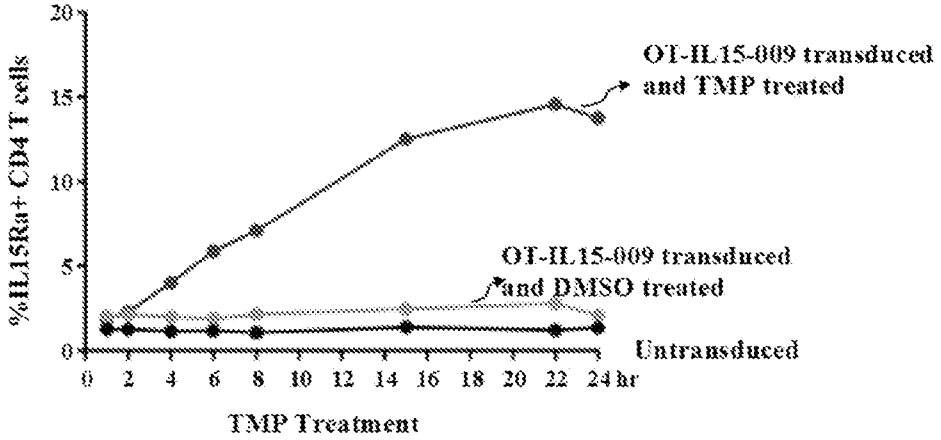


Figure 40

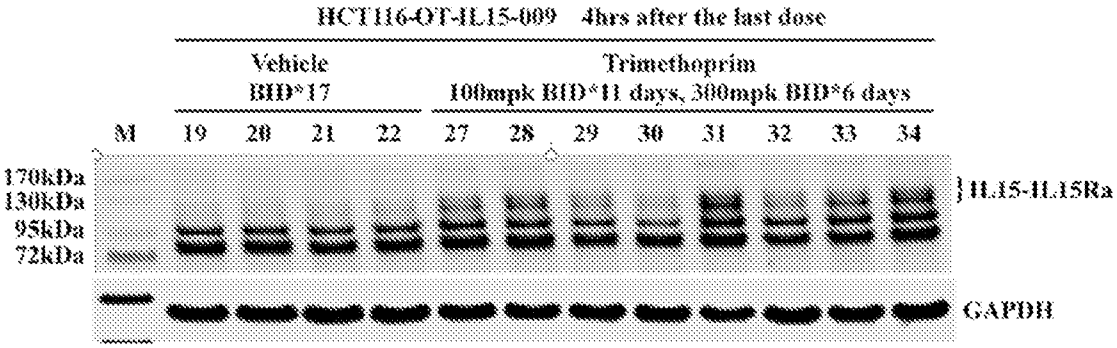


Figure 41

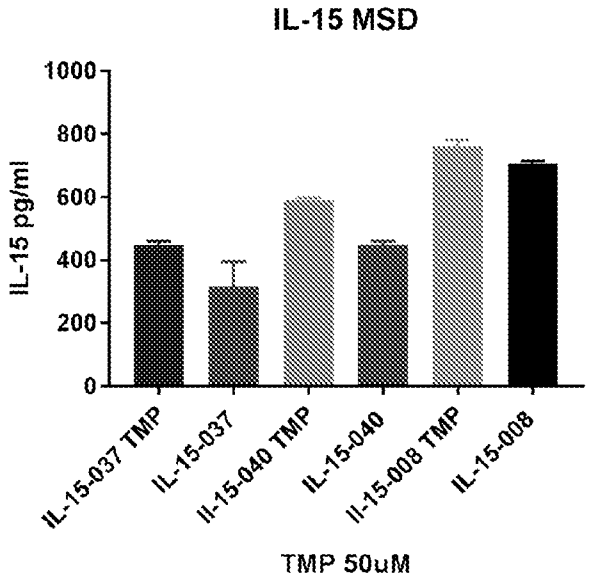
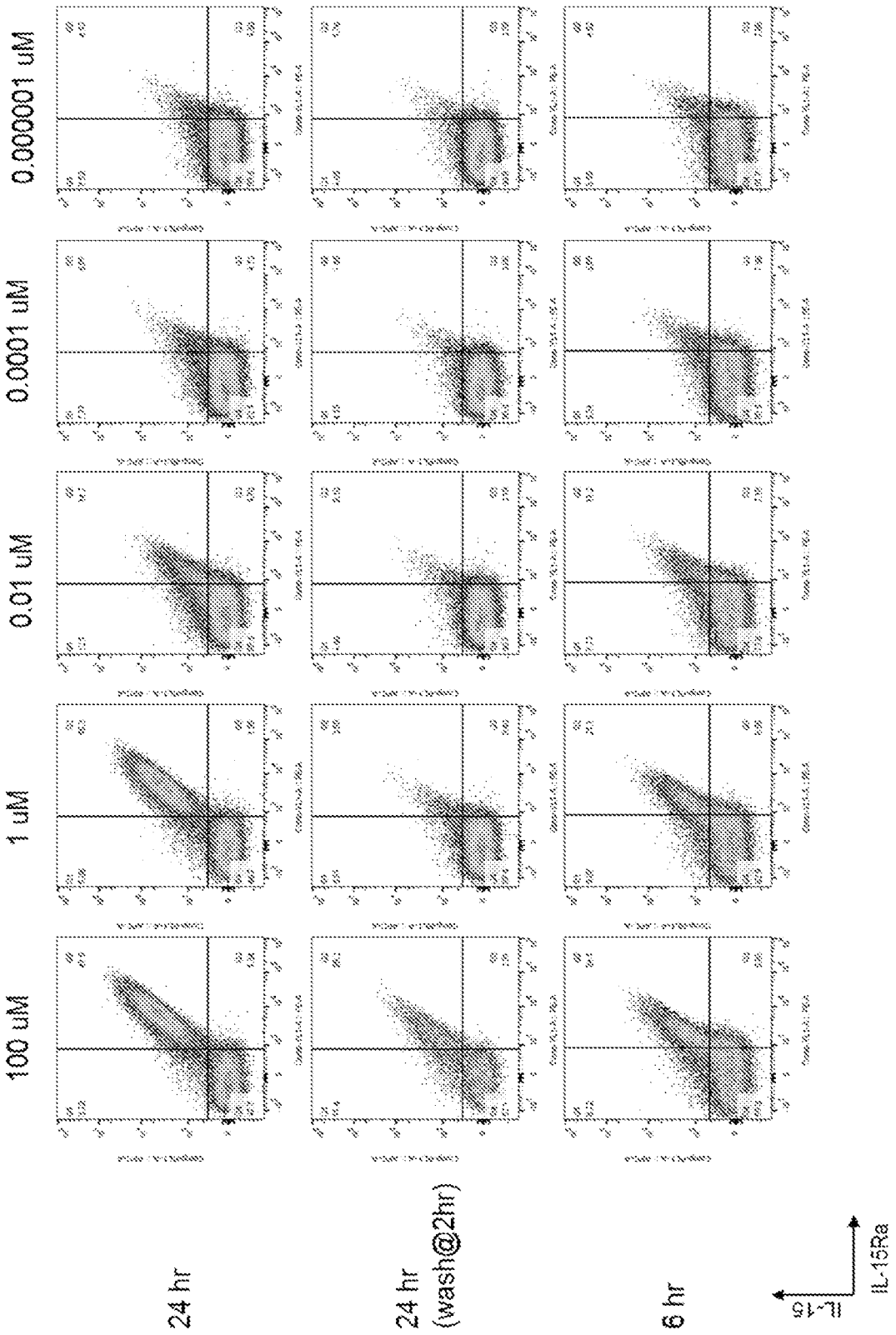


Figure 42A


100 uM
1 uM
0.01 uM
0.0001 uM
0.000001 uM
24 hr
24 hr (wash@2hr)
6 hr
IL-15
IL-15Ra

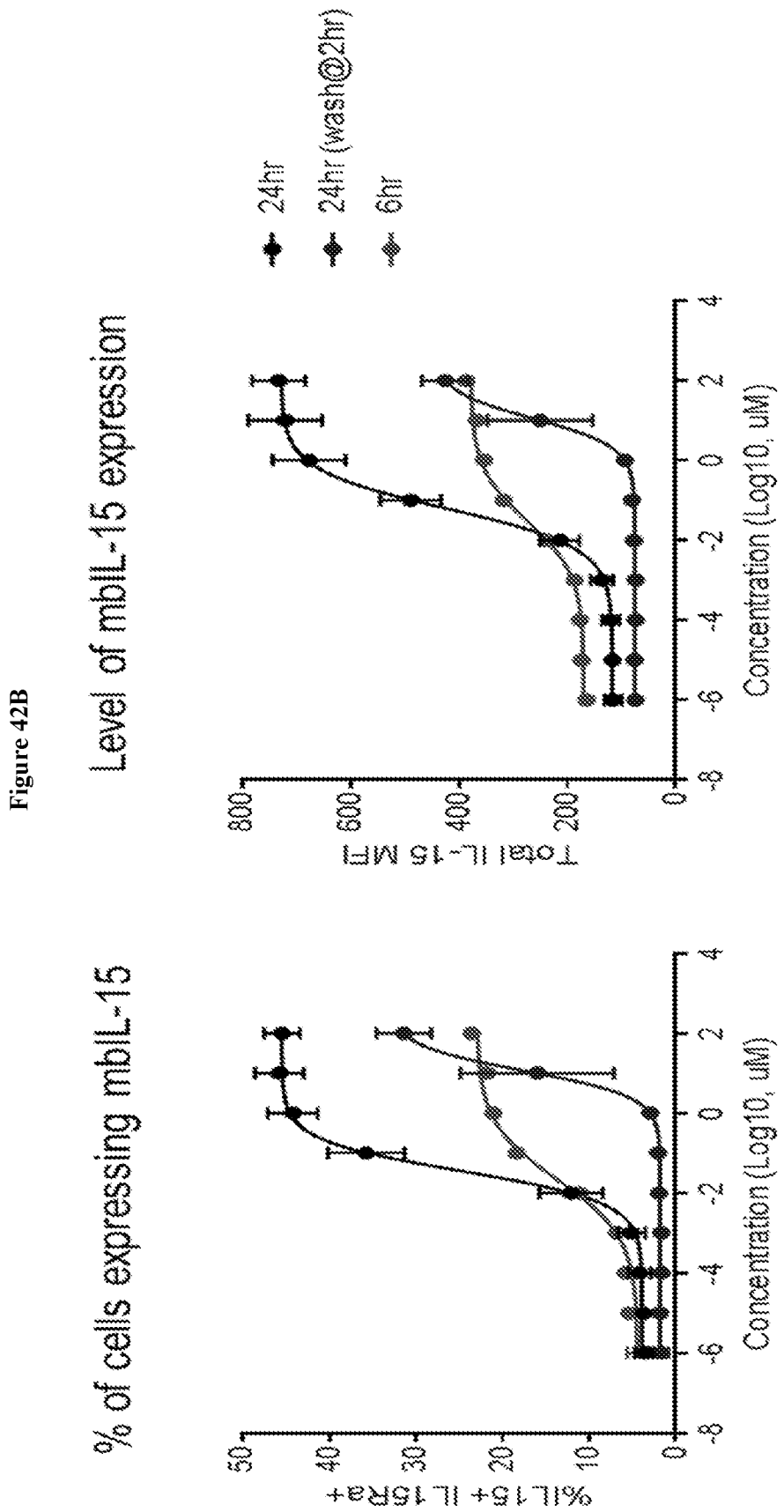

Level of mbIL-15 expression
24hr
24hr (wash@2hr)
6hr
Total IL-15 MFI
800
600
400
200
0
-8
-6
-4
-2
0
2
4
Concentration (Log10, uM)
% of cells expressing mbIL-15
%IL15+ IL15Ra+
50
40
30
20
10
0
-8
-6
-4
-2
0
2
4
Concentration (Log10, uM)

Figure 42B

Figure 43A

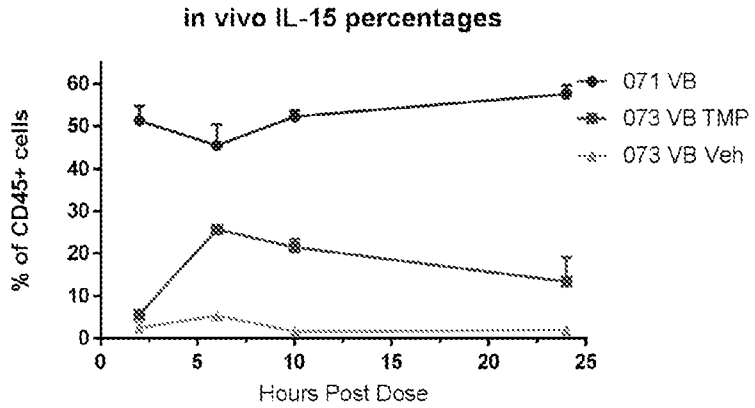


Figure 43B

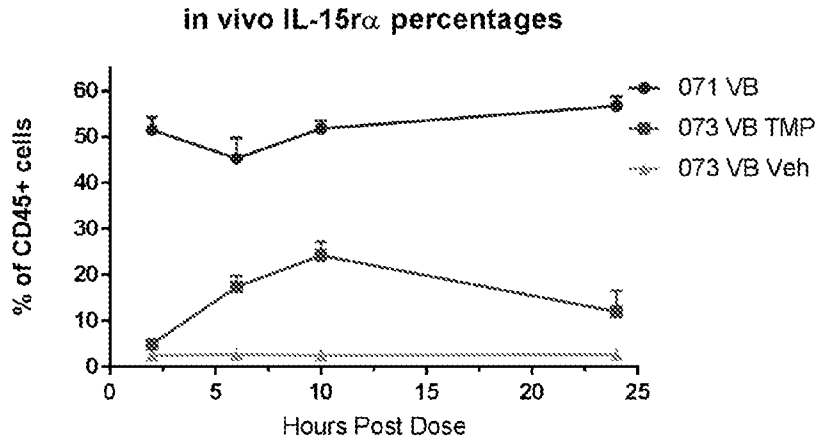


Figure 43C

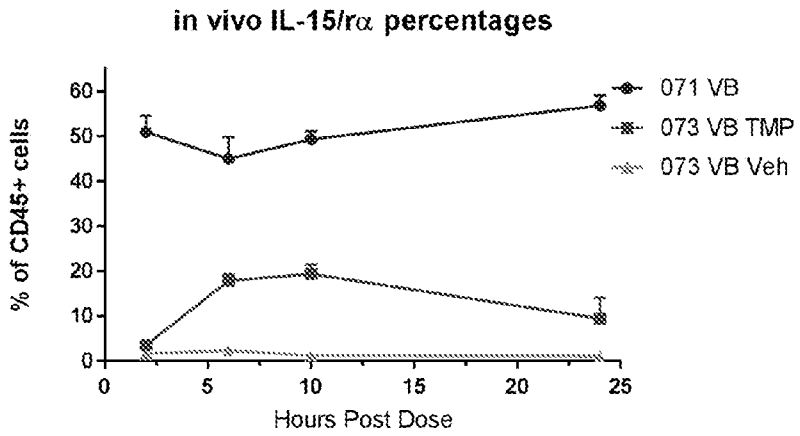

in vivo IL-15/rα percentages
% of CD45+ cells
60
50
40
30
20
10
0
0
5
10
15
20
25
Hours Post Dose
071 VB
073 VB TMP
073 VB Veh

Figure 43D

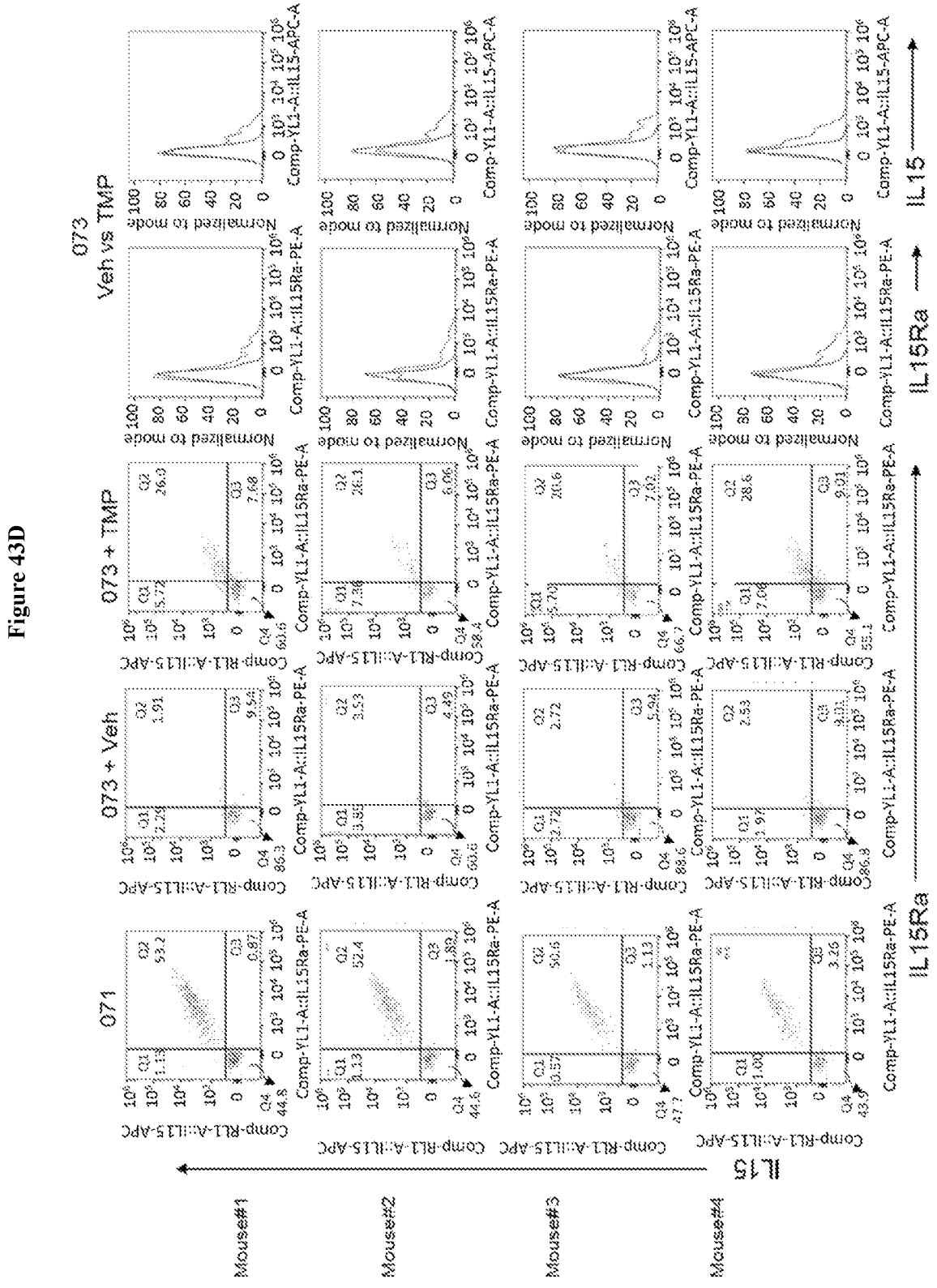

071
073 + Veh
073 + TMP
073
Veh vs TMP
Mouse#1
Mouse#2
Mouse#3
Mouse#4
IL15
IL15Ra
Comp-RL1-A::IL15-APC
Comp-YL1-A::IL15Ra-PE-A
Comp-YL1-A::IL15-APC-A
Normalized to mode

Figure 43E

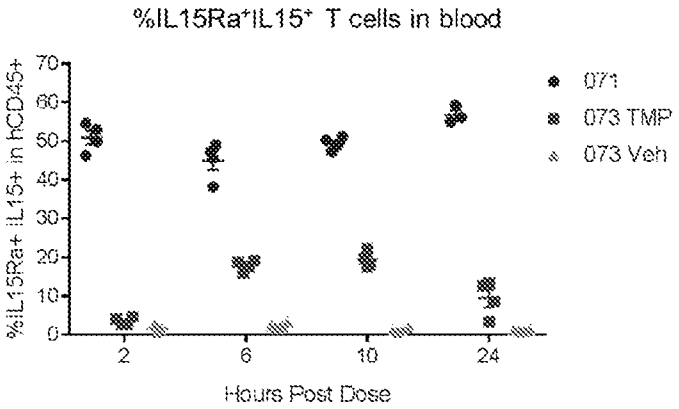


Figure 43F

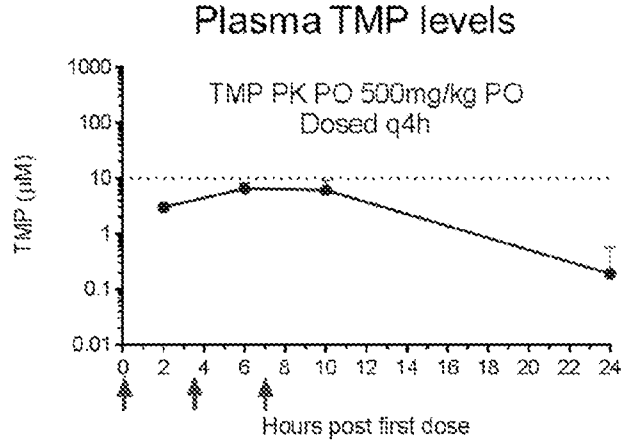
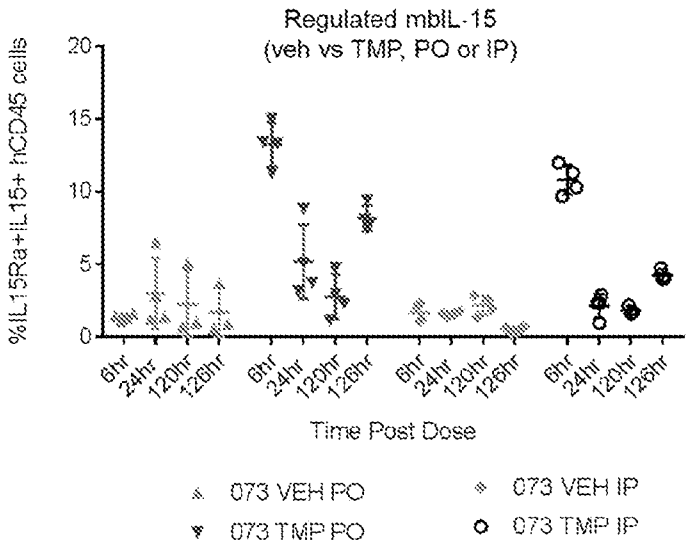


Figure 44



INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/20741

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:

   a. ☒ forming part of the international application as filed:

      ☒ in the form of an Annex C/ST.25 text file.

      ☐ on paper or in the form of an image file.

   b. ☐ furnished together with the international application under PCT Rule 13*ter*.1(a) for the purposes of international search only in the form of an Annex C/ST.25 text file.

   c. ☐ furnished subsequent to the international filing date for the purposes of international search only:

      ☐ in the form of an Annex C/ST.25 text file (Rule 13*ter*.1(a)).

      ☐ on paper or in the form of an image file (Rule 13*ter*.1(b) and Administrative Instructions, Section 713).

2. ☐ In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that forming part of the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US18/20741

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.: 27-45
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 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.:
1-5, 8-11, 12/9-11, 13/12/9-11, 14/13/12/9-11, 15-16, 18-23 SEQ ID NOs: 3, 49, 81, 339, 375, 426

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

International application No.
PCT/US18/20741

A. CLASSIFICATION OF SUBJECT MATTER

IPC - C07K 14/505, 14/705, 14/725, 16/18, 16/28, 16/30, 16/46; A61K 38/17, 38/43, 35/14 (2018.01)

CPC - C07K 14/505, 14/705, 16/18, 16/28, 16/30, 16/46, 16/2896; A61K 35/14, 38/17, 38/43, 39/395, 48/00; C12N 5/10, 15/62, 15/63, 15/85; A61P 35/02

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	WO 2016/134284 A1 (UNIVERSITY OF FLORIDA RESEARCH FOUNDATION, INC.) 25 August 2016; page 2, lines 13-15; page 5, lines 9-10; page 6, line 29; page 10, lines 15-17; page 11, lines 20-23; page 12, line 11; page 13, line 18; page 15, lines 23-24; page 16, line 10; page 26, line 20; page 39, lines 3-6, 18-19; page 42, lines 1-8; page 55, lines 14-17; page 107, line 28; Figure 1	1-4, 8-11, 12/9-11, 13/12/9-11, 15-16, 18, 20-22 ------- 5, 14/13/12/9-11, 19, 23
Y	US 2008/0280830 A1 (CHOI, SY et al.) 13 November 2008; paragraph [0004], [0011]	5
Y	US 2015/0307564 A1 (THE CALIFORNIA INSTITUTE FOR BIOMEDICAL RESEARCH) 29 October 2015; paragraphs [0004], [0060]	14/13/9-11, 23
Y	WO 2005/012493 A2 (IMMUNOMEDICS, INC.) 10 February 2005; page 54, lines 25-30	14/13/12/9-11, 23
Y	WO 2016/113203 A1 (PIERIS AG) 21 July 2016; page 5, 2nd paragraph	19
A	US 2013/0266551 A1 (ST. JUDE CHILDREN'S RESEARCH HOSPITAL, INC.) 10 October 2013: entire document	1-5, 8-11, 12/9-11, 13/12/9-11, 14/13/12/9-11, 15-16, 18-23
A	WO 2012/079000 A1 (THE TRUSTEES OF THE UNIVERSITY OF PENNSYLVANIA) 14 June 2012; entire document	1-5, 8-11, 12/9-11, 13/12/9-11, 14/13/12/9-11, 15-16, 18-23

☒ 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
21 May 2018 (21.05.2018)	21 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/US18/20741

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2013/059593 A1 (THE UNITED STATES OF AMERICA, AS REPRESENTED BY THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES) 25 April 2013; entire document	1-5, 8-11, 12/9-11, 13/12/9-11, 14/13/12/9-11, 15-16, 18-23
P, X	WO 2017/180587 A2 (OBSIDIAN THERAPEUTICS, INC.) 19 October 2017; entire document	1-5, 8-11, 12/9-11, 13/12/9-11, 14/13/12/9-11, 15-16, 18-23
P, X	WO 2018/023025 A1 (NOVARTIS AG et al.) 01 February 2018; entire document	1-5, 8-11, 12/9-11, 13/12/9-11, 14/13/12/9-11, 15-16, 18-23

INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/US18/20741

-***-Continued from Box No. 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 1-26, FKBP, a chimeric antigen receptor (CAR) encompassing a scFv and SEQ ID NOs: 3, 49, 81, 339, 375, and 426 are directed toward a composition for inducing an immune response in a cell or a subject.

The composition will be searched to the extent it encompasses an immunotherapeutic agent encompassing a chimeric antigen receptor encompassing a scFv (immunotherapeutic agent) comprising a heavy chain variable region encompassing SEQ ID NO: 49 (VH), a light chain variable region encompassing SEQ ID NO: 81 (VL), a signaling domain encompassing SEQ ID NO: 339 (signaling domain), a transmembrane domain sequence encompassing SEQ ID NO: 375 (TM domain); a hinge domain encompassing SEQ ID NO: 426 (hinge); and a SRE encompassing destabilizing domain encompassing FKBP (SEQ ID NO: 3)(SRE). Applicant is invited to elect additional immunotherapeutic agent(s), with (where applicable) a fully specified sequence for the full length agent (i.e. no optional or variable residues or substituents) represented by a SEQ ID NO: or SEQ ID NO: with specified substitution(s) at specified site(s) therein, for the immunotherapeutic agent(s), or associated set(s) of specified SEQ ID NO(s), representing the sub-sequences of the agent(s) (e.g. heavy and light chain variable region sequence pairs; signaling domain sequence, costimulatory domain sequence, transmembrane domain sequence, hinge domain sequence), and/or to elect additional SRE(s), with specified SEQ ID NO(s), or with specified mutation (s) to a SEQ ID NO: associated therewith, such that the sequence of the elected SRE(s) are fully specified, to be searched. Additional immunotherapeutic agent and/or SRE sequence(s) will be searched upon the payment of additional fees. It is believed that claims 1, 2 (in-part), 3 (in-part), 4 (in-part), 5 (in-part), 8 (in-part), 9 (in-part), 10 (in-part), 11 (in-part), 12 (in-part), 13 (in-part), 14 (in-part), 15 (in-part), 16 (in-part), 18 (in-part), 19 (in-part), 20 (in-part), 21 (in-part), 22 (in-part), and 23 (in-part) encompass this first named invention and thus these claims will be searched without fee to the extent that they encompass SEQ ID NO: 49 (VH), SEQ ID NO: 81 (VL), SEQ ID NO: 339 (signaling domain), SEQ ID NO: 375 (TM domain); SEQ ID NO: 426 (hinge), and FKBP (SEQ ID NO: 3)(SRE). Applicants must specify the claims that encompass any additionally elected agent(s) and/or SRE(s) and 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 a SRE encompassing human DHFR SEQ ID NO: 1 (SRE).

Group II, Claims 46-50 are directed toward a method of identifying a domain of a CD19 antigen which will not bind the FMC63 antibody (FMC63-distinct CD19 binding domain), said method comprising: (a) preparing a composition comprising a CD19 antigen, (b) contacting the composition in (a) with saturating levels of FMC63 antibody, (c) contact the composition of step (b) with one or more selected members of a library of potential CD19 binders; and (d) identifying a binding domain on the CD19 antigen based on the differential binding of the selected members of the library of CD19 binders compared to the binding of FMC63.

The inventions listed as Groups I+ and II do not relate to a single general inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: the special technical features of Groups I+ include SEQ ID NO: 3, not present in Group II; the special technical features of Group II include an FMC63 antibody, not present in any of Groups I+.

No technical features are shared between Groups I+ and II, accordingly, these groups lack unity a priori.

No technical features are shared between the agent and/or SRE sequences 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; 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]).

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.