

PEGYLATED IL-2 FOR SUPPRESSING ADAPTIVE IMMUNE RESPONSE TO GENE THERAPY**CROSS-REFERENCE TO RELATED APPLICATIONS**

[0001] This application claims the benefit of priority of US Provisional Patent Application No. 63/625,113, filed January 25, 2024, and US Provisional Patent Application No. 63/641,189, filed May 1, 2024, which are each incorporated by reference herein in its entirety for all purposes.

FIELD OF THE DISCLOSURE

[0002] This disclosure provides methods of delivering a gene therapy agent to a cell of a subject, methods of treating an individual in need thereof with a gene therapy agent, methods of increasing expression of a gene therapy agent, methods of reducing an immune response to a gene therapy agent, and methods of preventing immune-related adverse events in a subject in which an IL-2 conjugate is administered. In some embodiments, the IL-2 conjugate expands Treg cells, reduces an immune response to the gene therapy agent, facilitates increased expression of the gene therapy agent, and/or prevents immune-related adverse events in a subject receiving the gene therapy agent.

SEQUENCE LISTING

[0003] The present application is filed with a Sequence Listing in electronic format. The Sequence Listing is provided as a file entitled "01183-0317-00PCT.xml" created on January 22, 2025, which is 40,250 bytes in size. The information in the electronic format of the sequence listing is incorporated herein by reference in its entirety.

INTRODUCTION AND SUMMARY

[0004] One of the current challenges with adeno-associated virus (AAV)-based and lipid nanoparticle (LNP)-based gene therapy is the host immune responses, resulting in immune-related adverse events (irAEs) and reduced therapeutic efficacy. Adaptive immune responses develop cytotoxic T-cell responses after exposure to AAV capsid and its transgene, and transduced cells get destroyed by the activated cytotoxic T cells (Ertl, 2022), resulting in the irAEs and loss of transgene expression. Similarly, expression of LNP-delivered transgene (cdDNA or RNA) can elicit cytotoxic T-cell responses to the therapeutic protein, leading to death of transduced cells and related toxicities. Regulatory T cells play crucial roles in regulating activation of immune cells including cytotoxic T cells. Therefore, it was hypothesized that by

selectively and transiently expanding regulatory T cells, cytotoxic T-cell activation by the AAV and LNP can be hampered, leading to better safety and transgene expression. IL-2 is a crucial cytokine for regulatory T cells to proliferate. However, it is challenging to use recombinant human IL-2 (rhIL-2) to expand regulatory T cells in vivo as other lymphocytes, such as cytotoxic T cells, also express IL-2 receptors (IL-2Rs). Although regulatory T cells bind to IL-2 more strongly compared to other lymphocytes due to their constitutive expression of IL-2R alpha (Hernandez et al., 2022), its therapeutic window is very narrow to achieve selective expansion of regulatory T cells. In addition, rhIL-2 has shown a very poor half-life in vivo (<90 min.), which requires multiple intravenous (IV) infusions for a therapeutic purpose (see, e.g., www.accessdata.fda.gov/drugsatfda_docs/label/2012/103293s5130lbl.pdf). Accordingly, there is a need for improved methods of delivering a gene therapy agent to a cell of a subject, methods of treating an individual in need thereof with a gene therapy agent, methods of increasing expression of a gene therapy agent, methods of reducing an immune response to a gene therapy agent, and methods of preventing immune-related adverse events in a subject. The present disclosure aims to meet one or more of these needs, provide other benefits, or at least provide the public with a useful choice.

[0005] The present inventors explored use of an IL-2 conjugate comprising a conjugating moiety in the above methods. IL-2 conjugates as described herein can prevent or reduce its binding to IL-2R beta, and can result in selective expansion of regulatory T cells in that the IL-2 conjugate can have reduced binding to other lymphocytes, such as cytotoxic T cells (i.e., cytotoxic T lymphocytes (CTL)). In addition, IL-2 conjugates as described herein can exhibit a longer serum half-life compared to IL-2, which allows routes of administration other than IV infusion, and can lead to better patient compliance. There has been a mouse study applying regulatory T cells to AAV gene therapy to modulate immune responses (Arjomandnejad M, Sylvia K, Blackwood M, Nixon T, Tang Q, Muhuri M, Gruntman AM, Gao G, Flotte TR, Keeler AM. *Mol Ther Methods Clin Dev.* 2021 Oct 28;23:490-506.), but its application of regulatory T cells is in a manner of adoptive cell transfer, which increases complexity and regulatory issues relative to methods described herein, which do not require adoptive cell transfer.

[0006] The present inventors have selectively expanded regulatory T cells with Compound A as a pre-treatment or a combination treatment while administering AAV-based or LNP-based gene therapies to mitigate irAEs and enhance transgene expression in various animal models, such as mice, rats, and non-human primates.

[0007] The following embodiments are encompassed. Embodiment 1 is a method of delivering a gene therapy agent to a cell of a subject, comprising administering an IL-2 conjugate to the

subject, wherein the gene therapy agent is administered to the subject before, concurrently with, or after the IL-2 conjugate, wherein the IL-2 conjugate comprises an amino acid sequence having at least 80% sequence identity to SEQ ID NO: 1, in which at least one amino acid residue in the IL-2 conjugate is replaced by an unnatural amino acid linked to a conjugating moiety, and the unnatural amino acid linked to the conjugating moiety is positioned in the amino acid sequence so as to preferentially reduce binding of the IL-2 conjugate to IL-2R $\beta\gamma$ relative to IL-2R $\alpha\beta\gamma$, or is at position P1, T2, S3, S4, S5, T6, K7, K8, Q10, L11, E14, H15, L17, L18, D19, Q21, M22, N25, G26, N28, N29, Y30, K31, K34, T36, M45, P46, K47, A49, T50, E51, L52, K53, H54, Q56, E59, E66, N70, Q73, S74, K75, N76, F77, H78, R80, P81, R82, D83, S86, N87, I88, V90, I91, L93, E94, K96, G97, S98, E99, T100, T101, F102, M103, C104, E105, Y106, A107, D108, E109, T110, A111, T112, E115, N118, R119, T122, F123, S124, Q125, S126, S129, T130, L131, or T132 in reference to the sequence of SEQ ID NO: 1.

[0008] Embodiment 2 is a method of treating an individual in need thereof with a gene therapy agent, comprising administering an IL-2 conjugate to the subject, wherein the gene therapy agent is administered to the subject before, concurrently with, or after the IL-2 conjugate, and the IL-2 conjugate comprises an amino acid sequence having at least 80% sequence identity to SEQ ID NO: 1, in which at least one amino acid residue in the IL-2 conjugate is replaced by an unnatural amino acid linked to a conjugating moiety, and the unnatural amino acid linked to the conjugating moiety is positioned in the amino acid sequence so as to preferentially reduce binding of the IL-2 conjugate to IL-2R $\beta\gamma$ relative to IL-2R $\alpha\beta\gamma$, or is at position P1, T2, S3, S4, S5, T6, K7, K8, Q10, L11, E14, H15, L17, L18, D19, Q21, M22, N25, G26, N28, N29, Y30, K31, K34, T36, M45, P46, K47, A49, T50, E51, L52, K53, H54, Q56, E59, E66, N70, Q73, S74, K75, N76, F77, H78, R80, P81, R82, D83, S86, N87, I88, V90, I91, L93, E94, K96, G97, S98, E99, T100, T101, F102, M103, C104, E105, Y106, A107, D108, E109, T110, A111, T112, E115, N118, R119, T122, F123, S124, Q125, S126, S129, T130, L131, or T132 in reference to the sequence of SEQ ID NO: 1.

[0009] Embodiment 3 is a method of increasing expression of a gene therapy agent, comprising: administering an IL-2 conjugate to a subject; wherein the gene therapy agent is administered to the subject before, concurrently with, or after the IL-2 conjugate; and wherein the IL-2 conjugate comprises an amino acid sequence having at least 80% sequence identity to SEQ ID NO: 1, in which at least one amino acid residue in the IL-2 conjugate is replaced by an unnatural amino acid linked to a conjugating moiety, and the unnatural amino acid linked to the conjugating moiety is positioned in the amino acid sequence so as to

preferentially reduce binding of the IL-2 conjugate to IL-2R $\beta\gamma$ relative to IL-2R $\alpha\beta\gamma$, or is at position P1, T2, S3, S4, S5, T6, K7, K8, Q10, L11, E14, H15, L17, L18, D19, Q21, M22, N25, G26, N28, N29, Y30, K31, K34, T36, M45, P46, K47, A49, T50, E51, L52, K53, H54, Q56, E59, E66, N70, Q73, S74, K75, N76, F77, H78, R80, P81, R82, D83, S86, N87, I88, V90, I91, L93, E94, K96, G97, S98, E99, T100, T101, F102, M103, C104, E105, Y106, A107, D108, E109, T110, A111, T112, E115, N118, R119, T122, F123, S124, Q125, S126, S129, T130, L131, or T132 in reference to the sequence of SEQ ID NO: 1.

[0010] Embodiment 4 is a method of reducing an immune response to a gene therapy agent, comprising:

administering an IL-2 conjugate to a subject;

wherein the gene therapy agent is administered to the subject before, concurrently with, or after the IL-2 conjugate; and

wherein the IL-2 conjugate comprises an amino acid sequence having at least 80% sequence identity to SEQ ID NO: 1, in which at least one amino acid residue in the IL-2 conjugate is replaced by an unnatural amino acid linked to a conjugating moiety, and the unnatural amino acid linked to the conjugating moiety is positioned in the amino acid sequence so as to preferentially reduce binding of the IL-2 conjugate to IL-2R $\beta\gamma$ relative to IL-2R $\alpha\beta\gamma$, or is at position P1, T2, S3, S4, S5, T6, K7, K8, Q10, L11, E14, H15, L17, L18, D19, Q21, M22, N25, G26, N28, N29, Y30, K31, K34, T36, M45, P46, K47, A49, T50, E51, L52, K53, H54, Q56, E59, E66, N70, Q73, S74, K75, N76, F77, H78, R80, P81, R82, D83, S86, N87, I88, V90, I91, L93, E94, K96, G97, S98, E99, T100, T101, F102, M103, C104, E105, Y106, A107, D108, E109, T110, A111, T112, E115, N118, R119, T122, F123, S124, Q125, S126, S129, T130, L131, or T132 in reference to the sequence of SEQ ID NO: 1.

[0011] Embodiment 5 is a method of preventing immune-related adverse events in a subject, comprising:

administering an IL-2 conjugate to a subject;

wherein a gene therapy agent is administered to the subject before, concurrently with, or after the IL-2 conjugate; and

wherein the IL-2 conjugate comprises an amino acid sequence having at least 80% sequence identity to SEQ ID NO: 1, in which at least one amino acid residue in the IL-2 conjugate is replaced by an unnatural amino acid linked to a conjugating moiety, and the unnatural amino acid linked to the conjugating moiety is positioned in the amino acid sequence so as to preferentially reduce binding of the IL-2 conjugate to IL-2R $\beta\gamma$ relative to IL-2R $\alpha\beta\gamma$, or is at position P1, T2, S3, S4, S5, T6, K7, K8, Q10, L11, E14, H15, L17, L18, D19, Q21, M22, N25,

G26, N28, N29, Y30, K31, K34, T36, M45, P46, K47, A49, T50, E51, L52, K53, H54, Q56, E59, E66, N70, Q73, S74, K75, N76, F77, H78, R80, P81, R82, D83, S86, N87, I88, V90, I91, L93, E94, K96, G97, S98, E99, T100, T101, F102, M103, C104, E105, Y106, A107, D108, E109, T110, A111, T112, E115, N118, R119, T122, F123, S124, Q125, S126, S129, T130, L131, or T132 in reference to the sequence of SEQ ID NO: 1.

[0012] Embodiment 6 is the method of any one of the preceding embodiments, wherein the method further comprises, before the administration of the gene therapy agent and the IL-2 conjugate to the subject, a) incubating immune cells from the subject with the gene therapy agent and b) analyzing the immune cells for the expression of one or more activation biomarkers or increased expression of one or more activation biomarkers, wherein expression or increased expression of the one or more activation biomarkers following incubation with the gene therapy agent identifies the subject as being in need of the IL-2 conjugate.

[0013] Embodiment 7 is a method for selecting a subject for treatment with a gene therapy agent and an IL-2 conjugate, the method comprising a) incubating immune cells from the subject with the gene therapy agent, b) analyzing the immune cells for the expression of one or more activation biomarkers or increased expression of one or more activation biomarkers, wherein expression or increased expression of the one or more activation biomarkers following incubation with the gene therapy agent identifies the subject for treatment with the gene therapy agent and the IL-2 conjugate, and c) selecting the subject identified in step b) for treatment with the gene therapy agent and the IL-2 conjugate;

wherein the IL-2 conjugate comprises an amino acid sequence having at least 80% sequence identity to SEQ ID NO: 1, in which at least one amino acid residue in the IL-2 conjugate is replaced by an unnatural amino acid linked to a conjugating moiety, and the unnatural amino acid linked to the conjugating moiety is positioned in the amino acid sequence so as to preferentially reduce binding of the IL-2 conjugate to IL-2R $\beta\gamma$ relative to IL-2R $\alpha\beta\gamma$, or is at position P1, T2, S3, S4, S5, T6, K7, K8, Q10, L11, E14, H15, L17, L18, D19, Q21, M22, N25, G26, N28, N29, Y30, K31, K34, T36, M45, P46, K47, A49, T50, E51, L52, K53, H54, Q56, E59, E66, N70, Q73, S74, K75, N76, F77, H78, R80, P81, R82, D83, S86, N87, I88, V90, I91, L93, E94, K96, G97, S98, E99, T100, T101, F102, M103, C104, E105, Y106, A107, D108, E109, T110, A111, T112, E115, N118, R119, T122, F123, S124, Q125, S126, S129, T130, L131, or T132 in reference to the sequence of SEQ ID NO: 1.

[0014] Embodiment 8 is method of the immediately preceding embodiment, further comprising steps of administering the IL-2 conjugate to the subject identified in step b), and administering the gene therapy agent to the subject identified in step b).

[0015] Embodiment 9 is the method of any one of embodiments 6-8, wherein the immune cell is a lymphocyte, a T cell, a CD8+ T cell, an effector T cell, a cytotoxic T cell, or an NK cell.

[0016] Embodiment 10 is the method of any one of the preceding embodiments, wherein the unnatural amino acid is linked to the conjugating moiety through a linker.

[0017] Embodiment 11 is the method of the immediately preceding embodiment, wherein the linker comprises a homobifunctional linker, a heterobifunctional linker, a cleavable or a non-cleavable dipeptide linker, a maleimide group, a spacer, or a combination thereof.

[0018] Embodiment 12 is the method of any one of the preceding embodiments, wherein the unnatural amino acid is a substituted lysine, is a substituted phenylalanine, is a substituted histidine, is a substituted cysteine, comprises an azido group, comprises an alkyne group, comprises an aldehyde group, comprises an aromatic side chain, or comprises a ketone group.

[0019] Embodiment 13 is the method of any one of the preceding embodiments, wherein the at least one unnatural amino acid comprises N6-azidoethoxy-L-lysine, N6-((2-azidoethoxy)-carbonyl)-L-lysine, N6-propargylethoxy-L-lysine (PraK), BCN-L-lysine, norbornene lysine, TCO-lysine, methyltetrazine lysine, allyloxycarbonyllysine, p-acetyl-L-phenylalanine, p-azidomethyl-L-phenylalanine (pAMF), p-iodo-L-phenylalanine, m-acetylphenylalanine, p-propargyloxyphenylalanine, p-propargyl-phenylalanine, 3-methyl-phenylalanine, fluorinated phenylalanine, isopropyl-L-phenylalanine, p-azido-L-phenylalanine, p-acyl-L-phenylalanine, p-benzoyl-L-phenylalanine, p-bromophenylalanine, p-amino-L-phenylalanine, isopropyl-L-phenylalanine, O-allyltyrosine, O-methyl-L-tyrosine, O-4-allyl-L-tyrosine, 4-propyl-L-tyrosine, phosphotyrosine, L-3-(2-naphthyl)alanine, 2-amino-3-((2-((3-(benzyloxy)-3-oxopropyl)amino)ethyl)sulfonyl)propanoic acid, or 2-amino-3-(phenylsulfonyl)propanoic acid.

[0020] Embodiment 14 is the method of any one of the preceding embodiments, wherein the unnatural amino acid is an azido-substituted lysine.

[0021] Embodiment 15 is the method of any one of the preceding embodiments, wherein the unnatural amino acid is N6-((2-azidoethoxy)-carbonyl)-L-lysine.

[0022] Embodiment 16 is the method of any one of the preceding embodiments, wherein the conjugating moiety comprises a water-soluble polymer.

[0023] Embodiment 17 is the method of the immediately preceding embodiment, wherein the water-soluble polymer comprises polyethylene glycol (PEG), poly(propylene glycol) (PPG), copolymers of ethylene glycol and propylene glycol, poly(oxyethylated polyol), poly(olefinic alcohol), poly(vinylpyrrolidone), poly(hydroxyalkylmethacrylamide), poly(hydroxyalkylmethacrylate), poly(saccharides), poly(α -hydroxy acid), poly(vinyl alcohol), polyphosphazene, polyoxazolines (POZ), poly(N-acryloylmorpholine), or a combination thereof.

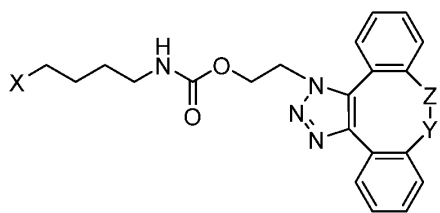
[0024] Embodiment 18 is the method of the immediately preceding embodiment, wherein the conjugating moiety comprises PEG.

[0025] Embodiment 19 is the method of the immediately preceding embodiment, wherein the conjugating moiety is PEG having a molecular weight of about 10-85 kDa or selected from about 10kDa, 15kDa, 20kDa, 25kDa, 30kDa, 35kDa, 40kDa, 45kDa, 50kDa, 55 kDa, 60kDa, 65kDa, 70kDa, 75 kDa, 80kDa, and 85 kDa.

[0026] Embodiment 20 is the method of any one of the preceding embodiments, wherein the conjugating moiety is PEG having a molecular weight of about 20-70 kDa or selected from about 20kDa, 25kDa, 30kDa, 35kDa, 40kDa, 45kDa, 50kDa, 55 kDa, 60kDa, 65kDa, and 70kDa.

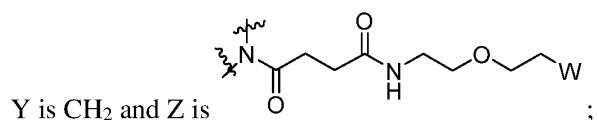
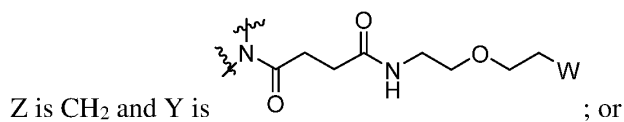
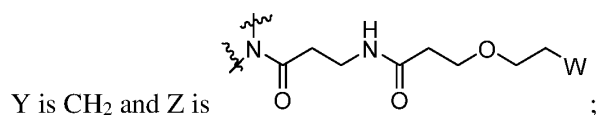
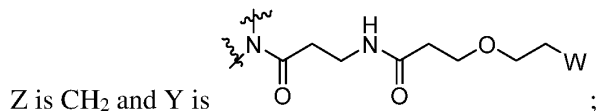
[0027] Embodiment 21 is the method of any one of the preceding embodiments, wherein the conjugating moiety is PEG having a molecular weight of about 30-60 kDa or selected from about 30kDa, 35kDa, 40kDa, 45kDa, 50kDa, 55 kDa, and 60kDa.

[0028] Embodiment 22 is the method of any one of the preceding embodiments, wherein the amino acid linked to the conjugating moiety has the structure of Formula (I):



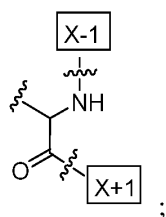
Formula (I);

wherein:



W is a PEG group; and

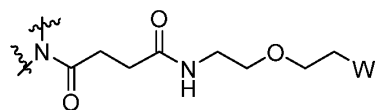
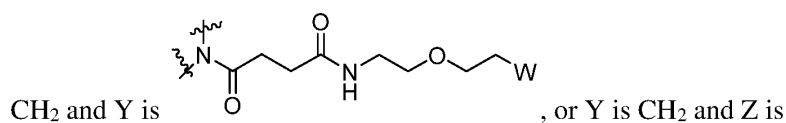
X has the structure:



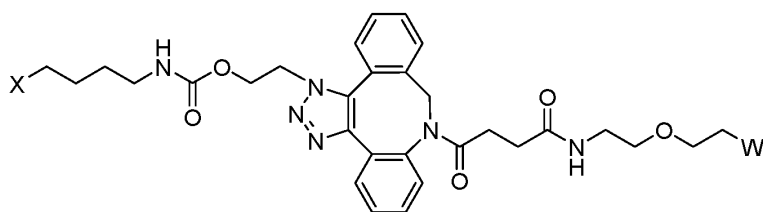
X-1 indicates the point of attachment to the preceding amino acid residue; and

X+1 indicates the point of attachment to the following amino acid residue.

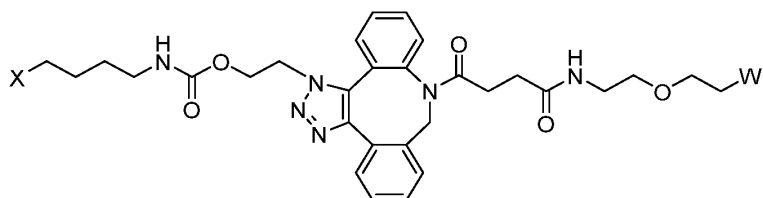
[0029] Embodiment 23 is the method of the immediately preceding embodiment, wherein Z is



[0030] Embodiment 24 is the method of the immediately preceding embodiment, wherein the structure of Formula (I) has the structure of Formula (IV) or Formula (V):



Formula (IV);



Formula (V);

wherein:

W is a PEG group having a molecular weight of about 5-60 kDa or about 5 kDa, 10 kDa, 15 kDa, 20 kDa, 25 kDa, 30 kDa, 35 kDa, 40 kDa, 45 kDa, 50 kDa, or 60 kDa.

[0031] Embodiment 25 is the method of any one of the preceding embodiments, wherein the IL-2 conjugate further comprises an alanine or methionine N-terminal to the first amino acid of the sequence having at least 80% sequence identity to SEQ ID NO: 1.

[0032] Embodiment 26 is the method of any one of the preceding embodiments, wherein the IL-2 conjugate comprises an amino acid sequence having at least 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 1.

[0033] Embodiment 27 is the method of any one of the preceding embodiments, wherein the IL-2 conjugate comprises the amino acid sequence of SEQ ID NO: 1 in which position P1, T2, S3, S4, S5, T6, K7, K8, Q10, L11, E14, H15, L17, L18, D19, Q21, M22, N25, G26, N28, N29, Y30, K31, K34, T36, M45, P46, K47, A49, T50, E51, L52, K53, H54, Q56, E59, E66, N70, Q73, S74, K75, N76, F77, H78, R80, P81, R82, D83, S86, N87, I88, V90, I91, L93, E94, K96, G97, S98, E99, T100, T101, F102, M103, C104, E105, Y106, A107, D108, E109, T110, A111, T112, E115, N118, R119, T122, F123, S124, Q125, S126, S129, T130, L131, or T132 is replaced with the unnatural amino acid.

[0034] Embodiment 28 is the method of any one of the preceding embodiments, wherein position K8, L11, E14, H15, L18, D19, M22, N87, E99, or D108 in reference to the sequence of SEQ ID NO: 1 is replaced with the unnatural amino acid.

[0035] Embodiment 29 is the method of the immediately preceding embodiment, wherein position L18 in reference to the sequence of SEQ ID NO: 1 is replaced with the unnatural amino acid.

[0036] Embodiment 30 is the method of embodiment 28, wherein position H15 in reference to the sequence of SEQ ID NO: 1 is replaced with the unnatural amino acid.

[0037] Embodiment 31 is the method of any one of the preceding embodiments, wherein the IL-2 conjugate is capable of expanding CD4⁺ T regulatory (Treg) cells.

[0038] Embodiment 32 is the method of any one of the preceding embodiments, wherein the unnatural amino acid and/or the conjugating moiety impairs or blocks the receptor signaling potency of the IL-2 conjugate to IL-2R $\beta\gamma$, or reduces recruitment of IL-2R γ subunit to an IL-2/IL-2R β complex.

[0039] Embodiment 33 is the method of any one of the preceding embodiments, wherein the IL-2 conjugate has a receptor signaling potency to IL-2R $\beta\gamma$ that is lower than a receptor signaling potency of wild-type IL-2 to IL-2R $\beta\gamma$.

[0040] Embodiment 34 is the method of any one of the preceding embodiments, wherein the IL-2 conjugate has a receptor signaling potency to IL-2R $\alpha\beta\gamma$ that is greater than or equal to a receptor signaling potency of wild-type IL-2 to IL-2R $\alpha\beta\gamma$.

[0041] Embodiment 35 is the method of any one of the preceding embodiments, wherein the IL-2 conjugate expands a CD4⁺ Treg population in the subject.

[0042] Embodiment 36 is the method of any one of the preceding embodiments, wherein the IL-2 conjugate suppresses CD8+ T cell proliferation in the subject.

[0043] Embodiment 37 is the method of any one of the preceding embodiments, wherein the IL-2 conjugate suppresses effector memory CD8+ T cell proliferation in the subject.

[0044] Embodiment 38 is the method of any one of the preceding embodiments, wherein the gene therapy agent comprises a vector and the IL-2 conjugate suppresses vector-specific IFN γ -secreting CD8+ T cells in the subject.

[0045] Embodiment 39 is the method of any one of the preceding embodiments, wherein the gene therapy agent encodes a transgene product and the IL-2 conjugate suppresses transgene-product-specific IFN γ -secreting CD8+ T cells in the subject.

[0046] Embodiment 40 is the method of any one of the preceding embodiments, wherein the gene therapy agent encodes a transgene product and the IL-2 conjugate suppresses production of antibodies against the transgene product.

[0047] Embodiment 41 is the method of any one of the preceding embodiments, wherein the gene therapy agent encodes a transgene product and the IL-2 conjugate suppresses production of IgG1 antibodies against the transgene product.

[0048] Embodiment 42 is the method of any one of the preceding embodiments, wherein the gene therapy agent encodes a transgene product and the IL-2 conjugate prolongs the expression of the transgene product in the subject relative to a subject that is administered the gene therapy agent and without the IL-2 conjugate.

[0049] Embodiment 43 is the method of the immediately preceding embodiment, wherein the prolonged expression of the transgene product is at least about 5 weeks, about 6 weeks, about 8 weeks, about 12 weeks, about 14 weeks, or about 16 weeks.

[0050] Embodiment 44 is the method of any one of the preceding embodiments, wherein the gene therapy agent comprises a viral vector.

[0051] Embodiment 45 is the method of embodiment 44, wherein the IL-2 conjugate suppresses production of antibodies against the viral vector.

[0052] Embodiment 46 is the method of embodiment 44 or embodiment 45, wherein the IL-2 conjugate suppresses production of antibodies against a capsid protein of the viral vector.

[0053] Embodiment 47 is the method of any one of embodiments 44-46, wherein the viral vector is an adeno-associated viral (AAV) particle.

[0054] Embodiment 48 is the method of the immediately preceding embodiment, wherein the AAV particle comprises an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an

AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAVrh32.33 capsid, an AAV-XL32 capsid, an AAV-XL32.1 capsid, an AAV LK03 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV / human bocavirus virus 1), an AAV2HBKO capsid, an AAVPHP.B capsid or an AAVPHP.eB capsid, or a functional variant thereof.

[0055] Embodiment 49 is the method of the immediately preceding embodiment, wherein the AAV capsid comprises a tyrosine mutation, a heparin binding mutation, or an HBKO mutation.

[0056] Embodiment 50 is the method of any one of embodiments 47-49, wherein the AAV viral particle comprises an AAV genome comprising one or more inverted terminal repeats (ITRs), wherein the one or more ITRs is an AAV1 ITR, an AAV2 ITR, an AAV3 ITR, an AAV4 ITR, an AAV5 ITR, an AAV6 ITR, an AAV7 ITR, an AAV8 ITR, an AAVrh8 ITR, an AAV9 ITR, an AAV10 ITR, an AAVrh10 ITR, an AAV11 ITR, or an AAV12 ITR.

[0057] Embodiment 51 is the method of the immediately preceding embodiment, wherein the one or more ITRs and the capsid of the AAV particle are derived from the same AAV serotype.

[0058] Embodiment 52 is the method of the immediately preceding embodiment, wherein the one or more ITRs and the capsid of the AAV particles are derived from different AAV serotypes.

[0059] Embodiment 53 is the method of any one of embodiments 44-46, wherein the viral vector is an adenoviral particle.

[0060] Embodiment 54 is the method of the immediately preceding embodiment, wherein the adenoviral particle comprises a capsid from Adenovirus serotype 2, 1, 5, 6, 19, 3, 11, 7, 14, 16, 21, 12, 18, 31, 8, 9, 10, 13, 15, 17, 19, 20, 22, 23, 24-30, 37, 40, 41, AdHu2, AdHu3, AdHu4, , AdHu24, AdHu26, AdHu34, AdHu35, AdHu36, AdHu37, AdHu41, AdHu48, AdHu49, AdHu50, AdC6, AdC7, AdC69, bovine Ad type 3, canine Ad type 2, ovine Ad, or porcine Ad type 3, or a functional variant thereof.

[0061] Embodiment 55 is the method of any one of embodiments 44-46, wherein the viral vector is a lentiviral particle.

[0062] Embodiment 56 is the method of the immediately preceding embodiment, wherein the lentiviral particle is pseudotyped with vesicular stomatitis virus (VSV), lymphocytic choriomeningitis virus (LCMV), Ross river virus (RRV), Ebola virus, Marburg virus, Mokala virus, Rabies virus, RD114, or a functional variant thereof.

[0063] Embodiment 57 is the method of any one of embodiments 44-46, wherein the viral vector is a Herpes simplex virus (HSV) particle.

[0064] Embodiment 58 is the method of the immediately preceding embodiment, wherein the HSV particle is an HSV-1 particle or an HSV-2 particle, or a functional variant thereof.

[0065] Embodiment 59 is the method of any one of embodiments 1-41, wherein the gene therapy agent comprises a lipid nanoparticle.

[0066] Embodiment 60 is the method of any one of the preceding embodiments, wherein the gene therapy agent comprises a nucleic acid encoding a heterologous transgene.

[0067] Embodiment 61 is the method of the immediately preceding embodiment, wherein the heterologous transgene is operably linked to a promoter.

[0068] Embodiment 62 is the method of the immediately preceding embodiment, wherein the promoter is a constitutive promoter, a tissue- specific promoter, or an inducible promoter.

[0069] Embodiment 63 is the method of any one of embodiments 60-62, wherein the nucleic acid comprises closed-end DNA (ceDNA).

[0070] Embodiment 64 is the method of embodiment 60, wherein the nucleic acid comprises mRNA.

[0071] Embodiment 65 is the method of any one of the preceding embodiments, wherein the gene therapy agent is administered to the subject concurrently with the IL-2 conjugate.

[0072] Embodiment 66 is the method of any one of embodiments 1-64, wherein the gene therapy agent is administered to the subject before the IL-2 conjugate.

[0073] Embodiment 67 is the method of the immediately preceding embodiment, wherein the gene therapy agent is administered less than 14 days or less than 7 days before the IL-2 conjugate.

[0074] Embodiment 68 is the method of any one of embodiments 1-64, wherein the gene therapy agent is administered to the subject after the IL-2 conjugate.

[0075] Embodiment 69 is the method of the immediately preceding embodiment, wherein the gene therapy agent is administered less than 7 days, less than 3 days, or less than 1 day after the IL-2 conjugate.

[0076] Embodiment 70 is the method of any one of embodiments 1-64, wherein the IL-2 conjugate is administered before, at the same time, or after administration of the gene therapy agent.

[0077] Embodiment 71 is the method of any one of the preceding embodiments, wherein the individual has a disease or disorder suitable for treatment by gene therapy.

[0078] Embodiment 72 is the method of the immediately preceding embodiment, wherein the disease or disorder is a monogenic disease or disorder.

[0079] Embodiment 73 is the method of any one of the preceding embodiments, wherein the gene therapy agent is administered intravenously, intraperitoneally, intra-arterially, intramuscularly, subcutaneously, intracranially, intra-CSF, intra-DRG, intracerebroventricularly, intraocularly, intracisterna magna, or intrahepatically.

[0080] Embodiment 74 is the method of any one of the preceding embodiments, wherein the IL-2 conjugate is administered parenterally and/or systemically.

[0081] Embodiment 75 is the method of any one of the preceding embodiments, wherein the IL-2 conjugate is administered intravenously, intraperitoneally, intra-arterially, intramuscularly, subcutaneously, intracranially, intra-CSF, intra-DRG, intracerebroventricularly, intraocularly, intracisterna magna, or intrahepatically.

[0082] Embodiment 76 is the method of any one of the preceding embodiments, wherein the subject is a mammal.

[0083] Embodiment 77 is the method of any one of the preceding embodiments, wherein the subject is a primate.

[0084] Embodiment 78 is the method of any one of the preceding embodiments, wherein the subject is a human.

[0085] Embodiment 79 is the method of any one of the preceding embodiments, wherein the IL-2 conjugate is administered about 1, 2, 3, 4, 5, 6, or 7 days before the gene therapy agent.

[0086] Embodiment 80 is the method of any one of embodiments 1-78, wherein the IL-2 conjugate is administered about 1, 2, 3, or 4 days after the gene therapy agent.

[0087] Embodiment 81 is the method of any one of embodiments 1-78, wherein the IL-2 conjugate is administered on the same day as the gene therapy agent.

[0088] Embodiment 82 is the method of any one of the preceding embodiments, wherein the IL-2 conjugate is administered at a dose of about 0.02-0.5 mg/kg about 0.03-0.4 mg/kg, about 0.04-0.1 mg/kg, or about 0.05-0.08 mg/kg.

[0089] Embodiment 83 is the method of the immediately preceding embodiment, wherein the IL-2 conjugate is administered at a dose of about 0.05 mg/kg.

[0090] Embodiment 84 is the method of embodiment 82, wherein the IL-2 conjugate is administered at a dose of about 0.08 mg/kg.

[0091] Embodiment 85 is the method of embodiment 82, wherein the IL-2 conjugate is administered at a dose of about 0.3 mg/kg.

[0092] Embodiment 86 is a use of an IL-2 conjugate for the manufacture of a medicament for use in the method of any one of the preceding embodiments.

[0093] Embodiment 87 is an IL-2 conjugate for use in the method of any one of embodiments 1-85.

BRIEF DESCRIPTION OF THE DRAWINGS

[0094] **FIG. 1** provides data of the kinetics of CD4 Treg expansion by Compound A in mice as measured by percentage of CD4 Tregs. The graph shows the CD4 Treg expansion in the peripheral blood after mice received 0.3 mg/kg of Compound A subcutaneously.

[0095] **FIGs. 2A and 2B** provide data that Compound A effectively suppresses the expansion of effector memory CD8 T cells (CD8 T_{EM}; **FIG. 2A**) and LacZ-specific CD8T cells stimulated by AAVrh32.33-LacZ administration in PBMC in mice (**FIG. 2B**).

[0096] **FIGs. 3A and 3B** provide data that Compound A effectively suppresses both AAV capsid-specific IFN γ secreting CD8 T (**FIG. 3A**) and LacZ-specific IFN γ secreting CD8 T cells (**FIG. 3B**) in the spleen in mice.

[0097] **FIGs. 4A and 4B** provide data that Compound A enhances the expression of the OVA gene delivered by AAVrh32.33 vector (**FIG. 4A**) and suppresses anti-OVA IgG1 production (**FIG. 4B**) in mice, indicating converse correlation between the level of OVA and anti-OVA IgG1 in serum. Results showed that Compound A enhanced the OVA expression while suppressing anti-OVA IgG1 production.

[0098] **FIGs. 5A, 5B and 5C** show graphs demonstrating that Compound A significantly increases the CD4 Treg population (**FIG. 5A**) and effectively suppresses CD8 T cell proliferation (**FIG. 5B**) and effector memory CD8 T-cell expansion (CD8 T_{EM}; **FIG. 5C**) stimulated by AAVrh10-EGFP administration in PBMC in rats.

[0099] **FIGs. 6A and 6B** show data that Compound A administration significantly increases the CD4 Treg population (**FIG. 6A**) as measured by percentage of CD4 Treg cells out of singlet cells and enhances the expression of the OVA gene delivered by AAVrh32.33 vector measured in serum (**FIG. 6B**) in non-human primates.

[0100] **FIGs. 7A and 7B** show the dose response curves of an exemplary IL-2 variant for pSTAT5 signaling in human LRS primary cell (**FIG. 7A**) and proliferation response in mouse CTLL-2 populations (**FIG. 7B**).

[0101] **FIG. 8** shows the plasma concentration profiles of IL-2 conjugates K9_30kD, L19_30kD, N88R/D109_30kD, H16_30kD, Q126_30kD, and N88_30kD (all dosed at 0.9 mg/kg) following dosing in C57/BL6 mice from Example 5.

[0102] **FIG. 9** shows the mean fold change of Treg (% in singlets) following the dosing of IL-2 conjugates in C57/BL6 mice from Example 5.

[0103] **FIG. 10** shows the proportion of the Treg (CD3+ CD4+ CD25+ FoxP3+) cell population within the total cell population (singlets) of IL-2 conjugates in C57/BL6 mice from Example 5.

[0104] **FIGs. 11A and 11B** show the proportion of the CD8+ T cell (CD3+ CD4- CD8+) population within the total cell population (singlets) following a single dose of IL-2 conjugates. **FIG. 11A** shows the proportion of the CD8+ T cell population (CD3+ CD4- CD8+) within the total cell population (singlets) following a single dose of IL-2 conjugates K9_30kD, L19_30kD, Q126_30kD, and H16_30kD in C57/BL6 mice from Example 5. **FIG. 11B** shows the proportion of the CD8+ T cell (CD3+ CD4- CD8+) population within the total cell population (singlets) following a single dose of IL-2 conjugates E100_30kD, N88R/D109_30kD, T123_30kD, N88_30kD, and V91_30kD in C57/BL6 mice from Example 5.

[0105] **FIG. 12** shows the plasma concentration profiles of IL-2 conjugates following dosing in Cynomolgus monkey from Example 5.

[0106] **FIG. 13** shows the proportion of the Treg cell population within the total blood cell population (singlets) in Cynomolgus monkeys following dosing with IL-2 conjugates from Example 5.

[0107] **FIG. 14** shows the proportion of the CD8+ T cell population within the total blood cell population (singlets) in Cynomolgus monkeys following dosing with IL-2 conjugates from Example 5.

[0108] **FIG. 15** shows the plots of plasma concentration versus time for the H16_30kD variant in non-human primates at doses of 0.12 mg/kg and 0.67 mg/kg from Example 5, wherein the 0.12 mg/kg dose is shown as the lower trace, while the 0.67 mg/kg dose is shown as the upper trace.

[0109] **FIG. 16** shows the plots of plasma concentration versus time for the H16_30kDa variant and the H16_50kDa variant in non-human primates at a dose of 0.12 mg/kg, and the H16_50kDa variant at a dose of 0.2 mg/kg from Example 5, wherein the trace for the 30 kDa variant is shown as the lower trace (squares) and the trace for the 50 kDa variant is shown as the upper trace (triangles).

[0110] **FIG. 17** shows plots of Treg percent in singles versus time post-dose in non-human primates from Example 5 for the H16_30kDa variant at a dose of 0.12 mg/kg, and the H16_50kDa variant at a dose of 0.2 mg/kg, wherein the trace for the vehicle is the lower trace (squares), the trace for the 30 kDa variant is shown in the middle trace, and the trace for the 50 kDa variant is shown in the upper trace.

[0111] **FIG. 18** shows the study design of Example 5 to assess the effects of H16_50kD on delayed-type hypersensitivity (DTH) in C57BL/6 mice. DTH in mice was induced with keyhole

limpet hemocyanin (KLH) (challenge at Day 7 following sensitization at Day 1 via subcutaneous injection) with dosing of H16_50kD (Day 0 and 3), at a dose of 0.03 mg/kg, 0.1 mg/kg, and 0.3 mg/kg from Example 5.

[0112] FIGs. 19A, 19B, and 19C show changes in ear thickness measurements and blood immunotypes of C57BL/6 mice from Example 5. **FIG. 19A** shows Area Under Curve (AUC) of increased ear thickness as compared to the mice with KLH challenge on Day 7 only. **FIG. 19B** shows changes in ear thickness measurements in the C57BL/6 mice prior to KLH challenge (on Day 7) and then subsequently on Days 8, 9 and 10. **FIG. 19C** shows changes over time in the relative percentage of CD4+ T cells within CD25+FoxP3+ cell population in whole blood samples from the mice. “KLH only” indicates KLH challenge on Day 7 only (without sensitization on Day 1) with dosing of vehicle only. “Vehicle” indicates KLH sensitization (Day 1) and challenge (Day 7) with dosing of vehicle only. “0.03” indicates KLH sensitization and challenge with dosing of H16_50kD at a dose of 0.03 mg/kg. “0.1” indicates KLH sensitization and challenge with dosing of H16_50kD at a dose of 0.1 mg/kg. “0.3” indicates KLH sensitization and challenge with dosing of H16_50kD at a dose of 0.3 mg/kg. “CsA” indicates KLH sensitization and challenge with dosing of Cyclosporine A. See also Table 10 in Example 5.

[0113] FIGs. 20A, 20B, and 20C show changes over time in the relative percentage of CD4+CD25+FoxP3+ cells within CD45+ cell population (**FIG. 20A**), within TCRβ+ cell population (**FIG. 20B**), and within CD4+ cell population (**FIG. 20C**) in whole blood samples from the mice from Example 5.

[0114] FIG. 21 shows the absolute counts of CD4+CD25+FoxP3+ cells on Day 10 in whole blood samples from the mice from Example 5.

[0115] FIGs. 22A and 22B provide data for Treg, CD8+ T cells, CD4+ T cells, NK cell, and B cell expansion by Compound A in mice. The Treg expansion as measured by percentage of CD4+ T cells in the peripheral blood was measured at the indicated times after mice received 0.3 mg/kg of Compound A subcutaneously (**FIG. 22A**). The CD4 Treg, CD8+ T cell, CD4+ T cell, NK cell, and B cell expansion as measured by fold change in the peripheral blood was measured at the indicated times after mice received 0.3 mg/kg of Compound A subcutaneously (**FIG. 22B**)

[0116] FIGs. 23A, 23B, and 23C provide data showing that Compound A effectively suppresses the expansion of effector memory CD8 T cells (CD8 T_{EM}; **FIG. 23A**) that would otherwise occur following AAV administration. Compound A effectively suppresses both AAV capsid-specific IFNγ secreting CD8 T cells (**FIG. 23B**) and LacZ-specific IFNγ secreting CD8 T cells (**FIG. 23C**) following ex vivo spleen restimulation at day 21.

[0117] **FIGs. 24A and 24B** show graphs demonstrating that Compound A mitigates CD8⁺ T cell responses and enhances transgene levels following gene therapy. Compound A enhances the expression of the OVA gene delivered by AAVrh32.33 vector (**FIG. 24A**) in mice as measured by serum OVA concentration. Compound A significantly suppresses effector memory CD8 T-cell expansion (CD8 T_{EM}; **FIG. 24B**) in mice after delivery of the OVA gene by AAVrh32.33 vector.

[0118] **FIGs. 25A, 25B and 25C** show graphs demonstrating that Compound A significantly increases the CD4 Treg population (**FIG. 25A**) and effectively suppresses effector memory CD8 T-cell expansion (CD8 T_{EM}; **FIG. 25B**) and CD8 T cell proliferation (**FIG. 25C**) stimulated by AAVrh10-EGFP administration in PBMC in rats.

[0119] **FIGs. 26A, 26B, and 26C** show data that Compound A administration significantly increases the CD4 Treg population (**FIG. 26A**) as measured by percentage of CD4 Treg cells out of singlet cells and enhances the expression of the OVA gene delivered by AAVrh32.33 vector measured in serum (**FIG. 26C**) in non-human primates relative to the expression of the OVA gene delivered by AAVrh32.33 vector without Compound A measured in serum (**FIG. 26B**) in non-human primates. Each line in **FIGs. 26B and 26C** represents serum OVA levels for an individual animal.

[0120] **FIGs. 27A, 27B, 27C, and 27D** show the longitudinal analysis of serum levels of anti AAV and anti-OVA IgG. **FIGs. 27A and 27B**: Wild-type C57BL/6 mice (N_≥5 per group) were treated with rAAV1-OVA 2x10¹¹ ventrogluteal (VG) or AAVrh32.33-OVA 5x10¹⁰ VG intramuscularly with or without a single subcutaneous dose of 0.3 mg/kg Compound A. Serum samples were collected via in-life serial bleeding at different time points over 16 weeks of the study. Levels of anti-AAV1 IgG (**FIG. 27A**), and anti-OVA IgG (**FIG. 27B**) in the serum were measured by ELISA. **FIGs. 27C and 27D**: Cynomolgus macaques (N_≥4 per group) were treated with rAAV1-OVA 3x10¹² VG intramuscularly with or without a single subcutaneous dose of 0.08 mg/kg Compound A. Serum samples were collected via in-life serial bleeding at different time points over 12 weeks of the study. Levels of anti-AAV1 IgG (**FIG. 27C**) and anti-OVA IgG (**FIG. 27D**) in the serum were measured by ELISA. Data is represented as mean ± SEM. Dotted line represents lower limit of detection. Statistical analysis performed by 2way ANOVA with test for repeated measures. **p* < 0.05, ***p* < 0.01, *****p* < 0.0001.

[0121] **FIG. 28** shows data from Cynomolgus macaques treated with either AAVrh32.33 or rAAV1 expressing the OVA transgene intramuscularly with or without a single subcutaneous dose of Compound A with a dose up to 0.08 mg/kg. Immune cell analysis and/or serum transgene levels were measured serially via in-life bleeding. OVA protein concentrations in the

serum of non-human primates (NHPs) were compared. Kaplan-Meier plot was generated for combined data from two studies comparing the duration for which the OVA expression lasts once expressed after AAV gene therapy. Statistical analysis performed by Cox proportional regression for combined data to determine the hazard ratio of the AAV treatment over the AAV + Compound A treatment group.

[0122] FIGs. 29A, 29B, 29C, and 29D show that wild-type C57BL/6 mice were treated with AAVrh32.33-LacZ 2×10^{11} ventrogluteal (VG) intramuscularly with or without a single subcutaneous dose of 0.3 mg/kg Compound A. The single dose of Compound A was administered either 4 days prior to AAV gene therapy (pre-treatment) or co-administered at the same time of AAV gene therapy (co-treatment). Mice from each group were sacrificed after at 21 days post AAV gene therapy treatment and CD8+ T cell immune responses were measured in the splenocytes by analyzing the proportion of proliferating CD8+ T cells (**FIG. 29A**) and the proportion of CD8+ T effector memory (CD8+T_{EM}) cells (**FIG. 29B**). Proliferating CD8+ T cells were identified by CD8+ T cells expressing the proliferative marker Ki67. CD8+T_{EM} cells were identified as CD62L+ and CD44+ cells within the CD8 +T cell population (**FIG. 29C** and **FIG. 29D**). Splenocytes were restimulated ex-vivo for 6 hours with 1 µg/ml of either AAVrh32.33 peptide pool to determine AAV capsid specific T-cell responses (**FIG. 29C**) or 1 µg/ml of beta-galactosidase peptide pool to determine transgene specific T-cell responses (**FIG. 29D**). IFN γ expressing CD8+ T cell responses were measured in the splenocytes by Intracellular cytokine staining assay. Data is represented as mean $\pm$ SD. Statistical analysis performed by two-way ANOVA with test for multiple comparisons. * $p < 0.05$, ** $p < 0.01$, **** $p < 0.0001$.

DETAILED DESCRIPTION OF CERTAIN EMBODIMENTS

[0123] This description and exemplary embodiments should not be taken as limiting. For the purposes of this specification and appended claims, unless otherwise indicated, all numbers expressing quantities, percentages, or proportions, and other numerical values used in the specification and claims, are to be understood as being modified in all instances by the term “about,” to the extent they are not already so modified. “About” indicates a degree of variation that does not substantially affect the properties of the described subject matter, e.g., within 10%, 5%, 2%, or 1%. Accordingly, unless indicated to the contrary, the numerical parameters set forth in the following specification and attached claims are approximations that may vary depending upon the desired properties sought to be obtained. At the very least, and not as an attempt to limit

the application of the doctrine of equivalents to the scope of the claims, each numerical parameter should at least be construed in light of the number of reported significant digits and by applying ordinary rounding techniques.

[0124] Reference will now be made in detail to certain embodiments of the invention. While the invention will be described in conjunction with such embodiments, it will be understood that they are not intended to limit the invention to those embodiments. On the contrary, the invention is intended to cover all alternatives, modifications, and equivalents, which may be included within the invention as defined by the appended claims.

[0125] Before describing the present teachings in detail, it is to be understood that the disclosure is not limited to specific compositions or process steps, as such may vary. It should be noted that, as used in this specification and the appended claims, the singular form “a”, “an” and “the” include plural references unless the context clearly dictates otherwise. Thus, for example, reference to “a nucleic acid” includes a plurality of nucleic acids, reference to “a cell” includes a plurality of cells, and the like.

[0126] Numeric ranges are inclusive of the numbers defining the range. Measured and measurable values are understood to be approximate, taking into account significant digits and the error associated with the measurement. Also, the use of “comprise”, “comprises”, “comprising”, “contain”, “contains”, “containing”, “include”, “includes”, and “including” are not intended to be limiting. It is to be understood that both the foregoing general description and detailed description are exemplary and explanatory only and are not restrictive of the teachings.

[0127] Unless specifically noted in the above specification, embodiments in the specification that recite “comprising” various components are also contemplated as “consisting of” or “consisting essentially of” the recited components; embodiments in the specification that recite “consisting of” various components are also contemplated as “comprising” or “consisting essentially of” the recited components; and embodiments in the specification that recite “consisting essentially of” various components are also contemplated as “consisting of” or “comprising” the recited components (this interchangeability does not apply to the use of these terms in the claims).

[0128] The section headings used herein are for organizational purposes and are not to be construed as limiting the disclosed subject matter in any way. In the event that any document or other material incorporated by reference contradicts any explicit content of this specification, including definitions, this specification controls.

I. Definitions

[0129] As used herein, “peripheral blood mononuclear cells” or “PBMCs” refers to immune cells having a single, round nucleus that originate in bone marrow and are found in the peripheral circulation. Such cells include, e.g., lymphocytes (T cells, B cells, and NK cells) as well as monocytes, and are isolated from blood samples (such as from a whole blood sample collected from a subject) using density gradient centrifugation.

[0130] As used herein, “isolated” refers to a biological component (such as a nucleic acid molecule, protein, or cell) that has been substantially separated, produced apart from, or purified away from other components (for example, other components in a sample, cell, or organism in which the component naturally occurs). Nucleic acid molecules, proteins, or cells that have been “isolated” include those purified using standard purification methods. The term “isolated” or “purified” does not require absolute purity; rather, it is intended as a relative term. Thus, for example, an isolated biological component is one in which the biological component is more enriched in a preparation than the biological component is in its natural environment within a cell, organism, sample, or production vessel (for example, a cell culture system). For example, an isolated biological component can represent at least 50%, such as at least 70%, at least 80%, at least 90%, at least 95%, or greater, of the total biological component content of the preparation.

[0131] As used herein, “subject” refers to an animal, such as a member of a mammalian species (e.g., human) or avian (e.g., bird) species, or other organism, such as a plant. More specifically, a subject can be a vertebrate, e.g., a mammal such as a mouse, a primate, a simian or a human. Animals include farm animals (e.g., production cattle, dairy cattle, poultry, horses, pigs, and the like), sport animals, and companion animals (e.g., pets or support animals). A subject can be a healthy individual, an individual that has or is suspected of having a disease or a predisposition to the disease, or an individual in need of therapy or suspected of needing therapy. The terms “individual” or “patient” are intended to be interchangeable with “subject”. For example, the subject can be an individual who is in need of gene therapy, e.g., due to having a disease such as an autoimmune disease or a developmental, neurological, or other genetic disorder. As another example, the subject can be a female individual who is pregnant or who is planning on becoming pregnant, who may have been diagnosed of or suspected of having a disease, e.g., a cancer, an auto-immune disease.

[0132] As used herein, the term “potency” refers to the amount of a cytokine (e.g., IL-2 polypeptide) required to produce a target effect. In some embodiments, the term “potency” refers to the amount of cytokine (e.g., IL-2 polypeptide) required to activate a target cytokine receptor (e.g., IL-2 receptor). In other instances, the term “potency” refers to the amount of cytokine (e.g.,

IL-2 polypeptide) required to activate a target cell population. In some embodiments, potency is measured as ED50 (Effective Dose 50), or the dose required to produce 50% of a maximal effect. In other cases, potency is measured as EC50 (Effective Concentration 50), or the dose required to produce the target effect in 50% of the population.

[0133] As used herein, an “IL-2 conjugate” is an IL-2 polypeptide attached (such as through a linker) to a conjugating moiety, e.g., comprising a PEG group; the IL-2 conjugate may be but is not necessarily in the form of a pharmaceutically acceptable salt, solvate, or hydrate. As described in detail elsewhere herein, the IL-2 polypeptide may comprise an unnatural amino acid, which can serve as the site of attachment to the conjugating moiety.

[0134] As used herein, the terms “operably linked” and “in functional connection with” with respect to promoters, refer to a relationship between a coding sequence and a promoter element. The promoter is operably linked or in functional connection with the coding sequence when expression from the coding sequence via transcription is regulated, or controlled by, the promoter element. The terms “operably linked” and “in functional connection with” are utilized interchangeably herein with respect to promoter elements.

[0135] As used herein, the term “gene therapy agent” refers to a nucleic acid (e.g., expression construct, miRNA, antisense, shRNA, siRNA) or a nucleic acid in combination with an agent used to deliver the nucleic acid to an individual or a cell to modify or manipulate the expression of one or more nucleic acids (e.g., gene, mRNA) in an individual or a cell to alter the biological properties of living cells. Examples of gene therapy agents include, but are not limited to, viral vectors (e.g., adeno-associated virus, adenovirus, lentivirus, Herpes simplex virus, baculovirus), bacterial vectors, and non-viral vectors (e.g., lipid nanoparticles encapsulating a therapeutic nucleic acid or plasmid DNAs (e.g., close ended DNA) comprising a therapeutic nucleic acid and/or encoding a therapeutic polypeptide).

[0136] As used herein, a “vector” refers to a recombinant plasmid or virus that comprises a nucleic acid to be delivered into a host cell, either in vitro or in vivo.

[0137] The term “polynucleotide” or “nucleic acid” as used herein refers to a polymeric form of nucleotides of any length, comprising ribonucleotides or deoxyribonucleotides. Thus, this term includes, but is not limited to, single-, double- or multi-stranded DNA or RNA, genomic DNA, cDNA, DNA-RNA hybrids, or a polymer comprising purine and pyrimidine bases, or other natural, chemically or biochemically modified, non-natural, or derivatized nucleotide bases. The backbone of the nucleic acid can comprise sugars and phosphate groups (as may typically be found in RNA or DNA), or modified or substituted sugar or phosphate groups. Alternatively, the backbone of the nucleic acid can comprise a polymer of synthetic subunits such as

phosphoramidates and thus can be an oligodeoxynucleoside phosphoramidate (P-NH₂) or a mixed phosphoramidate- phosphodiester oligomer. In addition, a double-stranded nucleic acid can be obtained from the single stranded polynucleotide product of chemical synthesis either by synthesizing the complementary strand and annealing the strands under appropriate conditions, or by synthesizing the complementary strand de novo using a DNA polymerase with an appropriate primer.

[0138] The terms “polypeptide” and “protein” are used interchangeably to refer to a polymer of amino acid residues, and are not limited to a minimum length. Such polymers of amino acid residues may contain natural or non-natural amino acid residues, and include, but are not limited to, peptides, oligopeptides, dimers, trimers, and multimers of amino acid residues. Both full-length proteins and fragments thereof are encompassed by the definition. The terms also include post-translational modifications of the polypeptide, for example, glycosylation, sialylation, acetylation, phosphorylation, and the like. Furthermore, for purposes of the present invention, a “polypeptide” refers to a protein which includes modifications, such as deletions, additions, and substitutions (which may be conservative in nature), to the native sequence, as long as the protein maintains the desired activity. These modifications may be deliberate, as through site-directed mutagenesis, or may be accidental, such as through mutations of hosts which produce the proteins or errors due to PCR amplification.

[0139] A “recombinant viral vector” refers to a recombinant polynucleotide vector comprising one or more heterologous sequences (a nucleic acid sequence that does not naturally occur in the virus from which the vector is derived, e.g., a sequence that is not of viral origin or from a different virus). In the case of recombinant AAV vectors, the recombinant nucleic acid is flanked by at least one, e.g., two, inverted terminal repeat sequences (ITRs).

[0140] A “recombinant AAV vector (rAAV vector)” refers to a polynucleotide vector comprising one or more heterologous sequences (i.e., nucleic acid sequence not of AAV origin) that are flanked by at least one, e.g., two, AAV inverted terminal repeat sequences (ITRs). Such rAAV vectors can be replicated and packaged into infectious viral particles when present in a host cell that has been infected with a suitable helper virus (or that is expressing suitable helper functions) and that is expressing AAV rep and cap gene products (i.e., AAV Rep and Cap proteins). When a rAAV vector is incorporated into a larger polynucleotide (e.g., in a chromosome or in another vector such as a plasmid used for cloning or transfection), then the rAAV vector may be referred to as a “pro-vector” which can be “rescued” by replication and encapsidation in the presence of AAV packaging functions and suitable helper functions. A rAAV vector can be in any of a number of forms, including, but not limited to, plasmids, linear

artificial chromosomes, complexed with lipids, encapsulated within liposomes, and, in embodiments, encapsidated in a viral particle, particularly an AAV particle. A rAAV vector can be packaged into an AAV virus capsid to generate a “recombinant adeno-associated viral particle (rAAV particle)”.

[0141] An “rAAV virus” or “rAAV viral particle” refers to a viral particle composed of at least one AAV capsid protein and an encapsidated rAAV vector genome.

[0142] A “recombinant adenoviral vector” refers to a polynucleotide vector comprising one or more heterologous sequences (i.e., nucleic acid sequence not of adenovirus origin) that are flanked by at least one adenovirus inverted terminal repeat sequence (ITR). In some embodiments, the recombinant nucleic acid is flanked by two inverted terminal repeat sequences (ITRs). Such recombinant viral vectors can be replicated and packaged into infectious viral particles when present in a host cell that is expressing essential adenovirus genes deleted from the recombinant viral genome (e.g., E1 genes, E2 genes, E4 genes, etc.). When a recombinant viral vector is incorporated into a larger polynucleotide (e.g., in a chromosome or in another vector such as a plasmid used for cloning or transfection), then the recombinant viral vector may be referred to as a “pro-vector” which can be “rescued” by replication and encapsidation in the presence of adenovirus packaging functions. A recombinant viral vector can be in any of a number of forms, including, but not limited to, plasmids, linear artificial chromosomes, complexed with lipids, encapsulated within liposomes, and encapsidated in a viral particle, for example, an adenovirus particle. A recombinant viral vector can be packaged into an adenovirus virus capsid to generate a “recombinant adenoviral particle.”

[0143] A “recombinant lentivirus vector” refers to a polynucleotide vector comprising one or more heterologous sequences (i.e., nucleic acid sequence not of lentivirus origin) that are flanked by at least one lentivirus terminal repeat sequences (LTRs). In some embodiments, the recombinant nucleic acid is flanked by two lentiviral terminal repeat sequences (LTRs). Such recombinant viral vectors can be replicated and packaged into infectious viral particles when present in a host cell that has been infected with a suitable helper function. A recombinant lentiviral vector can be packaged into a lentivirus capsid to generate a “recombinant lentiviral particle.”

[0144] A “recombinant herpes simplex vector (recombinant HSV vector)” refers to a polynucleotide vector comprising one or more heterologous sequences (i.e., nucleic acid sequence not of HSV origin) that are flanked by HSV terminal repeat sequences. Such recombinant viral vectors can be replicated and packaged into infectious viral particles when present in a host cell that has been infected with a suitable helper functions. When a recombinant

viral vector is incorporated into a larger polynucleotide (e.g., in a chromosome or in another vector such as a plasmid used for cloning or transfection), then the recombinant viral vector may be referred to as a “pro-vector” which can be “rescued” by replication and encapsidation in the presence of HSV packaging functions. A recombinant viral vector can be in any of a number of forms, including, but not limited to, plasmids, linear artificial chromosomes, complexed with lipids, encapsulated within liposomes, and encapsidated in a viral particle, for example, an HSV particle. A recombinant viral vector can be packaged into an HSV capsid to generate a “recombinant herpes simplex viral particle.”

[0145] “Solid lipid nanoparticles” (SLNs, sLNPs), or “lipid nanoparticles” (LNPs) as used herein refer to nanoparticles comprising lipids that can contain a payload. In some examples, there is only one phospholipid layer; furthermore, in some embodiments, the bulk of the interior of the particle is composed substantially of lipophilic substance and a payload. Payloads such as nucleic acids can be embedded in the interior. In some examples, the lipid nanoparticle is a liposome, which comprises a lipid bilayer and may comprise a hydrophilic or aqueous interior comprising a payload.

[0146] As used herein, the term “improving” as it relates to gene therapy may refer to the act of boosting, heightening, lengthening or otherwise increasing the expression of the therapeutic gene payload of a gene therapy agent. In some embodiments, an improved gene therapy is one where expression of the therapeutic gene payload of the gene therapy agent administered with an IL-2 conjugate is increased by greater than any of about 10%, 25%, 50%, 75%, or 100% compared to gene therapy administered without the IL-2 conjugate. In some embodiments, an improved gene therapy is one where time of expression of the therapeutic gene payload of the gene therapy agent administered with an IL-2 conjugate is lengthened by greater than any of about 10%, 25%, 50%, 75%, or 100% compared to gene therapy administered without the IL-2 conjugate. In some examples, gene therapy is improved by decreasing an immune response (e.g., an adaptive immune response) to the gene therapy agent. In some embodiments, an improved gene therapy is one where an immune response to gene therapy agent administered with an IL-2 conjugate is decreased by greater than any of about 10%, 25%, 50%, 75%, or 100% compared to gene therapy administered without the IL-2 conjugate. In some embodiments, the decrease in an immune response to a gene therapy agent is measured as a decrease in a cytokine signature following exposure of the gene therapy agent to immune cells in the presence of an IL-2 conjugate compared to exposure of the gene therapy agent to immune cells in the absence of the IL-2 conjugate.

[0147] As used herein, the term “modulating” as it refers to gene therapy may refer to the act of changing, altering, varying, improving or otherwise modifying the presence, or an activity of, a gene therapy agent. For example, modulating an immune response to a gene therapy agent may refer to any act leading to changing, altering, varying, improving or otherwise modifying an immune response to the gene therapy agent (e.g., decreasing, delaying and/or eliminating an immune response (e.g., an adaptive immune response) to the gene therapy agent).

[0148] As used herein, the term “cytokine signature” as it relates to an immune response (e.g., adaptive immune response) to a gene therapy agent refers to altered (e.g., increased, decreased) expression of one or more cytokines following exposure of an adaptive immune cell to a gene therapy agent. In some examples, the cytokines of the cytokine signature are specific to an interleukin-6 (IL-6); tumor necrosis factor-alpha (TNF- α); tumor necrosis factor-beta (TNF- β); interferon alpha (IFN- α); interleukin-10 (IL-10); interleukin-8 (IL-8); Regulated upon Activation, Normal T Cell Expressed and Presumably Secreted (RANTES); Granulocyte-macrophage colony-stimulating factor (GM-CSF); interferon gamma (IFN- γ); interferon gamma-induced protein 10 (IP-10); interleukin-1beta (IL-1 β); interleukin-2 (IL-2); and/or interleukin-4 (IL-4) pathway.

[0149] Adaptive immune cells are white blood cells that mediate adaptive immunity and include B cells, T cells, and NK cells. AAVs upon cell entry can evoke an immune response. The magnitude of this immune response may depend on AAV serotype and cell type. Once AAVs transduce a host immune cell they can engage immune receptors. Once these immune receptors are activated by viruses, they secrete cytokines that establish an anti-viral state within the infected cell and alert the neighboring cells.

[0150] Innate immune cells are white blood cells that mediate innate immunity and include basophils, dendritic cells, eosinophils, Langerhans cells, mast cells, monocytes and macrophages, neutrophils and NK cells. Different AAV capsids can enter these adaptive immune cells with different efficiencies often referred to as transduction efficiency. Some serotypes such as AAV1 are efficient at transducing certain immune cells like monocytes whereas other AAVs like AAV6 are efficient at transducing cells like dendritic cells (Grimm, D et al., J. Virol., 2008, 82(12):5887-5911). AAVs upon cell entry can evoke an immune response. The magnitude of this immune response is dependent on AAV serotype and cell type. Once AAVs transduce a host immune cell they can engage immune receptors such as TLRs (e.g., TLR9). Several studies using mouse models reveal that TLR9 is a key DNA sensor contributing to AAV immunogenicity (Zhu, J et al., J Clin Invest. 2009;119(8):2388-2398; Ashley SN et al., Cell. Immunol.2019, 346:103997). Once these TLRs are activated by viruses they secrete cytokines

that establish an anti-viral state within the infected cell and alert the neighboring cells. (Carty, M and Bowie, AG, Clin Exp Immunol, 2010, 161(3):397-406; Lester, SN and Li, K, J Mol Biol. 2014; 426(6):1246–1264; Fitzgerald, KA and Kagan, JC, Cell, 2020 180(6):1044-1066).

[0151] As used herein, the upregulation or downregulation of certain subset of cytokines is referred to as a “cytokine signature”. These cytokine signatures comprising three or more cytokines can be used as predictive markers for diseases and success of therapies. Examples of cytokine signatures are found in Zuniga, J et al., Int. J. Infect. Diseases, 2020, 94:4-11, Bergamaschi, C et al., Cell Reports, 2021, 36:109504; Del Valle, DM et al., Nat. Med. 2020, 26:1636-1643.

[0152] “Heterologous” means derived from a genotypically distinct entity from that of the rest of the entity to which it is compared or into which it is introduced or incorporated. For example, a nucleic acid introduced by genetic engineering techniques into a different cell type is a heterologous nucleic acid (and, when expressed, can encode a heterologous polypeptide). Similarly, a cellular sequence (e.g., a gene or portion thereof) that is incorporated into a viral vector is a heterologous nucleotide sequence with respect to the vector.

[0153] The term “transgene” refers to a nucleic acid that is introduced into a cell and is capable of being transcribed into RNA and optionally, translated and/or expressed under appropriate conditions. In some embodiments, it confers a desired property to a cell into which it was introduced, or otherwise leads to a desired therapeutic or diagnostic outcome. In another aspect, it may be transcribed into a molecule that mediates RNA interference, such as siRNA.

[0154] The terms “genome particles (gp),” “genome equivalents,” or “genome copies” as used in reference to a viral titer, refer to the number of virions containing the recombinant AAV DNA genome, regardless of infectivity or functionality. The number of genome particles in a particular vector preparation can be measured by procedures such as described in the Examples herein, or for example, in Clark et al. (1999) Hum. Gene Ther., 10:1031-1039; Veldwijk et al. (2002) Mol. Ther., 6:272-278.

[0155] The terms “infection unit (iu),” “infectious particle,” or “replication unit,” as used in reference to a viral titer, refer to the number of infectious and replication-competent recombinant AAV vector particles as measured by the infectious center assay, also known as replication center assay, as described, for example, in McLaughlin et al. (1988) J. Virol., 62:1963-1973.

[0156] The term “transducing unit (tu)” as used in reference to a viral titer, refers to the number of infectious recombinant AAV vector particles that result in the production of a functional transgene product as measured in functional assays such as described in Examples herein, or for

example, in Xiao et al. (1997) *Exp. Neurobiol.*, 144:113-124; or in Fisher et al. (1996) *J. Virol.*, 70:520-532 (LFU assay).

[0157] An “inverted terminal repeat” or “ITR” sequence is a term well understood in the art and refers to relatively short sequences found at the termini of viral genomes which are in opposite orientation.

[0158] An “AAV inverted terminal repeat (ITR)” sequence, a term well-understood in the art, is an approximately 145-nucleotide sequence that is present at both termini of the native single-stranded AAV genome. The outermost 125 nucleotides of the ITR can be present in either of two alternative orientations, leading to heterogeneity between different AAV genomes and between the two ends of a single AAV genome. The outermost 125 nucleotides also contains several shorter regions of self-complementarity (designated A, A', B, B', C, C' and D regions), allowing intrastrand base-pairing to occur within this portion of the ITR.

[0159] A “terminal resolution sequence” or “trs” is a sequence in the D region of the AAV ITR that is cleaved by AAV rep proteins during viral DNA replication. A mutant terminal resolution sequence is refractory to cleavage by AAV rep proteins. “AAV helper functions” refer to functions that allow AAV to be replicated and packaged by a host cell. AAV helper functions can be provided in any of a number of forms, including, but not limited to, helper virus or helper virus genes which aid in AAV replication and packaging. Other AAV helper functions are known in the art such as genotoxic agents.

[0160] “AAV helper functions” refer to functions that allow AAV to be replicated and packaged by a host cell. AAV helper functions can be provided in any of a number of forms, including, but not limited to, helper virus or helper virus genes which aid in AAV replication and packaging. Other AAV helper functions are known in the art such as genotoxic agents.

[0161] A “helper virus” for AAV refers to a virus that allows AAV (which is a defective parvovirus) to be replicated and packaged by a host cell. A number of such helper viruses have been identified, including adenoviruses, herpesviruses, poxviruses such as vaccinia, and baculovirus. The adenoviruses encompass a number of different subgroups, although Adenovirus type 5 of subgroup C (Ad5) is most commonly used. Numerous adenoviruses of human, non-human mammalian and avian origin are known and are available from depositories such as the ATCC. Viruses of the herpes family, which are also available from depositories such as ATCC, include, for example, herpes simplex viruses (HSV), Epstein-Barr viruses (EBV), cytomegaloviruses (CMV) and pseudorabies viruses (PRV). Baculoviruses available from depositories include *Autographa californica* nuclear polyhedrosis virus.

[0162] “Percent (%) sequence identity” with respect to a reference polypeptide or nucleic acid sequence is defined as the percentage of amino acid residues or nucleotides in a candidate sequence that are identical with the amino acid residues or nucleotides in the reference polypeptide or nucleic acid sequence, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent sequence identity, and not considering any conservative substitutions as part of the sequence identity. Alignment for purposes of determining percent amino acid or nucleic acid sequence identity can be achieved in various ways that are within the skill in the art, for instance, using publicly available computer software programs, for example, those described in Current Protocols in Molecular Biology (Ausubel et al., eds., 1987), Supp. 30, section 7.7.18, Table 7.7.1, and including BLAST, BLAST-2, ALIGN or Megalign (DNASTAR) software. A potential alignment program is ALIGN Plus (Scientific and Educational Software, Pennsylvania). Those skilled in the art can determine appropriate parameters for measuring alignment, including any algorithms needed to achieve maximal alignment over the full length of the sequences being compared. For purposes herein, the % amino acid sequence identity of a given amino acid sequence A to, with, or against a given amino acid sequence B (which can alternatively be phrased as a given amino acid sequence A that has or comprises a certain % amino acid sequence identity to, with, or against a given amino acid sequence B) is calculated as follows: 100 times the fraction X/Y , where X is the number of amino acid residues scored as identical matches by the sequence alignment program in that program's alignment of A and B, and where Y is the total number of amino acid residues in B. It will be appreciated that where the length of amino acid sequence A is not equal to the length of amino acid sequence B, the % amino acid sequence identity of A to B will not equal the % amino acid sequence identity of B to A. For purposes herein, the % nucleic acid sequence identity of a given nucleic acid sequence C to, with, or against a given nucleic acid sequence D (which can alternatively be phrased as a given nucleic acid sequence C that has or comprises a certain % nucleic acid sequence identity to, with, or against a given nucleic acid sequence D) is calculated as follows: 100 times the fraction W/Z , where W is the number of nucleotides scored as identical matches by the sequence alignment program in that program's alignment of C and D, and where Z is the total number of nucleotides in D. It will be appreciated that where the length of nucleic acid sequence C is not equal to the length of nucleic acid sequence D, the % nucleic acid sequence identity of C to D will not equal the % nucleic acid sequence identity of D to C.

[0163] An “effective amount” of an agent refers to an amount effective, at dosages and for periods of time necessary, to achieve the desired therapeutic result. For example, an effective amount of a gene therapy agent refers to an amount effective, at dosages and for periods of time

necessary, to achieve the desired gene therapeutic result. In another example, an effective amount of an IL-2 conjugate may refer to an amount effective, at dosages and for periods of time necessary, to achieve the desired result of improved gene therapy.

[0164] A “therapeutically effective amount” of a substance/molecule of the invention, (e.g., a gene therapy agent and/or an IL-2 conjugate) may vary according to factors such as the disease state, age, sex, and weight of the individual, and the ability of the substance/molecule, agonist or antagonist to elicit a desired response in the individual. A therapeutically effective amount is also one in which any toxic or detrimental effects of the substance/molecule are outweighed by the therapeutically beneficial effects.

[0165] The term “suitable control” as it refers to a cytokine signature is the expression of the cytokines in the cytokine signature from adaptive immune cells that are not incubated with the gene therapy agent or the expression of the cytokines in the cytokine signature from adaptive immune cells prior to incubation with the gene therapy agent.

[0166] Administration “in combination with” as it related to a gene therapy agent and a modulator of an adaptive immune response (e.g., an IL-2 conjugate) includes simultaneous (concurrent), consecutive or sequential administration in any order of the gene therapy agent and the modulator of an adaptive immune response (e.g., an IL-2 conjugate).

[0167] The term “concurrently” is used herein to refer to administration of a gene therapy agent and a modulator of the adaptive immune response (e.g., an IL-2 conjugate), where at least part of the administration overlaps in time. Accordingly, concurrent administration includes a dosing regimen when the administration of a gene therapy agent or a modulator of an adaptive immune response (e.g., an IL-2 conjugate) continues after discontinuing the administration of the other agent/modulator.

[0168] As used herein, “in conjunction with” refers to administration of one treatment modality in addition to another treatment modality. As such, “in conjunction with” refers to administration of one treatment modality (a gene therapy agent or a modulator of an adaptive immune response (e.g., an IL-2 conjugate)) before, during or after administration of the other treatment modality to the individual.

[0169] The terms “or a combination thereof” and “or combinations thereof” as used herein refers to any and all permutations and combinations of the listed terms preceding the term. For example, “A, B, C, or combinations thereof” is intended to include at least one of: A, B, C, AB, AC, BC, or ABC, and if order is important in a particular context, also BA, CA, CB, ACB, CBA, BCA, BAC, or CAB. Continuing with this example, expressly included are combinations that contain repeats of one or more item or term, such as BB, AAA, AAB, BBC, AAABCCCC,

CBBAAA, CABABB, and so forth. The skilled artisan will understand that typically there is no limit on the number of items or terms in any combination, unless otherwise apparent from the context.

[0170] “Or” is used in the inclusive sense, i.e., equivalent to “and/or,” unless the context requires otherwise.

II. Exemplary Methods

A. Overview

[0171] The present disclosure provides methods of delivering a gene therapy agent to a cell of a subject, treating an individual in need thereof with a gene therapy agent, increasing expression of a gene therapy agent, reducing an immune response to a gene therapy agent, preventing immune-related adverse events in a subject, selecting a subject for treatment with a gene therapy agent and an IL-2 conjugate, using an IL-2 conjugate. The IL-2 conjugate can be capable of selectively upregulating distinct population(s) of lymphocytes (e.g., CD4⁺ T regulatory cells), e.g., through cytokine/cytokine receptor signaling. In some embodiments, the amino acid sequence of the IL-2 conjugate has at least one amino acid residue replaced by an unnatural amino acid linked to a conjugating moiety. In some embodiments, the unnatural amino acid that is linked to the conjugating moiety is positioned in the amino acid sequence so as to preferentially reduce binding of the IL-2 conjugate to IL-2R $\beta\gamma$ relative to IL-2R $\alpha\beta\gamma$ or is at position P1, T2, S3, S4, S5, T6, K7, K8, Q10, L11, E14, H15, L17, L18, D19, Q21, M22, N25, G26, N28, N29, Y30, K31, K34, T36, M45, P46, K47, A49, T50, E51, L52, K53, H54, Q56, E59, E66, N70, Q73, S74, K75, N76, F77, H78, R80, P81, R82, D83, S86, N87, I88, V90, I91, L93, E94, K96, G97, S98, E99, T100, T101, F102, M103, C104, E105, Y106, A107, D108, E109, T110, A111, T112, E115, N118, R119, T122, F123, S124, Q125, S126, S129, T130, L131, or T132 in reference to the sequence of SEQ ID NO: 1.

[0172] In some embodiments, also described herein is a method of selectively upregulating CD4⁺ T regulatory cell through IL-2/IL-2R signaling. In some embodiments, the IL-2 conjugate suppresses CD8⁺ T cell proliferation in the subject. In some embodiments, the IL-2 conjugate suppresses effector memory CD8⁺ T cell proliferation in the subject. In some embodiments, IL-2 is an IL-2 conjugate, which interacts with the IL-2R $\alpha\beta\gamma$ complex and with a weakened IL-2R $\beta\gamma$ interaction relative to wild-type IL-2. In some embodiments, further described herein are methods of delivering a gene therapy agent to a cell of a subject with use of an IL-2 conjugate described herein. In additional embodiments, described herein are pharmaceutical compositions

and kits which comprise one or more IL-2 conjugates and/or gene therapy agents described herein, e.g., for use in the disclosed methods.

[0173] As shown in the examples, gene therapy resulted in a significant increase in proliferating and effector CD8+ T cell proportion in the spleen with which was mitigated by treatment with an IL-2 conjugate according to this disclosure. The mitigation of CD8+ T cell responses were not meaningfully different when the IL-2 conjugate was administered as a pre-treatment or given as a co-treatment. In addition, upon *ex vivo* restimulation of splenocytes with a gene therapy agent peptide pool and a transgene peptide pool, there was a significant increase in the proportion of IFN γ secreting CD8+ T cells in response to each set of peptides, which were mitigated by IL-2 conjugate treatment. There was no meaningful difference in mitigation of AAV and/or transgene specific CD8+ T cell responses when the IL-2 conjugate was given as a pre-treatment or as a co-treatment. Without wishing to be bound by any particular theory, this suggests that administering AAV gene therapy within the timeframe of the expansion of Tregs mediated by the IL-2 conjugate maintains the therapeutic benefit of the IL-2 conjugate of suppressing adverse immune responses to the gene therapy agent and/or the transgene.

B. Cytokine Conjugates

[0174] In some embodiments, the IL-2 conjugate used in the disclosed methods comprises an amino acid sequence having at least 80% sequence identity to SEQ ID NO: 1. In some embodiments, the amino acid sequence of the IL-2 conjugate has at least one amino acid residue replaced by an unnatural amino acid linked to a conjugating moiety. In some embodiments, the unnatural amino acid that is linked to the conjugating moiety is positioned in the amino acid sequence so as to preferentially reduce binding of the IL-2 conjugate to IL-2R $\beta\gamma$ relative to IL-2R $\alpha\beta\gamma$. In some embodiments, the unnatural amino acid linked to the conjugating moiety is positioned in the amino acid sequence at position P1, T2, S3, S4, S5, T6, K7, K8, Q10, L11, E14, H15, L17, L18, D19, Q21, M22, N25, G26, N28, N29, Y30, K31, K34, T36, M45, P46, K47, A49, T50, E51, L52, K53, H54, Q56, E59, E66, N70, Q73, S74, K75, N76, F77, H78, R80, P81, R82, D83, S86, N87, I88, V90, I91, L93, E94, K96, G97, S98, E99, T100, T101, F102, M103, C104, E105, Y106, A107, D108, E109, T110, A111, T112, E115, N118, R119, T122, F123, S124, Q125, S126, S129, T130, L131, or T132 in reference to the sequence of SEQ ID NO: 1.

[0175] Table 1 provides exemplary IL-2 sequences. The sequence of SEQ ID NO: 1 is aldesleukin, in which the first amino acid of wild-type IL-2 has been removed. An amino acid in SEQ ID NO: 1 or a sequence having a percentage identity thereto as described elsewhere herein

may be replaced with an unnatural amino acid. Exemplary sequences comprising an unnatural amino acid, indicated by X, are listed as SEQ ID NOs: 2-14.

Table 1

Name	Sequence	SEQ ID NO:
aldesleukin	PTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTF KFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRP RDLISNINVIVLELKGSETTFMCEYADETATIVEFLNRWITF SQSIISTLT	1
IL-2_K8X	PTSSSTK X TQLQLEHLLLDLQMILNGINNYKNPKLTRMLTF KFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRP RDLISNINVIVLELKGSETTFMCEYADETATIVEFLNRWITF SQSIISTLT	2
IL-2_H15X	PTSSSTKKTQLQLE X LLLDLQMILNGINNYKNPKLTRMLTF KFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRP RDLISNINVIVLELKGSETTFMCEYADETATIVEFLNRWITF SQSIISTLT	3
IL-2_L18X	PTSSSTKKTQLQLEHLL X DLQMILNGINNYKNPKLTRMLTF KFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRP RDLISNINVIVLELKGSETTFMCEYADETATIVEFLNRWITF SQSIISTLT	4
IL-2_D19X	PTSSSTKKTQLQLEHLL X LQMILNGINNYKNPKLTRMLTF KFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRP RDLISNINVIVLELKGSETTFMCEYADETATIVEFLNRWITF SQSIISTLT	5
IL-2_M22X	PTSSSTKKTQLQLEHLLLDLQ X ILNGINNYKNPKLTRMLTF KFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRP RDLISNINVIVLELKGSETTFMCEYADETATIVEFLNRWITF SQSIISTLT	6
IL-2_N25X	PTSSSTKKTQLQLEHLLLDLQMIL X GINNYKNPKLTRMLTF KFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRP RDLISNINVIVLELKGSETTFMCEYADETATIVEFLNRWITF SQSIISTLT	7
IL-2_N87X	PTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTF KFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRP RDLIS X INVIVLELKGSETTFMCEYADETATIVEFLNRWITF SQSIISTLT	8
IL-2_E99X	PTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTF KFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRP RDLISNINVIVLELKG SX TFMCEYADETATIVEFLNRWITF SQSIISTLT	9
IL-2_N118X	PTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTF KFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRP RDLISNINVIVLELKGSETTFMCEYADETATIVEFL X RWITF SQSIISTLT	10
IL-2_T122X	PTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTF KFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRP	11

	RDLISNINVIVLELKGSETTFMCEYADETATIVEFLNRWIXF SQSIISTLT	
IL-2_Q125X	PTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTF KFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRP RDLISNINVIVLELKGSETTFMCEYADETATIVEFLNRWITF SXSIISTLT	12
IL-2_S126X	PTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTF KFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRP RDLISNINVIVLELKGSETTFMCEYADETATIVEFLNRWITF SQXIISTLT	13
IL-2_T130X	PTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTF KFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLRP RDLISNINVIVLELKGSETTFMCEYADETATIVEFLNRWITF SQSIISXLT	14
IL-2 (homo sapiens) (mature form)	APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLT FKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLR PRDLISNINVIVLELKGSETTFMCEYADETATIVEFLNRWIT FCQSIISTLT	15
IL-2 (homo sapiens) (precursor) NCBI Accession No.: AAB46883.1	MYRMQLLSICIALSLALVTNSAPTSSSTKKTQLQLEHLLLDL QMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCLEE ELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSETTF MCEYADETATIVEFLNRWITFCQSIISTLT	16
CTP Peptide (30 amino acids)	FQSSSSKAPPPSLPSPSRLPGPSDTPILPQ	17
CTP Peptide (31 amino acids)	FQDSSSSKAPPPSLPSPSRLPGPSDTPILPQ	18
ITR sequence	CACTCCCTCTCTGCGCGCTCGCTCGCTCACTGAGGCC GGGCGACCAAAGGTCGCCCACGCCCGGGCTTTGCCCGG GCG	19
IL-2_C125S (in reference to SEQ ID NO: 15)	APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLT FKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLR PRDLISNINVIVLELKGSETTFMCEYADETATIVEFLNRWIT FSQSIISTLT	20
IL-2_H16X	APTSSSTKKTQLQLEXLLLDLQMILNGINNYKNPKLTRMLT FKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLR PRDLISNINVIVLELKGSETTFMCEYADETATIVEFLNRWIT FSQSIISTLT	21
IL-2_L19X	APTSSSTKKTQLQLEHLLXDLQMILNGINNYKNPKLTRMLT FKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLR PRDLISNINVIVLELKGSETTFMCEYADETATIVEFLNRWIT FSQSIISTLT	22
IL-2_N88X	APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLT FKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLR PRDLISXINVIVLELKGSETTFMCEYADETATIVEFLNRWIT FSQSIISTLT	23
IL- 2_N88R_D109X	APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLT FKFYMPKKATELKHLQCLEEELKPLEEVLNLAQSKNFHLR	24

	PRDLIS R INVIVLELKGSETTFMCEYAX E TATIVEFLNRWIT FSQSIISTLT	
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[0176] In some embodiments, the position of the at least one unnatural amino acid is selected from P1, T2, S3, S4, S5, T6, K7, K8, Q10, L11, E14, H15, L17, L18, D19, Q21, M22, N25, G26, N28, N29, Y30, K31, K34, T36, M45, P46, K47, A49, T50, E51, L52, K53, H54, Q56, E59, E66, N70, Q73, S74, K75, N76, F77, H78, R80, P81, R82, D83, S86, N87, I88, V90, I91, L93, E94, K96, G97, S98, E99, T100, T101, F102, M103, C104, E105, Y106, A107, D108, E109, T110, A111, T112, E115, N118, R119, T122, F123, S124, Q125, S126, S129, T130, L131, or T132, wherein the numbering of the amino acid residues is in reference to the sequence of SEQ ID NO: 1. In some embodiments, the position of the at least one unnatural amino acid is selected from K8, L11, E14, H15, L18, D19, M22, N87, E99, and D108, wherein the numbering of the amino acid residues is in reference to the sequence of SEQ ID NO: 1. In some embodiments, the position of the at least one unnatural amino acid is L18, wherein the numbering of the amino acid residues is in reference to the sequence of SEQ ID NO: 1. In some embodiments, the position of the at least one unnatural amino acid is D19, wherein the numbering of the amino acid residues is in reference to the sequence of SEQ ID NO: 1. In some embodiments, the position of the at least one unnatural amino acid is H15, wherein the numbering of the amino acid residues is in reference to the sequence of SEQ ID NO: 1.

[0177] In some embodiments, IL-2 conjugates modified at an amino acid position are provided. In some embodiments, the modification is to an unnatural amino acid. In some embodiments, described herein is an isolated and IL-2 conjugate that comprises at least one unnatural amino acid. In some embodiments, the IL-2 polypeptide is an isolated and purified mammalian IL-2, for example, a rodent IL-2 protein, or a human IL-2 protein. In some embodiments, the IL-2 polypeptide is a human IL-2 protein. In some embodiments, the IL-2 polypeptide comprises about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 1. In some embodiments, the IL-2 polypeptide comprises the sequence of SEQ ID NO: 1. In some embodiments, the IL-2 polypeptide consists of the sequence of SEQ ID NO: 1. In some embodiments, the IL-2 polypeptide comprises about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 2. In some embodiments, the IL-2 polypeptide comprises the sequence of SEQ ID NO: 2. In some embodiments, the IL-2 polypeptide consists of the sequence of SEQ ID NO: 2. In some embodiments, the IL-2 polypeptide comprises about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 3. In some embodiments, the IL-2 polypeptide comprises the sequence of SEQ ID NO: 3. In some embodiments, the IL-2 polypeptide consists of the sequence of SEQ ID NO: 3. In some

embodiments, the IL-2 polypeptide comprises about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 4. In some embodiments, the IL-2 polypeptide comprises the sequence of SEQ ID NO: 4. In some embodiments, the IL-2 polypeptide consists of the sequence of SEQ ID NO: 4. In some embodiments, the IL-2 polypeptide comprises about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 5. In some embodiments, the IL-2 polypeptide comprises the sequence of SEQ ID NO: 5. In some embodiments, the IL-2 polypeptide consists of the sequence of SEQ ID NO: 6. In some embodiments, the IL-2 polypeptide comprises about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 7. In some embodiments, the IL-2 polypeptide comprises the sequence of SEQ ID NO: 7. In some embodiments, the IL-2 polypeptide consists of the sequence of SEQ ID NO: 7. In some embodiments, the IL-2 polypeptide comprises about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 8. In some embodiments, the IL-2 polypeptide comprises the sequence of SEQ ID NO: 8. In some embodiments, the IL-2 polypeptide consists of the sequence of SEQ ID NO: 8.

[0178] In some embodiments, the IL-2 polypeptide comprises about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 9. In some embodiments, the IL-2 polypeptide comprises the sequence of SEQ ID NO: 9. In some embodiments, the IL-2 polypeptide consists of the sequence of SEQ ID NO: 9. In some embodiments, the IL-2 polypeptide comprises about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 10. In some embodiments, the IL-2 polypeptide comprises the sequence of SEQ ID NO: 10. In some embodiments, the IL-2 polypeptide consists of the sequence of SEQ ID NO: 10. In some embodiments, the IL-2 polypeptide comprises about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 11. In some embodiments, the IL-2 polypeptide comprises the sequence of SEQ ID NO: 11. In some embodiments, the IL-2 polypeptide consists of the sequence of SEQ ID NO: 11. In some embodiments, the IL-2 polypeptide comprises about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 12. In some embodiments, the IL-2 polypeptide comprises the sequence of SEQ ID NO: 12. In some embodiments, the IL-2 polypeptide consists of the sequence of SEQ ID NO: 12. In some embodiments, the IL-2 polypeptide comprises about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 13. In some embodiments, the IL-2 polypeptide comprises the sequence of SEQ ID NO: 13. In some embodiments, the IL-2 polypeptide consists of the sequence of SEQ ID NO: 13. In some embodiments, the IL-2 polypeptide comprises about 80%, 85%, 90%, 95%, 96%, 97%, 98%, or 99% sequence identity to SEQ ID NO: 14. In some embodiments, the IL-2 polypeptide comprises the sequence of SEQ

ID NO: 14. In some embodiments, the IL-2 polypeptide consists of the sequence of SEQ ID NO: 14.

[0179] In some embodiments, the IL-2 polypeptide is a truncated variant, e.g., relative to a wild-type IL-2, such as SEQ ID NO: 15 or 16. In some embodiments, the truncation is an N-terminal deletion. In some embodiments, the truncation is a C-terminal deletion. In some embodiments, the truncation comprises both N-terminal and C-terminal deletions. For example, the truncation can be a deletion of at least or about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, or more residues from either the N-terminus or the C-terminus, or both termini. In some embodiments, the IL-2 polypeptide comprises an N-terminal deletion of at least or about 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, or more residues. In some embodiments, the IL-2 polypeptide comprises an N-terminal deletion of at least or about 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 residues. In some embodiments, the IL-2 polypeptide comprises an N-terminal deletion of at least or about 2 residues. In some embodiments, the IL-2 polypeptide comprises an N-terminal deletion of at least or about 3 residues. In some embodiments, the IL-2 polypeptide comprises an N-terminal deletion of at least or about 4 residues. In some embodiments, the IL-2 polypeptide comprises an N-terminal deletion of at least or about 5 residues. In some embodiments, the IL-2 polypeptide comprises an N-terminal deletion of at least or about 6 residues. In some embodiments, the IL-2 polypeptide comprises an N-terminal deletion of at least or about 7 residues. In some embodiments, the IL-2 polypeptide comprises an N-terminal deletion of at least or about 8 residues. In some embodiments, the IL-2 polypeptide comprises an N-terminal deletion of at least or about 9 residues. In some embodiments, the IL-2 polypeptide comprises an N-terminal deletion of at least or about 10 residues.

[0180] In some embodiments, the IL-2 polypeptide comprises an amino acid addition in reference to, for example, SEQ ID NO: 1 or a sequence having at least 80% sequence identity to SEQ ID NO: 1. In some embodiments, the addition is an N-terminal addition. In some embodiments, the addition is a C-terminal addition. In some embodiments, the addition comprises both N-terminal and C-terminal additions. In some embodiments, the IL-2 polypeptide comprises an alanine or methionine N-terminal addition to the first amino acid of the sequence having at least 80% sequence identity to SEQ ID NO: 1.

[0181] In some embodiments, the IL-2 polypeptide is a functionally active fragment. In some embodiments, the functionally active fragment comprises IL-2 region 10-133, 20-133, 30-133, 10-130, 20-130, 30-130, 10-125, 20-125, 30-125, 1-130, or 1-125, wherein the residue positions are in reference to the positions in SEQ ID NO: 1. In some embodiments, the functionally active fragment comprises IL-2 region 10-133, wherein the residue positions are in reference to the

positions in SEQ ID NO: 1. In some embodiments, the functionally active fragment comprises IL-2 region 20-133, wherein the residue positions are in reference to the positions in SEQ ID NO: 1. In some embodiments, the functionally active fragment comprises IL-2 region 30-133, wherein the residue positions are in reference to the positions in SEQ ID NO: 1. In some embodiments, the functionally active fragment comprises IL-2 region 10-125, wherein the residue positions are in reference to the positions in SEQ ID NO: 1. In some embodiments, the functionally active fragment comprises IL-2 region 20-125, wherein the residue positions are in reference to the positions in SEQ ID NO: 1. In some embodiments, the functionally active fragment comprises IL-2 region 1-130, wherein the residue positions are in reference to the positions in SEQ ID NO: 1. In some embodiments, the functionally active fragment comprises IL-2 region 1-125, wherein the residue positions are in reference to the positions in SEQ ID NO: 1.

[0182] In some embodiments, described herein is an IL-2 conjugate that comprises an isolated, purified, and IL-2 conjugate and a conjugating moiety. In some embodiments, the IL-2 conjugate has a decreased affinity to an IL-2 receptor $\beta\gamma$ (IL-2R $\beta\gamma$) subunit relative to a wild-type IL-2 polypeptide. In some embodiments, the conjugating moiety is bound to an amino acid residue that interacts with IL-2R $\beta\gamma$ (e.g., at the IL-2/ IL-2R $\beta\gamma$ interface). In some embodiments, the conjugating moiety is bound to an amino acid residue that is proximal to the IL-2/ IL-2R $\beta\gamma$ interface (e.g., about 5Å, about 10Å, about 15Å, or about 20Å away from the IL-2/ IL-2R $\beta\gamma$ interface). As used herein, the residues involved in the IL-2/ IL-2R $\beta\gamma$ interface comprise IL-2 residues that form hydrophobic interactions, hydrogen bonds, or ionic interactions with residues from the IL-2R $\beta\gamma$ subunit.

[0183] In some embodiments, the conjugating moiety is bound to an amino acid residue selected from an amino acid position P1, T2, S3, S4, S5, T6, K7, K8, Q10, L11, E14, H15, L17, L18, D19, Q21, M22, N25, G26, N28, N29, Y30, K31, K34, T36, M45, P46, K47, A49, T50, E51, L52, K53, H54, Q56, E59, E66, N70, Q73, S74, K75, N76, F77, H78, R80, P81, R82, D83, S86, N87, I88, V90, I91, L93, E94, K96, G97, S98, E99, T100, T101, F102, M103, C104, E105, Y106, A107, D108, E109, T110, A111, T112, E115, N118, R119, T122, F123, S124, Q125, S126, S129, T130, L131, or T132 in reference to the sequence of SEQ ID NO: 1. In some embodiments, the conjugating moiety is bound to an amino acid residue selected from an amino acid position K8, L11, E14, H15, L18, D19, M22, N87, E99, or D108 in reference to the sequence of SEQ ID NO: 1. In some embodiments, the conjugating moiety is bound to an amino acid residue selected from an amino acid position L18 in reference to the sequence of SEQ ID NO: 1. In some embodiments, the conjugating moiety is bound to an amino acid residue selected

from an amino acid position H15 in reference to the sequence of SEQ ID NO: 1. In some embodiments, the conjugating moiety is bound to an amino acid residue selected from an amino acid position D19 in reference to the sequence of SEQ ID NO: 1.

[0184] In some embodiments, the IL-2 conjugate further comprises an additional mutation. In some embodiments, the additional mutation is at an amino acid position selected from K8, L11, E14, H15, L18, D19, M22, N87, E99, or D108 in reference to the sequence of SEQ ID NO: 1. In such cases, the amino acid is conjugated to an additional conjugating moiety for increase in serum half-life, stability, or a combination thereof. Alternatively, the amino acid is first mutated to an unnatural amino acid prior to binding to the additional conjugating moiety.

[0185] In some embodiments, the receptor signaling potency is measured by an ED50 value. In some embodiments, the IL-2 conjugate provides a first ED50 value for activating IL-2 $\beta\gamma$ signaling complex and a second ED50 value for activating IL-2 $\alpha\beta\gamma$ signaling complex, and wherein a difference between the first ED50 and the second ED50 value is less than 10-fold. In some embodiments, the IL-2 conjugate provides a first ED50 value for activating IL-2 $\beta\gamma$ signaling complex and a second ED50 value for activating IL-2 $\alpha\beta\gamma$ signaling complex, and wherein a difference between the first ED50 and the second ED50 value is less than 5-fold. In some embodiments, the difference is less than 9-fold, less than 8-fold, less than 7-fold, less than 6-fold, less than 5-fold, less than 4-fold, less than 3-fold, less than 2-fold, less than 75%, less than 50%, or less than 25%.

[0186] In some embodiments, the conjugating moiety is linked to the N-terminus or the C-terminus of an IL-2 polypeptide, either directly or indirectly through a linker peptide. In some embodiments, the conjugating moiety (e.g., a polymer, a protein, or a peptide) is genetically fused to the IL-2, at the N-terminus or the C-terminus of IL-2, and either directly or indirectly through a linker peptide. In some embodiments, the conjugating moiety is linked to the N-terminus or the C-terminus amino acid residue. In some embodiments, the conjugating moiety is linked to a reactive group that is bound to the N-terminus or C-terminus amino acid residue.

[0187] In some embodiments, the IL-2 conjugate with reduced binding affinity to IL-2R $\beta\gamma$ is capable of expanding CD4⁺ T regulatory cell populations. In some embodiments, the IL-2 conjugate with reduced binding affinity to IL-2R $\beta\gamma$ is capable of suppressing CD8⁺ T cell populations, including CD8⁺ T effector memory cell populations, vector-specific IFN γ -secreting CD8⁺ T cells, and transgene-product-specific IFN γ -secreting CD8⁺ T cells. In some embodiments, the IL-2 conjugate with reduced binding affinity to IL-2R $\beta\gamma$ is capable of suppressing the production of antibodies against the transgene product and the production of

IgG1 antibodies against the transgene product. In some embodiments, the conjugating moiety impairs or blocks binding of IL-2 with IL-2R $\beta\gamma$.

[0188] In some embodiments, activation of CD4⁺ T regulatory cell population via the IL-2R $\alpha\beta\gamma$ complex by the IL-2 conjugate retains significant potency of activation of said cell population relative to a wild-type IL-2 polypeptide. In some embodiments, the activation by the IL-2 conjugate is equivalent to that of the wild-type IL-2 polypeptide. In other instances, the activation by the IL-2 conjugate is higher than that of the wild-type IL-2 polypeptide. In some embodiments, the receptor signaling potency of the IL-2 conjugate to the IL-2R $\alpha\beta\gamma$ complex is higher than a receptor signaling potency of the wild-type IL-2 polypeptide to the IL-2R $\alpha\beta\gamma$ complex. In some embodiments, the receptor signaling potency of the IL-2 conjugate is at least 50% higher than the respective potency of the wild-type IL-2 polypeptide. In some embodiments, the receptor signaling potency of the IL-2 conjugate is about or at least 100%, 150%, 200%, 250%, 300%, 350%, 400%, 450%, 500%, 550%, 600%, 650%, 700%, 750%, 800%, 850%, 900%, 950%, 1000%, 1500%, 2000%, 3000%, 4000%, 5000%, 6000%, 7000%, 8000%, 9000%, 10000%, 15000%, 20000%, 30000%, 40000%, 50000%, 100000%, or higher than the respective potency of the wild-type IL-2 polypeptide. In such cases, the dose or concentration of the IL-2 conjugate used for achieving a similar level of activation of the CD4⁺ T regulatory cell population as a wild-type IL-2 polypeptide is lower than a dose or concentration used for the wild-type IL-2 polypeptide.

[0189] In some embodiments, activation of CD4⁺ T regulatory cell population via the IL-2R $\alpha\beta\gamma$ complex by the IL-2 conjugate retains significant potency of activation of said cell population by a wild-type IL-2 polypeptide. In some embodiments, the receptor signaling potency of the IL-2 conjugate the IL-2R $\beta\gamma$ complex is lower than a receptor signaling potency of the wild-type IL-2 polypeptide the IL-2R $\beta\gamma$ complex. In some embodiments, the receptor signaling potency of the IL-2 conjugate is about or at least 25%, 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 20-fold, or 50-fold lower than the respective potency of the wild-type IL-2 polypeptide.

[0190] In some embodiments, the IL-2 conjugate exhibits a first receptor signaling potency to IL-2R $\alpha\beta\gamma$ and a second receptor signaling potency to IL-2R $\beta\gamma$. In some embodiments, the first receptor signaling potency to IL-2R $\alpha\beta\gamma$ is an improved potency relative to a wild-type IL-2 polypeptide. In some embodiments, the second receptor signaling potency to IL-2R $\beta\gamma$ is an impaired potency relative to the wild-type IL-2 polypeptide. In some embodiments, the IL-2 conjugate exhibits a first receptor signaling potency to IL-2R $\alpha\beta\gamma$ and a second receptor signaling potency to IL-2R $\beta\gamma$, and wherein the first receptor signaling potency is at least 50%, 1-fold, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 20-fold, 30-fold, 50-fold, 100-

fold, 500-fold, 1000-fold, or higher than the second receptor signaling potency. In some embodiments, the first receptor signaling potency is at least 50% or higher than the second receptor signaling potency. In some embodiments, the first receptor signaling potency is at least 50% higher than the second receptor signaling potency. In some embodiments, the first receptor signaling potency is at least 2-fold or higher than the second receptor signaling potency. In some embodiments, the first receptor signaling potency is at least 5-fold or higher than the second receptor signaling potency. In some embodiments, the first receptor signaling potency is at least 10-fold or higher than the second receptor signaling potency. In some embodiments, the first receptor signaling potency is at least 20-fold or higher than the second receptor signaling potency. In some embodiments, the first receptor signaling potency is at least 50-fold or higher than the second receptor signaling potency. In some embodiments, the first receptor signaling potency is at least 100-fold or higher than the second receptor signaling potency. In some embodiments, the first receptor signaling potency is at least 500-fold or higher than the second receptor signaling potency. In some embodiments, the first receptor signaling potency is at least 1000-fold or higher than the second receptor signaling potency. In some embodiments, the first receptor signaling potency of the modified IL-2 polypeptide is higher than a receptor signaling potency of the wild-type IL-2 polypeptide to the IL-2R $\alpha\beta\gamma$, and the second receptor signaling potency of the modified IL-2 polypeptide is lower than a receptor signaling potency of the wild-type IL-2 polypeptide to the IL-2R $\beta\gamma$. In some embodiments, both receptor signaling potencies are lower than their respective potencies in a wild-type IL-2 polypeptide. In other cases, both receptor signaling potencies are higher than their respective potencies in a wild-type IL-2 polypeptide.

[0191] In some embodiments, the IL-2 conjugate decreases a toxic adverse event in a subject administered with the IL-2 conjugate. Exemplary toxic adverse events include eosinophilia, capillary leak, and vascular leak syndrome (VLS). In some embodiments, the IL-2 conjugate decreases the occurrence of a toxic adverse event in the subject by about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99%, or about 100%, relative to a second subject administered with a wild-type IL-2 or aldesleukin. In some embodiments, the IL-2 conjugate decreases the severity of a toxic adverse event in the subject by about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99%, or about 100%, relative to a second subject administered with a wild-type IL-2 or aldesleukin.

[0192] In some embodiments, the toxic adverse event is eosinophilia. In some embodiments, the IL-2 conjugate decreases the occurrence of eosinophilia in the subject by about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99%, or about 100%, relative to a second subject

administered with a wild-type IL-2 or aldesleukin. In some embodiments, the IL-2 conjugate decreases the severity of eosinophilia in the subject by about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99%, or about 100%, relative to a second subject administered with a wild-type IL-2 or aldesleukin.

[0193] In some embodiments, the toxic adverse event is capillary leak. In some embodiments, the IL-2 conjugate decreases the occurrence of capillary leak in the subject by about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99%, or about 100%, relative to a second subject administered with a wild-type IL-2 or aldesleukin. In some embodiments, the IL-2 conjugate decreases the severity of capillary leak in the subject by about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99%, or about 100%, relative to a second subject administered with a wild-type IL-2 or aldesleukin.

[0194] In some embodiments, the toxic adverse event is VLS. In some embodiments, the IL-2 conjugate decreases the occurrence of VLS in the subject by about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99%, or about 100%, relative to a second subject administered with a wild-type IL-2 or aldesleukin. In some embodiments, the IL-2 conjugate decreases the severity of VLS in the subject by about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99%, or about 100%, relative to a second subject administered with a wild-type IL-2 or aldesleukin.

[0195] In some embodiments, the IL-2 conjugate has a plasma half-life of at least 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 12 hours, 15 hours, 18 hours, 24 hours, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, or more. In some embodiments, the IL-2 conjugate has a plasma half-life of at least 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 12 hours, 15 hours, 18 hours, 24 hours, or more.

[0196] In some embodiments, the IL-2 conjugate has a plasma half-life of from about 1 hour to about 7 days, from about 12 hours to about 7 days, from about 18 hours to about 7 days, from about 24 hours to about 7 days, from about 1 hours to about 5 days, from about 12 hours to about 5 days, from about 24 hours to about 5 days, from about 2 days to about 5 days, or from about 2 days to about 3 days.

[0197] In some embodiments, the IL-2 conjugate has a plasma half-life of from about 1 hour to about 18 hours, from about 1 hour to about 12 hours, from about 2 hours to about 10 hours, from about 2 hours to about 8 hours, from about 4 hours to about 18 hours, from about 4 hours to about 12 hours, from about 4 hours to about 10 hours, from about 4 hours to about 8 hours, from about 6 hours to about 18 hours, from about 6 hours to about 12 hours, from about 6 hours to

about 10 hours, from about 6 hours to about 8 hours, from about 8 hours to about 18 hours, from about 8 hours to about 12 hours, or from about 8 hours to about 10 hours.

[0198] In some embodiments, the IL-2 conjugate has a plasma half-life that is capable of proliferating and/or expanding a CD4⁺ T regulatory cells but does not exert a deleterious effect such as apoptosis.

[0199] In some embodiments, the IL-2 conjugate has an extended plasma half-life, e.g., by at least 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 12 hours, 15 hours, 18 hours, 24 hours, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, or more relative to a wild-type IL-2. In some embodiments, the IL-2 conjugate has an extended plasma half-life, e.g., by at least 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 7 hours, 8 hours, 9 hours, 10 hours, 12 hours, 15 hours, 18 hours, 24 hours, or more relative to a wild-type IL-2 or aldesleukin.

[0200] In some embodiments, described herein is an IL-2 conjugate comprising an unnatural amino acid covalently attached to a conjugating moiety, wherein the unnatural amino acid is located in region 1-132, and wherein the region 1-132 is in reference to residues P1-T132 of the sequence of SEQ ID NO: 1 or the unnatural amino acid is located in region 8-108, and wherein the region 8-108 is in reference to residues K8-D108 of the sequence of SEQ ID NO: 1.

[0201] In some embodiments, the IL-2 conjugate comprises a mutation at L18 in reference to residue position 18 of the sequence of SEQ ID NO: 1, and comprises a conjugating moiety comprising a PEG having a molecular weight of about 2 kDa to about 60 kDa. In some embodiments, the molecular weight comprises about 30 kDa. In some embodiments, the molecular weight comprises about 35 kDa. In some embodiments, the molecular weight comprises about 40 kDa. In some embodiments, the molecular weight comprises about 45 kDa. In some embodiments, the molecular weight comprises about 50 kDa. In some embodiments, the molecular weight comprises about 55 kDa. In some embodiments, the molecular weight comprises about 60 kDa. In some embodiments, the molecular weight of the PEG determines, at least in part, the in vivo plasma half-life of the IL-2 conjugate. In some embodiments, the PEG corresponds with a longer in vivo plasma half-life of the IL-2 conjugate, as compared to the in vivo plasma half-life of a smaller PEG. In some embodiments, the PEG corresponds with a shorter in vivo plasma half-life of the IL-2 conjugate, as compared to the in vivo plasma half-life of a larger PEG. In some embodiments, the molecular weight of the PEG does not affect, nor has minimal effect, on the receptor signaling potency of the IL-2 conjugate to the IL-2 α or IL-2 $\alpha\beta\gamma$ signaling complexes. In some embodiments, the molecular weight of the PEG does not affect, or has minimal effect, on the desired reduced binding of the IL-2 conjugate to IL-2R $\alpha\beta\gamma$ or the

maintained binding with IL-2R $\alpha\beta\gamma$ signaling complex, wherein the reduced binding to IL-2R $\beta\gamma$ is compared to binding between a wild-type IL-2 polypeptide and IL-2R $\beta\gamma$. In some embodiments, the molecular weight of the PEG does not affect the formation of the modified IL-2 polypeptide/IL-2R $\alpha\beta\gamma$ complex, wherein the reduced binding to IL-2R $\beta\gamma$ is compared to binding between a wild-type IL-2 polypeptide and IL-2R $\beta\gamma$.

[0202] In some embodiments, the IL-2 conjugate comprises a mutation at H15 in reference to residue position 15 of the sequence of SEQ ID NO: 1, comprises a conjugating moiety comprising a PEG having a molecular weight of about 2 kDa to about 60 kDa. In some embodiments, the molecular weight comprises about 30 kDa. In some embodiments, the molecular weight comprises about 35 kDa. In some embodiments, the molecular weight comprises about 40 kDa. In some embodiments, the molecular weight comprises about 45 kDa. In some embodiments, the molecular weight comprises about 50 kDa. In some embodiments, the molecular weight comprises about 55 kDa. In some embodiments, the molecular weight comprises about 60 kDa.

[0203] In some embodiments, the conjugating moiety is bound to an amino acid residue selected from an amino acid position P1, T2, S3, S4, S5, T6, K7, K8, Q10, L11, E14, H15, L17, L18, D19, Q21, M22, N25, G26, N28, N29, Y30, K31, K34, T36, M45, P46, K47, A49, T50, E51, L52, K53, H54, Q56, E59, E66, N70, Q73, S74, K75, N76, F77, H78, R80, P81, R82, D83, S86, N87, I88, V90, I91, L93, E94, K96, G97, S98, E99, T100, T101, F102, M103, C104, E105, Y106, A107, D108, E109, T110, A111, T112, E115, N118, R119, T122, F123, S124, Q125, S126, S129, T130, L131, and T132, in which the numbering of the amino acid residues is in reference to the sequence of SEQ ID NO: 1. In some embodiments, the amino acid position is selected from K8, L11, E14, H15, L18, D19, M22, N87, E99, and D108. In some embodiments, the amino acid position is selected from L18 and H15. In some embodiments, the amino acid position is at K8. In some embodiments, the amino acid position is at L11. In some embodiments, the amino acid position is at E14. In some embodiments, the amino acid position is at H15. In some embodiments, the amino acid position is at L18. In some embodiments, the amino acid position is at D19. In some embodiments, the amino acid position is at M22. In some embodiments, the amino acid position is at N87. In some embodiments, the amino acid position is at E99. In some embodiments, the amino acid position is at D108.

[0204] In some embodiments, the IL-2 conjugate further comprises an additional mutation. In such cases, the amino acid is conjugated to an additional conjugating moiety for increase in serum half-life, stability, or a combination thereof. Alternatively, the amino acid is first mutated to an unnatural amino acid prior to binding to the additional conjugating moiety.

[0205] In some embodiments, the IL-2 conjugate has a decreased binding affinity to IL-2 receptor β (IL-2R β) subunit, IL-2 receptor γ (IL-2R γ) subunit, or a combination thereof, of the IL-2R $\alpha\beta\gamma$ complex, relative to a wild-type IL-2 polypeptide. In some embodiments, the decreased affinity of the IL-2 conjugate to IL-2 receptor β (IL-2R β) subunit, IL-2 receptor γ (IL-2R γ) subunit, or a combination thereof, relative to a wild-type IL-2 polypeptide, is about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99%, or greater than 99%.

[0206] In some embodiments, the decreased binding affinity of the IL-2 conjugate to IL-2 receptor β (IL-2R β) subunit, IL-2 receptor γ (IL-2R γ) subunit, or a combination thereof, relative to a wild-type IL-2 polypeptide, is about 25%, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 30-fold, 50-fold, 100-fold, 200-fold, 300-fold, 400-fold, 500-fold, 1,000-fold, or more.

[0207] In some embodiments, the IL-2 conjugate has a reduced IL-2R $\beta\gamma$ subunit recruitment to the IL-2/IL-2R $\beta\gamma$ complex. In some embodiments, the reduced recruitment is compared to an IL-2R $\beta\gamma$ subunit recruitment by an equivalent IL-2 polypeptide without the unnatural amino acid (e.g., a wild-type IL-2 polypeptide). In some embodiments, the decrease in IL-2R $\beta\gamma$ subunit recruitment is about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99%, or greater than 99% decrease relative to an equivalent IL-2 polypeptide without the unnatural amino acid modification (e.g., a wild-type IL-2 polypeptide).

[0208] In some embodiments, the decrease in IL-2R $\beta\gamma$ subunit recruitment is about 50%, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 30-fold, 50-fold, 100-fold, 200-fold, 300-fold, 400-fold, 500-fold, 1,000-fold, or more relative to an equivalent IL-2 polypeptide without the unnatural amino acid modification (e.g., a wild-type IL-2 polypeptide). In some embodiments, the IL-2 conjugate further has an increase in IL-2R $\alpha\beta\gamma$ recruitment.

[0209] In some embodiments, the IL-2 conjugate has an increase in IL-2R $\alpha\beta\gamma$ recruitment to the IL-2 polypeptide. In some embodiments, the reduced recruitment is compared to an IL-2R $\alpha\beta\gamma$ recruitment by an equivalent IL-2 polypeptide without the unnatural amino acid (e.g., a wild-type IL-2 polypeptide). In some embodiments, the increase in IL-2R $\alpha\beta\gamma$ recruitment is about 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95%, 99%, or greater than 99% increase relative to an equivalent IL-2 polypeptide without the unnatural amino acid modification. In some embodiments, the IL-2 conjugate further has a decrease in recruitment of an IL-2R $\beta\gamma$.

[0210] In some embodiments, the increase in IL-2R $\alpha\beta\gamma$ recruitment is about 50%, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 30-fold, 50-fold, 100-fold, 200-fold, 300-fold, 400-fold, 500-fold, 1,000-fold, or more relative to an equivalent IL-2 polypeptide without the unnatural amino acid modification (e.g., a wild-type IL-2 polypeptide). In some

embodiments, the IL-2 conjugate further has a decrease in recruitment of an IL-2R γ subunit to an IL-2/IL-2R β complex.

[0211] In some embodiments, an IL-2 conjugate described herein has a decrease in receptor signaling potency to IL-2R $\beta\gamma$. In some embodiments, the decrease in receptor signaling potency is about 50%, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 30-fold, 50-fold, 100-fold, 200-fold, 300-fold, 400-fold, 500-fold, 1000-fold, or more to IL-2R $\beta\gamma$ relative to a wild-type IL-2 polypeptide.

[0212] In some embodiments, the receptor signaling potency is measured by an EC50 value. In some embodiments, the decrease in receptor signaling potency is an increase in EC50. In some embodiments, the increase in EC50 is about 50%, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 30-fold, 50-fold, 100-fold, 200-fold, 300-fold, 400-fold, 500-fold, 1000-fold, or more relative to a wild-type IL-2 polypeptide.

[0213] In some embodiments, the receptor signaling potency is measured by an ED50 value. In some embodiments, the decrease in receptor signaling potency is an increase in ED50. In some embodiments, the increase in ED50 is about 50%, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 30-fold, 50-fold, 100-fold, 200-fold, 300-fold, 400-fold, 500-fold, 1000-fold, or more relative to a wild-type IL-2 polypeptide.

[0214] In some embodiments, an IL-2 conjugate described herein has an expanded therapeutic window compared to a therapeutic window of a wild-type IL-2 polypeptide. In some embodiments, the expanded therapeutic window is due to a decrease in binding between the IL-2 conjugate and interleukin 2 receptor $\beta\gamma$ (IL-2R $\beta\gamma$), a decrease in receptor signaling potency to IL-2R $\beta\gamma$, a decrease in recruitment of an IL-2R $\beta\gamma$ subunit to the IL-2/IL-2R $\alpha\beta\gamma$ complex, or an increase in recruitment of an IL-2R $\alpha\beta\gamma$ to the IL-2 polypeptide. In some embodiments, the IL-2 conjugate does not have an impaired activation of interleukin 2 $\alpha\beta\gamma$ receptor (IL-2R $\alpha\beta\gamma$).

[0215] In some embodiments, the IL-2 conjugate exhibits a first receptor signaling potency to an IL-2 $\beta\gamma$ signaling complex and a second receptor signaling potency to an IL-2 $\alpha\beta\gamma$ signaling complex, and wherein a difference between the first receptor signaling potency and the second receptor signaling potency is at least 50%. In some embodiments, the difference is at least 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 20-fold, 30-fold, 40-fold, 50-fold, 60-fold, 70-fold, 80-fold, 90-fold, 100-fold, 200-fold, 300-fold, 400-fold, 500-fold, 1000-fold, or more. In some embodiments, the first receptor signaling potency is less than the second receptor signaling potency. In some embodiments, the first receptor signaling potency is at least 25%, 2-fold, 3-fold, 4-fold, 5-fold, 6-fold, 7-fold, 8-fold, 9-fold, 10-fold, 20-fold, 30-fold, 50-fold, 100-fold, 500-fold, 1000-fold, or lower than the second receptor signaling potency. In some

embodiments, the IL-2 conjugate has a lower receptor signaling potency to an IL-2 $\beta\gamma$ signaling complex than a second receptor signaling potency to an IL-2 $\alpha\beta\gamma$ signaling complex. In some embodiments, the first receptor signaling potency of the IL-2 conjugate is at least 25% lower than a receptor signaling potency of the wild-type IL-2 polypeptide. In some embodiments, the first receptor signaling potency of the IL-2 conjugate is at least 25%, 2-fold, 3-fold, 4-fold, 5-fold, 10-fold, 20-fold, 50-fold, 100-fold, 200-fold, or 500-fold lower than a receptor signaling potency of the wild-type IL-2 polypeptide. In some embodiments, the first receptor signaling potency and the second receptor signaling potency are both lower than the respective potencies of the wild-type IL-2 polypeptide, but the first receptor signaling potency is lower than the second receptor signaling potency. In some embodiments, the difference between the first receptor signaling potency and the second receptor signaling potency increases the therapeutic window for the IL-2 conjugate.

[0216] In some embodiments, the conjugating moiety impairs or blocks the receptor signaling potency of IL-2 with IL-2R $\beta\gamma$, or reduces recruitment of the IL-2R β subunit and/or the IL-2R γ subunit to the IL-2/IL-2R $\beta\gamma$ complex. In some embodiments, the IL-2 conjugate further has a decrease in recruitment of an IL-2R γ subunit to an IL-2/IL-2R β complex.

[0217] In some embodiments, the IL-2 conjugate with the decrease in receptor signaling potency to IL-2R $\beta\gamma$ is capable of expanding CD4⁺ T regulatory (Treg) cells.

[0218] In some embodiments, CD4⁺ Treg cell proliferation by the modified IL-2/IL-2R $\alpha\beta\gamma$ complex is equivalent or greater to that of a wild-type IL-2 polypeptide.

[0219] In some embodiments, the IL-2/IL-2R $\alpha\beta\gamma$ complex induces proliferation of the CD4⁺ Treg cells to a population that is sufficient to modulate a disease course in an animal model.

1. Natural and Unnatural Amino acids

[0220] In some embodiments, an unnatural amino acid is not conjugated with a conjugating moiety. In some embodiments, a cytokine described herein comprises an unnatural amino acid, wherein the cytokine is conjugated to the protein, wherein the point of attachment is not the unnatural amino acid.

[0221] In some embodiments, an amino acid residue described herein (e.g., within a cytokine such as IL-2) is mutated to an unnatural amino acid prior to binding to a conjugating moiety. In some embodiments, the mutation to an unnatural amino acid prevents or minimizes a self-antigen response of the immune system. As used herein, the term "unnatural amino acid" or "non-canonical amino acid" refers to an amino acid other than the 20 amino acids that occur naturally in protein. Non-limiting examples of unnatural amino acids include: p-acetyl-L-phenylalanine, p-iodo-L-phenylalanine, p-methoxyphenylalanine, O-methyl-L-tyrosine, p-

propargyloxyphenylalanine, p-propargyl-phenylalanine, L-3-(2-naphthyl)alanine, 3-methyl-phenylalanine, O- 4-allyl-L-tyrosine, 4-propyl-L-tyrosine, tri-O-acetyl-GlcNAc-serine, L-Dopa, fluorinated phenylalanine, isopropyl-L-phenylalanine, o-azido-L-phenylalanine, m-azido-L-phenylalanine, p-azido-L-phenylalanine, p-acyl-L-phenylalanine, p-benzoyl-L-phenylalanine, p-Boronophenylalanine, O-propargyltyrosine, L-phosphoserine, phosphoserine, phosphonotyrosine, p-bromophenylalanine, selenocysteine, p-amino-L-phenylalanine, isopropyl-L-phenylalanine, N6-(2-azidoethoxy)-carbonyl-L-lysine (AzK), an unnatural analogue of a tyrosine amino acid; an unnatural analogue of a glutamine amino acid; an unnatural analogue of a phenylalanine amino acid; an unnatural analogue of a serine amino acid; an unnatural analogue of a threonine amino acid; an unnatural analogue of a lysine amino acid; an alkyl, aryl, acyl, azido, cyano, halo, hydrazine, hydrazide, hydroxyl, alkenyl, alkynyl, ether, thiol, sulfonyl, seleno, ester, thioacid, borate, boronate, phospho, phosphono, phosphine, heterocyclic, enone, imine, aldehyde, hydroxylamine, keto, or amino substituted amino acid, or a combination thereof; an amino acid with a photoactivatable cross-linker; a spin-labeled amino acid; a fluorescent amino acid; a metal binding amino acid; a metal-containing amino acid; a radioactive amino acid; a photocaged and/or photoisomerizable amino acid; a biotin or biotin-analogue containing amino acid; a keto containing amino acid; an amino acid comprising polyethylene glycol or polyether; a heavy atom substituted amino acid; a chemically cleavable or photocleavable amino acid; an amino acid with an elongated side chain; an amino acid containing a toxic group; a sugar substituted amino acid; a carbon-linked sugar-containing amino acid; a redox-active amino acid; an α -hydroxy containing acid; an amino thio acid; an α , α disubstituted amino acid; a β -amino acid; a cyclic amino acid other than proline or histidine, and an aromatic amino acid other than phenylalanine, tyrosine or tryptophan. In some embodiments, the unnatural amino acid is an azido-substituted lysine.

[0222] In some embodiments, the unnatural amino acid comprises a selective reactive group, or a reactive group for site-selective labeling of a target polypeptide. In some embodiments, the chemistry is a biorthogonal reaction (e.g., biocompatible and selective reactions). In some embodiments, the chemistry is a Cu(I)-catalyzed or “copper-free” alkyne-azide triazole-forming reaction, the Staudinger ligation, inverse-electron-demand Diels-Alder (IEDDA) reaction, “photo-click” chemistry, or a metal-mediated process such as olefin metathesis and Suzuki-Miyaura or Sonogashira cross-coupling.

[0223] In some embodiments, the unnatural amino acid comprises a photoreactive group, which crosslinks, upon irradiation with, e.g., UV.

[0224] In some embodiments, the unnatural amino acid comprises a photo-caged amino acid.

[0225] In some embodiments, the unnatural amino acid is a para-substituted, meta-substituted, or an ortho-substituted amino acid derivative.

[0226] In some embodiments, the unnatural amino acid comprises p-acetyl-L-phenylalanine, o-azidomethyl-L-phenylalanine, m-azidomethyl-L-phenylalanine, p-azidomethyl-L-phenylalanine (pAMF), p-iodo-L-phenylalanine, O-methyl-L-tyrosine, p-methoxyphenylalanine, p-propargyloxyphenylalanine, o-propargyl-phenylalanine, m-propargyl-phenylalanine, p-propargyl-phenylalanine, L-3-(2-naphthyl)alanine, 3-methyl-phenylalanine, O- 4-allyl-L-tyrosine, 4-propyl-L-tyrosine, tri-O-acetyl-GlcNAc-p-serine, L-Dopa, fluorinated phenylalanine, isopropyl-L-phenylalanine, p-azido-L-phenylalanine, p-acyl-L-phenylalanine, p-benzoyl-L-phenylalanine, L-phosphoserine, phosphoserine, phosphotyrosine, p-bromophenylalanine, p-amino-L-phenylalanine, or isopropyl-L-phenylalanine.

[0227] In some embodiments, the unnatural amino acid is 3-aminotyrosine, 3-nitrotyrosine, 3,4-dihydroxy-phenylalanine, or 3-iodotyrosine.

[0228] In some embodiments, the unnatural amino acid is phenylselenocysteine.

[0229] In some embodiments, the unnatural amino acid is a benzophenone, ketone, iodide, methoxy, acetyl, benzoyl, or azide containing phenylalanine derivative.

[0230] In some embodiments, the unnatural amino acid is a benzophenone, ketone, iodide, methoxy, acetyl, benzoyl, or azide containing lysine derivative.

[0231] In some embodiments, the unnatural amino acid comprises an aromatic side chain.

[0232] In some embodiments, the unnatural amino acid does not comprise an aromatic side chain.

[0233] In some embodiments, the unnatural amino acid comprises an azido group.

[0234] In some embodiments, the unnatural amino acid comprises a Michael-acceptor group. In some embodiments, Michael-acceptor groups comprise an unsaturated moiety capable of forming a covalent bond through a 1,2-addition reaction. In some embodiments, Michael-acceptor groups comprise electron-deficient alkenes or alkynes. In some embodiments, Michael-acceptor groups include but are not limited to alpha,beta unsaturated: ketones, aldehydes, sulfoxides, sulfones, nitriles, imines, or aromatics.

[0235] In some embodiments, the unnatural amino acid is dehydroalanine.

[0236] In some embodiments, the unnatural amino acid comprises an aldehyde or ketone group.

[0237] In some embodiments, the unnatural amino acid is a lysine derivative comprising an aldehyde or ketone group.

[0238] In some embodiments, the unnatural amino acid is a lysine derivative comprising one or more O, N, Se, or S atoms at the beta, gamma, or delta position. In some embodiments, the

unnatural amino acid is a lysine derivative comprising O, N, Se, or S atoms at the gamma position.

[0239] In some embodiments, the unnatural amino acid is a lysine derivative wherein the epsilon N atom is replaced with an oxygen atom.

[0240] In some embodiments, the unnatural amino acid is a lysine derivative that is not naturally-occurring post-translationally modified lysine.

[0241] In some embodiments, the unnatural amino acid is an amino acid comprising a side chain, wherein the sixth atom from the alpha position comprises a carbonyl group. In some embodiments, the unnatural amino acid is an amino acid comprising a side chain, wherein the sixth atom from the alpha position comprises a carbonyl group, and the fifth atom from the alpha position is a nitrogen. In some embodiments, the unnatural amino acid is an amino acid comprising a side chain, wherein the seventh atom from the alpha position is an oxygen atom.

[0242] In some embodiments, the unnatural amino acid is a serine derivative comprising selenium. In some embodiments, the unnatural amino acid is selenoserine (2-amino-3-hydroselenopropanoic acid). In some embodiments, the unnatural amino acid is 2-amino-3-((2-((3-(benzyloxy)-3-oxopropyl)amino)ethyl)selanyl)propanoic acid. In some embodiments, the unnatural amino acid is 2-amino-3-(phenylselanyl)propanoic acid. In some embodiments, the unnatural amino acid comprises selenium, wherein oxidation of the selenium results in the formation of an unnatural amino acid comprising an alkene.

[0243] In some embodiments, the unnatural amino acid comprises a cyclooctynyl group.

[0244] In some embodiments, the unnatural amino acid comprises a transcycloctenyl group.

[0245] In some embodiments, the unnatural amino acid comprises a norbornenyl group.

[0246] In some embodiments, the unnatural amino acid comprises a cyclopropenyl group.

[0247] In some embodiments, the unnatural amino acid comprises a diazirine group.

[0248] In some embodiments, the unnatural amino acid comprises a tetrazine group.

[0249] In some embodiments, the unnatural amino acid is a lysine derivative, wherein the side-chain nitrogen is carbamylated. In some embodiments, the unnatural amino acid is a lysine derivative, wherein the side-chain nitrogen is acylated. In some embodiments, the unnatural amino acid is 2-amino-6-[[tert-butoxy)carbonyl]amino]hexanoic acid. In some embodiments, the unnatural amino acid is 2-amino-6-[[tert-butoxy)carbonyl]amino]hexanoic acid. In some embodiments, the unnatural amino acid is N6-Boc-N6-methyllysine. In some embodiments, the unnatural amino acid is N6-acetyllysine. In some embodiments, the unnatural amino acid is pyrrolysine. In some embodiments, the unnatural amino acid is N6-trifluoroacetyllysine. In some embodiments, the unnatural amino acid is 2-amino-6-[[benzyloxy)carbonyl]amino]hexanoic

acid. In some embodiments, the unnatural amino acid is 2-amino-6-[(p-iodobenzoyloxy)carbonyl]amino}hexanoic acid. In some embodiments, the unnatural amino acid is 2-amino-6-[(p-nitrobenzoyloxy)carbonyl]amino}hexanoic acid. In some embodiments, the unnatural amino acid is N6-prolyllysine. In some embodiments, the unnatural amino acid is 2-amino-6-[(cyclopentyloxy)carbonyl]amino}hexanoic acid. In some embodiments, the unnatural amino acid is N6-(cyclopentanecarbonyl)lysine. In some embodiments, the unnatural amino acid is N6-(tetrahydrofuran-2-carbonyl)lysine. In some embodiments, the unnatural amino acid is N6-(3-ethynyltetrahydrofuran-2-carbonyl)lysine. In some embodiments, the unnatural amino acid is N6-((prop-2-yn-1-yloxy)carbonyl)lysine. In some embodiments, the unnatural amino acid is 2-amino-6-[(2-azidocyclopentyloxy)carbonyl]amino}hexanoic acid. In some embodiments, the unnatural amino acid is N6-(2-azidoethoxy)-carbonyl-lysine. In some embodiments, the unnatural amino acid is 2-amino-6-[(2-nitrobenzoyloxy)carbonyl]amino}hexanoic acid. In some embodiments, the unnatural amino acid is 2-amino-6-[(2-cyclooctynyloxy)carbonyl]amino}hexanoic acid. In some embodiments, the unnatural amino acid is N6-(2-aminobut-3-ynoyl)lysine. In some embodiments, the unnatural amino acid is 2-amino-6-((2-aminobut-3-ynoyl)oxy)hexanoic acid. In some embodiments, the unnatural amino acid is N6-(allyloxy)carbonyl)lysine. In some embodiments, the unnatural amino acid is N6-(butenyl-4-oxycarbonyl)lysine. In some embodiments, the unnatural amino acid is N6-(pentenyl-5-oxycarbonyl)lysine. In some embodiments, the unnatural amino acid is N6-((but-3-yn-1-yloxy)carbonyl)-lysine. In some embodiments, the unnatural amino acid is N6-((pent-4-yn-1-yloxy)carbonyl)-lysine. In some embodiments, the unnatural amino acid is N6-(thiazolidine-4-carbonyl)lysine. In some embodiments, the unnatural amino acid is 2-amino-8-oxononanoic acid. In some embodiments, the unnatural amino acid is 2-amino-8-oxooctanoic acid. In some embodiments, the unnatural amino acid is N6-(2-oxoacetyl)lysine.

[0250] In some embodiments, the unnatural amino acid is N6-propionyllysine. In some embodiments, the unnatural amino acid is N6-butyryllysine. In some embodiments, the unnatural amino acid is N6-(but-2-enoyl)lysine. In some embodiments, the unnatural amino acid is N6-((bicyclo[2.2.1]hept-5-en-2-yloxy)carbonyl)lysine. In some embodiments, the unnatural amino acid is N6-((spiro[2.3]hex-1-en-5-ylmethoxy)carbonyl)lysine. In some embodiments, the unnatural amino acid is N6-(((4-(1-(trifluoromethyl)cycloprop-2-en-1-yl)benzyl)oxy)carbonyl)lysine. In some embodiments, the unnatural amino acid is N6-((bicyclo[2.2.1]hept-5-en-2-ylmethoxy)carbonyl)lysine. In some embodiments, the unnatural amino acid is cysteinyllysine. In some embodiments, the unnatural amino acid is N6-((1-(6-nitrobenzo[d][1,3]dioxol-5-yl)ethoxy)carbonyl)lysine. In some embodiments, the unnatural

amino acid is N6-((2-(3-methyl-3H-diazirin-3-yl)ethoxy)carbonyl)lysine. In some embodiments, the unnatural amino acid is N6-((3-(3-methyl-3H-diazirin-3-yl)propoxy)carbonyl)lysine. In some embodiments, the unnatural amino acid is N6-((meta nitrobenzyloxy)N6-methylcarbonyl)lysine. In some embodiments, the unnatural amino acid is N6-((bicyclo[6.1.0]non-4-yn-9-ylmethoxy)carbonyl)-lysine. In some embodiments, the unnatural amino acid is N6-((cyclohept-3-en-1-yl)oxy)carbonyl)-L-lysine.

[0251] In some embodiments, the unnatural amino acid is 2-amino-3-((((benzyloxy)carbonyl)amino)methyl)selanylpropanoic acid.

[0252] In some embodiments, the unnatural amino acid is incorporated into the cytokine (e.g., the IL polypeptide) by a repurposed amber, opal, or ochre stop codon.

[0253] In some embodiments, the unnatural amino acid is incorporated into the cytokine (e.g., the IL polypeptide) by a 4-base codon.

[0254] In some embodiments, the unnatural amino acid is incorporated into the cytokine (e.g., the IL polypeptide) by a repurposed rare sense codon.

[0255] In some embodiments, the unnatural amino acid is incorporated into the cytokine (e.g., the IL polypeptide) by a synthetic codon comprising an unnatural nucleic acid.

[0256] In some embodiments, the unnatural amino acid is incorporated into the cytokine by an orthogonal, modified synthetase/tRNA pair. Such orthogonal pairs comprise an unnatural synthetase that is capable of charging the unnatural tRNA with the unnatural amino acid, while minimizing charging of a) other endogenous amino acids onto the unnatural tRNA and b) unnatural amino acids onto other endogenous tRNAs. Such orthogonal pairs comprise tRNAs that are capable of being charged by the unnatural synthetase, while avoiding being charged with a) other endogenous amino acids by endogenous synthetases. In some embodiments, such pairs are identified from various organisms, such as bacteria, yeast, Archaea, or human sources. In some embodiments, an orthogonal synthetase/tRNA pair comprises components from a single organism. In some embodiments, an orthogonal synthetase/tRNA pair comprises components from two different organisms. In some embodiments, an orthogonal synthetase/tRNA pair comprising components that prior to modification, promote translation of two different amino acids. In some embodiments, an orthogonal synthetase is a modified alanine synthetase. In some embodiments, an orthogonal synthetase is a modified arginine synthetase. In some embodiments, an orthogonal synthetase is a modified asparagine synthetase. In some embodiments, an orthogonal synthetase is a modified aspartic acid synthetase. In some embodiments, an orthogonal synthetase is a modified cysteine synthetase. In some embodiments, an orthogonal synthetase is a modified glutamine synthetase. In some embodiments, an orthogonal synthetase

is a modified glutamic acid synthetase. In some embodiments, an orthogonal synthetase is a modified alanine synthetase. In some embodiments, an orthogonal synthetase is a modified histidine synthetase. In some embodiments, an orthogonal synthetase is a modified leucine synthetase. In some embodiments, an orthogonal synthetase is a modified isoleucine synthetase. In some embodiments, an orthogonal synthetase is a modified lysine synthetase. In some embodiments, an orthogonal synthetase is a modified methionine synthetase. In some embodiments, an orthogonal synthetase is a modified phenylalanine synthetase. In some embodiments, an orthogonal synthetase is a modified proline synthetase. In some embodiments, an orthogonal synthetase is a modified serine synthetase. In some embodiments, an orthogonal synthetase is a modified threonine synthetase. In some embodiments, an orthogonal synthetase is a modified tryptophan synthetase. In some embodiments, an orthogonal synthetase is a modified tyrosine synthetase. In some embodiments, an orthogonal synthetase is a modified valine synthetase. In some embodiments, an orthogonal synthetase is a modified phosphoserine synthetase. In some embodiments, an orthogonal tRNA is a modified alanine tRNA. In some embodiments, an orthogonal tRNA is a modified arginine tRNA. In some embodiments, an orthogonal tRNA is a modified asparagine tRNA. In some embodiments, an orthogonal tRNA is a modified aspartic acid tRNA. In some embodiments, an orthogonal tRNA is a modified cysteine tRNA. In some embodiments, an orthogonal tRNA is a modified glutamine tRNA. In some embodiments, an orthogonal tRNA is a modified glutamic acid tRNA. In some embodiments, an orthogonal tRNA is a modified alanine glycine. In some embodiments, an orthogonal tRNA is a modified histidine tRNA. In some embodiments, an orthogonal tRNA is a modified leucine tRNA. In some embodiments, an orthogonal tRNA is a modified isoleucine tRNA. In some embodiments, an orthogonal tRNA is a modified lysine tRNA. In some embodiments, an orthogonal tRNA is a modified methionine tRNA. In some embodiments, an orthogonal tRNA is a modified phenylalanine tRNA. In some embodiments, an orthogonal tRNA is a modified proline tRNA. In some embodiments, an orthogonal tRNA is a modified serine tRNA. In some embodiments, an orthogonal tRNA is a modified threonine tRNA. In some embodiments, an orthogonal tRNA is a modified tryptophan tRNA. In some embodiments, an orthogonal tRNA is a modified tyrosine tRNA. In some embodiments, an orthogonal tRNA is a modified valine tRNA. In some embodiments, an orthogonal tRNA is a modified phosphoserine tRNA.

[0257] In some embodiments, the unnatural amino acid is incorporated into the cytokine (e.g., the IL polypeptide) by an aminoacyl (aaRS or RS)-tRNA synthetase-tRNA pair. Exemplary aaRS-tRNA pairs include, but are not limited to, *Methanococcus jannaschii* (Mj-Tyr) aaRS/tRNA pairs, *E. coli* TyrRS (Ec-Tyr)/B. stearothermophilus tRNA^{CUA} pairs, *E. coli* LeuRS

(Ec-Leu)/*B. stearothermophilus* tRNACUA pairs, and pyrrolysyl-tRNA pairs. In some embodiments, the unnatural amino acid is incorporated into the cytokine (e.g., the IL polypeptide) by a Mj-TyrRS/tRNA pair. Exemplary UAAs that can be incorporated by a Mj-TyrRS/tRNA pair include, but are not limited to, para-substituted phenylalanine derivatives such as p-aminophenylalanine and p-methoxyphenylalanine; meta-substituted tyrosine derivatives such as 3-aminotyrosine, 3-nitrotyrosine, 3,4-dihydroxyphenylalanine, and 3-iodotyrosine; phenylselenocysteine; p-boronophenylalanine; and o-nitrobenzyltyrosine.

[0258] In some embodiments, the unnatural amino acid is incorporated into the cytokine (e.g., the IL polypeptide) by a Ec-Tyr/tRNACUA or a Ec-Leu/tRNACUA pair. Exemplary UAAs that can be incorporated by a Ec-Tyr/tRNACUA or a Ec-Leu/tRNACUA pair include, but are not limited to, phenylalanine derivatives containing benzophenone, ketone, iodide, or azide substituents; O-propargyltyrosine; α -aminocaprylic acid, O-methyl tyrosine, O-nitrobenzyl cysteine; and 3-(naphthalene-2-ylamino)-2-amino-propanoic acid.

[0259] In some embodiments, the unnatural amino acid is incorporated into the cytokine (e.g., the IL polypeptide) by a pyrrolysyl-tRNA pair. In some embodiments, the PylRS is obtained from an archaeobacterial, e.g., from a methanogenic archaeobacterial. In some embodiments, the PylRS is obtained from *Methanosarcina barkeri*, *Methanosarcina mazei*, or *Methanosarcina acetivorans*. Exemplary UAAs that can be incorporated by a pyrrolysyl-tRNA pair include, but are not limited to, amide and carbamate substituted lysines such as 2-amino-6-((R)-tetrahydrofuran-2-carboxamido)hexanoic acid, N- ϵ -D-prolyl-L-lysine, and N- ϵ -cyclopentylloxycarbonyl-L-lysine; N- ϵ -Acryloyl-L-lysine; N- ϵ -[(1-(6-nitrobenzo[d][1,3]dioxol-5-yl)ethoxy)carbonyl]-L-lysine; and N- ϵ -(1-methylcyclopro-2-enecarboxamido)lysine. In some embodiments, the IL-2 conjugates disclosed herein may be prepared by use of *M. mazei* Pyl tRNA which is selectively charged with a non-natural amino acid such as N6-(2-azidoethoxy)-carbonyl-L-lysine (AzK) by the *M. barkeri* pyrrolysyl-tRNA synthetase (Mb PylRS). Other methods are known to those of ordinary skill in the art, such as those disclosed in Zhang et al., Nature 2017, 551(7682): 644-647.

[0260] In some embodiments, an unnatural amino acid is incorporated into a cytokine described herein (e.g., the IL polypeptide) by a synthetase disclosed in US 9,988,619 and US 9,938,516. Exemplary UAAs that can be incorporated by such synthetases include para-methylazido-L-phenylalanine, aralkyl, heterocyclyl, heteroaralkyl unnatural amino acids, and others. In some embodiments, such UAAs comprise pyridyl, pyrazinyl, pyrazolyl, triazolyl, oxazolyl, thiazolyl, thiophenyl, or other heterocycle. Such amino acids in some embodiments comprise azides, tetrazines, or other chemical group capable of conjugation to a coupling partner, such as a water-

soluble moiety. In some embodiments, such synthetases are expressed and used to incorporate UAAs into cytokines in-vivo. In some embodiments, such synthetases are used to incorporate UAAs into cytokines using a cell-free translation system.

[0261] In some embodiments, an unnatural amino acid is incorporated into a cytokine described herein (e.g., the IL polypeptide) by a naturally occurring synthetase. In some embodiments, an unnatural amino acid is incorporated into a cytokine by an organism that is auxotrophic for one or more amino acids. In some embodiments, synthetases corresponding to the auxotrophic amino acid are capable of charging the corresponding tRNA with an unnatural amino acid. In some embodiments, the unnatural amino acid is selenocysteine, or a derivative thereof. In some embodiments, the unnatural amino acid is selenomethionine, or a derivative thereof. In some embodiments, the unnatural amino acid is an aromatic amino acid, wherein the aromatic amino acid comprises an aryl halide, such as an iodide. In embodiments, the unnatural amino acid is structurally similar to the auxotrophic amino acid.

[0262] In some embodiments, the unnatural amino acid comprises an unnatural amino acid described and illustrated in, for example, in International Publication Number WO 2021/050554 A1, which is hereby incorporated by reference in its entirety.

[0263] In some embodiments, the unnatural amino acid comprises a lysine or phenylalanine derivative or analogue. In some embodiments, the unnatural amino acid comprises a lysine derivative or a lysine analogue. In some embodiments, the unnatural amino acid comprises a pyrrolysine (Pyl). In some embodiments, the unnatural amino acid comprises a phenylalanine derivative or a phenylalanine analogue. In some embodiments, the unnatural amino acid is an unnatural amino acid described in Wan, et al., "Pyrrolysyl-tRNA synthetase: an ordinary enzyme but an outstanding genetic code expansion tool," *Biochim Biophys Acta* 1844(6): 1059-4070 (2014).

[0264] In some embodiments, an unnatural amino acid incorporated into a cytokine described herein (e.g., the IL polypeptide) is disclosed in US 9,840,493; US 9,682,934; US 2017/0260137; US 9,938,516; or US 2018/0086734. Exemplary UAAs that can be incorporated by such synthetases include para-methylazido-L-phenylalanine, aralkyl, heterocyclyl, and heteroaralkyl, and lysine derivative unnatural amino acids. In some embodiments, such UAAs comprise pyridyl, pyrazinyl, pyrazolyl, triazolyl, oxazolyl, thiazolyl, thiophenyl, or other heterocycle. Such amino acids in some embodiments comprise azides, tetrazines, or other chemical group capable of conjugation to a coupling partner, such as a water soluble moiety. In some embodiments, a UAA comprises an azide attached to an aromatic moiety via an alkyl linker. In some embodiments, an alkyl linker is a C1-C10 linker. In some embodiments, a UAA comprises

a tetrazine attached to an aromatic moiety via an alkyl linker. In some embodiments, a UAA comprises a tetrazine attached to an aromatic moiety via an amino group. In some embodiments, a UAA comprises a tetrazine attached to an aromatic moiety via an alkylamino group. In some embodiments, a UAA comprises an azide attached to the terminal nitrogen (e.g., N6 of a lysine derivative, or N5, N4, or N3 of a derivative comprising a shorter alkyl side chain) of an amino acid side chain via an alkyl chain. In some embodiments, a UAA comprises a tetrazine attached to the terminal nitrogen of an amino acid side chain via an alkyl chain. In some embodiments, a UAA comprises an azide or tetrazine attached to an amide via an alkyl linker. In some embodiments, the UAA is an azide or tetrazine-containing carbamate or amide of 3-aminoalanine, serine, lysine, or derivative thereof. In some embodiments, such UAAs are incorporated into cytokines in-vivo. In some embodiments, such UAAs are incorporated into cytokines in a cell-free system.

2. Conjugating Moieties

[0265] In some embodiments, disclosed herein are conjugating moieties that are bound to one or more cytokines (e.g., interleukins, IFNs, or TNFs) described supra. In some embodiments, the conjugating moiety is a molecule that perturbs the interaction of a cytokine with its receptor. In some embodiments, the conjugating moiety is any molecule that when bound to the cytokine, enables the cytokine conjugate to modulate an immune response. In some embodiments, the conjugating moiety is bound to the cytokine through a covalent bond. In some embodiments, a cytokine described herein is attached to a conjugating moiety with a triazole group. In some embodiments, a cytokine described herein is attached to a conjugating moiety with a dihydropyridazine or pyridazine group. In some embodiments, the conjugating moiety comprises a water-soluble polymer. In other instances, the conjugating moiety comprises a protein or a binding fragment thereof. In additional instances, the conjugating moiety comprises a peptide. In additional instances, the conjugating moiety comprises a nucleic acid. In additional instances, the conjugating moiety comprises a small molecule. In additional instances, the conjugating moiety comprises a bioconjugate (e.g., a TLR agonist such as a TLR1, TLR2, TLR3, TLR4, TLR5, TLR6, TLR7, TLR8, or TLR9 agonist; or a synthetic ligand such as Pam3Cys, CFA, MALP2, Pam2Cys, FSL-1, Hib-OMPC, Poly I:C, poly A:U, AGP, MPL A, RC-529, MDF2 β , CFA, or Flagellin). In some embodiments, the conjugating moiety increases serum half-life, and/or improves stability. In some embodiments, the conjugating moiety reduces cytokine interaction with one or more cytokine receptor domains or subunits. In additional cases, the conjugating moiety blocks cytokine interaction with one or more cytokine domains or subunits with its cognate receptor(s). In some embodiments, cytokine conjugates described herein comprise

multiple conjugating moieties. In some embodiments, a conjugating moiety is attached to an unnatural amino acid in the cytokine peptide. In some embodiments, a cytokine conjugate is attached to an unnatural amino acid in the cytokine peptide. In some embodiments, a conjugating moiety is attached to the N or C terminal amino acid of the cytokine peptide. Various combinations sites are disclosed herein, for example, a first conjugating moiety is attached to an unnatural amino acid in the cytokine peptide, and a second conjugating moiety is attached to the N or C terminal amino acid of the cytokine peptide. In some embodiments, a single conjugating moiety is attached to multiple residues of the cytokine peptide (e.g. a staple). In some embodiments, a conjugating moiety is attached to both the N and C terminal amino acids of the cytokine peptide.

[0266] In some embodiments, a conjugating moiety described herein is a water-soluble polymer. In some embodiments, the water-soluble polymer is a nonpeptidic, nontoxic, and biocompatible. As used herein, a substance is considered biocompatible if the beneficial effects associated with use of the substance alone or with another substance (e.g., an active agent such as a cytokine moiety) in connection with living tissues (e.g., administration to a patient) outweighs any deleterious effects as evaluated by a clinician, e.g., a physician, a toxicologist, or a clinical development specialist. In some embodiments, a water-soluble polymer is further non-immunogenic. In some embodiments, a substance is considered non-immunogenic if the intended use of the substance in vivo does not produce an undesired immune response (e.g., the formation of antibodies) or, if an immune response is produced, that such a response is not deemed clinically significant or important as evaluated by a clinician, e.g., a physician, a toxicologist, or a clinical development specialist.

[0267] In some embodiments, the water-soluble polymer is characterized as having from about 2 to about 300 termini. Exemplary water soluble polymers include, but are not limited to, poly(alkylene glycols) such as polyethylene glycol ("PEG"), poly(propylene glycol) ("PPG"), copolymers of ethylene glycol and propylene glycol and the like, poly(oxyethylated polyol), poly(olefinic alcohol), poly(vinylpyrrolidone), poly(hydroxyalkylmethacrylamide), poly(hydroxyalkylmethacrylate), poly(saccharides), poly(α -hydroxy acid), poly(vinyl alcohol) (PVA), polyacrylamide (PAAm), poly(N-(2-hydroxypropyl) methacrylamide) (PHPMA), polydimethylacrylamide (PDAAm), polyphosphazene, polyoxazolines ("POZ") (which are described in WO 2008/106186), poly(N-acryloylmorpholine), and combinations of any of the foregoing.

[0268] In some embodiments, the water-soluble polymer is not limited to a particular structure. In some embodiments, the water-soluble polymer is linear (e.g., an end capped, e.g., alkoxy PEG

or a bifunctional PEG), branched or multi-armed (e.g., forked PEG or PEG attached to a polyol core), a dendritic (or star) architecture, each with or without one or more degradable linkages. Moreover, the internal structure of the water-soluble polymer can be organized in any number of different repeat patterns and can be selected from the group consisting of homopolymer, alternating copolymer, random copolymer, block copolymer, alternating tripolymer, random tripolymer, and block tripolymer.

[0269] In some embodiments, the water-soluble polymer is represented by a length of repeating polymeric units, for example, a number n of polyethylene glycol units. In some embodiments, the water-soluble polymer has the structure:

[0270] , wherein the wavy line indicates attachment to a linker, reactive group, or unnatural amino acid, and n is 1-5000. In some embodiments, the water-soluble polymer has the structure:

[0271] , wherein the wavy line indicates attachment to a linker, reactive group, or unnatural amino acid, “Cap” indicates a capping group (for example, such as $-\text{OCH}_3$, $-\text{O}(\text{C1-C6 alkyl})$, $-\text{SMe}$, $-\text{S}(\text{C1-C6 alkyl})$, $-\text{CO}_2\text{H}$, $-\text{CO}_2(\text{C1-C6 alkyl})$, $-\text{CONH}_2$, $-\text{CONH}(\text{C1-C6 alkyl})$, $-\text{CON}(\text{C1-C6 alkyl})_2$, $-\text{NH}_2$, $-\text{SH}$, or OH) and n is 1-5000. In some embodiments, n is 100-2000, 200-1000, 300-750, 400-600, 450-550, 400-2000, 750-3000, or 100-750. In some embodiments, n is about 100, 200, 300, 400, 500, 600, 700, 800, 900, or about 1000. In some embodiments, n is at least 100, 200, 300, 400, 500, 600, 700, 800, 900, or at least 1000. In some embodiments, n is no more than 100, 200, 300, 400, 500, 600, 700, 800, 900, or no more than 1000. In some embodiments, the n is represented as an average length of the water-soluble polymer.

[0272] In some embodiments, the weight-average molecular weight of the water-soluble polymer in the IL-2 conjugate is from about 100 Daltons to about 150 kDa. Exemplary ranges include, for example, weight-average molecular weights in the range of greater than 5 kDa to about 100 kDa, in the range of from about 6 kDa to about 90 kDa, in the range of from about 10 kDa to about 85 kDa, in the range of greater than 10 kDa to about 85 kDa, in the range of from about 20 kDa to about 85 kDa, in the range of from about 53 kDa to about 85 kDa, in the range of from about 25 kDa to about 120 kDa, in the range of from about 29 kDa to about 120 kDa, in the range of from about 35 kDa to about 120 kDa, and in the range of from about 40 kDa to about 120 kDa.

[0273] Exemplary weight-average molecular weights for the water-soluble polymer include about 100 Daltons, about 200 Daltons, about 300 Daltons, about 400 Daltons, about 500 Daltons, about 600 Daltons, about 700 Daltons, about 750 Daltons, about 800 Daltons, about 900 Daltons, about 1 kDa, about 1.5 kDa, about 2 kDa, about 2.2 kDa, about 2.5 kDa, about 3 kDa, about 4 kDa, about 4.4 kDa, about 4.5 kDa, about 5 kDa, about 5.5 kDa, about 6 kDa, about 7 kDa, about

7.5 kDa, about 8 kDa, about 9 kDa, about 10 kDa, about 11 kDa, about 12 kDa, about 13 kDa, about 14 kDa, about 15 kDa, about 20 kDa, about 22.5 kDa, about 25 kDa, about 30 kDa, about 35 kDa, about 40 kDa, about 45 kDa, about 50 kDa, about 55 kDa, about 60 kDa, about 65 kDa, about 70 kDa, and about 75 kDa. Branched versions of the water-soluble polymer (e.g., a branched 40 kDa water-soluble polymer comprised of two 20 kDa polymers) having a total molecular weight of any of the foregoing can also be used. In one or more embodiments, the conjugate will not have any PEG moieties attached, either directly or indirectly, with a PEG having a weight average molecular weight of less than about 6 kDa.

[0274] PEGs will typically comprise a number of (OCH₂CH₂) monomers [or (CH₂CH₂O) monomers, depending on how the PEG is defined]. As used herein, the number of repeating units is identified by the subscript “n” in “(OCH₂CH₂)_n.” Thus, the value of (n) typically falls within one or more of the following ranges: from 2 to about 3400, from about 100 to about 2300, from about 100 to about 2270, from about 136 to about 2050, from about 225 to about 1930, from about 450 to about 1930, from about 1200 to about 1930, from about 568 to about 2727, from about 660 to about 2730, from about 795 to about 2730, from about 795 to about 2730, from about 909 to about 2730, and from about 1,200 to about 1,900. For any given polymer in which the molecular weight is known, it is possible to determine the number of repeating units (i.e., “n”) by dividing the total weight-average molecular weight of the polymer by the molecular weight of the repeating monomer.

[0275] In some embodiments, the water-soluble polymer is an end-capped polymer, that is, a polymer having at least one terminus capped with a relatively inert group, such as a lower C1-6 alkoxy group, or a hydroxyl group. When the polymer is PEG, for example, a methoxy-PEG (commonly referred to as mPEG) may be used, which is a linear form of PEG wherein one terminus of the polymer is a methoxy (—OCH₃) group, while the other terminus is a hydroxyl or other functional group that can be optionally chemically modified.

[0276] In some embodiments, the PEG group comprising the IL-2 conjugates disclosed herein is a linear or branched PEG group. In some embodiments, the PEG group is a linear PEG group. In some embodiments, the PEG group is a branched PEG group. In some embodiments, the PEG group is a methoxy PEG group. In some embodiments, the PEG group is a linear or branched methoxy PEG group. In some embodiments, the PEG group is a linear methoxy PEG group. In some embodiments, the PEG group is a branched methoxy PEG group. In some embodiments, the PEG group is a linear or branched PEG group having an average molecular weight of from about 100 Daltons to about 150 kDa. Exemplary ranges include, for example, weight-average molecular weights in the range of greater than 5 kDa to about 100 kDa, in the range of from

about 6 kDa to about 90 kDa, in the range of from about 10 kDa to about 85 kDa, in the range of greater than 10 kDa to about 85 kDa, in the range of from about 20 kDa to about 85 kDa, in the range of from about 53 kDa to about 85 kDa, in the range of from about 25 kDa to about 120 kDa, in the range of from about 29 kDa to about 120 kDa, in the range of from about 35 kDa to about 120 kDa, and in the range of from about 40 kDa to about 120 kDa. Exemplary weight-average molecular weights for the PEG group include about 100 Daltons, about 200 Daltons, about 300 Daltons, about 400 Daltons, about 500 Daltons, about 600 Daltons, about 700 Daltons, about 750 Daltons, about 800 Daltons, about 900 Daltons, about 1 kDa, about 1.5 kDa, about 2 kDa, about 2.2 kDa, about 2.5 kDa, about 3 kDa, about 4 kDa, about 4.4 kDa, about 4.5 kDa, about 5 kDa, about 5.5 kDa, about 6 kDa, about 7 kDa, about 7.5 kDa, about 8 kDa, about 9 kDa, about 10 kDa, about 11 kDa, about 12 kDa, about 13 kDa, about 14 kDa, about 15 kDa, about 20 kDa, about 22.5 kDa, about 25 kDa, about 30 kDa, about 35 kDa, about 40 kDa, about 45 kDa, about 50 kDa, about 55 kDa, about 60 kDa, about 65 kDa, about 70 kDa, about 75 kDa, about 80 kDa, about 90 kDa, about 95 kDa, and about 100 kDa. In some embodiments, the PEG group is a linear PEG group having an average molecular weight as disclosed above. In some embodiments, the PEG group is a branched PEG group having an average molecular weight as disclosed above. In some embodiments, the PEG group comprising the IL-2 conjugates disclosed herein is a linear or branched PEG group having a defined molecular weight $\pm 10\%$, or 15% or 20% or 25% . For example, included within the scope of the present disclosure are IL-2 conjugates comprising a PEG group having a molecular weight of 30,000 Da ± 3000 Da, or 30,000 Da $\pm 4,500$ Da, or 30,000 Da $\pm 6,000$ Da.

[0277] In some embodiments, the PEG group comprising the IL-2 conjugates disclosed herein is a linear or branched PEG group having an average molecular weight of from about 5 kDa to about 60 kDa. In some embodiments, the PEG group is a linear or branched PEG group having an average molecular weight of about 5 kDa, about 5.5 kDa, about 6 kDa, about 7 kDa, about 7.5 kDa, about 8 kDa, about 9 kDa, about 10 kDa, about 11 kDa, about 12 kDa, about 13 kDa, about 14 kDa, about 15 kDa, about 20 kDa, about 22.5 kDa, about 25 kDa, about 30 kDa, about 35 kDa, about 40 kDa, about 45 kDa, about 50 kDa, about 55 kDa, about 60 kDa, about 65 kDa, about 70 kDa, about 75 kDa, about 80 kDa, about 90 kDa, about 95 kDa, and about 100 kDa. In some embodiments, the PEG group is a linear or branched PEG group having an average molecular weight of about 5 kDa, about 10 kDa, about 20 kDa, about 30 kDa, about 50 kDa, or about 60 kDa. In some embodiments, the PEG group is a linear or branched PEG group having an average molecular weight of about 5 kDa, about 30 kDa, about 50 kDa, or about 60 kDa. In some embodiments, the PEG group is a linear PEG group having an average molecular of about

5 kDa, about 10 kDa, about 20 kDa, about 30 kDa, about 50 kDa, or about 60 kDa. In some embodiments, the PEG group is a branched PEG group having an average molecular weight of about 5 kDa, about 10 kDa, about 20 kDa, about 30 kDa, about 50 kDa, or about 60 kDa.

[0278] In some embodiments, the PEG group comprising the IL-2 conjugates disclosed herein is a linear methoxy PEG group having an average molecular weight of from about 5 kDa to about 60 kDa. In some embodiments, the PEG group is a linear methoxy PEG group having an average molecular weight of about 5 kDa, about 5.5 kDa, about 6 kDa, about 7 kDa, about 7.5 kDa, about 8 kDa, about 9 kDa, about 10 kDa, about 11 kDa, about 12 kDa, about 13 kDa, about 14 kDa, about 15 kDa, about 20 kDa, about 22.5 kDa, about 25 kDa, about 30 kDa, about 35 kDa, about 40 kDa, about 45 kDa, about 50 kDa, about 55 kDa, about 60 kDa, about 65 kDa, about 70 kDa, about 75 kDa, about 80 kDa, about 90 kDa, about 95 kDa, and about 100 kDa. In some embodiments, the PEG group is a linear methoxy PEG group having an average molecular weight of about 5 kDa, about 10 kDa, about 20 kDa, about 30 kDa, about 50 kDa, or about 60 kDa. In some embodiments, the PEG group is a linear methoxy PEG group having an average molecular weight of about 5 kDa, about 30 kDa, about 50 kDa, or about 60 kDa. In some embodiments, the PEG group is a linear methoxy PEG group having an average molecular of about 5 kDa, about 10 kDa, about 20 kDa, about 30 kDa, about 50 kDa, or about 60 kDa. In some embodiments, the PEG group is a linear methoxy PEG group having an average molecular of about 30 kDa. In some embodiments, the PEG group is a linear methoxy PEG group having an average molecular of about 50 kDa. In some embodiments, the PEG group is a linear methoxy PEG group having an average molecular of about 60 kDa. In some embodiments, the PEG group comprising the IL-2 conjugates disclosed herein is a linear methoxy PEG group having a defined molecular weight $\pm 10\%$, or 15% or 20% or 25% . For example, included within the scope of the present disclosure are IL-2 conjugates comprising a linear methoxy PEG group having a molecular weight of 30,000 Da ± 3000 Da, or 30,000 Da $\pm 4,500$ Da, or 30,000 Da $\pm 6,000$ Da.

[0279] In some embodiments, the PEG group comprising the IL-2 conjugates disclosed herein is a branched methoxy PEG group having an average molecular weight of from about 5 kDa to about 60 kDa. In some embodiments, the PEG group is a branched methoxy PEG group having an average molecular weight of about 5 kDa, about 5.5 kDa, about 6 kDa, about 7 kDa, about 7.5 kDa, about 8 kDa, about 9 kDa, about 10 kDa, about 11 kDa, about 12 kDa, about 13 kDa, about 14 kDa, about 15 kDa, about 20 kDa, about 22.5 kDa, about 25 kDa, about 30 kDa, about 35 kDa, about 40 kDa, about 45 kDa, about 50 kDa, about 55 kDa, about 60 kDa, about 65 kDa, about 70 kDa, about 75 kDa, about 80 kDa, about 90 kDa, about 95 kDa, and about 100 kDa. In

some embodiments, the PEG group is a branched methoxy PEG group having an average molecular weight of about 5 kDa, about 10 kDa, about 20 kDa, about 30 kDa, about 50 kDa, or about 60 kDa. In some embodiments, the PEG group is a branched methoxy PEG group having an average molecular weight of about 5 kDa, about 30 kDa, about 50 kDa, or about 60 kDa. In some embodiments, the PEG group is a branched methoxy PEG group having an average molecular of about 5 kDa, about 10 kDa, about 20 kDa, about 30 kDa, about 50 kDa, or about 60 kDa. In some embodiments, the PEG group is a branched methoxy PEG group having an average molecular of about 5 kDa, about 10 kDa, about 20 kDa, about 30 kDa, about 50 kDa, or about 60 kDa. In some embodiments, the PEG group comprising the IL-2 conjugates disclosed herein is a branched methoxy PEG group having a defined molecular weight $\pm 10\%$, or 15% or 20% or 25% . For example, included within the scope of the present disclosure are IL-2 conjugates comprising a branched methoxy PEG group having a molecular weight of 30,000 Da ± 3000 Da, or 30,000 Da $\pm 4,500$ Da, or 30,000 Da $\pm 6,000$ Da.

[0280] In some embodiments, exemplary water-soluble polymers include, but are not limited to, linear or branched discrete PEG (dPEG) from Quanta Biodesign, Ltd; linear, branched, or forked PEGs from Nektar Therapeutics; and Y-shaped PEG derivatives from JenKem Technology.

[0281] In some embodiments, an IL-2 polypeptide described herein is conjugated to a water-soluble polymer selected from poly(alkylene glycols) such as polyethylene glycol ("PEG"), poly(propylene glycol) ("PPG"), copolymers of ethylene glycol and propylene glycol and the like, poly(oxyethylated polyol), poly(olefinic alcohol), poly(vinylpyrrolidone), poly(hydroxyalkylmethacrylamide), poly(hydroxyalkylmethacrylate), poly(saccharides), poly(α -hydroxy acid), poly(vinyl alcohol) (PVA), polyacrylamide (PAAm), polydimethylacrylamide (PDAAm), poly(N-(2-hydroxypropyl) methacrylamide) (PHPMA), polyphosphazene, polyoxazolines ("POZ"), poly(N-acryloylmorpholine), and a combination thereof. In some embodiments, the IL-2 polypeptide is conjugated to PEG (e.g., PEGylated). In some embodiments, the IL-2 polypeptide is conjugated to PPG. In some embodiments, the IL-2 polypeptide is conjugated to POZ. In some embodiments, the IL-2 polypeptide is conjugated to PVP.

[0282] In some embodiments, a water-soluble polymer comprises a polyglycerol (PG). In some embodiments, the polyglycerol is a hyperbranched PG (HPG) (e.g., as described by Imran, et al. "Influence of architecture of high molecular weight linear and branched polyglycerols on their biocompatibility and biodistribution," *Biomaterials* 33:9135–9147 (2012)). In other cases, the polyglycerol is a linear PG (LPG). In additional cases, the polyglycerol is a midfunctional PG, a linear-block-hyperbranched PG (e.g., as described by Wurm et. Al., "Squaric acid mediated

synthesis and biological activity of a library of linear and hyperbranched poly(glycerol)–protein conjugates,” *Biomacromolecules* 13:1161–1171 (2012)), or a side-chain functional PG (e.g., as described by Li, et. al., “Synthesis of linear polyether polyol derivatives as new materials for bioconjugation,” *Bioconjugate Chem.* 20:780–789 (2009).

[0283] In some embodiments, a cytokine (e.g., an interleukin, IFN, or TNF) polypeptide described herein is conjugated to a PG, e.g., a HPG, a LPG, a midfunctional PG, a linear-block-hyperbranched PG, or a side-chain functional PG. In some embodiments, the cytokine is an IL-2 polypeptide. In some embodiments, the IL-2 polypeptide is conjugated to a PG, a midfunctional PG, a linear-block-hyperbranched PG.

[0284] In some embodiments, a water-soluble polymer is a degradable synthetic PEG alternative. Exemplary degradable synthetic PEG alternatives include, but are not limited to, poly[oligo(ethylene glycol)methyl methacrylate] (POEGMA); backbone modified PEG derivatives generated by polymerization of telechelic, or di-end-functionalized PEG-based macromonomers; PEG derivatives comprising comonomers comprising degradable linkage such as poly[(ethylene oxide)-co-(methylene ethylene oxide)][P(EO-co-MEO)], cyclic ketene acetals such as 5,6-benzo-2-methylene-1,3-dioxepane (BMDO), 2-methylene-1,3-dioxepane (MDO), and 2-methylene-4-phenyl-1,3-dioxolane (MPDL) copolymerized with OEGMA; or poly-(ϵ -caprolactone)-graft-poly(ethylene oxide) (PCL-g-PEO).

[0285] In some embodiments, a cytokine (e.g., an interleukin, IFN, or TNF) polypeptide described herein is conjugated to a degradable synthetic PEG alternative, such as for example, POEGM; backbone modified PEG derivatives generated by polymerization of telechelic, or di-end-functionalized PEG-based macromonomers; P(EO-co-MEO); cyclic ketene acetals such as BMDO, MDO, and MPDL copolymerized with OEGMA; or PCL-g-PEO. In some embodiments, the cytokine is an IL-2 polypeptide. In some embodiments, the IL-2 polypeptide is conjugated to a degradable synthetic PEG alternative, such as for example, POEGM; backbone modified PEG derivatives generated by polymerization of telechelic, or di-end-functionalized PEG-based macromonomers; P(EO-co-MEO); cyclic ketene acetals such as BMDO, MDO, and MPDL copolymerized with OEGMA; or PCL-g-PEO.

[0286] In some embodiments, a water-soluble polymer comprises a poly(zwitterions). Exemplary poly(zwitterions) include, but are not limited to, poly(sulfobetaine methacrylate) (PSBMA), poly(carboxybetaine methacrylate) (PCBMA), and poly(2-methacryloyloxyethyl phosphorylcholine) (PMPC). In some embodiments, a cytokine (e.g., an interleukin, IFN, or TNF) polypeptide described herein is conjugated to a poly(zwitterion) such as PSBMA, PCBMA, or PMPC. In some embodiments, the cytokine is an IL-2 polypeptide. In some

embodiments, the IL-2 polypeptide is conjugated to a poly(zwitterion) such as PSBMA, PCBMA, or PMPC.

[0287] In some embodiments, a water-soluble polymer comprises a polycarbonate. Exemplary polycarbonates include, but are not limited to, pentafluorophenyl 5-methyl-2-oxo-1,3-dioxane-5-carboxylate (MTC-OC6F5). In some embodiments, a cytokine (e.g., an interleukin, IFN, or TNF) polypeptide described herein is conjugated to a polycarbonate such as MTC-OC6F5. In some embodiments, the cytokine is an IL-2 polypeptide. In some embodiments, the IL-2 polypeptide is conjugated to a polycarbonate such as MTC-OC6F5.

[0288] In some embodiments, a water-soluble polymer comprises a polymer hybrid, such as for example, a polycarbonate/PEG polymer hybrid, a peptide/protein-polymer conjugate, or a hydroxyl containing and/or zwitterionic derivatized polymer (e.g., a hydroxyl containing and/or zwitterionic derivatized PEG polymer). In some embodiments, a cytokine (e.g., an interleukin, IFN, or TNF) polypeptide described herein is conjugated to a polymer hybrid such as a polycarbonate/PEG polymer hybrid, a peptide/protein-polymer conjugate, or a hydroxyl containing and/or zwitterionic derivatized polymer (e.g., a hydroxyl containing and/or zwitterionic derivatized PEG polymer). In some embodiments, the cytokine is an IL-2 polypeptide. In some embodiments, the IL-2 polypeptide is conjugated to a polymer hybrid such as a polycarbonate/PEG polymer hybrid, a peptide/protein-polymer conjugate, or a hydroxyl containing and/or zwitterionic derivatized polymer (e.g., a hydroxyl containing and/or zwitterionic derivatized PEG polymer).

[0289] In some embodiments, a water-soluble polymer comprises a polysaccharide. Exemplary polysaccharides include, but are not limited to, dextran, polysialic acid (PSA), hyaluronic acid (HA), amylose, heparin, heparan sulfate (HS), dextrin, or hydroxyethyl-starch (HES). In some embodiments, a cytokine (e.g., an interleukin, IFN, or TNF) polypeptide is conjugated to a polysaccharide. In some embodiments, an IL-2 polypeptide is conjugated to dextran. In some embodiments, an IL-2 polypeptide is conjugated to PSA. In some embodiments, an IL-2 polypeptide is conjugated to HA. In some embodiments, an IL-2 polypeptide is conjugated to amylose. In some embodiments, an IL-2 polypeptide is conjugated to heparin. In some embodiments, an IL-2 polypeptide is conjugated to HS. In some embodiments, an IL-2 polypeptide is conjugated to dextrin. In some embodiments, an IL-2 polypeptide is conjugated to HES.

[0290] In some embodiments, a water-soluble polymer comprises a glycan. Exemplary classes of glycans include N-linked glycans, O-linked glycans, glycolipids, O-GlcNAc, and glycosaminoglycans. In some embodiments, a cytokine (e.g., an interleukin, IFN, or TNF)

polypeptide is conjugated to a glycan. In some embodiments, an IL-2 polypeptide is conjugated to N-linked glycans. In some embodiments, an IL-2 polypeptide is conjugated to O-linked glycans. In some embodiments, an IL-2 polypeptide is conjugated to glycolipids. In some embodiments, an IL-2 polypeptide is conjugated to O-GlcNAc. In some embodiments, an IL-2 polypeptide is conjugated to glycosaminoglycans.

[0291] In some embodiments, a water-soluble polymer comprises a polyoxazoline polymer. A polyoxazoline polymer is a linear synthetic polymer, and similar to PEG, comprises a low polydispersity. In some embodiments, a polyoxazoline polymer is a polydispersed polyoxazoline polymer, characterized with an average molecule weight. In some embodiments, the average molecule weight of a polyoxazoline polymer includes, for example, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 4500, 5000, 5500, 6000, 6500, 7000, 7500, 8000, 10,000, 12,000, 20,000, 35,000, 40,000, 50,000, 60,000, 100,000, 200,000, 300,000, 400,000, or 500,000 Da. In some embodiments, a polyoxazoline polymer comprises poly(2-methyl 2-oxazoline) (PMOZ), poly(2-ethyl 2-oxazoline) (PEOZ), or poly(2-propyl 2-oxazoline) (PPOZ). In some embodiments, a cytokine (e.g., an interleukin, IFN, or TNF) polypeptide is conjugated to a polyoxazoline polymer. In some embodiments, an IL-2 polypeptide is conjugated to a polyoxazoline polymer. In some embodiments, an IL-2 polypeptide is conjugated to PMOZ. In some embodiments, an IL-2 polypeptide is conjugated to PEOZ. In some embodiments, an IL-2 polypeptide is conjugated to PPOZ.

[0292] In some embodiments, a water-soluble polymer comprises a polyacrylic acid polymer. In some embodiments, a cytokine (e.g., an interleukin, IFN, or TNF) polypeptide is conjugated to a polyacrylic acid polymer. In some embodiments, an IL-2 polypeptide is conjugated to a polyacrylic acid polymer.

[0293] In some embodiments, a water-soluble polymer comprises polyamine. Polyamine is an organic polymer comprising two or more primary amino groups. In some embodiments, a polyamine includes a branched polyamine, a linear polyamine, or cyclic polyamine. In some embodiments, a polyamine is a low-molecular-weight linear polyamine. Exemplary polyamines include putrescine, cadaverine, spermidine, spermine, ethylene diamine, 1,3-diaminopropane, hexamethylenediamine, tetraethylmethylenediamine, and piperazine. In some embodiments, a cytokine (e.g., an interleukin, IFN, or TNF) polypeptide is conjugated to a polyamine. In some embodiments, an IL-2 polypeptide is conjugated to polyamine. In some embodiments, an IL-2 polypeptide is conjugated to putrescine, cadaverine, spermidine, spermine, ethylene diamine, 1,3-diaminopropane, hexamethylenediamine, tetraethylmethylenediamine, or piperazine.

[0294] In some embodiments, a water-soluble polymer is described in US Patent Nos. 7,744,861, 8,273,833, and 7,803,777. In some embodiments, a cytokine (e.g., an interleukin, IFN, or TNF) polypeptide is conjugated to a linker described in US Patent No. 7,744,861, 8,273,833, or 7,803,777. In some embodiments, an IL-2 polypeptide is conjugated to a linker described in US Patent No. 7,744,861, 8,273,833, or 7,803,777.

[0295] In some embodiments, a conjugating moiety described herein is a lipid. In some embodiments, the lipid is a fatty acid. In some embodiments, the fatty acid is a saturated fatty acid. In other cases, the fatty acid is an unsaturated fatty acid. Exemplary fatty acids include, but are not limited to, fatty acids comprising from about 6 to about 26 carbon atoms, from about 6 to about 24 carbon atoms, from about 6 to about 22 carbon atoms, from about 6 to about 20 carbon atoms, from about 6 to about 18 carbon atoms, from about 20 to about 26 carbon atoms, from about 12 to about 26 carbon atoms, from about 12 to about 24 carbon atoms, from about 12 to about 22 carbon atoms, from about 12 to about 20 carbon atoms, or from about 12 to about 18 carbon atoms. In some embodiments, the lipid binds to one or more serum proteins, thereby increasing serum stability and/or serum half-life.

[0296] In some embodiments, the lipid is conjugated to IL-2. In some embodiments, the lipid is a fatty acid, e.g., a saturated fatty acid or an unsaturated fatty acid. In some embodiments, the fatty acid is from about 6 to about 26 carbon atoms, from about 6 to about 24 carbon atoms, from about 6 to about 22 carbon atoms, from about 6 to about 20 carbon atoms, from about 6 to about 18 carbon atoms, from about 20 to about 26 carbon atoms, from about 12 to about 26 carbon atoms, from about 12 to about 24 carbon atoms, from about 12 to about 22 carbon atoms, from about 12 to about 20 carbon atoms, or from about 12 to about 18 carbon atoms. In some embodiments, the fatty acid comprises about 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or 26 carbon atoms in length. In some embodiments, the fatty acid comprises caproic acid (hexanoic acid), enanthic acid (heptanoic acid), caprylic acid (octanoic acid), pelargonic acid (nonanoic acid), capric acid (decanoic acid), undecylic acid (undecanoic acid), lauric acid (dodecanoic acid), tridecylic acid (tridecanoic acid), myristic acid (tetradecanoic acid), pentadecylic acid (pentadecanoic acid), palmitic acid (hexadecanoic acid), margaric acid (heptadecanoic acid), stearic acid (octadecanoic acid), nonadecylic acid (nonadecanoic acid), arachidic acid (eicosanoic acid), heneicosylic acid (heneicosanoic acid), behenic acid (docosanoic acid), tricosylic acid (tricosanoic acid), lignoceric acid (tetracosanoic acid), pentacosylic acid (pentacosanoic acid), or cerotic acid (hexacosanoic acid).

[0297] In some embodiments, the IL-2 lipid conjugate enhances serum stability and/or serum half-life.

[0298] In some embodiments, a conjugating moiety described herein is a protein or a binding fragment thereof. Exemplary proteins include albumin, transferrin, or transthyretin. In some embodiments, the protein or a binding fragment thereof comprises an antibody, or its binding fragments thereof. In some embodiments, a cytokine conjugate comprises a protein or a binding fragment thereof. In some embodiments, an IL-2 conjugate comprising a protein or a binding fragment thereof has an increased serum half-life, and/or stability. In some embodiments, an IL-2 conjugate comprising a protein or a binding fragment thereof has a reduced IL-2 interaction with one or more IL-2R subunits. In additional cases, the protein or a binding fragment thereof blocks IL-2 interaction with one or more IL-2R subunits.

[0299] In some embodiments, the conjugating moiety is albumin. Albumin is a family of water-soluble globular proteins. It is commonly found in blood plasma, comprising about 55-60% of all plasma proteins. Human serum albumin (HSA) is a 585 amino acid polypeptide in which the tertiary structure is divided into three domains, domain I (amino acid residues 1-195), domain II (amino acid residues 196-383), and domain III (amino acid residues 384-585). Each domain further comprises a binding site, which can interact either reversibly or irreversibly with endogenous ligands such as long- and medium-chain fatty acids, bilirubin, or heme, or exogenous compounds such as heterocyclic or aromatic compounds.

[0300] In some embodiments, a cytokine (e.g., an interleukin, IFN, or TNF) polypeptide is conjugated to albumin. In some embodiments, the cytokine polypeptide is conjugated to human serum albumin (HSA). In additional cases, the cytokine polypeptide is conjugated to a functional fragment of albumin.

[0301] In some embodiments, an IL-2 polypeptide is conjugated to albumin. In some embodiments, the IL-2 polypeptide is conjugated to human serum albumin (HSA). In additional cases, the IL-2 polypeptide is conjugated to a functional fragment of albumin.

[0302] In some embodiments, the conjugating moiety is transferrin. Transferrin is a 679 amino acid polypeptide that is about 80 kDa in size and comprises two Fe³⁺ binding sites with one at the N-terminal domain and the other at the C-terminal domain. In some embodiments, human transferrin has a half-life of about 7-12 days.

[0303] In some embodiments, a cytokine (e.g., an interleukin, IFN, or TNF) polypeptide is conjugated to transferrin. In some embodiments, the cytokine polypeptide is conjugated to human transferrin. In additional cases, the cytokine polypeptide is conjugated to a functional fragment of transferrin.

[0304] In some embodiments, an IL-2 polypeptide is conjugated to transferrin. In some embodiments, the IL-2 polypeptide is conjugated to human transferrin. In additional cases, the IL-2 polypeptide is conjugated to a functional fragment of transferrin.

[0305] In some embodiments, the conjugating moiety is transthyretin (TTR). Transthyretin is a transport protein located in the serum and cerebrospinal fluid which transports the thyroid hormone thyroxine (T4) and retinol-binding protein bound to retinol.

[0306] In some embodiments, a cytokine (e.g., an interleukin, IFN, or TNF) polypeptide is conjugated to transthyretin (via one of its termini or via an internal hinge region). In some embodiments, the cytokine polypeptide is conjugated to a functional fragment of transthyretin.

[0307] In some embodiments, an IL-2 polypeptide is conjugated to transthyretin (via one of its termini or via an internal hinge region). In some embodiments, the IL-2 polypeptide is conjugated to a functional fragment of transthyretin.

[0308] In some embodiments, the conjugating moiety is an antibody, or its binding fragments thereof. In some embodiments, an antibody or its binding fragments thereof comprise a humanized antibody or binding fragment thereof, murine antibody or binding fragment thereof, chimeric antibody or binding fragment thereof, monoclonal antibody or binding fragment thereof, monovalent Fab', divalent Fab2, F(ab)'3 fragments, single-chain variable fragment (scFv), bis-scFv, (scFv)2, diabody, minibody, nanobody, triabody, tetrabody, humabody, disulfide stabilized Fv protein (dsFv), single-domain antibody (sdAb), Ig NAR, camelid antibody or binding fragment thereof, bispecific antibody or binding fragment thereof, or a chemically modified derivative thereof.

[0309] In some embodiments, the conjugating moiety comprises a scFv, bis-scFv, (scFv)2, dsFv, or sdAb. In some embodiments, the conjugating moiety comprises a scFv. In some embodiments, the conjugating moiety comprises a bis-scFv. In some embodiments, the conjugating moiety comprises a (scFv)2. In some embodiments, the conjugating moiety comprises a dsFv. In some embodiments, the conjugating moiety comprises a sdAb.

[0310] In some embodiments, the conjugating moiety comprises an Fc portion of an antibody, e.g., of IgG, IgA, IgM, IgE, or IgD. In some embodiments, the moiety comprises an Fc portion of IgG (e.g., IgG1, IgG3, or IgG4).

[0311] In some embodiments, a cytokine (e.g., an interleukin, IFN, or TNF) polypeptide is conjugated to an antibody, or its binding fragments thereof. In some embodiments, the cytokine polypeptide is conjugated to a humanized antibody or binding fragment thereof, murine antibody or binding fragment thereof, chimeric antibody or binding fragment thereof, monoclonal antibody or binding fragment thereof, monovalent Fab', divalent Fab2, F(ab)'3 fragments, single-

chain variable fragment (scFv), bis-scFv, (scFv)₂, diabody, minibody, nanobody, triabody, tetrabody, humabody, disulfide stabilized Fv protein (dsFv), single-domain antibody (sdAb), Ig NAR, camelid antibody or binding fragment thereof, bispecific antibody or binding fragment thereof, or a chemically modified derivative thereof. In additional cases, the cytokine polypeptide is conjugated to an Fc portion of an antibody. In additional cases, the cytokine polypeptide is conjugated to an Fc portion of IgG (e.g., IgG1, IgG3, or IgG4).

[0312] In some embodiments, an IL-2 polypeptide is conjugated to an antibody, or its binding fragments thereof. In some embodiments, the IL-2 polypeptide is conjugated to a humanized antibody or binding fragment thereof, murine antibody or binding fragment thereof, chimeric antibody or binding fragment thereof, monoclonal antibody or binding fragment thereof, monovalent Fab', divalent Fab₂, F(ab')₃ fragments, single-chain variable fragment (scFv), bis-scFv, (scFv)₂, diabody, minibody, nanobody, triabody, tetrabody, humabody, disulfide stabilized Fv protein (dsFv), single-domain antibody (sdAb), Ig NAR, camelid antibody or binding fragment thereof, bispecific antibody or binding fragment thereof, or a chemically modified derivative thereof. In additional cases, the IL-2 polypeptide is conjugated to an Fc portion of an antibody. In additional cases, the IL-2 polypeptide is conjugated to an Fc portion of IgG (e.g., IgG1, IgG3, or IgG4).

[0313] In some embodiments, an IL-2 polypeptide is conjugated to a water-soluble polymer (e.g., PEG) and an antibody or binding fragment thereof. In some embodiments, the antibody or binding fragments thereof comprises a humanized antibody or binding fragment thereof, murine antibody or binding fragment thereof, chimeric antibody or binding fragment thereof, monoclonal antibody or binding fragment thereof, monovalent Fab', divalent Fab₂, F(ab')₃ fragments, single-chain variable fragment (scFv), bis-scFv, (scFv)₂, diabody, minibody, nanobody, triabody, tetrabody, humabody, disulfide stabilized Fv protein (dsFv), single-domain antibody (sdAb), Ig NAR, camelid antibody or binding fragment thereof, bispecific antibody or binding fragment thereof, or a chemically modified derivative thereof. In some embodiments, the antibody or binding fragments thereof comprises a scFv, bis-scFv, (scFv)₂, dsFv, or sdAb. In some embodiments, the antibody or binding fragments thereof comprises a scFv. In some embodiments, the antibody or binding fragment thereof guides the IL-2 conjugate to a target cell of interest and the water-soluble polymer enhances stability and/or serum half-life.

[0314] In some embodiments, one or more IL-2 polypeptide – water-soluble polymer (e.g., PEG) conjugates are further bound to an antibody or binding fragments thereof. In some embodiments, the ratio of the IL-2 conjugate to the antibody is about 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1, 10:1, 11:1, or 12:1. In some embodiments, the ratio of the IL-2 conjugate to the antibody is about

1:1. In other cases, the ratio of the IL-2 conjugate to the antibody is about 2:1, 3:1, or 4:1. In additional cases, the ratio of the IL-2 conjugate to the antibody is about 6:1 or higher.

[0315] In some embodiments, the one or more IL-2 polypeptide – water-soluble polymer (e.g., PEG) conjugates are directly bound to the antibody or binding fragments thereof. In other instances, the IL-2 conjugate is indirectly bound to the antibody or binding fragments thereof with a linker. Exemplary linkers include homobifunctional linkers, heterobifunctional linkers, maleimide-based linkers, zero-trace linkers, self-immolative linkers, spacers, and the like.

[0316] In some embodiments, the antibody or binding fragments thereof is bound either directly or indirectly to the IL-2 polypeptide portion of the IL-2 polypeptide – water-soluble polymer (e.g., PEG) conjugate. In such cases, the conjugation site of the antibody to the IL-2 polypeptide is at a site that will not impede binding of the IL-2 polypeptide with the IL-2R $\beta\gamma$. In additional cases, the conjugation site of the antibody to the IL-2 polypeptide is at a site that partially blocks binding of the IL-2 polypeptide with the IL-2R $\beta\gamma$. In additional cases, the conjugation site of the antibody to the IL-2 polypeptide is at a site that will impede or further impede binding of the IL-2 polypeptide with the IL-2R α . In other embodiments, the antibody or binding fragments thereof is bound either directly or indirectly to the water-soluble polymer portion of the IL-2 polypeptide – water-soluble polymer (e.g., PEG) conjugate.

[0317] In some embodiments, a conjugating moiety described herein is a peptide. In some embodiments, the peptide is a non-structured peptide. In some embodiments, a cytokine (e.g., an interleukin, IFN, or TNF) polypeptide is conjugated to a peptide. In some embodiments, the IL-2 conjugate comprising a peptide has an increased serum half-life, and/or stability. In some embodiments, the IL-2 conjugate comprising a peptide has a reduced IL-2 interaction with one or more IL-2R subunits. In additional cases, the peptide blocks IL-2 interaction with one or more IL-2R subunits.

[0318] In some embodiments, the conjugating moiety is a XTENT[™] peptide (Amunix Operating Inc.) and the modification is referred to as XTENylation. XTENylation is the genetic fusion of a nucleic acid encoding a polypeptide of interest with a nucleic acid encoding a XTENT[™] peptide (Amunix Operating Inc.), a long unstructured hydrophilic peptide comprising different percentage of six amino acids: Ala, Glu, Gly, Ser, and Thr. In some embodiments, a XTENT[™] peptide is selected based on properties such as expression, genetic stability, solubility, aggregation resistance, enhanced half-life, increased potency, and/or increased in vitro activity in combination with a polypeptide of interest. In some embodiments, a cytokine (e.g., an interleukin, IFN, or TNF) polypeptide is conjugated to a XTEN peptide. In some embodiments, an IL-2 polypeptide is conjugated to a XTEN peptide.

[0319] In some embodiments, the conjugating moiety is a glycine-rich homoamino acid polymer (HAP) and the modification is referred to as HAPylation. HAPylation is the genetic fusion of a nucleic acid encoding a polypeptide of interest with a nucleic acid encoding a glycine-rich homoamino acid polymer (HAP). In some embodiments, the HAP polymer comprises a (Gly4Ser)_n repeat motif and sometimes are about 50, 100, 150, 200, 250, 300, or more residues in length. In some embodiments, a cytokine (e.g., an interleukin, IFN, or TNF) polypeptide is conjugated to HAP. In some embodiments, an IL-2 polypeptide is conjugated to HAP.

[0320] In some embodiments, the conjugating moiety is a PAS polypeptide and the modification is referred to as PASylation. PASylation is the genetic fusion of a nucleic acid encoding a polypeptide of interest with a nucleic acid encoding a PAS polypeptide. A PAS polypeptide is a hydrophilic uncharged polypeptide consisting of Pro, Ala and Ser residues. In some embodiments, the length of a PAS polypeptide is at least about 100, 200, 300, 400, 500, or 600 amino acids. In some embodiments, a cytokine (e.g., an interleukin, IFN, or TNF) polypeptide is conjugated to a PAS polypeptide. In some embodiments, an IL-2 polypeptide is conjugated to a PAS polypeptide.

[0321] In some embodiments, the conjugating moiety is an elastin-like polypeptide (ELP) and the modification is referred to as ELPylation. ELPylation is the genetic fusion of a nucleic acid encoding a polypeptide of interest with a nucleic acid encoding an elastin-like polypeptide (ELPs). An ELP comprises a VPGxG repeat motif in which x is any amino acid except proline. In some embodiments, a cytokine (e.g., an interleukin, IFN, or TNF) polypeptide is conjugated to ELP. In some embodiments, an IL-2 polypeptide is conjugated to ELP.

[0322] In some embodiments, the conjugating moiety is a CTP peptide. A CTP peptide comprises a 30 or 31 amino acid residue peptide (FQSSSS*KAPPPS*LPSPS*RLPGPS*DTPILPQ (SEQ ID NO: 17) or FQDSSSS*KAPPPS*LPSPS*RLPGPS*DTPILPQ (SEQ ID NO: 18)) in which the S* denotes O-glycosylation sites (OPKO). In some embodiments, a CTP peptide is genetically fused to a cytokine polypeptide (e.g., an IL-2 polypeptide). In some embodiments, a cytokine polypeptide (e.g., an IL-2 polypeptide) is conjugated to a CTP peptide.

[0323] In some embodiments, a cytokine (e.g., an IL-2 polypeptide) is modified by glutamylation. Glutamylation (or polyglutamylation) is a reversible posttranslational modification of glutamate, in which the γ -carboxy group of glutamate forms a peptide-like bond with the amino group of a free glutamate in which the α -carboxy group extends into a polyglutamate chain.

[0324] In some embodiments, a cytokine (e.g., an IL-2 polypeptide) is modified by a gelatin-like protein (GLK) polymer. In some embodiments, the GLK polymer comprises multiple repeats of Gly-Xaa-Yaa wherein Xaa and Yaa primarily comprise proline and 4-hydroxyproline, respectively. In some embodiments, the GLK polymer further comprises amino acid residues Pro, Gly, Glu, Qln, Asn, Ser, and Lys. In some embodiments, the length of the GLK polymer is about 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 150 residues or longer.

[0325] In some embodiments, the conjugating moiety comprises an extracellular biomarker. In some embodiments, the extracellular biomarker is a tumor antigen. In some embodiments, exemplary extracellular biomarker comprises CD19, PSMA, B7-H3, B7-H6, CD70, CEA, CSPG4, EGFRvIII, EphA3, EpCAM, EGFR, ErbB2 (HER2), FAP, FR α , GD2, GD3, Lewis-Y, mesothelin, Muc1, Muc 16, ROR1, TAG72, VEGFR2, CD11, Gr-1, CD204, CD16, CD49b, CD3, CD4, CD8, and B220. In some embodiments, the conjugating moiety is bond or conjugated to the cytokine (e.g., IL-2). In some embodiments, the conjugating moiety is genetically fused, for example, at the N-terminus or the C-terminus, of the cytokine (e.g., IL-2).

[0326] In some embodiments, the conjugating moiety comprises a molecule from a post-translational modification. In some embodiments, examples of post-translational modification include myristoylation, palmitoylation, isoprenylation (or prenylation) (e.g., farnesylation or geranylgeranylation), glypiation, acylation (e.g., O-acylation, N-acylation, S-acylation), alkylation (e.g., additional of alkyl groups such as methyl or ethyl groups), amidation, glycosylation, hydroxylation, iodination, nucleotide addition, oxidation, phosphorylation, succinylation, sulfation, glycation, carbamylation, glutamylation, or deamidation. In some embodiments, the cytokine (e.g., IL-2) is modified by a post-translational modification such as myristoylation, palmitoylation, isoprenylation (or prenylation) (e.g., farnesylation or geranylgeranylation), glypiation, acylation (e.g., O-acylation, N-acylation, S-acylation), alkylation (e.g., additional of alkyl groups such as methyl or ethyl groups), amidation, glycosylation, hydroxylation, iodination, nucleotide addition, oxidation, phosphorylation, succinylation, sulfation, glycation, carbamylation, glutamylation, or deamidation.

3. Conjugation

[0327] In some embodiments, useful functional reactive groups for conjugating or binding a conjugating moiety to a cytokine polypeptide (e.g., an IL-2 polypeptide) described herein include, for example, zero or higher-order linkers. In some embodiments, an unnatural amino acid incorporated into an interleukin described herein comprises a functional reactive group. In some embodiments, a linker comprises a functional reactive group that reacts with an unnatural amino acid incorporated into an interleukin described herein. In some embodiments, a

conjugating moiety comprises a functional reactive group that reacts with an unnatural amino acid incorporated into an interleukin described herein. In some embodiments, a conjugating moiety comprises a functional reactive group that reacts with a linker (optionally pre-attached to a cytokine peptide) described herein. In some embodiments, a linker comprises a reactive group that reacts with an unnatural amino acid in a cytokine peptide described herein. In some embodiments, higher-order linkers comprise bifunctional linkers, such as homobifunctional linkers or heterobifunctional linkers. Exemplary homobifunctional linkers include, but are not limited to, Lomant's reagent dithiobis (succinimidylpropionate) DSP, 3,3'-dithiobis(sulfosuccinimidyl propionate) (DTSSP), disuccinimidyl suberate (DSS), bis(sulfosuccinimidyl)suberate (BS), disuccinimidyl tartrate (DST), disulfosuccinimidyl tartrate (sulfo DST), ethylene glycobis(succinimidylsuccinate) (EGS), disuccinimidyl glutarate (DSG), N,N'-disuccinimidyl carbonate (DSC), dimethyl adipimide (DMA), dimethyl pimelimide (DMP), dimethyl suberimide (DMS), dimethyl-3,3'-dithiobispropionimide (DTBP), 1,4-di-3'-(2'-pyridyldithio)propionamido)butane (DPDPB), bismaleimido-hexane (BMH), aryl halide-containing compound (DFDNB), such as e.g. 1,5-difluoro-2,4-dinitrobenzene or 1,3-difluoro-4,6-dinitrobenzene, 4,4'-difluoro-3,3'-dinitrophenylsulfone (DFDNPS), bis-[β-(4-azidosalicylamido)ethyl]disulfide (BASED), formaldehyde, glutaraldehyde, 1,4-butanediol diglycidyl ether, adipic acid dihydrazide, carbonyldiimidazole, o-toluidine, 3,3'-dimethylbenzidine, benzidine, α,α'-p-diaminodiphenyl, diiodo-p-xylene sulfonic acid, N,N'-ethylene-bis(iodoacetamide), or N,N'-hexamethylene-bis(iodoacetamide).

[0328] In some embodiments, the bifunctional linker comprises a heterobifunctional linker. Exemplary heterobifunctional linker include, but are not limited to, amine-reactive and sulfhydryl cross-linkers such as N-succinimidyl 3-(2-pyridyldithio)propionate (sPDP), long-chain N-succinimidyl 3-(2-pyridyldithio)propionate (LC-sPDP), water-soluble-long-chain N-succinimidyl 3-(2-pyridyldithio) propionate (sulfo-LC-sPDP), succinimidyl-oxycarbonyl-α-methyl-α-(2-pyridyldithio)toluene (sMPT), sulfosuccinimidyl-6-[α-methyl-α-(2-pyridyldithio)toluamido]hexanoate (sulfo-LC-sMPT), succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sMCC), sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-sMCC), m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBs), m-maleimidobenzoyl-N-hydroxysulfosuccinimide ester (sulfo-MBs), N-succinimidyl(4-iodoacetyl)aminobenzoate (sIAB), sulfosuccinimidyl(4-iodoacetyl)aminobenzoate (sulfo-sIAB), succinimidyl-4-(p-maleimidophenyl)butyrate (sMPB), sulfosuccinimidyl-4-(p-maleimidophenyl)butyrate (sulfo-sMPB), N-(γ-maleimidobutyryloxy)succinimide ester (GMBs), N-(γ-maleimidobutyryloxy)sulfosuccinimide

ester (sulfo-GMBs), succinimidyl 6-((iodoacetyl)amino)hexanoate (sIAX), succinimidyl 6-[6-(((iodoacetyl)amino)hexanoyl)amino]hexanoate (sIAXX), succinimidyl 4-(((iodoacetyl)amino)methyl)cyclohexane-1-carboxylate (sIAC), succinimidyl 6-(((4-iodoacetyl)amino)methyl)cyclohexane-1-carboxyl)amino) hexanoate (sIACX), p-nitrophenyl iodoacetate (NPIA), carbonyl-reactive and sulfhydryl-reactive cross-linkers such as 4-(4-N-maleimidophenyl)butyric acid hydrazide (MPBH), 4-(N-maleimidomethyl)cyclohexane-1-carboxyl-hydrazide-8 (M2C2H), 3-(2-pyridyldithio)propionyl hydrazide (PDPH), amine-reactive and photoreactive cross-linkers such as N-hydroxysuccinimidyl-4-azidosalicylic acid (NHs-AsA), N-hydroxysulfosuccinimidyl-4-azidosalicylic acid (sulfo-NHs-AsA), sulfosuccinimidyl-(4-azidosalicylamido)hexanoate (sulfo-NHs-LC-AsA), sulfosuccinimidyl-2-(p-azidosalicylamido)ethyl-1,3'-dithiopropionate (sAsD), N-hydroxysuccinimidyl-4-azidobenzoate (HsAB), N-hydroxysulfosuccinimidyl-4-azidobenzoate (sulfo-HsAB), N-succinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate (sANPAH), sulfosuccinimidyl-6-(4'-azido-2'-nitrophenylamino)hexanoate (sulfo-sANPAH), N-5-azido-2-nitrobenzoyloxysuccinimide (ANB-NOs), sulfosuccinimidyl-2-(m-azido-o-nitrobenzamido)-ethyl-1,3'-dithiopropionate (sAND), N-succinimidyl-4(4-azidophenyl)1,3'-dithiopropionate (sADP), N-sulfosuccinimidyl(4-azidophenyl)-1,3'-dithiopropionate (sulfo-sADP), sulfosuccinimidyl 4-(p-azidophenyl)butyrate (sulfo-sAPB), sulfosuccinimidyl 2-(7-azido-4-methylcoumarin-3-acetamide)ethyl-1,3'-dithiopropionate (sAED), sulfosuccinimidyl 7-azido-4-methylcoumain-3-acetate (sulfo-sAMCA), p-nitrophenyl diazopyruvate (pNPDP), p-nitrophenyl-2-diazo-3,3,3-trifluoropropionate (PNP-DTP), sulfhydryl-reactive and photoreactive cross-linkers such as 1-(p-Azidosalicylamido)-4-(iodoacetamido)butane (AsIB), N-[4-(p-azidosalicylamido)butyl]-3'-(2'-pyridyldithio)propionamide (APDP), benzophenone-4-iodoacetamide, benzophenone-4-maleimide carbonyl-reactive and photoreactive cross-linkers such as p-azidobenzoyl hydrazide (ABH), carboxylate-reactive and photoreactive cross-linkers such as 4-(p-azidosalicylamido)butylamine (AsBA), and arginine-reactive and photoreactive cross-linkers such as p-azidophenyl glyoxal (APG).

[0329] In some embodiments, the reactive functional group comprises a nucleophilic group that is reactive to an electrophilic group present on a binding moiety (e.g., on a conjugating moiety or on IL-2). Exemplary electrophilic groups include carbonyl groups—such as aldehyde, ketone, carboxylic acid, ester, amide, enone, acyl halide or acid anhydride. In some embodiments, the reactive functional group is aldehyde. Exemplary nucleophilic groups include hydrazide, oxime, amino, hydrazine, thiosemicarbazone, hydrazine carboxylate, and arylhydrazide. In some

embodiments, an unnatural amino acid incorporated into an interleukin described herein comprises an electrophilic group.

[0330] In some embodiments, the linker is a cleavable linker. In some embodiments, the linker is a non-cleavable linker. In some embodiments, the non-cleavable linker is a dipeptide linker. In some embodiments, the cleavable linker is a dipeptide linker. In some embodiments, the dipeptide linker is valine-citrulline (Val-Cit), phenylalanine-lysine (Phe-Lys), valine-alanine (Val-Ala) and valine-lysine (Val-Lys). In some embodiments, the dipeptide linker is valine-citrulline.

[0331] In some embodiments, the linker is a peptide linker comprising, e.g., at least 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 20, 25, 30, 35, 40, 45, 50, or more amino acids. In some embodiments, the peptide linker comprises at most 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 20, 25, 30, 35, 40, 45, 50, or less amino acids. In additional cases, the peptide linker comprises about 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 15, 20, 25, 30, 35, 40, 45, or 50 amino acids.

[0332] In some embodiments, the linker comprises a self-immolative linker moiety. In some embodiments, the self-immolative linker moiety comprises p-aminobenzyl alcohol (PAB), p-aminobenzyloxycarbonyl (PABC), or derivatives or analogs thereof. In some embodiments, the linker comprises a dipeptide linker moiety and a self-immolative linker moiety. In some embodiments, the self-immolative linker moiety is such as described in U.S. Patent No. 9089614 and WIPO Application No. WO2015038426.

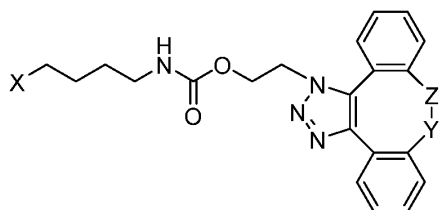
[0333] In some embodiments, the cleavable linker is glucuronide. In some embodiments, the cleavable linker is an acid-cleavable linker. In some embodiments, the acid-cleavable linker is hydrazine. In some embodiments, the cleavable linker is a reducible linker.

[0334] In some embodiments, the linker comprises a maleimide group. In some embodiments, the maleimide group is also referred to as a maleimide spacer. In some embodiments, the maleimide group further comprises a caproic acid, forming maleimidocaproyl (mc). In some embodiments, the linker comprises maleimidocaproyl (mc). In some embodiments, linker is maleimidocaproyl (mc). In other instances, the maleimide group comprises a maleimidomethyl group, such as succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sMCC) or sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (sulfo-sMCC) described above.

[0335] In some embodiments, the maleimide group is a self-stabilizing maleimide. In some embodiments, the self-stabilizing maleimide utilizes diaminopropionic acid (DPR) to incorporate a basic amino group adjacent to the maleimide to provide intramolecular catalysis of thiosuccinimide ring hydrolysis, thereby eliminating maleimide from undergoing an elimination

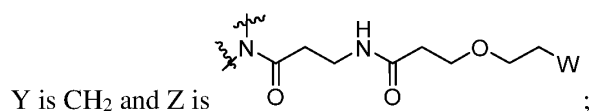
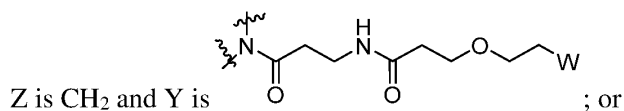
reaction through a retro-Michael reaction. In some embodiments, the self-stabilizing maleimide is a maleimide group described in Lyon, et al., “Self-hydrolyzing maleimides improve the stability and pharmacological properties of antibody-drug conjugates,” Nat. Biotechnol. 32(10):1059-1062 (2014). In some embodiments, the linker comprises a self-stabilizing maleimide. In some embodiments, the linker is a self-stabilizing maleimide.

[0336] Described herein are IL-2 conjugates having the structure of Formula (I):



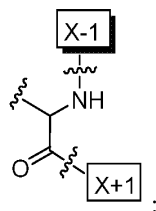
Formula (I);

wherein:



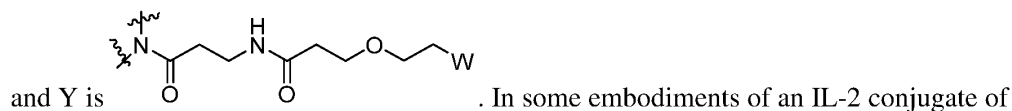
W is a PEG group having an average molecular weight selected from 5kDa, 10kDa, 15kDa, 20kDa, 25kDa, 30kDa, 35kDa, 40kDa, 45kDa, and 50kDa; and

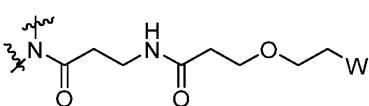
X has the structure:

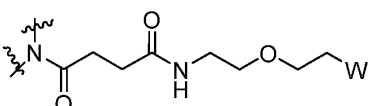


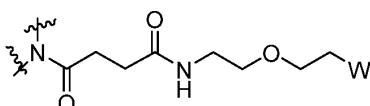
X-1 indicates the point of attachment to the preceding amino acid residue; and

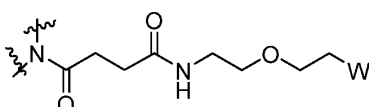
[0337] X+1 indicates the point of attachment to the following amino acid residue. In some embodiments, X is an amino acid position of a recombinant human IL-2, wherein the amino acid position is in reference to the positions in SEQ ID NO: 1; or a pharmaceutically acceptable salt, solvate, or hydrate thereof. In some embodiments of an IL-2 conjugate of Formula (I), Z is CH₂



Formula (I), Y is CH₂ and Z is . Further provided herein are IL-2

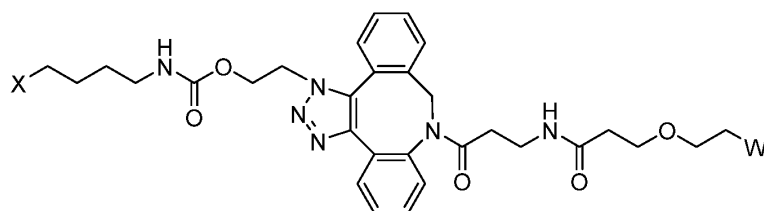
conjugates wherein Z is CH₂ and Y is , or a pharmaceutically acceptable salt, solvate, or hydrate thereof. Further provided herein are IL-2 conjugates wherein

Z is CH₂ and Y is , and W is a PEG group having an average molecular weight selected from 15kDa, 20kDa, 25kDa, 30kDa, 35kDa, 40kDa, 45kDa, 50kDa, 55 kDa, and 60kDa. Further provided herein are IL-2 conjugates wherein Y is CH₂ and Z is

. In some embodiments of an IL-2 conjugate of Formula (I), the PEG group has an average molecular weight selected from 5kDa, 10kDa, 30kDa, 40kDa, 45 kDa, 50kDa, 55kDa, and 60kDa. Here and throughout, embodiments of Z and Y also encompass a pharmaceutically acceptable salt, solvate, or hydrate thereof. Further provided herein are IL-2 conjugates wherein the PEG group has an average molecular weight of 30kDa. Further provided herein are IL-2 conjugates wherein the PEG group has an average molecular weight of 35kDa. Further provided herein are IL-2 conjugates wherein the PEG group has an average molecular weight of 40kDa. Further provided herein are IL-2 conjugates wherein the PEG group has an average molecular weight of 45kDa. Further provided herein are IL-2 conjugates wherein the PEG group has an average molecular weight of 50kDa. Further provided herein are IL-2 conjugates wherein the PEG group has an average molecular weight of 55kDa. Further provided herein are IL-2 conjugates wherein the PEG group has an average molecular weight of 60kDa. Further provided herein are IL-2 conjugates wherein the position of the structure of Formula (I) in the amino acid sequence of the IL-2 conjugate is selected from P1, T2, S3, S4, S5, T6, K7, K8, Q10, L11, E14, H15, L17, L18, D19, Q21, M22, N25, G26, N28, N29, Y30, K31, K34, T36, M45, P46, K47, A49, T50, E51, L52, K53, H54, Q56, E59, E66, N70, Q73, S74, K75, N76, F77, H78, R80, P81, R82, D83, S86, N87, I88, V90, I91, L93, E94, K96, G97, S98, E99, T100, T101, F102, M103, C104, E105, Y106, A107, D108, E109, T110, A111, T112, E115, N118, R119, T122, F123, S124, Q125, S126, S129, T130, L131, and T132. Further described herein are IL-2 conjugates, wherein the position of the structure of Formula (VI) or (VII), or a mixture of (VI) and (VII), in the amino acid sequence of the IL-2 conjugate is selected from K8, L11, E14, H15,

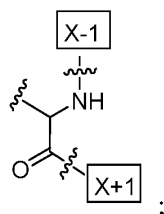
L18, D19, M22, N87, E99, or D108. In some embodiments of an IL-2 conjugate of Formula (I), X is selected from P1, T2, S3, S4, S5, T6, K7, K8, Q10, L11, E14, H15, L17, L18, D19, Q21, M22, N25, G26, N28, N29, Y30, K31, K34, T36, M45, P46, K47, A49, T50, E51, L52, K53, H54, Q56, E59, E66, N70, Q73, S74, K75, N76, F77, H78, R80, P81, R82, D83, S86, N87, I88, V90, I91, L93, E94, K96, G97, S98, E99, T100, T101, F102, M103, C104, E105, Y106, A107, D108, E109, T110, A111, T112, E115, N118, R119, T122, F123, S124, Q125, S126, S129, T130, L131, and T132. In some embodiments of an IL-2 conjugate of Formula (I), X is selected from K8, L11, E14, H15, L18, D19, M22, N87, E99, and D108. In some embodiments an IL-2 conjugate of Formula (I) comprises the sequence of any one of SEQ ID NOs: 2-14 or a sequence having at least 80% sequence identity to SEQ ID NO: 1. In some embodiments an IL-2 conjugate of Formula (I) comprises the sequence of SEQ ID NO: 1.

[0338] Described herein are IL-2 conjugates having the structure of Formula (II):



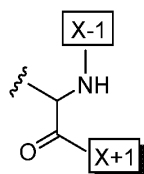
Formula (II);

wherein W is a PEG group having an average molecular weight selected from 5kDa, 10kDa, 15kDa, 20kDa, 25kDa, 30kDa, 35kDa, 40kDa, 45kDa, 50kDa, 55kDa, and 60kDa; and X has the structure:



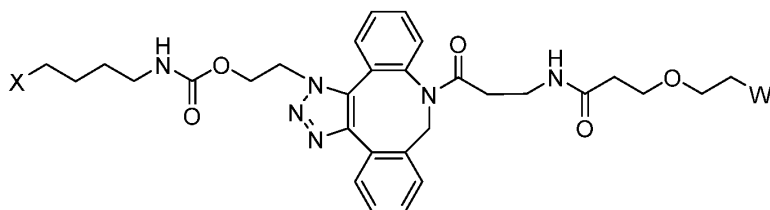
X-1 indicates the point of attachment to the preceding amino acid residue; and

X+1 indicates the point of attachment to the following amino acid residue. In some embodiments, X is an amino acid position having the structure:



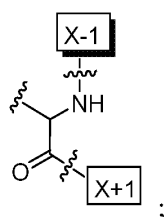
of a recombinant human IL-2 selected from K8, L11, E14, H15, L18, D19, M22, N87, E99, or D108, wherein the amino acid position is in reference to the positions of the sequence of SEQ ID NO: 1.

[0339] Described herein are IL-2 conjugates having the structure of Formula (III):



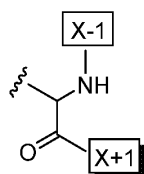
Formula (III);

wherein W is a PEG group having an average molecular weight selected from 5kDa, 10kDa, 15kDa, 20kDa, 25kDa, 30kDa, 35kDa, 40kDa, 45kDa, 50kDa, 55kDa, and 60kDa; and X has the structure:



X-1 indicates the point of attachment to the preceding amino acid residue; and

X+1 indicates the point of attachment to the following amino acid residue. In some embodiments, X is an amino acid position having the structure:



of a recombinant human IL-2 selected from K8, L11, E14, H15, L18, D19, M22, N87, E99, or D108, wherein the amino acid is in reference to the positions of the sequence of SEQ ID NO: 1.

[0340] Described herein are IL-2 conjugates comprising the amino acid sequence of any one of SEQ ID NOS: 2-14 or a sequence having at least 80% sequence identity to SEQ ID NO: 1, wherein [AzK_PEG50kDa] has the structure of Formula (II) or Formula (III), or a mixture of Formula (II) and Formula (III). Further described herein are IL-2 conjugates wherein the ratio of the amount of the structure of Formula (II) to the amount of the structure of Formula (III) comprising the total amount of [AzK_PEG50kDa] in the IL-2 conjugate is about 1:1. Further described herein are IL-2 conjugates wherein the ratio of the amount of the structure of Formula (II) to the amount of the structure of Formula (III) comprising the total amount of [AzK_PEG50kDa] in the IL-2 conjugate is greater than 1:1. Further described herein are IL-2 conjugates wherein the ratio of the amount of the structure of Formula (II) to the amount of the structure of Formula (III) comprising the total amount of [AzK_PEG50kDa] in the IL-2

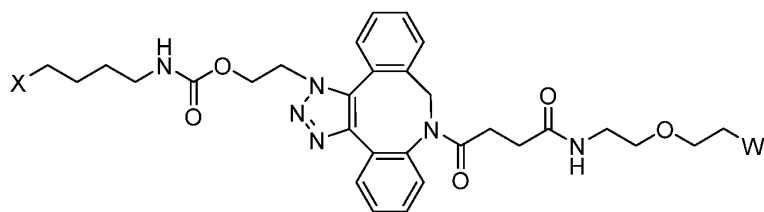
conjugate is less than 1:1. Further described herein are IL-2 conjugates wherein the ratio of the amount of the structure of Formula (II) to the amount of the structure of Formula (III) comprising the total amount of [AzK_PEG30kDa] in the IL-2 conjugate is about 1:1. Further described herein are IL-2 conjugates wherein the ratio of the amount of the structure of Formula (II) to the amount of the structure of Formula (III) comprising the total amount of [AzK_PEG30kDa] in the IL-2 conjugate is greater than 1:1. Further described herein are IL-2 conjugates wherein the ratio of the amount of the structure of Formula (II) to the amount of the structure of Formula (III) comprising the total amount of [AzK_PEG30kDa] in the IL-2 conjugate is less than 1:1.

[0341] In some embodiments, the IL-2 conjugate is a pharmaceutically acceptable salt, solvate, or hydrate thereof. Here and throughout, embodiments of Formula (II) and/or (III) also encompass a pharmaceutically acceptable salt, solvate, or hydrate thereof. Further described herein are IL-2 conjugates wherein the [AzK_PEG] is a mixture of Formula (II) and Formula (III). In some embodiments of an IL-2 conjugate of Formula (II) and/or Formula (III), X is selected from P1, T2, S3, S4, S5, T6, K7, K8, Q10, L11, E14, H15, L17, L18, D19, Q21, M22, N25, G26, N28, N29, Y30, K31, K34, T36, M45, P46, K47, A49, T50, E51, L52, K53, H54, Q56, E59, E66, N70, Q73, S74, K75, N76, F77, H78, R80, P81, R82, D83, S86, N87, I88, V90, I91, L93, E94, K96, G97, S98, E99, T100, T101, F102, M103, C104, E105, Y106, A107, D108, E109, T110, A111, T112, E115, N118, R119, T122, F123, S124, Q125, S126, S129, T130, L131, and T132 of recombinant human IL-2. In some embodiments of an IL-2 conjugate of Formula (II) and/or Formula (III), X is selected from K8, L11, E14, H15, L18, D19, M22, N87, E99, and D108 of recombinant human IL-2. In some embodiments of an IL-2 conjugate of Formula (II) and/or Formula (III), the PEG group has an average molecular weight of about 10 kDa, about 20 kDa, about 30 kDa, about 35 kDa, about 50 kDa, about 60 kDa, and X is selected from K8, L11, E14, H15, L18, D19, M22, N87, E99, and D108 of recombinant human IL-2.

[0342] Further described herein are IL-2 conjugates wherein the IL-2 conjugate has the amino acid sequence of any one of SEQ ID NOS: 2-14 or a sequence having at least 80% sequence identity to SEQ ID NO: 1. Further described herein are IL-2 conjugates wherein W is a PEG group having an average molecular weight selected from 5kDa, 10kDa, 15kDa, 20kDa, 25kDa, 30kDa, 35kDa, 40kDa, 45kDa, 50kDa, 55kDa, or 60kDa. Further described herein are IL-2 conjugates wherein W is a PEG group having an average molecular weight selected from 25kDa, 30kDa, 35kDa, 40kDa, 45kDa, 50kDa, 55kDa, or 60kDa. Further described herein are IL-2 conjugates wherein W is a PEG group having an average molecular weight of 30kDa. Further provided herein are IL-2 conjugates wherein the PEG group has an average molecular weight of

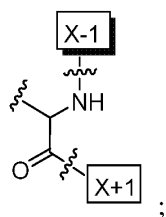
35kDa. Further provided herein are IL-2 conjugates wherein the PEG group has an average molecular weight of 40kDa. Further provided herein are IL-2 conjugates wherein the PEG group has an average molecular weight of 45kDa. Further provided herein are IL-2 conjugates wherein the PEG group has an average molecular weight of 50kDa. Further provided herein are IL-2 conjugates wherein the PEG group has an average molecular weight of 55kDa. Further provided herein are IL-2 conjugates wherein the PEG group has an average molecular weight of 60kDa. Further described herein are IL-2 conjugates wherein the IL-2 conjugate has the amino acid sequence of any one of SEQ ID NOS: 2-14 or a sequence having at least 80% sequence identity to SEQ ID NO: 1. Further described herein are IL-2 conjugates wherein the IL-2 conjugate has the amino acid sequence of SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14. Further described herein are IL-2 conjugates wherein W is a PEG group having an average molecular weight selected from 5kDa, 10kDa, 15kDa, 20kDa, 25kDa, 30kDa, 35kDa, 40kDa, 45kDa, 50kDa, 55kDa, or 60kDa. Further described herein are IL-2 conjugates wherein W is a PEG group having an average molecular weight selected from 50kDa and 30kDa.

[0343] Described herein are IL-2 conjugates having the structure of Formula (IV):



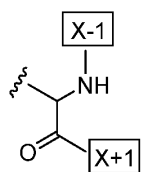
Formula (IV);

wherein W is a PEG group having an average molecular weight selected from 5kDa, 10kDa, 15kDa, 20kDa, 25kDa, 30kDa, 35kDa, 40kDa, 45kDa, 50kDa, 55 kDa, and 60kDa; and X has the structure:



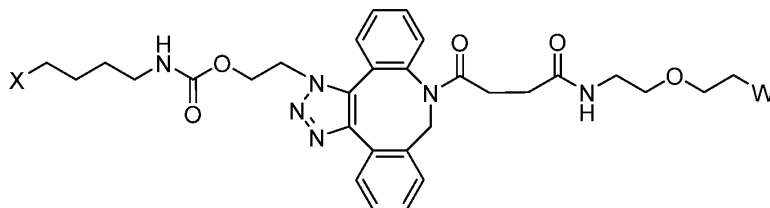
X-1 indicates the point of attachment to the preceding amino acid residue; and

X+1 indicates the point of attachment to the following amino acid residue. In some embodiments, X is an amino acid position having the structure:



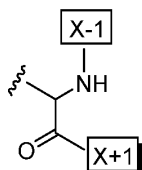
of a recombinant human IL-2 selected from K8, L11, E14, H15, L18, D19, M22, N87, E99, or D108, wherein the amino acid position is in reference to the positions of the sequence of SEQ ID NO: 1.

Described herein are IL-2 conjugates having the structure of Formula (V):

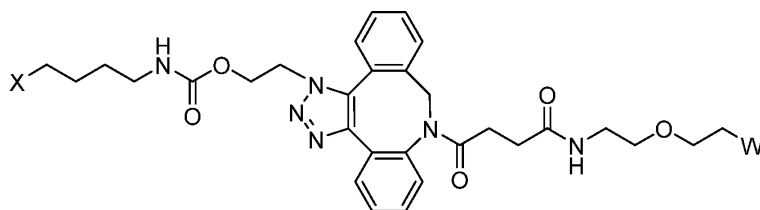


Formula (V);

wherein W is a PEG group having an average molecular weight selected from 5kDa, 10kDa, 15kDa, 20kDa, 25kDa, 30kDa, 35kDa, 40kDa, 45kDa, 50kDa, 55 kDa, and 60kDa; and X is an amino acid position having the structure



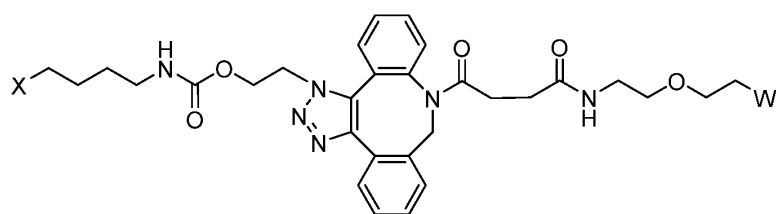
[0344] of a recombinant human IL-2 selected from K8, L11, E14, H15, L18, D19, M22, N87, E99, or D108, wherein the amino acid is in reference to the positions of the sequence of SEQ ID NO: 1. Here and throughout, embodiments of Formula (IV) and/or (V) also encompass a pharmaceutically acceptable salt, solvate, or hydrate thereof. Further described herein are IL-2 conjugates wherein the [AzK_L1_PEG] is a mixture of Formula (IV) and Formula (V). Further described herein are IL-2 conjugates wherein the [AzK_L1_PEG] has the structure of Formula (IV):



Formula (IV)

[0345] Here and throughout, the structure of Formula (IV) encompasses pharmaceutically acceptable salts, solvates, or hydrates thereof. Further described herein are IL-2 conjugates wherein the IL-2 conjugate has the amino acid sequence of any one of SEQ ID NOs: 2-14 or a

sequence having at least 80% sequence identity to SEQ ID NO: 1. Further described herein are IL-2 conjugates wherein W is a PEG group having an average molecular weight selected from 5kDa, 10kDa, 15kDa, 20kDa, 25kDa, 30kDa, 35kDa, 40kDa, 45kDa, 50kDa, 55kDa, or 60kDa. Further described herein are IL-2 conjugates wherein W is a PEG group having an average molecular weight selected from 50kDa and 30kDa. Further described herein are IL-2 conjugates wherein W is a PEG group having an average molecular weight of 5kDa. Further described herein are IL-2 conjugates wherein W is a PEG group having an average molecular weight of 30kDa. Further described herein are IL-2 conjugates wherein the IL-2 conjugate has the amino acid sequence of SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13 or 14. Further described herein are IL-2 conjugates wherein W is a PEG group having an average molecular weight selected from 5kDa, 10kDa, 15kDa, 20kDa, 25kDa, 30kDa, 35kDa, 40kDa, 45kDa, 50kDa, 55kDa, or 60kDa. Further described herein are IL-2 conjugates wherein W is a PEG group having an average molecular weight selected from 5kDa and 30kDa. Further described herein are IL-2 conjugates wherein W is a PEG group having an average molecular weight of 30 kDa. Further provided herein are IL-2 conjugates wherein the PEG group has an average molecular weight of 35 kDa. Further provided herein are IL-2 conjugates wherein the PEG group has an average molecular weight of 40kDa. Further provided herein are IL-2 conjugates wherein the PEG group has an average molecular weight of 45kDa. Further provided herein are IL-2 conjugates wherein the PEG group has an average molecular weight of 50kDa. Further provided herein are IL-2 conjugates wherein the PEG group has an average molecular weight of 55kDa. Further provided herein are IL-2 conjugates wherein the PEG group has an average molecular weight of 60kDa. Further described herein are IL-2 conjugates wherein the [AzK_L1_PEG] has the structure of Formula (V)



Formula (V)

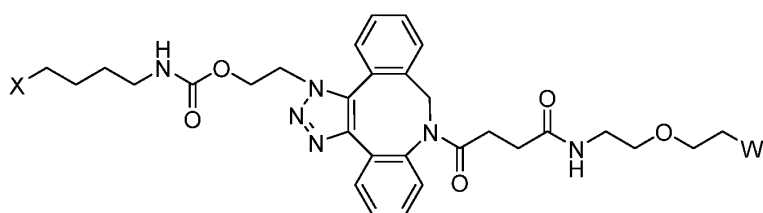
[0346] Here and throughout, the structure of Formula (V) encompasses pharmaceutically acceptable salts, solvates, or hydrates thereof. Further described herein are IL-2 conjugates wherein the IL-2 conjugate has the amino acid sequence of any one of SEQ ID NOS: 2-14 or a sequence having at least 80% sequence identity to SEQ ID NO: 1. Further described herein are IL-2 conjugates wherein W is a PEG group having an average molecular weight selected from

5kDa, 10kDa, 15kDa, 20kDa, 25kDa, 30kDa, 35kDa, 40kDa, 45kDa, 50kDa, 55kDa, or 60kDa. Further described herein are IL-2 conjugates wherein W is a PEG group having an average molecular weight selected from 50kDa and 30kDa. Further described herein are IL-2 conjugates wherein W is a PEG group having an average molecular weight of 30 kDa. Further provided herein are IL-2 conjugates wherein the PEG group has an average molecular weight of 35 kDa. Further provided herein are IL-2 conjugates wherein the PEG group has an average molecular weight of 40kDa. Further provided herein are IL-2 conjugates wherein the PEG group has an average molecular weight of 45kDa. Further provided herein are IL-2 conjugates wherein the PEG group has an average molecular weight of 50kDa. Further provided herein are IL-2 conjugates wherein the PEG group has an average molecular weight of 55kDa. Further provided herein are IL-2 conjugates wherein the PEG group has an average molecular weight of 60kDa. Further described herein are IL-2 conjugates wherein the IL-2 conjugate has the amino acid sequence of SEQ ID NO: 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, or 14.

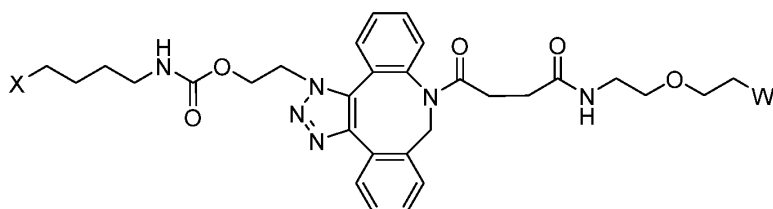
[0347] Further described herein are IL-2 conjugates wherein the ratio of the amount of the structure of Formula (IV) to the amount of the structure of Formula (V) comprising the total amount of [AzK_L1_PEG] in the IL-2 conjugate is about 1:1. Further described herein are IL-2 conjugates wherein the ratio of the amount of the structure of Formula (IV) to the amount of the structure of Formula (V) comprising the total amount of [AzK_L1_PEG] in the IL-2 conjugate is greater than 1:1. Further described herein are IL-2 conjugates wherein the ratio of the amount of the structure of Formula (IV) to the amount of the structure of Formula (V) comprising the total amount of [AzK_L1_PEG] in the IL-2 conjugate is less than 1:1. Further described herein are IL-2 conjugates wherein W is a linear or branched PEG group. Further described herein are IL-2 conjugates wherein W is a linear PEG group. Further described herein are IL-2 conjugates wherein W is a branched PEG group. Further described herein are IL-2 conjugates wherein W is a methoxy PEG group. Further described herein are IL-2 conjugates wherein the methoxy PEG group is linear or branched. Further described herein are IL-2 conjugates wherein the methoxy PEG group is linear. Further described herein are IL-2 conjugates wherein the methoxy PEG group is branched.

Described herein are IL-2 conjugates comprising the amino acid sequence of any one of SEQ ID NOs: 2-14 or a sequence having at least 80% sequence identity to SEQ ID NO: 1

[AzK_L1_PEG50kDa] has the structure of Formula (IV) or Formula (V), or a mixture of Formula (IV) and Formula (V):



Formula (IV);

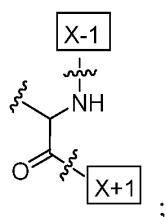


Formula (V);

wherein:

W is a PEG group having an average molecular weight of 50kDa; and

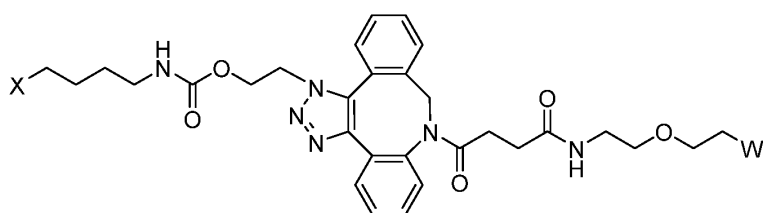
X has the structure:



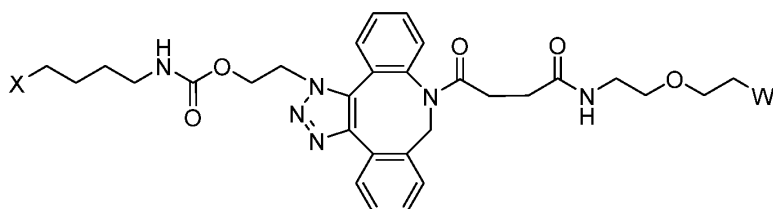
X-1 indicates the point of attachment to the preceding amino acid residue; and

X+1 indicates the point of attachment to the following amino acid residue.

Further described herein are IL-2 conjugates wherein the ratio of the amount of the structure of Formula (IV) to the amount of the structure of Formula (V) comprising the total amount of [AzK_L1_PEG50kDa] in the IL-2 conjugate is about 1:1. Further described herein are IL-2 conjugates wherein the ratio of the amount of the structure of Formula (IV) to the amount of the structure of Formula (V) comprising the total amount of [AzK_L1_PEG50kDa] in the IL-2 conjugate is greater than 1:1. Further described herein are IL-2 conjugates wherein the ratio of the amount of the structure of Formula (IV) to the amount of the structure of Formula (V) comprising the total amount of [AzK_L1_PEG50kDa] in the IL-2 conjugate is less than 1:1. Described herein are IL-2 conjugates comprising the amino acid sequence of any one of SEQ ID NOs: 2-14 or a sequence having at least 80% sequence identity to SEQ ID NO: 1 [AzK_L1_PEG30kDa] has the structure of Formula (IV) or Formula (V), or is a mixture of the structures of Formula (IV) and Formula (V):



Formula (IV);

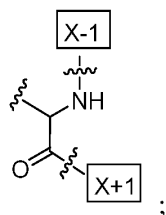


Formula (V);

wherein:

W is a PEG group having an average molecular weight of 30kDa; and

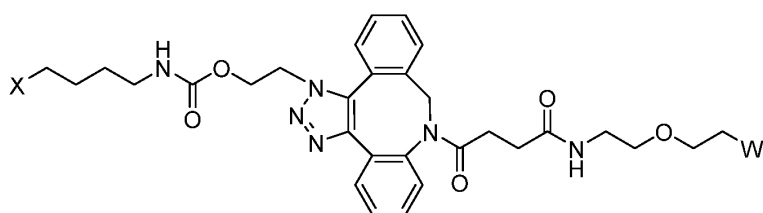
X has the structure:



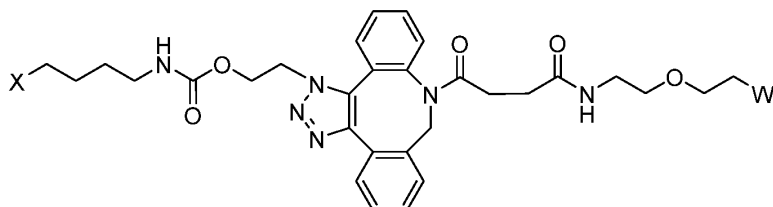
X-1 indicates the point of attachment to the preceding amino acid residue; and

X+1 indicates the point of attachment to the following amino acid residue.

[0348] Further described herein are IL-2 conjugates wherein the ratio of the amount of the structure of Formula (IV) to the amount of the structure of Formula (V) comprising the total amount of [AzK_L1_PEG30kDa] in the IL-2 conjugate is about 1:1. Further described herein are IL-2 conjugates wherein the ratio of the amount of the structure of Formula (IV) to the amount of the structure of Formula (V) comprising the total amount of [AzK_L1_PEG30kDa] in the IL-2 conjugate is greater than 1:1. Further described herein are IL-2 conjugates wherein the ratio of the amount of the structure of Formula (IV) to the amount of the structure of Formula (V) comprising the total amount of [AzK_L1_PEG30kDa] in the IL-2 conjugate is less than 1:1. Described herein are IL-2 conjugates comprising the amino acid sequence of any one of SEQ ID NOS: 2-14 or a sequence having at least 80% sequence identity to SEQ ID NO: 1 [Azk_L1_PEG] is a mixture of the structures of Formula (IV) and Formula (V):



Formula (IV);

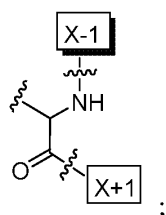


Formula (V);

wherein:

W is a PEG group having an average molecular weight selected from 5kDa, 10kDa, 15kDa, 20kDa, 25kDa, 30kDa, 35kDa, 40kDa, 45kDa, 50kDa, 55 kDa, and 60kDa; and

X has the structure:



X-1 indicates the point of attachment to the preceding amino acid residue; and

X+1 indicates the point of attachment to the following amino acid residue.

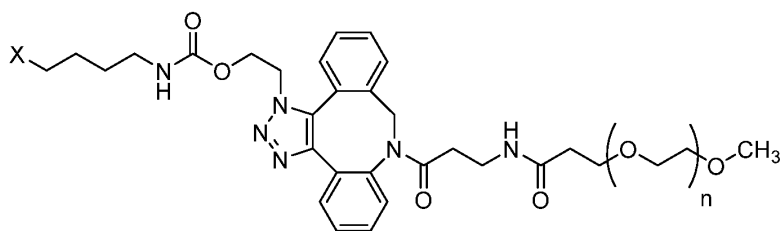
[0349] In some embodiments, the IL-2 conjugate is a pharmaceutically acceptable salt, solvate, or hydrate thereof. Further described herein are IL-2 conjugates wherein the ratio of the amount of the structure of Formula (IV) to the amount of the structure of Formula (V) comprising the total amount of [AzK_L1_PEG] in the IL-2 conjugate is about 1:1. Further described herein are IL-2 conjugates wherein the ratio of the amount of the structure of Formula (IV) to the amount of the structure of Formula (V) comprising the total amount of [AzK_L1_PEG] in the IL-2 conjugate is greater than 1:1. Further described herein are IL-2 conjugates wherein the ratio of the amount of the structure of Formula (IV) to the amount of the structure of Formula (V) comprising the total amount of [AzK_L1_PEG] in the IL-2 conjugate is less than 1:1. Further described herein are IL-2 conjugates wherein W is a linear or branched PEG group. Further described herein are IL-2 conjugates wherein W is a linear PEG group. Further described herein are IL-2 conjugates wherein W is a branched PEG group. Further described herein are IL-2

conjugates wherein W is a methoxy PEG group. Further described herein are IL-2 conjugates wherein the methoxy PEG group is linear or branched. Further described herein are IL-2 conjugates wherein the methoxy PEG group is linear. Further described herein are IL-2 conjugates wherein the methoxy PEG group is branched.

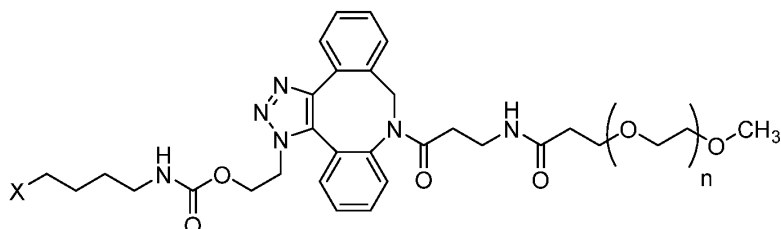
[0350] In some embodiments of an IL-2 conjugate of Formula (IV) and/or Formula (V), X is selected from P1, T2, S3, S4, S5, T6, K7, K8, Q10, L11, E14, H15, L17, L18, D19, Q21, M22, N25, G26, N28, N29, Y30, K31, K34, T36, M45, P46, K47, A49, T50, E51, L52, K53, H54, Q56, E59, E66, N70, Q73, S74, K75, N76, F77, H78, R80, P81, R82, D83, S86, N87, I88, V90, I91, L93, E94, K96, G97, S98, E99, T100, T101, F102, M103, C104, E105, Y106, A107, D108, E109, T110, A111, T112, E115, N118, R119, T122, F123, S124, Q125, S126, S129, T130, L131, and T132 of recombinant human IL-2. In some embodiments of an IL-2 conjugate of Formula (IV) and/or Formula (V), X is selected from K8, L11, E14, H15, L18, D19, M22, N87, E99, and D108 of recombinant human IL-2. In some embodiments of an IL-2 conjugate of Formula (IV) and/or Formula (V), the PEG group has an average molecular weight of about 10 kDa, about 20 kDa, about 30 kDa, about 35 kDa, about 50 kDa, about 60 kDa, and X is selected from K8, L11, E14, H15, L18, D19, M22, N87, E99, and D108 of recombinant human IL-2.

[0351] In some embodiments an IL-2 conjugate of Formula (IV) or Formula (V) comprises the sequence of any one of SEQ ID NOs: 2-14 or a sequence having at least 80% sequence identity to SEQ ID NO: 1.

[0352] Described herein are IL-2 conjugates comprising the amino acid sequence of SEQ ID NO: 1 in which at least one amino acid residue in the IL-2 conjugate is replaced by the structure of Formula (VI) or (VII), or a mixture of (VI) and (VII):



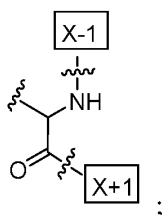
Formula (VI);



Formula (VII);

wherein:

n is an integer in the range from about 2 to about 5000; and

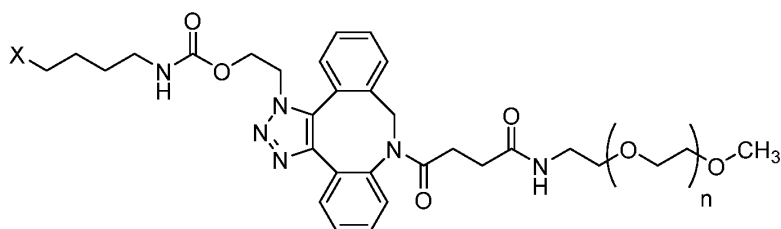


X has the structure:

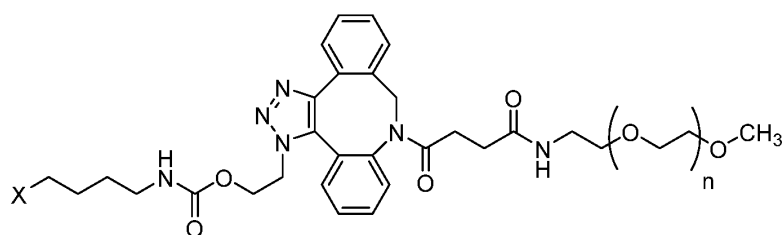
X-1 indicates the point of attachment to the preceding amino acid residue; and

[0353] X+1 indicates the point of attachment to the following amino acid residue. In some embodiments, the IL-2 conjugate is a pharmaceutically acceptable salt, solvate, or hydrate thereof. Here and throughout, embodiments of Formula (VI) and/or (VII) also encompass a pharmaceutically acceptable salt, solvate, or hydrate thereof. Further described herein are IL-2 conjugates wherein the position of the structure of Formula (VI) or (VII), or a mixture of (VI) and (VII), in the amino acid sequence of the IL-2 conjugate is selected from P1, T2, S3, S4, S5, T6, K7, K8, Q10, L11, E14, H15, L17, L18, D19, Q21, M22, N25, G26, N28, N29, Y30, K31, K34, T36, M45, P46, K47, A49, T50, E51, L52, K53, H54, Q56, E59, E66, N70, Q73, S74, K75, N76, F77, H78, R80, P81, R82, D83, S86, N87, I88, V90, I91, L93, E94, K96, G97, S98, E99, T100, T101, F102, M103, C104, E105, Y106, A107, D108, E109, T110, A111, T112, E115, N118, R119, T122, F123, S124, Q125, S126, S129, T130, L131, and T132. Further described herein are IL-2 conjugates, wherein the position of the structure of Formula (VI) or (VII), or a mixture of (VI) and (VII), in the amino acid sequence of the IL-2 conjugate is selected from K8, L11, E14, H15, L18, D19, M22, N87, E99, or D108. Further described herein are IL-2 conjugates wherein the position of the structure of Formula (VI) or (VII), or a mixture of (VI) and (VII), in the amino acid sequence of the IL-2 conjugate is selected from H15 and L18.

[0354] Described herein are IL-2 conjugates comprising the amino acid sequence of SEQ ID NO: 1 in which at least one amino acid residue in the IL-2 conjugate is replaced by the structure of Formula (VIII) or (IX), or a mixture of (VIII) and (IX):



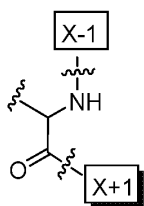
Formula (VIII);

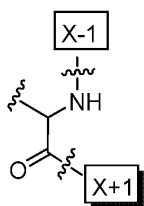


Formula (IX);

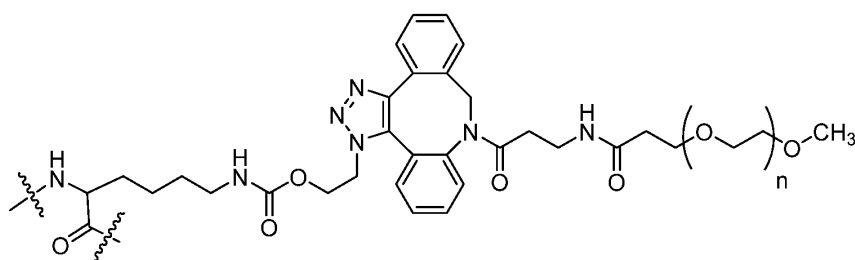
wherein:

n is an integer in the range from about 2 to about 5000; and

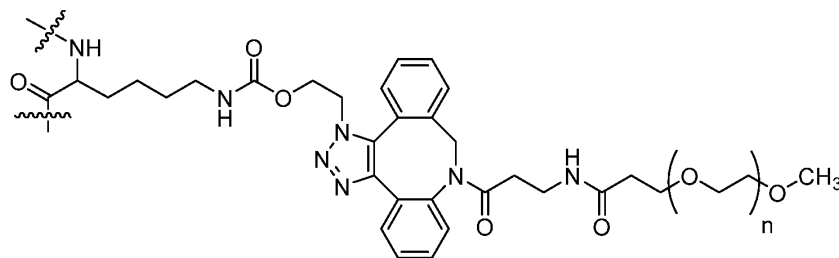


X has the structure: , X-1 indicates the point of attachment to the preceding amino acid residue; and X+1 indicates the point of attachment to the following amino acid residue. In some embodiments, the IL-2 conjugate is a pharmaceutically acceptable salt, solvate, or hydrate thereof. Here and throughout, embodiments of Formula (VIII) and/or (IX) also encompass a pharmaceutically acceptable salt, solvate, or hydrate thereof. Further described herein are IL-2 conjugates wherein the position of the structure of Formula (VIII) or (IX), or a mixture of (VIII) and (IX), in the amino acid sequence of the IL-2 conjugate is selected from P1, T2, S3, S4, S5, T6, K7, K8, Q10, L11, E14, H15, L17, L18, D19, Q21, M22, N25, G26, N28, N29, Y30, K31, K34, T36, M45, P46, K47, A49, T50, E51, L52, K53, H54, Q56, E59, E66, N70, Q73, S74, K75, N76, F77, H78, R80, P81, R82, D83, S86, N87, I88, V90, I91, L93, E94, K96, G97, S98, E99, T100, T101, F102, M103, C104, E105, Y106, A107, D108, E109, T110, A111, T112, E115, N118, R119, T122, F123, S124, Q125, S126, S129, T130, L131, and T132. Further described herein are IL-2 conjugates, wherein the position of the structure of Formula (VIII) or (IX), or a mixture of (VIII) and (IX), in the amino acid sequence of the IL-2 conjugate is selected from K8, L11, E14, H15, L18, D19, M22, N87, E99, or D108. Further described herein are IL-2 conjugates wherein the position of the structure of Formula (VIII) or (IX), or a mixture of (VIII) and (IX), in the amino acid sequence of the IL-2 conjugate is selected from H15 and L18.

[0355] Described herein are IL-2 conjugates comprising the amino acid sequence of SEQ ID NO: 1 in which at least one amino acid residue in the IL-2 conjugate is replaced by the structure of Formula (X) or (XI), or a mixture of (X) and (XI):



Formula (X);



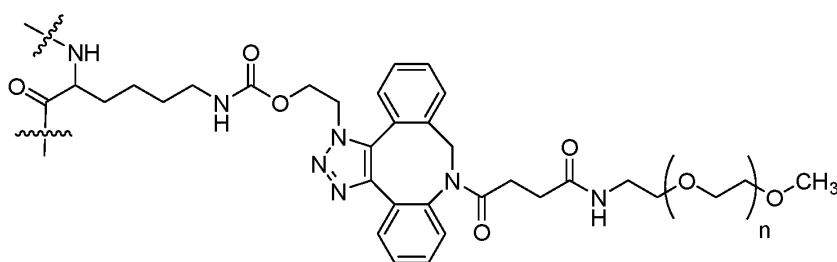
Formula (XI);

wherein:

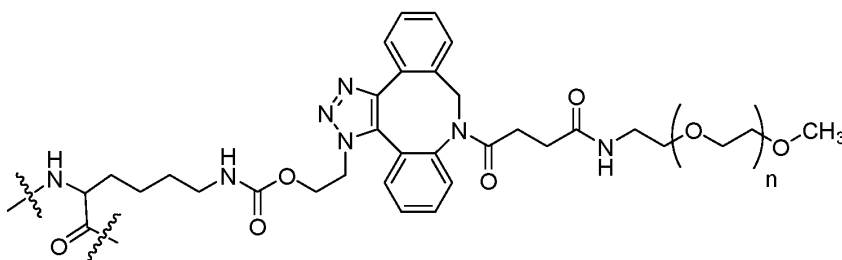
n is an integer in the range from about 2 to about 5000; and

the wavy lines indicate covalent bonds to amino acid residues within SEQ ID NO: 1 that are not replaced. Here and throughout, embodiments of Formula (X) and/or (XI) also encompass a pharmaceutically acceptable salt, solvate, or hydrate thereof. Further described herein are IL-2 conjugates wherein the position of the structure of Formula (X) or (XI), or a mixture of (X) and (XI), in the amino acid sequence of the IL-2 conjugate is selected from P1, T2, S3, S4, S5, T6, K7, K8, Q10, L11, E14, H15, L17, L18, D19, Q21, M22, N25, G26, N28, N29, Y30, K31, K34, T36, M45, P46, K47, A49, T50, E51, L52, K53, H54, Q56, E59, E66, N70, Q73, S74, K75, N76, F77, H78, R80, P81, R82, D83, S86, N87, I88, V90, I91, L93, E94, K96, G97, S98, E99, T100, T101, F102, M103, C104, E105, Y106, A107, D108, E109, T110, A111, T112, E115, N118, R119, T122, F123, S124, Q125, S126, S129, T130, L131, and T132. Further described herein are IL-2 conjugates, wherein the position of the structure of Formula (X) or (XI), or a mixture of (X) and (XI), in the amino acid sequence of the IL-2 conjugate is selected from K8, L11, E14, H15, L18, D19, M22, N87, E99, or D108. Further described herein are IL-2 conjugates wherein the position of the structure of Formula (X) or (XI), or a mixture of (X) and (XI), in the amino acid sequence of the IL-2 conjugate is selected from H15 and L18.

[0356] Described herein are IL-2 conjugates comprising the amino acid sequence of SEQ ID NO: 1 in which at least one amino acid residue in the IL-2 conjugate is replaced by the structure of Formula (XII) or (XIII), or a mixture of (XII) and (XIII):



Formula (XII);



Formula (XIII);

wherein:

n is an integer in the range from about 2 to about 5000; and

[0357] the wavy lines indicate covalent bonds to amino acid residues within SEQ ID NO: 1 that are not replaced. Here and throughout, embodiments of Formula (XII) and/or (XIII) also encompass a pharmaceutically acceptable salt, solvate, or hydrate thereof. Further described herein are IL-2 conjugates wherein the position of the structure of Formula (XII) or (XIII), or a mixture of (XII) and (XIII), in the amino acid sequence of the IL-2 conjugate is selected from P1, T2, S3, S4, S5, T6, K7, K8, Q10, L11, E14, H15, L17, L18, D19, Q21, M22, N25, G26, N28, N29, Y30, K31, K34, T36, M45, P46, K47, A49, T50, E51, L52, K53, H54, Q56, E59, E66, N70, Q73, S74, K75, N76, F77, H78, R80, P81, R82, D83, S86, N87, I88, V90, I91, L93, E94, K96, G97, S98, E99, T100, T101, F102, M103, C104, E105, Y106, A107, D108, E109, T110, A111, T112, E115, N118, R119, T122, F123, S124, Q125, S126, S129, T130, L131, and T132. Further described herein are IL-2 conjugates, wherein the position of the structure of Formula (XII) or (XIII), or a mixture of (XII) and (XIII), in the amino acid sequence of the IL-2 conjugate is selected from K8, L11, E14, H15, L18, D19, M22, N87, E99, or D108. Further described herein are IL-2 conjugates wherein the position of the structure of Formula (XII) or (XIII), or a mixture of (XII) and (XIII), in the amino acid sequence of the IL-2 conjugate is selected from H15 and L18.

[0358] Described herein are pharmaceutical compositions of Formula (I), Formula (IV), or Formula (V). In some embodiments, a pharmaceutical compositions of Formula (I), Formula

(IV), or Formula (V) comprises a sequence comprising any one of SEQ ID NOS: 2-14 or a sequence having at least 80% sequence identity to SEQ ID NO: 1. In some embodiments, a pharmaceutical compositions of Formula (I), Formula (IV), or Formula (V) comprises a sequence comprising any one of SEQ ID NO: 4. Described herein are pharmaceutical compositions of Formula (I), Formula (VI), or Formula (VII). In some embodiments, a pharmaceutical compositions of Formula (I), Formula (VI), or Formula (VII) comprises a sequence comprising any one of SEQ ID NOS: 2-14 or a sequence having at least 80% sequence identity to SEQ ID NO: 1. In some embodiments, a pharmaceutical compositions of Formula (I), Formula (VI), or Formula (VII) comprises a sequence comprising any one of SEQ ID NO: 4. Described herein are pharmaceutical compositions of Formula (I), Formula (VIII), or Formula (IX). In some embodiments, a pharmaceutical compositions of Formula (I), Formula (VIII), or Formula (IX) comprises a sequence comprising any one of SEQ ID NOS: 2-14 or a sequence having at least 80% sequence identity to SEQ ID NO: 1. In some embodiments, a pharmaceutical compositions of Formula (I), Formula (VIII), or Formula (IX) comprises a sequence comprising any one of SEQ ID NO: 4. Described herein are pharmaceutical compositions of Formula (I), Formula (X), or Formula (XI). In some embodiments, a pharmaceutical compositions of Formula (I), Formula (X), or Formula (XI) comprises a sequence comprising any one of SEQ ID NOS: 2-14 or a sequence having at least 80% sequence identity to SEQ ID NO: 1. In some embodiments, a pharmaceutical compositions of Formula (I), Formula (X), or Formula (XI) comprises a sequence comprising any one of SEQ ID NO: 4. Described herein are pharmaceutical compositions of Formula (XII), Formula (XIII), or Formula (V). In some embodiments, a pharmaceutical compositions of Formula (I), Formula (XII), or Formula (XIII) comprises a sequence comprising any one of SEQ ID NOS: 2-14 or a sequence having at least 80% sequence identity to SEQ ID NO: 1. In some embodiments, a pharmaceutical compositions of Formula (I), Formula (XII), or Formula (XIII) comprises a sequence comprising any one of SEQ ID NO: 4.

[0359] In some embodiments described herein, a conjugation reaction described herein comprises an inverse-electron demand cycloaddition reaction comprising a diene and a dienophile. In some embodiments, the diene comprises a tetrazine. In some embodiments, the dienophile comprises an alkene. In some embodiments, the dienophile comprises an alkyne. In some embodiments, the alkyne is a strained alkyne. In some embodiments, the alkene is a strained diene. In some embodiments, the alkyne is a trans-cyclooctyne. In some embodiments, the alkyne is a cyclooctene. In some embodiments, the alkene is a cyclopropene. In some embodiments, the alkene is a fluorocyclopropene. In some embodiments, a conjugation reaction

described herein results in the formation of a cytokine peptide attached to a linker or conjugation moiety via a 6-membered ring heterocycle comprising two nitrogen atoms in the ring.

[0360] In some embodiments described herein, a conjugation reaction described herein comprises an olefin metathesis reaction. In some embodiments, a conjugation reaction described herein comprises reaction of an alkene and an alkyne with a ruthenium catalyst. In some embodiments, a conjugation reaction described herein comprises reaction of two alkenes with a ruthenium catalyst. In some embodiments, a conjugation reaction described herein comprises reaction of two alkynes with a ruthenium catalyst. In some embodiments, a conjugation reaction described herein comprises reaction of an alkene or alkyne with a ruthenium catalyst and an amino acid comprising an allyl group. In some embodiments, a conjugation reaction described herein comprises reaction of an alkene or alkyne with a ruthenium catalyst and an amino acid comprising an allyl sulfide or selenide. In some embodiments, a ruthenium catalyst is Hoveyda-Grubbs 2nd generation catalyst. In some embodiments, an olefin metathesis reaction comprises reaction of one or more strained alkenes or alkynes.

[0361] In some embodiments described herein, a conjugation reaction described herein comprises a cross-coupling reaction. In some embodiments, cross-coupling reactions comprise transition metal catalysts, such as iridium, gold, ruthenium, rhodium, palladium, nickel, platinum, or other transition metal catalyst and one or more ligands. In some embodiments, transition metal catalysts are water-soluble. In some embodiments described herein, a conjugation reaction described herein comprises a Suzuki-Miyaura cross-coupling reaction. In some embodiments described herein, a conjugation reaction described herein comprises reaction of an aryl halide (or triflate, or tosylate), an aryl or alkenyl boronic acid, and a palladium catalyst. In some embodiments described herein, a conjugation reaction described herein comprises a Sonogashira cross-coupling reaction. In some embodiments described herein, a conjugation reaction described herein comprises reaction of an aryl halide (or triflate, or tosylate), an alkyne, and a palladium catalyst. In some embodiments, cross-coupling reactions result in attachment of a linker or conjugating moiety to a cytokine peptide via a carbon-carbon bond.

[0362] In some embodiments described herein, a conjugation reaction described herein comprises a deprotection or “uncaging” reaction of a reactive group prior to conjugation. In some embodiments, a conjugation reaction described herein comprises uncaging of a reactive group with light, followed by a conjugation reaction. In some embodiments, a reactive group is protected with an aralkyl moiety comprising one or more nitro groups. In some embodiments, uncaging of a reactive group results in a free amine, sulfide, or other reactive group. In some

embodiments, a conjugation reaction described herein comprises uncaging of a reactive group with a transition metal catalyst, followed by a conjugation reaction. In some embodiments, the transition metal catalyst comprises palladium and one or more ligands. In some embodiments, a reactive group is protected with an allyl moiety. In some embodiments, a reactive group is protected with an allylic carbamate. In some embodiments, a reactive group is protected with a propargylic moiety. In some embodiments, a reactive group is protected with a propargyl carbamate. In some embodiments, a reactive group is protected with a dienophile, wherein exposure to a diene (such as a tetrazine) results in deprotection of the reactive group.

[0363] In some embodiments described herein, a conjugation reaction described herein comprises a ligand-directed reaction, wherein a ligand (optionally) attached to a reactive group) facilitates the site of conjugation between the reactive group and the cytokine peptide. In some embodiments, the ligand is cleaved during or after reaction of the cytokine peptide with the reactive group. In some embodiments, the conjugation site of the cytokine peptide is an unnatural amino acid described herein. In some embodiments the reactive group comprises a leaving group, such as an electron-poor aryl or heteroaryl group. In some embodiments the reactive group comprises a leaving group, such as an electron-poor alkyl group that is displaced by the cytokine peptide. In some embodiments, a conjugation reaction described herein comprises a reaction of a radical trapping agent with a radical species. In some embodiments, a conjugation reaction described herein comprises an oxidative radical addition reaction. In some embodiments, a radical trapping agent is an arylamine. In some embodiments, a radical species is a tyrosyl radical. In some embodiments, radical species are generated by a ruthenium catalyst (such as [Ru(bpy)₃]) and light.

[0364] Enzymatic reactions are optionally used for conjugation reactions described herein. Exemplary enzymatic conjugations include SortA-mediated conjugation, a TGs-mediated conjugation, or an FGE-mediated conjugation. In some embodiments, a conjugation reaction described herein comprises native protein ligation (NPL) of a terminal 1-amino-2-thio group with a thioester to form an amide bond.

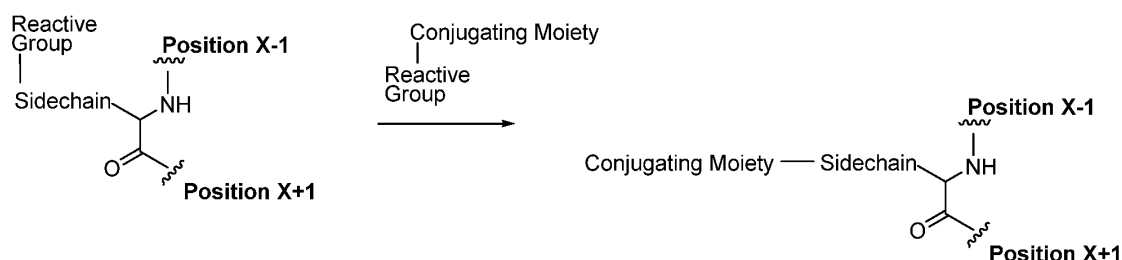
[0365] Various conjugation reactions are described herein for reacting a linker or conjugating moiety with a cytokine peptide, wherein the reaction occurs with an unnatural amino acid in the cytokine peptide. In some embodiments, a conjugation reaction comprises formation of a disulfide bond at an unnatural amino acid residue. In some embodiments, a conjugation reaction comprises a 1,4 Michael addition reaction of an unnatural amino acid. In some embodiments, a conjugation reaction comprises a cyanobenzothiazole ligation of an unnatural amino acid. In some embodiments, a conjugation reaction comprises crosslinking with an acetone moiety, such

as 1,3-dichloro-2-propionone. In some embodiments, a conjugation reaction comprises a 1,4 Michael addition to a dehydroalanine, formed by reaction of an unnatural amino acid with O-mesitylenesulfonylhydroxylamine. In some embodiments a conjugation reaction comprises reaction of an unnatural amino acid with a triazolinedione (TAD), or TAD derivative. In some embodiments a conjugation reaction comprises reaction of an unnatural amino acid with a rhodium carbenoid.

[0366] Various conjugation reactions are used to conjugate linkers, conjugation moieties, and unnatural amino acids incorporated into cytokine peptides described herein. Such conjugation reactions are often compatible with aqueous conditions, such as “bioorthogonal” reactions. In some embodiments, conjugation reactions are mediated by chemical reagents such as catalysts, light, or reactive chemical groups found on linkers, conjugation moieties, or unnatural amino acids. In some embodiments, conjugation reactions are mediated by enzymes. In some embodiments, a conjugation reaction used herein is described in Gong, Y., Pan, L. *Tett. Lett.* 2015, 56, 2123. In some embodiments, a conjugation reaction used herein is described in Chen, X.; Wu, Y-W. *Org. Biomol. Chem.* 2016, 14, 5417.

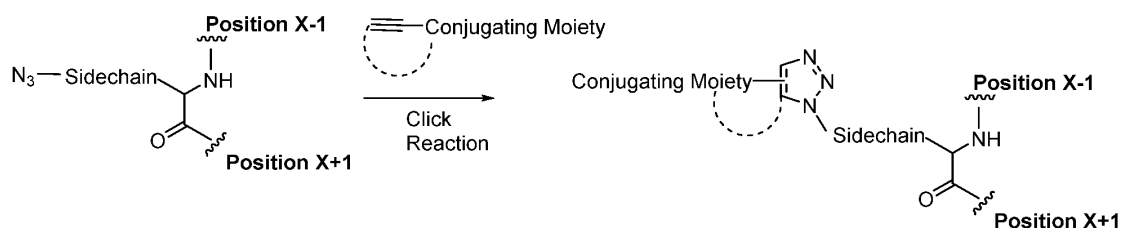
[0367] In some embodiments described herein, a conjugation reaction described herein comprises a 1,3-dipolar cycloaddition reaction. In some embodiments, the 1,3-dipolar cycloaddition reaction comprises reaction of an azide and a phosphine (“Click” reaction). In some embodiments, the conjugation reaction is catalyzed by copper. In some embodiments, a conjugation reaction described herein results in cytokine peptide comprising a linker or conjugation moiety attached via a triazole. In some embodiments, a conjugation reaction described herein comprises reaction of an azide with a strained olefin. In some embodiments, a conjugation reaction described herein comprises reaction of an azide with a strained alkyne. In some embodiments, a conjugation reaction described herein comprises reaction of an azide with a cycloalkyne, for example DBCO.

[0368] In some embodiments described herein, a conjugation reaction described herein comprises:

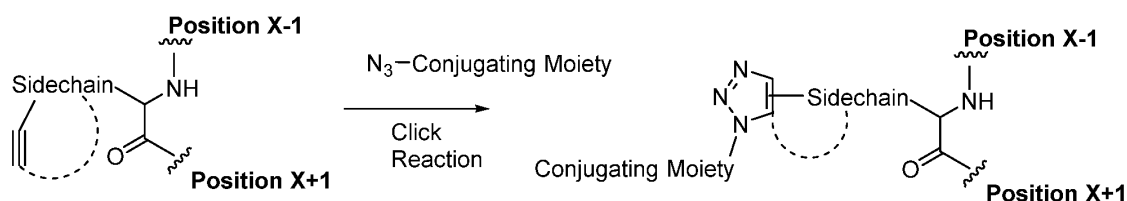


, wherein X is the position in the IL-2 conjugate comprising an unnatural amino acid, such as in

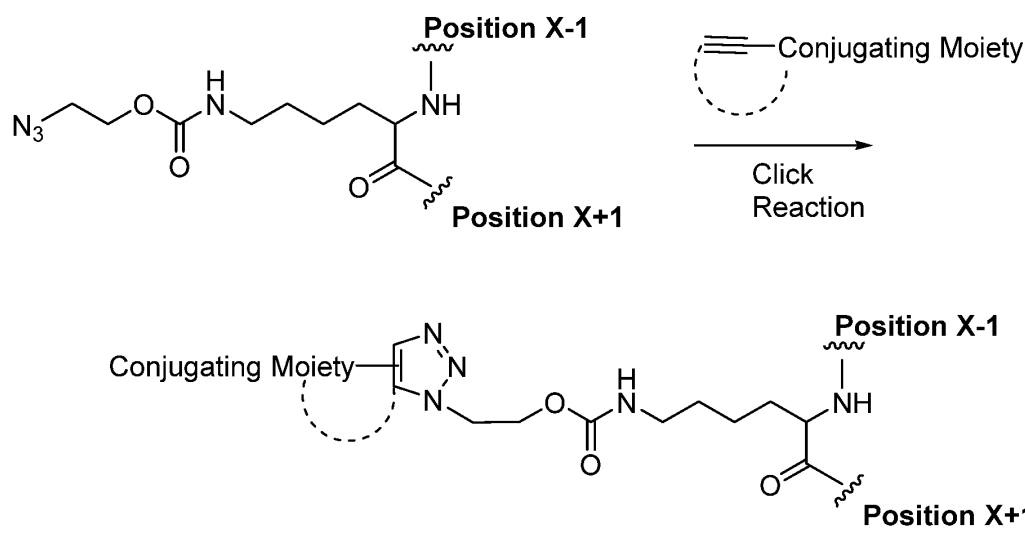
any one of SEQ ID NOS: 2 to 14 or a sequence having at least 80% sequence identity to SEQ ID NO: 1. In some embodiments, the conjugating moiety comprises water soluble polymer. In some embodiments, a reactive group comprises an alkyne or azide. In some embodiments described herein, a conjugation reaction described herein comprises:



, wherein X is the position in the IL-2 conjugate comprising an unnatural amino acid, such as in any one of SEQ ID NOS: 2 to 14 or a sequence having at least 80% sequence identity to SEQ ID NO: 1. In some embodiments described herein, a conjugation reaction described herein comprises:



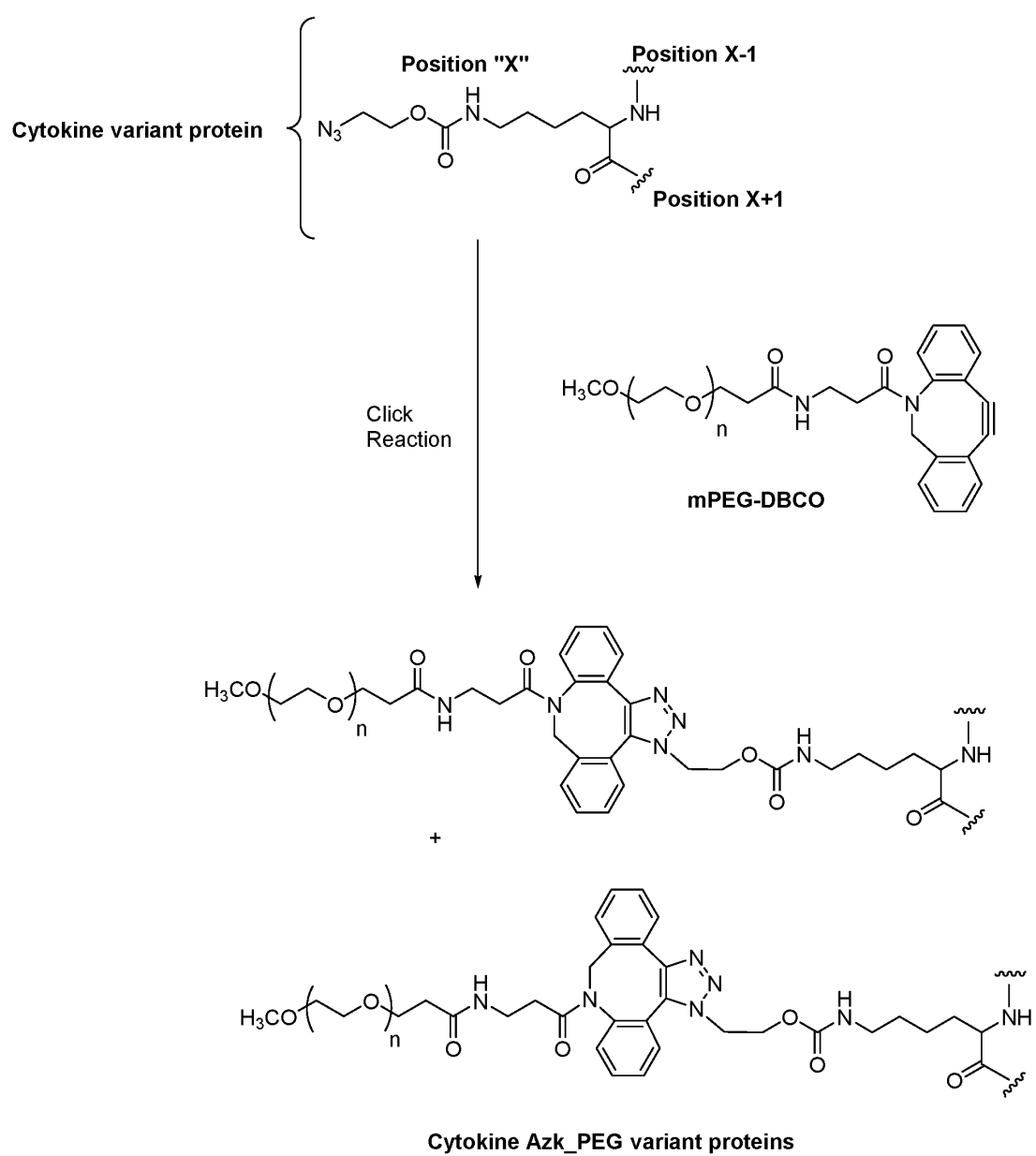
, wherein X is the position in the IL-2 conjugate comprising an unnatural amino acid, such as in any one of SEQ ID NOS: 2 to 14 or a sequence having at least 80% sequence identity to SEQ ID NO: 1. In some embodiments described herein, a conjugation reaction described herein comprises:

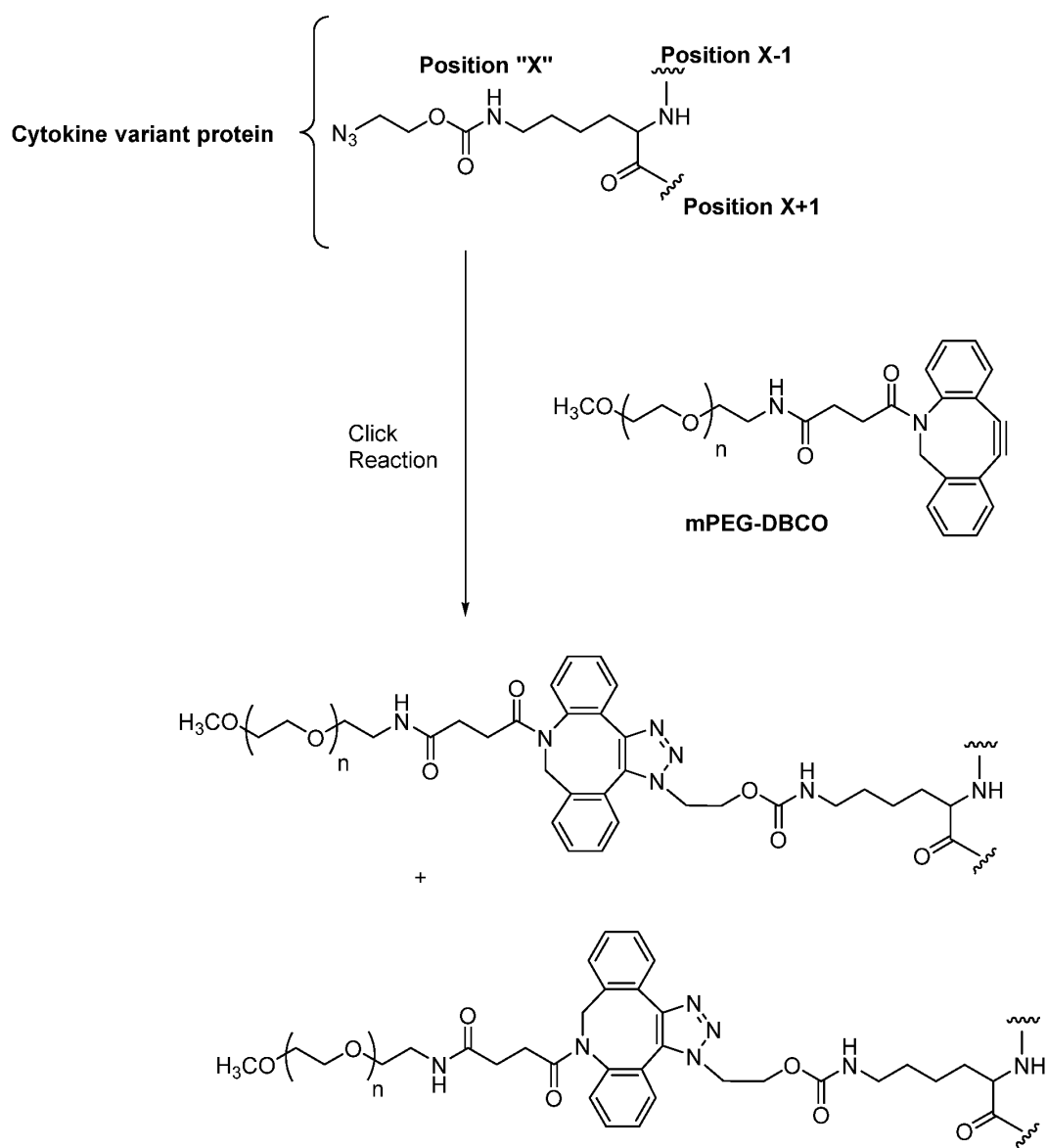


wherein X is the position in the IL-2 conjugate comprising an unnatural amino acid, such as in

any one of SEQ ID NOS: 2 to 14 or a sequence having at least 80% sequence identity to SEQ ID NO: 1.

[0369] In some embodiments described herein, a conjugation reaction described herein comprises a cycloaddition reaction between an azide moiety, such as that contained in a protein containing an amino acid residue derived from N6-((2-azidoethoxy)-carbonyl)-L-lysine (AzK), and a strained cycloalkyne, such as that derived from DBCO, which is a chemical moiety comprising a dibenzocyclooctyne group. PEG groups comprising a DBCO moiety are commercially available or may be prepared by methods known to those of ordinary skill in the art.





[0370] Conjugation reactions such as a click reaction described herein may generate a single regioisomer, or a mixture of regioisomers. In some embodiments, the ratio of regioisomers is about 1:1. In some embodiments the ratio of regioisomers is about 2:1. In some embodiments the ratio of regioisomers is about 1.5:1. In some embodiments the ratio of regioisomers is about 1.2:1. In some embodiments the ratio of regioisomers is about 1.1:1. In some embodiments the ratio of regioisomers is greater than 1:1.

4. Cytokine Polypeptide Production

[0371] In some embodiments, the IL-2 conjugates described herein, either containing an unnatural amino acid mutation, are generated recombinantly or are synthesized chemically. In some embodiments, IL-2 conjugates described herein are generated recombinantly, for example, either by a host cell system, or in a cell-free system.

[0372] In some embodiments, IL-2 conjugates are generated recombinantly through a host cell system. In some embodiments, the host cell is a eukaryotic cell (e.g., mammalian cell, insect cells, yeast cells or plant cell) or a prokaryotic cell (e.g., gram-positive bacterium or a gram-negative bacterium). In some embodiments, a eukaryotic host cell is a mammalian host cell. In some embodiments, a mammalian host cell is a stable cell line, or a cell line that has incorporated a genetic material of interest into its own genome and has the capability to express the product of the genetic material after many generations of cell division. In other cases, a mammalian host cell is a transient cell line, or a cell line that has not incorporated a genetic material of interest into its own genome and does not have the capability to express the product of the genetic material after many generations of cell division.

[0373] Exemplary mammalian host cells include 293T cell line, 293A cell line, 293FT cell line, 293F cells, 293 H cells, A549 cells, MDCK cells, CHO DG44 cells, CHO-S cells, CHO-K1 cells, Expi293F™ cells, Flp-In™ T-REx™ 293 cell line, Flp-In™-293 cell line, Flp-In™-3T3 cell line, Flp-In™-BHK cell line, Flp-In™-CHO cell line, Flp-In™-CV-1 cell line, Flp-In™-Jurkat cell line, FreeStyle™ 293-F cells, FreeStyle™ CHO-S cells, GripTite™ 293 MSR cell line, GS-CHO cell line, HepaRG™ cells, T-REx™ Jurkat cell line, Per.C6 cells, T-REx™-293 cell line, T-REx™-CHO cell line, and T-REx™-HeLa cell line.

[0374] In some embodiments, a eukaryotic host cell is an insect host cell. Exemplary insect host cell include Drosophila S2 cells, Sf9 cells, Sf21 cells, High Five™ cells, and expresSF+® cells.

[0375] In some embodiments, a eukaryotic host cell is a yeast host cell. Exemplary yeast host cells include Pichia pastoris yeast strains such as GS115, KM71H, SMD1168, SMD1168H, and X-33, and Saccharomyces cerevisiae yeast strain such as INVSc1.

[0376] In some embodiments, a eukaryotic host cell is a plant host cell. In some embodiments, the plant cells comprise a cell from algae. Exemplary plant cell lines include strains from Chlamydomonas reinhardtii 137c, or Synechococcus elongatus PPC 7942.

[0377] In some embodiments, a host cell is a prokaryotic host cell. Exemplary prokaryotic host cells include BL21, Mach1™, DH10B™, TOP10, DH5α, DH10Bac™, OmniMax™, MegaX™, DH12S™, INV110, TOP10F', INVαF, TOP10/P3, ccdB Survival, PIR1, PIR2, Stbl2™, Stbl3™, or Stbl4™.

[0378] In some embodiments, suitable polynucleic acid molecules or vectors for the production of an IL-2 polypeptide described herein include any suitable vectors derived from either a eukaryotic or prokaryotic source. Exemplary polynucleic acid molecules or vectors include vectors from bacteria (e.g., *E. coli*), insects, yeast (e.g., *Pichia pastoris*), algae, or mammalian source. Bacterial vectors include, for example, pACYC177, pASK75, pBAD vector series, pBADM vector series, pET vector series, pETM vector series, pGEX vector series, pHAT, pHAT2, pMal-c2, pMal-p2, pQE vector series, pRSET A, pRSET B, pRSET C, pTrcHis2 series, pZA31-Luc, pZE21-MCS-1, pFLAG ATS, pFLAG CTS, pFLAG MAC, pFLAG Shift-12c, pTAC-MAT-1, pFLAG CTC, or pTAC-MAT-2.

[0379] Insect vectors include, for example, pFastBac1, pFastBac DUAL, pFastBac ET, pFastBac HTa, pFastBac HTb, pFastBac HTc, pFastBac M30a, pFastBac M30b, pFastBac M30c, pVL1392, pVL1393, pVL1393 M10, pVL1393 M11, pVL1393 M12, FLAG vectors such as pPolh-FLAG1 or pPolh-MAT 2, or MAT vectors such as pPolh-MAT1, or pPolh-MAT2.

[0380] Yeast vectors include, for example, Gateway® pDEST™ 14 vector, Gateway® pDEST™ 15 vector, Gateway® pDEST™ 17 vector, Gateway® pDEST™ 24 vector, Gateway® pYES-DEST52 vector, pBAD-DEST49 Gateway® destination vector, pAO815 *Pichia* vector, pFLD1 *Pichia pastoris* vector, pGAPZA, B, & C *Pichia pastoris* vector, pPIC3.5K *Pichia* vector, pPIC6 A, B, & C *Pichia* vector, pPIC9K *Pichia* vector, pTEF1/Zeo, pYES2 yeast vector, pYES2/CT yeast vector, pYES2/NT A, B, & C yeast vector, or pYES3/CT yeast vector.

[0381] Algae vectors include, for example, pChlamy-4 vector or MCS vector.

[0382] Mammalian vectors include, for example, transient expression vectors or stable expression vectors. Exemplary mammalian transient expression vectors include p3xFLAG-CMV 8, pFLAG-Myc-CMV 19, pFLAG-Myc-CMV 23, pFLAG-CMV 2, pFLAG-CMV 6a,b,c, pFLAG-CMV 5.1, pFLAG-CMV 5a,b,c, p3xFLAG-CMV 7.1, pFLAG-CMV 20, p3xFLAG-Myc-CMV 24, pCMV-FLAG-MAT1, pCMV-FLAG-MAT2, pBICEP-CMV 3, or pBICEP-CMV 4. Exemplary mammalian stable expression vectors include pFLAG-CMV 3, p3xFLAG-CMV 9, p3xFLAG-CMV 13, pFLAG-Myc-CMV 21, p3xFLAG-Myc-CMV 25, pFLAG-CMV 4, p3xFLAG-CMV 10, p3xFLAG-CMV 14, pFLAG-Myc-CMV 22, p3xFLAG-Myc-CMV 26, pBICEP-CMV 1, or pBICEP-CMV 2.

[0383] In some embodiments, a cell-free system is used for the production of a cytokine (e.g., IL-2) polypeptide described herein. In some embodiments, a cell-free system comprises a mixture of cytoplasmic and/or nuclear components from a cell and is suitable for in vitro nucleic acid synthesis. In some embodiments, a cell-free system utilizes prokaryotic cell components. In other instances, a cell-free system utilizes eukaryotic cell components. Nucleic acid synthesis is

obtained in a cell-free system based on, for example, *Drosophila* cell, *Xenopus* egg, Archaea, or HeLa cells. Exemplary cell-free systems include *E. coli* S30 Extract system, *E. coli* T7 S30 system, or PURExpress®, XpressCF, and XpressCF+.

[0384] Cell-free translation systems variously comprise components such as plasmids, mRNA, DNA, tRNAs, synthetases, release factors, ribosomes, chaperone proteins, translation initiation and elongation factors, natural and/or unnatural amino acids, and/or other components used for protein expression. Such components are optionally modified to improve yields, increase synthesis rate, increase protein product fidelity, or incorporate unnatural amino acids. In some embodiments, cytokines described herein are synthesized using cell-free translation systems described in US 8,778,631; US 2017/0283469; US 2018/0051065; US 2014/0315245; or US 8,778,631. In some embodiments, cell-free translation systems comprise modified release factors, or even removal of one or more release factors from the system. In some embodiments, cell-free translation systems comprise a reduced protease concentration. In some embodiments, cell-free translation systems comprise modified tRNAs with re-assigned codons used to code for unnatural amino acids. In some embodiments, the synthetases described herein for the incorporation of unnatural amino acids are used in cell-free translation systems. In some embodiments, tRNAs are pre-loaded with unnatural amino acids using enzymatic or chemical methods before being added to a cell-free translation system. In some embodiments, components for a cell-free translation system are obtained from modified organisms, such as modified bacteria, yeast, or other organism.

[0385] In some embodiments, a cytokine (e.g., IL-2) polypeptide is generated as a circularly permuted form, either via an expression host system or through a cell-free system.

[0386] An orthogonal or expanded genetic code can be used in the present disclosure, in which one or more specific codons present in the nucleic acid sequence of a cytokine (e.g., IL-2) polypeptide are allocated to encode the unnatural amino acid so that it can be genetically incorporated into the cytokine (e.g., IL-2) by using an orthogonal tRNA synthetase/tRNA pair. The orthogonal tRNA synthetase/tRNA pair is capable of charging a tRNA with an unnatural amino acid and is capable of incorporating that unnatural amino acid into the polypeptide chain in response to the codon.

[0387] In some embodiments, the codon is the codon amber, ochre, opal or a quadruplet codon. In some embodiments, the codon corresponds to the orthogonal tRNA which will be used to carry the unnatural amino acid. In some embodiments, the codon is amber. In other cases, the codon is an orthogonal codon.

[0388] In some embodiments, the codon is a quadruplet codon, which can be decoded by an orthogonal ribosome ribo-Q1. In some embodiments, the quadruplet codon is as illustrated in Neumann, *et al.*, “Encoding multiple unnatural amino acids via evolution of a quadruplet-decoding ribosome,” *Nature*, **464**(7287): 441-444 (2010).

[0389] In some embodiments, a codon used in the present disclosure is a recoded codon, e.g., a synonymous codon or a rare codon that is replaced with alternative codon. In some embodiments, the recoded codon is as described in Napolitano, *et al.*, “Emergent rules for codon choice elucidated by editing rare arginine codons in *Escherichia coli*,” *PNAS*, **113**(38): E5588-5597 (2016). In some embodiments, the recoded codon is as described in Ostrov *et al.*, “Design, synthesis, and testing toward a 57-codon genome,” *Science* **353**(6301): 819-822 (2016).

[0390] In some embodiments, unnatural nucleic acids are utilized leading to incorporation of one or more unnatural amino acids into the cytokine (e.g., IL-2). Exemplary unnatural nucleic acids include, but are not limited to, uracil-5-yl, hypoxanthin-9-yl (I), 2-aminoadenin-9-yl, 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl and other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil and cytosine, 5-propynyl uracil and cytosine, 6-azo uracil, cytosine and thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl and other 5-substituted uracils and cytosines, 7-methylguanine and 7-methyladenine, 8-azaguanine and 8-azaadenine, 7-deazaguanine and 7-deazaadenine and 3-deazaguanine and 3-deazaadenine. Certain unnatural nucleic acids, such as 5-substituted pyrimidines, 6-azapyrimidines and N-2 substituted purines, N-6 substituted purines, O-6 substituted purines, 2-aminopropyladenine, 5-propynyluracil, 5-propynylcytosine, 5-methylcytosine, those that increase the stability of duplex formation, universal nucleic acids, hydrophobic nucleic acids, promiscuous nucleic acids, size-expanded nucleic acids, fluorinated nucleic acids, 5-substituted pyrimidines, 6-azapyrimidines and N-2, N-6 and O-6 substituted purines, including 2-aminopropyladenine, 5-propynyluracil and 5-propynylcytosine. 5-methylcytosine (5-me-C), 5-hydroxymethyl cytosine, xanthine, hypoxanthine, 2-aminoadenine, 6-methyl, other alkyl derivatives of adenine and guanine, 2-propyl and other alkyl derivatives of adenine and guanine, 2-thiouracil, 2-thiothymine and 2-thiocytosine, 5-halouracil, 5-halocytosine, 5-propynyl ($-C\equiv C-CH_3$) uracil, 5-propynyl cytosine, other alkynyl derivatives of pyrimidine nucleic acids, 6-azo uracil, 6-azo cytosine, 6-azo thymine, 5-uracil (pseudouracil), 4-thiouracil, 8-halo, 8-amino, 8-thiol, 8-thioalkyl, 8-hydroxyl and other 8-substituted adenines and guanines, 5-halo particularly 5-bromo, 5-trifluoromethyl,

other 5-substituted uracils and cytosines, 7-methylguanine, 7-methyladenine, 2-F-adenine, 2-amino-adenine, 8-azaguanine, 8-azaadenine, 7-deazaguanine, 7-deazaadenine, 3-deazaguanine, 3-deazaadenine, tricyclic pyrimidines, phenoxazine cytidine([5,4-b][1,4]benzoxazin-2(3H)-one), phenothiazine cytidine (1H- pyrimido[5,4-b][1,4]benzothiazin-2(3H)-one), G-clamps, phenoxazine cytidine (*e.g.* 9- (2-aminoethoxy)-H-pyrimido[5,4-b][1,4]benzoxazin-2(3H)-one), carbazole cytidine (2H-pyrimido[4,5- b]indol-2-one), pyridoindole cytidine (H-pyrido[3',2':4,5]pyrrolo[2,3-d]pyrimidin-2-one), those in which the purine or pyrimidine base is replaced with other heterocycles, 7-deaza-adenine, 7-deazaguanosine, 2-aminopyridine, 2-pyridone, azacytosine, 5-bromocytosine, bromouracil, 5-chlorocytosine, chlorinated cytosine, cyclocytosine, cytosine arabinoside, 5-fluorocytosine, fluoropyrimidine, fluorouracil, 5,6-dihydrocytosine, 5-iodocytosine, hydroxyurea, iodouracil, 5-nitrocytosine, 5- bromouracil, 5-chlorouracil, 5-fluorouracil, and 5-iodouracil, 2-amino-adenine, 6-thio-guanine, 2-thio-thymine, 4-thio-thymine, 5-propynyl-uracil, 4-thio-uracil, N4-ethylcytosine, 7-deazaguanine, 7-deaza-8-azaguanine, 5-hydroxycytosine, 2'-deoxyuridine, 2-amino-2'-deoxyadenosine, and those described in U.S. Patent Nos. 3,687,808; 4,845,205; 4,910,300; 4,948,882; 5,093,232; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121; 5,596,091; 5,614,617; 5,645,985; 5,681,941; 5,750,692; 5,763,588; 5,830,653 and 6,005,096; WO 99/62923; Kandimalla et al., (2001) Bioorg. Med. Chem. 9:807-813; The Concise Encyclopedia of Polymer Science and Engineering, Kroschwitz, J.I., Ed., John Wiley & Sons, 1990, 858- 859; Englisch et al., Angewandte Chemie, International Edition, 1991, 30, 613; and Sanghvi, Chapter 15, Antisense Research and Applications, Crooke and Lebleu Eds., CRC Press, 1993, 273-288. Additional base modifications can be found, for example, in U.S. Pat. No. 3,687,808; Englisch et al., Angewandte Chemie, International Edition, 1991, 30, 613; and Sanghvi, Chapter 15, Antisense Research and Applications, pages 289-302, Crooke and Lebleu ed., CRC Press, 1993.

[0391] Unnatural nucleic acids comprising various heterocyclic bases and various sugar moieties (and sugar analogs) are available in the art, and the nucleic acids in some embodiments include one or several heterocyclic bases other than the principal five base components of naturally-occurring nucleic acids. For example, the heterocyclic base includes, in some embodiments, uracil-5-yl, cytosin-5-yl, adenin-7-yl, adenin-8-yl, guanin-7-yl, guanin-8-yl, 4- aminopyrrolo [2.3-d] pyrimidin-5-yl, 2-amino-4-oxopyrrolo [2, 3-d] pyrimidin-5-yl, 2- amino-4-oxopyrrolo [2.3-d] pyrimidin-3-yl groups, where the purines are attached to the sugar moiety of the nucleic acid via the 9-position, the pyrimidines via the 1 -position, the pyrrolopyrimidines via the 7-position and the pyrazolopyrimidines via the 1-position.

[0392] In some embodiments, nucleotide analogs are also modified at the phosphate moiety. Modified phosphate moieties include, but are not limited to, those with modification at the linkage between two nucleotides and contains, for example, a phosphorothioate, chiral phosphorothioate, phosphorodithioate, phosphotriester, aminoalkylphosphotriester, methyl and other alkyl phosphonates including 3'-alkylene phosphonate and chiral phosphonates, phosphinates, phosphoramidates including 3'-amino phosphoramidate and aminoalkylphosphoramidates, thionophosphoramidates, thionoalkylphosphonates, thionoalkylphosphotriesters, and boranophosphates. It is understood that these phosphate or modified phosphate linkage between two nucleotides are through a 3'-5' linkage or a 2'-5' linkage, and the linkage contains inverted polarity such as 3'-5' to 5'-3' or 2'-5' to 5'-2'. Various salts, mixed salts and free acid forms are also included. Numerous United States patents teach how to make and use nucleotides containing modified phosphates and include but are not limited to, 3,687,808; 4,469,863; 4,476,301; 5,023,243; 5,177,196; 5,188,897; 5,264,423; 5,276,019; 5,278,302; 5,286,717; 5,321,131; 5,399,676; 5,405,939; 5,453,496; 5,455,233; 5,466,677; 5,476,925; 5,519,126; 5,536,821; 5,541,306; 5,550,111; 5,563,253; 5,571,799; 5,587,361; and 5,625,050.

[0393] In some embodiments, unnatural nucleic acids include 2',3'-dideoxy-2',3'-dideohydro-nucleosides (PCT/US2002/006460), 5'-substituted DNA and RNA derivatives (PCT/US2011/033961; Saha et al., *J. Org Chem.*, 1995, 60, 788-789; Wang et al., *Bioorganic & Medicinal Chemistry Letters*, 1999, 9, 885-890; and Mikhailov et al., *Nucleosides & Nucleotides*, 1991, 10(1-3), 339-343; Leonid et al., 1995, 14(3-5), 901-905; and Eppacher et al., *Helvetica Chimica Acta*, 2004, 87, 3004-3020; PCT/JP2000/004720; PCT/JP2003/002342; PCT/JP2004/013216; PCT/JP2005/020435; PCT/JP2006/315479; PCT/JP2006/324484; PCT/JP2009/056718; PCT/JP2010/067560), or 5'-substituted monomers made as the monophosphate with modified bases (Wang et al., *Nucleosides Nucleotides & Nucleic Acids*, 2004, 23 (1 & 2), 317-337).

[0394] In some embodiments, unnatural nucleic acids include modifications at the 5'-position and the 2'-position of the sugar ring (PCT/US94/02993), such as 5'-CH₂-substituted 2'-O-protected nucleosides (Wu et al., *Helvetica Chimica Acta*, 2000, 83, 1127-1143 and Wu et al., *Bioconjugate Chem.* 1999, 10, 921-924). In some embodiments, unnatural nucleic acids include amide linked nucleoside dimers have been prepared for incorporation into oligonucleotides wherein the 3' linked nucleoside in the dimer (5' to 3') comprises a 2'-OCH₃ and a 5'-(S)-CH₃ (Mesmaeker et al., *Synlett*, 1997, 1287-1290). Unnatural nucleic acids can include 2'-substituted 5'-CH₂ (or O) modified nucleosides (PCT/US92/01020). Unnatural nucleic acids can include 5'-

methylenephosphonate DNA and RNA monomers, and dimers (Bohringer et al., Tet. Lett., 1993, 34, 2723-2726; Collingwood et al., Synlett, 1995, 7, 703-705; and Hutter et al., Helvetica Chimica Acta, 2002, 85, 2777-2806). Unnatural nucleic acids can include 5'-phosphonate monomers having a 2'-substitution (US2006/0074035) and other modified 5'-phosphonate monomers (WO1997/35869). Unnatural nucleic acids can include 5'-modified methylenephosphonate monomers (EP614907 and EP629633). Unnatural nucleic acids can include analogs of 5' or 6'-phosphonate ribonucleosides comprising a hydroxyl group at the 5' and/or 6'-position (Chen et al., Phosphorus, Sulfur and Silicon, 2002, 777, 1783-1786; Jung et al., Bioorg. Med. Chem., 2000, 8, 2501-2509; Gallier et al., Eur. J. Org. Chem., 2007, 925-933; and Hampton et al., J. Med. Chem., 1976, 19(8), 1029-1033). Unnatural nucleic acids can include 5'-phosphonate deoxyribonucleoside monomers and dimers having a 5'-phosphate group (Nawrot et al., Oligonucleotides, 2006, 16(1), 68-82). Unnatural nucleic acids can include nucleosides having a 6'-phosphonate group wherein the 5' or/and 6'-position is unsubstituted or substituted with a thio-tert-butyl group ($\text{SC}(\text{CH}_3)_3$) (and analogs thereof); a methyleneamino group (CH_2NH_2) (and analogs thereof) or a cyano group (CN) (and analogs thereof) (Fairhurst et al., Synlett, 2001, 4, 467-472; Kappler et al., J. Med. Chem., 1986, 29, 1030-1038; Kappler et al., J. Med. Chem., 1982, 25, 1179-1184; Vruthula et al., J. Med. Chem., 1987, 30, 888-894; Hampton et al., J. Med. Chem., 1976, 19, 1371-1377; Geze et al., J. Am. Chem. Soc., 1983, 105(26), 7638-7640; and Hampton et al., J. Am. Chem. Soc., 1973, 95(13), 4404-4414).

[0395] In some embodiments, unnatural nucleic acids also include modifications of the sugar moiety. In some embodiments, nucleic acids contain one or more nucleosides wherein the sugar group has been modified. Such sugar modified nucleosides may impart enhanced nuclease stability, increased binding affinity, or some other beneficial biological property. In certain embodiments, nucleic acids comprise a chemically modified ribofuranose ring moiety. Examples of chemically modified ribofuranose rings include, without limitation, addition of substituent groups (including 5' and/or 2' substituent groups; bridging of two ring atoms to form bicyclic nucleic acids (BNA); replacement of the ribosyl ring oxygen atom with S, N(R), or C(R₁)(R₂) (R = H, C₁-C₁₂ alkyl or a protecting group); and combinations thereof. Examples of chemically modified sugars can be found in WO2008/101157, US2005/0130923, and WO2007/134181.

[0396] In some embodiments, a modified nucleic acid comprises modified sugars or sugar analogs. Thus, in addition to ribose and deoxyribose, the sugar moiety can be pentose, deoxypentose, hexose, deoxyhexose, glucose, arabinose, xylose, lyxose, or a sugar "analog" cyclopentyl group. The sugar can be in a pyranosyl or furanosyl form. The sugar moiety may be the furanoside of ribose, deoxyribose, arabinose or 2'-O-alkylribose, and the sugar can be

attached to the respective heterocyclic bases either in [alpha] or [beta] anomeric configuration. Sugar modifications include, but are not limited to, 2'-alkoxy-RNA analogs, 2'-amino-RNA analogs, 2'-fluoro-DNA, and 2'-alkoxy- or amino-RNA/DNA chimeras. For example, a sugar modification may include 2'-O-methyl-uridine or 2'-O-methyl-cytidine. Sugar modifications include 2'-O-alkyl-substituted deoxyribonucleosides and 2'-O-ethyleneglycol like ribonucleosides. The preparation of these sugars or sugar analogs and the respective

"nucleosides" wherein such sugars or analogs are attached to a heterocyclic base (nucleic acid base) is known. Sugar modifications may also be made and combined with other modifications.

[0397] Modifications to the sugar moiety include natural modifications of the ribose and deoxy ribose as well as unnatural modifications. Sugar modifications include, but are not limited to, the following modifications at the 2' position: OH; F; O-, S-, or N-alkyl; O-, S-, or N-alkenyl; O-, S- or N-alkynyl; or O-alkyl-O-alkyl, wherein the alkyl, alkenyl and alkynyl may be substituted or unsubstituted C₁ to C₁₀, alkyl or C₂ to C₁₀ alkenyl and alkynyl. 2' sugar modifications also include but are not limited to -O[(CH₂)_nO]_mCH₃, -O(CH₂)_nOCH₃, -O(CH₂)_nNH₂, -O(CH₂)_nCH₃, -O(CH₂)_nONH₂, and -O(CH₂)_nON[(CH₂)_nCH₃]₂, where n and m are from 1 to about 10.

[0398] Other modifications at the 2' position include but are not limited to: C₁ to C₁₀ lower alkyl, substituted lower alkyl, alkaryl, aralkyl, O-alkaryl, O-aralkyl, SH, SCH₃, OCN, Cl, Br, CN, CF₃, OCF₃, SOCH₃, SO₂CH₃, ONO₂, NO₂, N₃, NH₂, heterocycloalkyl, heterocycloalkaryl, aminoalkylamino, polyalkylamino, substituted silyl, an RNA cleaving group, a reporter group, an intercalator, a group for improving the pharmacokinetic properties of an oligonucleotide, or a group for improving the pharmacodynamic properties of an oligonucleotide, and other substituents having similar properties. Similar modifications may also be made at other positions on the sugar, particularly the 3' position of the sugar on the 3' terminal nucleotide or in 2'-5' linked oligonucleotides and the 5' position of the 5' terminal nucleotide. Modified sugars also include those that contain modifications at the bridging ring oxygen, such as CH₂ and S.

Nucleotide sugar analogs may also have sugar mimetics such as cyclobutyl moieties in place of the pentofuranosyl sugar. There are numerous United States patents that teach the preparation of such modified sugar structures and which detail and describe a range of base modifications, such as U.S. Patent Nos. 4,981,957; 5,118,800; 5,319,080; 5,359,044; 5,393,878; 5,446,137; 5,466,786; 5,514,785; 5,519,134; 5,567,811; 5,576,427; 5,591,722; 5,597,909; 5,610,300; 5,627,053; 5,639,873; 5,646,265; 5,658,873; 5,670,633; 4,845,205; 5,130,302; 5,134,066; 5,175,273; 5,367,066; 5,432,272; 5,457,187; 5,459,255; 5,484,908; 5,502,177; 5,525,711; 5,552,540; 5,587,469; 5,594,121; 5,596,091; 5,614,617; 5,681,941; and 5,700,920, each of which is herein incorporated by reference in its entirety.

[0399] Examples of nucleic acids having modified sugar moieties include, without limitation, nucleic acids comprising 5'-vinyl, 5'-methyl (R or S), 4'-S, 2'-F, 2'-OCH₃, and 2'-O(CH₂)₂OCH₃ substituent groups. The substituent at the 2' position can also be selected from allyl, amino, azido, thio, O-allyl, O-(C₁-C₁₀ alkyl), OCF₃, O(CH₂)₂SCH₃, O(CH₂)₂-O-N(R_m)(R_n), and O-CH₂-C(=O)-N(R_m)(R_n), where each R_m and R_n is, independently, H or substituted or unsubstituted C₁-C₁₀ alkyl.

[0400] In certain embodiments, nucleic acids described herein include one or more bicyclic nucleic acids. In certain such embodiments, the bicyclic nucleic acid comprises a bridge between the 4' and the 2' ribosyl ring atoms. In certain embodiments, nucleic acids provided herein include one or more bicyclic nucleic acids wherein the bridge comprises a 4' to 2' bicyclic nucleic acid. Examples of such 4' to 2' bicyclic nucleic acids include, but are not limited to, one of the formulae: 4'-(CH₂)-O-2' (LNA); 4'-(CH₂)-S-2'; 4'-(CH₂)₂-O-2' (ENA); 4'-CH(CH₃)-O-2' and 4'-CH(CH₂OCH₃)-O-2', and analogs thereof (see, U.S. Patent No. 7,399,845); 4'-C(CH₃)(CH₃)-O-2' and analogs thereof, (see WO2009/006478, WO2008/150729, US2004/0171570, U.S. Patent No. 7,427,672, Chattopadhyaya et al., J. Org. Chem., 209, 74, 118-134, and WO2008/154401). Also see, for example: Singh et al., Chem. Commun., 1998, 4, 455-456; Koshkin et al., Tetrahedron, 1998, 54, 3607-3630; Wahlestedt et al., Proc. Natl. Acad. Sci. U. S. A., 2000, 97, 5633-5638; Kumar et al., Bioorg. Med. Chem. Lett., 1998, 8, 2219-2222; Singh et al., J. Org. Chem., 1998, 63, 10035-10039; Srivastava et al., J. Am. Chem. Soc., 2007, 129(26) 8362-8379; Elayadi et al., Curr. Opinion Inven. Drugs, 2001, 2, 558-561; Braasch et al., Chem. Biol, 2001, 8, 1-7; Oram et al., Curr. Opinion Mol. Ther., 2001, 3, 239-243; U.S. Patent Nos. 4,849,513; 5,015,733; 5,118,800; 5,118,802; 7,053,207; 6,268,490; 6,770,748; 6,794,499; 7,034,133; 6,525,191; 6,670,461; and 7,399,845; International Publication Nos. WO2004/106356, WO1994/14226, WO2005/021570, WO2007/090071, and WO2007/134181; U.S. Patent Publication Nos. US2004/0171570, US2007/0287831, and US2008/0039618; U.S. Provisional Application Nos. 60/989,574, 61/026,995, 61/026,998, 61/056,564, 61/086,231, 61/097,787, and 61/099,844; and International Applications Nos. PCT/US2008/064591, PCT US2008/066154, PCT US2008/068922, and PCT/DK98/00393.

[0401] In certain embodiments, nucleic acids comprise linked nucleic acids. Nucleic acids can be linked together using any inter nucleic acid linkage. The two main classes of inter nucleic acid linking groups are defined by the presence or absence of a phosphorus atom. Representative phosphorus containing inter nucleic acid linkages include, but are not limited to, phosphodiester, phosphotriester, methylphosphonates, phosphoramidate, and phosphorothioates (P=S). Representative non-phosphorus containing inter nucleic acid linking

groups include, but are not limited to, methylenemethylimino ($-\text{CH}_2-\text{N}(\text{CH}_3)-\text{O}-\text{CH}_2-$), thiodiester ($-\text{O}-\text{C}(\text{O})-\text{S}-$), thionocarbamate ($-\text{O}-\text{C}(\text{O})(\text{NH})-\text{S}-$); siloxane ($-\text{O}-\text{Si}(\text{H})_2-\text{O}-$); and N,N^* -dimethylhydrazine ($-\text{CH}_2-\text{N}(\text{CH}_3)-\text{N}(\text{CH}_3)$). In certain embodiments, inter nucleic acids linkages having a chiral atom can be prepared as a racemic mixture, as separate enantiomers, *e.g.*, alkylphosphonates and phosphorothioates. Unnatural nucleic acids can contain a single modification. Unnatural nucleic acids can contain multiple modifications within one of the moieties or between different moieties.

[0402] Backbone phosphate modifications to nucleic acid include, but are not limited to, methyl phosphonate, phosphorothioate, phosphoramidate (bridging or non-bridging), phosphotriester, phosphorodithioate, phosphodithioate, and boranophosphate, and may be used in any combination. Other non- phosphate linkages may also be used.

[0403] In some embodiments, backbone modifications (*e.g.*, methylphosphonate, phosphorothioate, phosphoroamidate and phosphorodithioate internucleotide linkages) can confer immunomodulatory activity on the modified nucleic acid and/or enhance their stability *in vivo*.

[0404] In some embodiments, a phosphorous derivative (or modified phosphate group) is attached to the sugar or sugar analog moiety in and can be a monophosphate, diphosphate, triphosphate, alkylphosphonate, phosphorothioate, phosphorodithioate, phosphoramidate or the like. Exemplary polynucleotides containing modified phosphate linkages or non-phosphate linkages can be found in Peyrottes et al., 1996, *Nucleic Acids Res.* 24: 1841-1848; Chaturvedi et al., 1996, *Nucleic Acids Res.* 24:2318-2323; and Schultz et al., (1996) *Nucleic Acids Res.* 24:2966-2973; Matteucci, 1997, "Oligonucleotide Analogs: an Overview" in *Oligonucleotides as Therapeutic Agents*, (Chadwick and Cardew, ed.) John Wiley and Sons, New York, NY; Zon, 1993, "Oligonucleoside Phosphorothioates" in *Protocols for Oligonucleotides and Analogs, Synthesis and Properties*, Humana Press, pp. 165-190; Miller et al., 1971, *JACS* 93:6657-6665; Jager et al., 1988, *Biochem.* 27:7247-7246; Nelson et al., 1997, *JOC* 62:7278-7287; U.S. Patent No. 5,453,496; and Micklefield, 2001, *Curr. Med. Chem.* 8: 1157-1179.

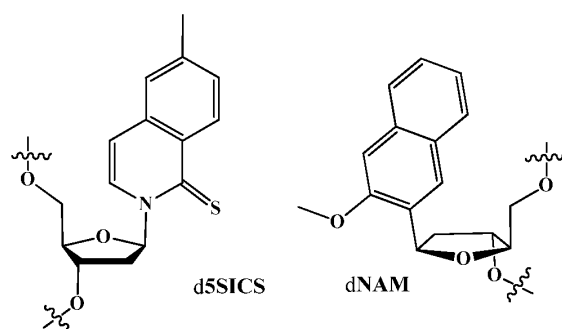
[0405] In some embodiments, backbone modification comprises replacing the phosphodiester linkage with an alternative moiety such as an anionic, neutral or cationic group. Examples of such modifications include: anionic internucleoside linkage; $\text{N}3'$ to $\text{P}5'$ phosphoramidate modification; boranophosphate DNA; prooligonucleotides; neutral internucleoside linkages such as methylphosphonates; amide linked DNA; methylene(methylimino) linkages; formacetal and thioformacetal linkages; backbones containing sulfonyl groups; morpholino oligos; peptide nucleic acids (PNA); and positively charged deoxyribonucleic guanine (DNG) oligos

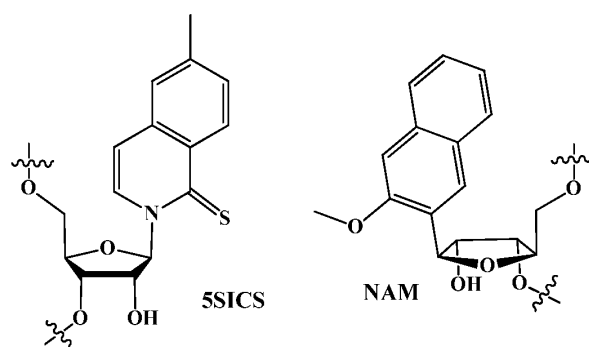
(Micklefield, 2001, Current Medicinal Chemistry 8: 1157-1179). A modified nucleic acid may comprise a chimeric or mixed backbone comprising one or more modifications, *e.g.* a combination of phosphate linkages such as a combination of phosphodiester and phosphorothioate linkages.

[0406] Substitutes for the phosphate include, for example, short chain alkyl or cycloalkyl internucleoside linkages, mixed heteroatom and alkyl or cycloalkyl internucleoside linkages, or one or more short chain heteroatomic or heterocyclic internucleoside linkages. These include those having morpholino linkages (formed in part from the sugar portion of a nucleoside); siloxane backbones; sulfide, sulfoxide and sulfone backbones; formacetyl and thioformacetyl backbones; methylene formacetyl and thioformacetyl backbones; alkene containing backbones; sulfamate backbones; methyleneimino and methylenehydrazino backbones; sulfonate and sulfonamide backbones; amide backbones; and others having mixed N, O, S and CH₂ component parts. Numerous United States patents disclose how to make and use these types of phosphate replacements and include but are not limited to U.S. Patent Nos. 5,034,506; 5,166,315; 5,185,444; 5,214,134; 5,216,141; 5,235,033; 5,264,562; 5,264,564; 5,405,938; 5,434,257; 5,466,677; 5,470,967; 5,489,677; 5,541,307; 5,561,225; 5,596,086; 5,602,240; 5,610,289; 5,602,240; 5,608,046; 5,610,289; 5,618,704; 5,623,070; 5,663,312; 5,633,360; 5,677,437; and 5,677,439. It is also understood in a nucleotide substitute that both the sugar and the phosphate moieties of the nucleotide can be replaced, by for example an amide type linkage (aminoethylglycine) (PNA). United States Patent Nos. 5,539,082; 5,714,331; and 5,719,262 teach how to make and use PNA molecules, each of which is herein incorporated by reference. See also Nielsen et al., Science, 1991, 254, 1497-1500. It is also possible to link other types of molecules (conjugates) to nucleotides or nucleotide analogs to enhance for example, cellular uptake. Conjugates can be chemically linked to the nucleotide or nucleotide analogs. Such conjugates include but are not limited to lipid moieties such as a cholesterol moiety (Letsinger et al., Proc. Natl. Acad. Sci. USA, 1989, 86, 6553-6556), cholic acid (Manoharan et al., Bioorg. Med. Chem. Lett., 1994, 4, 1053-1060), a thioether, *e.g.*, hexyl-S-tritylthiol (Manoharan et al., Ann. KY. Acad. Sci., 1992, 660, 306-309; Manoharan et al., Bioorg. Med. Chem. Lett., 1993, 3, 2765-2770), a thiocholesterol (Oberhauser et al., Nucl. Acids Res., 1992, 20, 533-538), an aliphatic chain, *e.g.*, dodecandiol or undecyl residues (Saison-Behmoaras et al., EMBOJ, 1991, 10, 1111-1118; Kabanov et al., FEBS Lett., 1990, 259, 327-330; Svinarchuk et al., Biochimie, 1993, 75, 49-54), a phospholipid, *e.g.*, di-hexadecyl-rac-glycerol or triethylammonium l-di-O-hexadecyl-rac-glycero-S-H-phosphonate (Manoharan et al., Tetrahedron Lett., 1995, 36, 3651-3654; Shea et al., Nucl. Acids Res., 1990, 18, 3777-3783), a polyamine or a polyethylene glycol

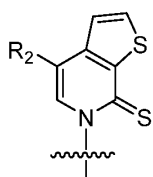
chain (Manoharan et al., Nucleosides & Nucleotides, 1995, 14, 969-973), or adamantane acetic acid (Manoharan et al., Tetrahedron Lett., 1995, 36, 3651-3654), a palmityl moiety (Mishra et al., Biochem. Biophys. Acta, 1995, 1264, 229-237), or an octadecylamine or hexylamino-carbonyl-oxycholesterol moiety (Crooke et al., J. Pharmacol. Exp. Ther., 1996, 277, 923-937). Numerous United States patents teach the preparation of such conjugates and include, but are not limited to U.S. Patent Nos. 4,828,979; 4,948,882; 5,218,105; 5,525,465; 5,541,313; 5,545,730; 5,552,538; 5,578,717; 5,580,731; 5,580,731; 5,591,584; 5,109,124; 5,118,802; 5,138,045; 5,414,077; 5,486,603; 5,512,439; 5,578,718; 5,608,046; 4,587,044; 4,605,735; 4,667,025; 4,762,779; 4,789,737; 4,824,941; 4,835,263; 4,876,335; 4,904,582; 4,958,013; 5,082,830; 5,112,963; 5,214,136; 5,082,830; 5,112,963; 5,214,136; 5,245,022; 5,254,469; 5,258,506; 5,262,536; 5,272,250; 5,292,873; 5,317,098; 5,371,241; 5,391,723; 5,416,203; 5,451,463; 5,510,475; 5,512,667; 5,514,785; 5,565,552; 5,567,810; 5,574,142; 5,585,481; 5,587,371; 5,595,726; 5,597,696; 5,599,923; 5,599,928 and 5,688,941.

[0407] In some embodiments, the unnatural nucleic acids further form unnatural base pairs. Exemplary unnatural nucleotides capable of forming an unnatural DNA or RNA base pair (UBP) under conditions *in vivo* includes, but is not limited to, TPT3, dTPT3, 5SICS, d5SICS, NaM, dNaM, CNMO, dCNMO, and combinations thereof. Other examples of unnatural nucleotides capable of forming unnatural UBPs that may be used to prepare the IL-2 conjugates disclosed herein may be found in Dien et al., J Am Chem Soc., 2018, 140:16115–16123; Feldman et al., J Am Chem Soc, 2017, 139:11427–11433; Ledbetter et al., J Am Chem Soc., 2018, 140:758-765; Dhami et al., Nucleic Acids Res. 2014, 42:10235-10244; Malyshev et al., Nature, 2014, 509:385-388; Betz et al., J Am Chem Soc., 2013, 135:18637-18643; Lavergne et al., J Am Chem Soc. 2013, 135:5408-5419; and Malyshev et al. Proc Natl Acad Sci USA, 2012, 109:12005-12010. In some embodiments, unnatural nucleotides include:





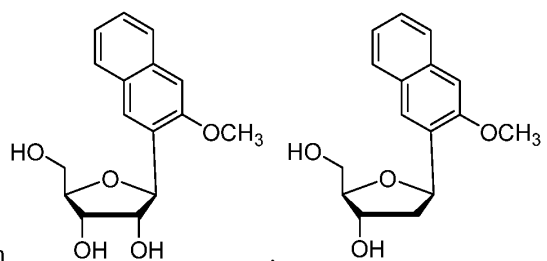
[0408] In some embodiments, the unnatural nucleotides that may be used to prepare the IL-2 conjugates disclosed herein may be derived from a compound of the formula



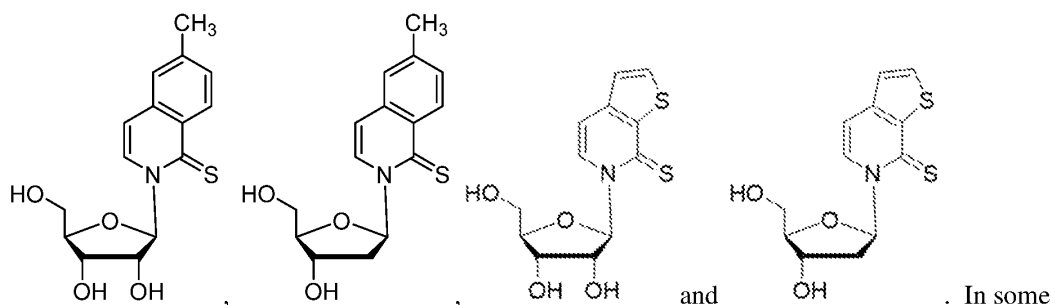
wherein R₂ is selected from the group consisting of hydrogen, alkyl, alkenyl, alkynyl, methoxy, methanethiol, methaneseleno, halogen, cyano, and azido; and

the wavy line indicates a bond to a ribosyl or 2'-deoxyribosyl, wherein the 5'-hydroxy group of the ribosyl or 2'-deoxyribosyl moiety is in free form, or is optionally bonded to a monophosphate, a diphosphate, or a triphosphate group.

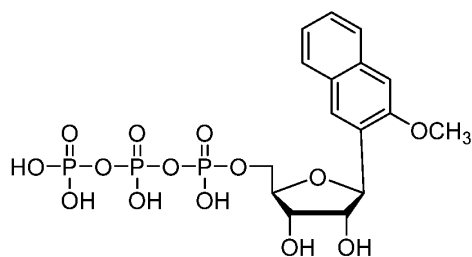
[0409] In some embodiments, the unnatural nucleotides that may be used to prepare the IL-2



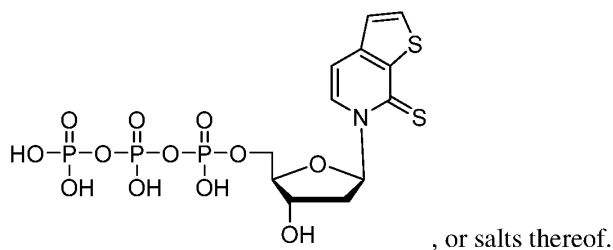
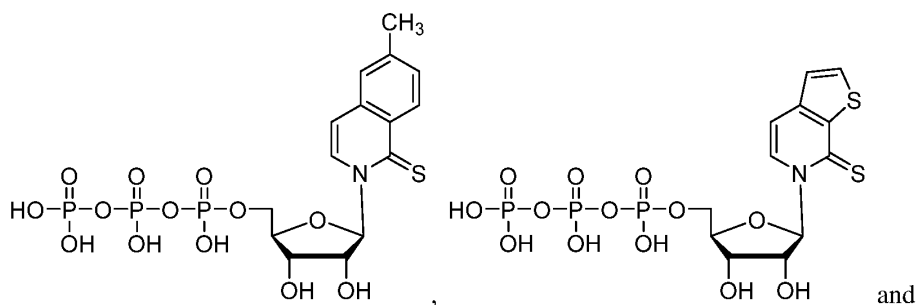
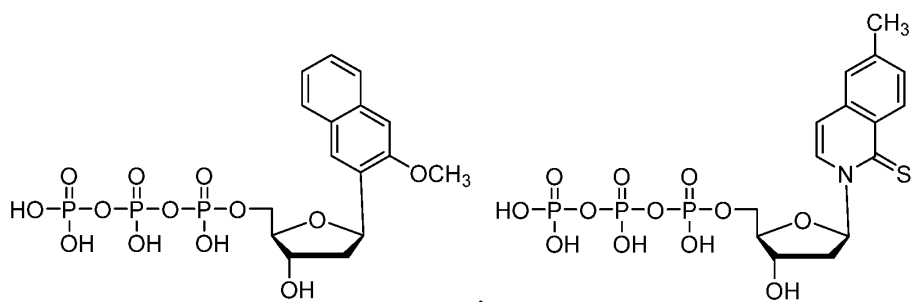
conjugates disclosed herein may be derived from



embodiments, the unnatural nucleotides that may be used to prepare the IL-2 conjugates disclosed



herein include



, or salts thereof.

[0410] In some embodiments, an unnatural base pair generates an unnatural amino acid described in Dumas *et al.*, “Designing logical codon reassignment – Expanding the chemistry in biology,” *Chemical Science*, **6**: 50-69 (2015).

[0411] In some embodiments, the unnatural amino acid is incorporated into the cytokine (e.g., the IL polypeptide) by a synthetic codon comprising an unnatural nucleic acid. In some embodiments, the unnatural amino acid is incorporated into the cytokine by an orthogonal, modified synthetase/tRNA pair. Such orthogonal pairs comprise an unnatural synthetase that is capable of charging the unnatural tRNA with the unnatural amino acid, while minimizing charging of a) other endogenous amino acids onto the unnatural tRNA and b) unnatural amino acids onto other endogenous tRNAs. Such orthogonal pairs comprise tRNAs that are capable of

being charged by the unnatural synthetase, while avoiding being charged with a) other endogenous amino acids by endogenous synthetases. In some embodiments, such pairs are identified from various organisms, such as bacteria, yeast, Archaea, or human sources. In some embodiments, an orthogonal synthetase/tRNA pair comprises components from a single organism. In some embodiments, an orthogonal synthetase/tRNA pair comprises components from two different organisms. In some embodiments, an orthogonal synthetase/tRNA pair comprising components that prior to modification, promote translation of two different amino acids. In some embodiments, an orthogonal synthetase is a modified alanine synthetase. In some embodiments, an orthogonal synthetase is a modified arginine synthetase. In some embodiments, an orthogonal synthetase is a modified asparagine synthetase. In some embodiments, an orthogonal synthetase is a modified aspartic acid synthetase. In some embodiments, an orthogonal synthetase is a modified cysteine synthetase. In some embodiments, an orthogonal synthetase is a modified glutamine synthetase. In some embodiments, an orthogonal synthetase is a modified glutamic acid synthetase. In some embodiments, an orthogonal synthetase is a modified alanine glycine. In some embodiments, an orthogonal synthetase is a modified histidine synthetase. In some embodiments, an orthogonal synthetase is a modified leucine synthetase. In some embodiments, an orthogonal synthetase is a modified isoleucine synthetase. In some embodiments, an orthogonal synthetase is a modified lysine synthetase. In some embodiments, an orthogonal synthetase is a modified methionine synthetase. In some embodiments, an orthogonal synthetase is a modified phenylalanine synthetase. In some embodiments, an orthogonal synthetase is a modified proline synthetase. In some embodiments, an orthogonal synthetase is a modified serine synthetase. In some embodiments, an orthogonal synthetase is a modified threonine synthetase. In some embodiments, an orthogonal synthetase is a modified tryptophan synthetase. In some embodiments, an orthogonal synthetase is a modified tyrosine synthetase. In some embodiments, an orthogonal synthetase is a modified valine synthetase. In some embodiments, an orthogonal synthetase is a modified phosphoserine synthetase. In some embodiments, an orthogonal tRNA is a modified alanine tRNA. In some embodiments, an orthogonal tRNA is a modified arginine tRNA. In some embodiments, an orthogonal tRNA is a modified asparagine tRNA. In some embodiments, an orthogonal tRNA is a modified aspartic acid tRNA. In some embodiments, an orthogonal tRNA is a modified cysteine tRNA. In some embodiments, an orthogonal tRNA is a modified glutamine tRNA. In some embodiments, an orthogonal tRNA is a modified glutamic acid tRNA. In some embodiments, an orthogonal tRNA is a modified alanine glycine. In some embodiments, an orthogonal tRNA is a modified histidine tRNA. In some embodiments, an orthogonal tRNA is a modified leucine tRNA. In some

embodiments, an orthogonal tRNA is a modified isoleucine tRNA. In some embodiments, an orthogonal tRNA is a modified lysine tRNA. In some embodiments, an orthogonal tRNA is a modified methionine tRNA. In some embodiments, an orthogonal tRNA is a modified phenylalanine tRNA. In some embodiments, an orthogonal tRNA is a modified proline tRNA. In some embodiments, an orthogonal tRNA is a modified serine tRNA. In some embodiments, an orthogonal tRNA is a modified threonine tRNA. In some embodiments, an orthogonal tRNA is a modified tryptophan tRNA. In some embodiments, an orthogonal tRNA is a modified tyrosine tRNA. In some embodiments, an orthogonal tRNA is a modified valine tRNA. In some embodiments, an orthogonal tRNA is a modified phosphoserine tRNA.

[0412] In some embodiments, the unnatural amino acid is incorporated into the cytokine (e.g., the IL polypeptide) by an aminoacyl (aaRS or RS)-tRNA synthetase-tRNA pair. Exemplary aaRS-tRNA pairs include, but are not limited to, *Methanococcus jannaschii* (*Mj-Tyr*) aaRS/tRNA pairs, *E. coli* TyrRS (*Ec-Tyr*)/*B. stearothermophilus* tRNA_{CUA} pairs, *E. coli* LeuRS (*Ec-Leu*)/*B. stearothermophilus* tRNA_{CUA} pairs, and pyrrolysyl-tRNA pairs. In some embodiments, the unnatural amino acid is incorporated into the cytokine (e.g., the IL polypeptide) by a *Mj-Tyr*RS/tRNA pair. Exemplary UAAs that can be incorporated by a *Mj-Tyr*RS/tRNA pair include, but are not limited to, para-substituted phenylalanine derivatives such as *p*-aminophenylalanine and *p*-methoxyphenylalanine; meta-substituted tyrosine derivatives such as 3-aminotyrosine, 3-nitrotyrosine, 3,4-dihydroxyphenylalanine, and 3-iodotyrosine; phenylselenocysteine; *p*-boronophenylalanine; and *o*-nitrobenzyltyrosine.

[0413] In some embodiments, the unnatural amino acid is incorporated into the cytokine (e.g., the IL polypeptide) by a *Ec-Tyr*/tRNA_{CUA} or a *Ec-Leu*/tRNA_{CUA} pair. Exemplary UAAs that can be incorporated by a *Ec-Tyr*/tRNA_{CUA} or a *Ec-Leu*/tRNA_{CUA} pair include, but are not limited to, phenylalanine derivatives containing benzophenone, ketone, iodide, or azide substituents; *O*-propargyltyrosine; α -aminocaprylic acid, *O*-methyl tyrosine, *O*-nitrobenzyl cysteine; and 3-(naphthalene-2-ylamino)-2-amino-propanoic acid.

[0414] In some embodiments, the unnatural amino acid is incorporated into the cytokine (e.g., the IL polypeptide) by a pyrrolysyl-tRNA pair. In some embodiments, the PylRS is obtained from an archaeobacterial, e.g., from a methanogenic archaeobacterial. In some embodiments, the PylRS is obtained from *Methanosarcina barkeri*, *Methanosarcina mazei*, or *Methanosarcina acetivorans*. Exemplary UAAs that can be incorporated by a pyrrolysyl-tRNA pair include, but are not limited to, amide and carbamate substituted lysines such as 2-amino-6-((R)-tetrahydrofuran-2-carboxamido)hexanoic acid, *N*- ϵ -D-prolyl-L-lysine, and *N*- ϵ -cyclopentylloxycarbonyl-L-lysine; *N*- ϵ -Acryloyl-L-lysine; *N*- ϵ -[(1-(6-nitrobenzo[d][1,3]dioxol-5-

yl)ethoxy)carbonyl]-L-lysine; and *N*-ε-(1-methylcyclopro-2-enecarboxamido)lysine. In some embodiments, the IL-2 conjugates disclosed herein may be prepared by use of *M. mazei* tRNA which is selectively charged with a non-natural amino acid such as *N*6-((2-azidoethoxy)-carbonyl)-L-lysine (AzK) by the *M. barkeri* pyrrolysyl-tRNA synthetase (*Mb* PylRS). Other methods are known to those of ordinary skill in the art, such as those disclosed in Zhang et al., *Nature* 2017, 551(7682): 644-647.

[0415] In some embodiments, an unnatural amino acid is incorporated into a cytokine described herein (e.g., the IL polypeptide) by a synthetase disclosed in US 9,988,619 and US 9,938,516.

[0416] The host cell into which the constructs or vectors disclosed herein are introduced is cultured or maintained in a suitable medium such that the tRNA, the tRNA synthetase and the protein of interest are produced. The medium also comprises the unnatural amino acid(s) such that the protein of interest incorporates the unnatural amino acid(s). In some embodiments, a nucleoside triphosphate transporter (NTT) from bacteria, plant, or algae is also present in the host cell. In some embodiments, the IL-2 conjugates disclosed herein are prepared by use of a host cell that expresses a NTT. In some embodiments, the nucleotide nucleoside triphosphate transporter used in the host cell may be selected from TpNTT1, TpNTT2, TpNTT3, TpNTT4, TpNTT5, TpNTT6, TpNTT7, TpNTT8 (*T. pseudonana*), PtNTT1, PtNTT2, PtNTT3, PtNTT4, PtNTT5, PtNTT6 (*P. tricornutum*), GsNTT (*Galdieria sulphuraria*), AtNTT1, AtNTT2 (*Arabidopsis thaliana*), CtNTT1, CtNTT2 (*Chlamydia trachomatis*), PamNTT1, PamNTT2 (*Protochlamydia amoebophila*), CcNTT (*Caedibacter caryophilus*), RpNTT1 (*Rickettsia prowazekii*). In some embodiments, the NTT is selected from PtNTT1, PtNTT2, PtNTT3, PtNTT4, PtNTT5, and PtNTT6. In some embodiments, the NTT is PtNTT1. In some embodiments, the NTT is PtNTT2. In some embodiments, the NTT is PtNTT3. In some embodiments, the NTT is PtNTT4. In some embodiments, the NTT is PtNTT5. In some embodiments, the NTT is PtNTT6. Other NTTs that may be used are disclosed in Zhang et al., *Nature* 2017, 551(7682): 644-647; Malyshev et al. *Nature* 2014 (509(7500), 385-388; and Zhang et al. *Proc Natl Acad Sci USA*, 2017, 114:1317–1322.

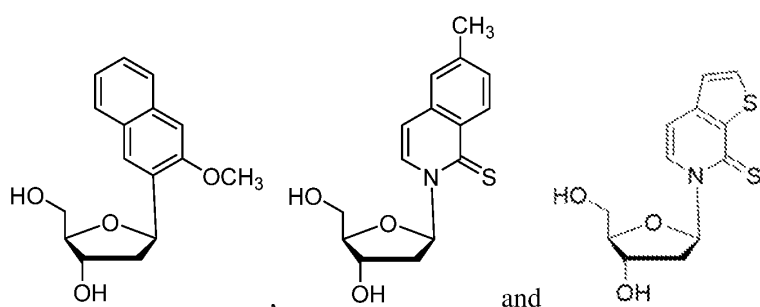
[0417] The orthogonal tRNA synthetase/tRNA pair charges a tRNA with an unnatural amino acid and incorporates the unnatural amino acid into the polypeptide chain in response to the codon. Exemplary aaRS-tRNA pairs include, but are not limited to, *Methanococcus jannaschii* (*Mj-Tyr*) aaRS/tRNA pairs, *E. coli* TyrRS (*Ec-Tyr*)/*B. stearothermophilus* tRNA_{CUA} pairs, *E. coli* LeuRS (*Ec-Leu*)/*B. stearothermophilus* tRNA_{CUA} pairs, and pyrrolysyl-tRNA pairs. Other aaRS-tRNA pairs that may be used according to the present disclosure include those derived from *M.*

mazei those described in Feldman et al., J Am Chem Soc., 2018 140:1447–1454; and Zhang et al. Proc Natl Acad Sci USA, 2017, 114:1317–1322.

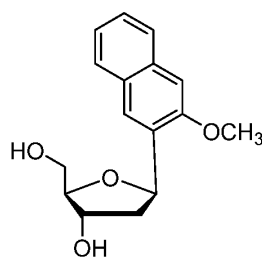
[0418] In some embodiments are provided methods of preparing the IL-2 conjugates disclosed herein in a cellular system that expresses a NTT and a tRNA synthetase. In some embodiments described herein, the NTT is selected from PtNTT1, PtNTT2, PtNTT3, PtNTT4, PtNTT5, and PtNTT6, and the tRNA synthetase is selected from *Methanococcus jannaschii*, *E. coli* TyrRS (*Ec-Tyr*)/*B. stearothermophilus*, and *M. mazei*. In some embodiments, the NTT is PtNTT1 and the tRNA synthetase is derived from *Methanococcus jannaschii*, *E. coli* TyrRS (*Ec-Tyr*)/*B. stearothermophilus*, or *M. mazei*. In some embodiments, the NTT is PtNTT2 and the tRNA synthetase is derived from *Methanococcus jannaschii*, *E. coli* TyrRS (*Ec-Tyr*)/*B. stearothermophilus*, or *M. mazei*. In some embodiments, the NTT is PtNTT3 and the tRNA synthetase is derived from *Methanococcus jannaschii*, *E. coli* TyrRS (*Ec-Tyr*)/*B. stearothermophilus*, or *M. mazei*. In some embodiments, the NTT is PtNTT3 and the tRNA synthetase is derived from *Methanococcus jannaschii*, *E. coli* TyrRS (*Ec-Tyr*)/*B. stearothermophilus*, or *M. mazei*. In some embodiments, the NTT is PtNTT4 and the tRNA synthetase is derived from *Methanococcus jannaschii*, *E. coli* TyrRS (*Ec-Tyr*)/*B. stearothermophilus*, or *M. mazei*. In some embodiments, the NTT is PtNTT5 and the tRNA synthetase is derived from *Methanococcus jannaschii*, *E. coli* TyrRS (*Ec-Tyr*)/*B. stearothermophilus*, or *M. mazei*. In some embodiments, the NTT is PtNTT6 and the tRNA synthetase is derived from *Methanococcus jannaschii*, *E. coli* TyrRS (*Ec-Tyr*)/*B. stearothermophilus*, or *M. mazei*.

[0419] In some embodiments, the IL-2 conjugates disclosed herein may be prepared in a cell, such as *E. coli*, comprising (a) nucleoside triphosphate transporter *PtNTT2* (including a truncated variant in which the first 65 amino acid residues of the full-length protein are deleted), (b) a plasmid comprising a double-stranded oligonucleotide that encodes an IL-2 variant having a desired amino acid sequence and that contains a unnatural base pair comprising a first unnatural nucleotide and a second unnatural nucleotide to provide a codon at the desired position at which an unnatural amino acid, such as *N*6-((2-azidoethoxy)-carbonyl)-L-lysine (AzK), will be incorporated, (c) a plasmid encoding a tRNA derived from *M. mazei* and which comprises an unnatural nucleotide to provide a recognized anticodon (to the codon of the IL-2 variant) in place of its native sequence, and (d) a plasmid encoding a *M. barkeri* derived pyrrolysyl-tRNA synthetase (*Mb* PylRS), which may be the same plasmid that encodes the tRNA or a different plasmid. In some embodiments, the cell is further supplemented with deoxyribo triphosphates comprising one or more unnatural bases. In some embodiments, the cell is further supplemented

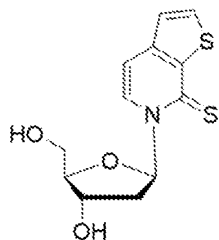
with ribo triphosphates comprising one or more unnatural bases. In some embodiments, the cells is further supplemented with one or more unnatural amino acids, such as *N*6-((2-azidoethoxy)-carbonyl)-L-lysine (AzK). In some embodiments, the double-stranded oligonucleotide that encodes the amino acid sequence of the desired IL-2 variant contains a codon AXC at, for example, at position 8, 11, 14, 15, 18, 19, 22, 87, 99, or 108 of the sequence that encodes the protein having SEQ ID NO: 1 (aldesleukin), wherein X is an unnatural nucleotide. In some embodiments, the cell further comprises a plasmid, which may be the protein expression plasmid or another plasmid, that encodes an orthogonal tRNA gene from *M. mazei* that comprises an AXC-matching anticodon GYT in place of its native sequence, wherein Y is an unnatural nucleotide that is complementary and may be the same or different as the unnatural nucleotide in the codon. In some embodiments, the unnatural nucleotide in the codon is different than and complimentary to the unnatural nucleotide in the anti-codon. In some embodiments, the unnatural nucleotide in the codon is the same as the unnatural nucleotide in the anti-codon. In some embodiments, the first unnatural nucleotide and second unnatural nucleotide of the unnatural base pair in the double-stranded oligonucleotide may be derived from



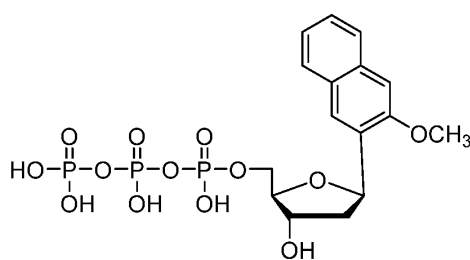
. In some embodiments, the first unnatural nucleotide and second unnatural nucleotide of the unnatural base pair in the



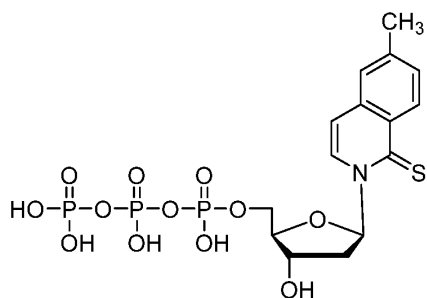
double-stranded oligonucleotide may be derived from



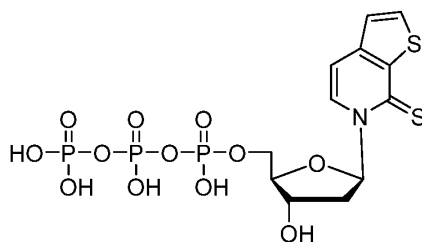
. In some embodiments, the triphosphates of the first and second unnatural



nucleotides include,

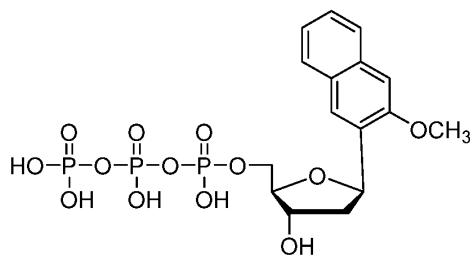


and

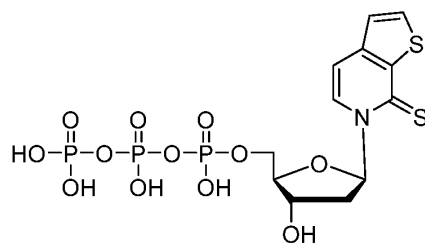


, or salts thereof.

In some embodiments, the triphosphates of the first and second unnatural nucleotides include,

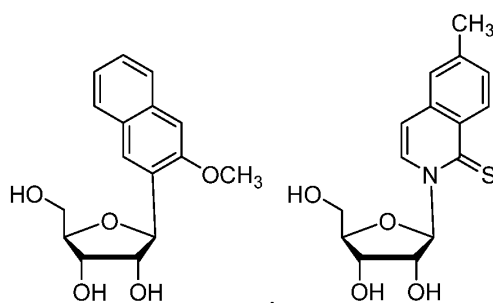


, and

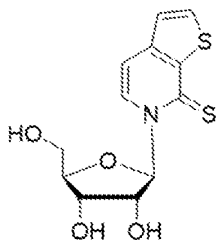


, or salts

thereof. In some embodiments, the mRNA derived the double-stranded oligonucleotide comprising a first unnatural nucleotide and a second unnatural nucleotide may comprise a codon

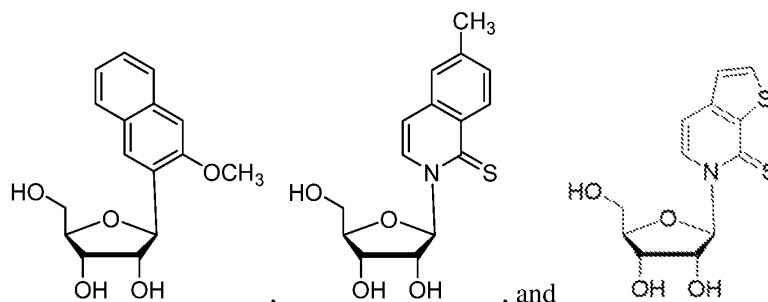


comprising an unnatural nucleotide derived from



and . In some embodiments, the *M. maei* tRNA may comprise an anti-codon comprising an unnatural nucleotide that recognizes the codon comprising the unnatural

nucleotide of the mRNA. The anti-codon in the *M. mazei* tRNA may comprise an unnatural



nucleotide derived from

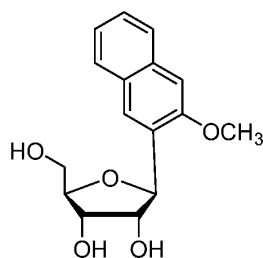
,

,

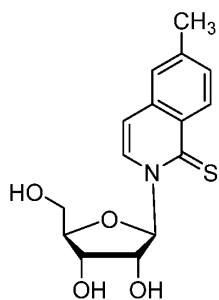
and

.

In some embodiments, the mRNA comprises an unnatural nucleotide derived from



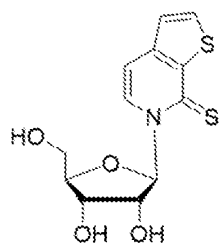
. In some embodiments, the mRNA comprises an unnatural nucleotide



derived from

.

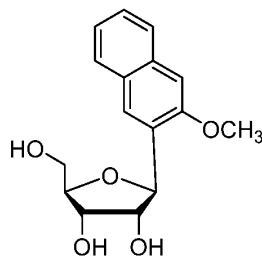
In some embodiments, the mRNA comprises an unnatural



nucleotide derived from

.

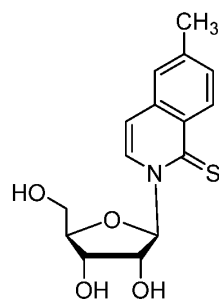
In some embodiments, the tRNA comprises an



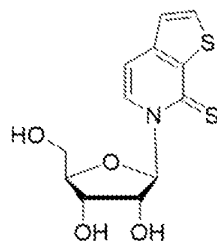
unnatural nucleotide derived from

.

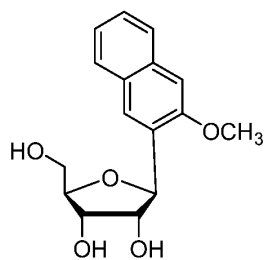
In some embodiments, the tRNA



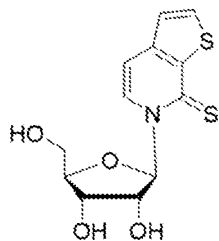
comprises an unnatural nucleotide derived from . In some embodiments, the



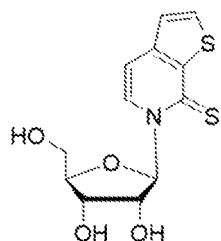
tRNA comprises an unnatural nucleotide derived from . In some
embodiments, the mRNA comprises an unnatural nucleotide derived from



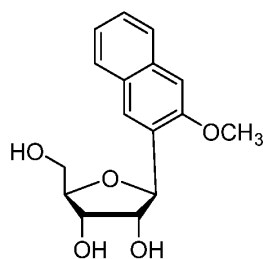
and the tRNA comprises an unnatural nucleotide derived from



. In some embodiments, the mRNA comprises an unnatural nucleotide



derived from and the tRNA comprises an unnatural nucleotide derived from



. The host cell is cultured in a medium containing appropriate nutrients, and is supplemented with (a) the triphosphates of the deoxyribo nucleosides comprising one or more unnatural bases that are necessary for replication of the plasmid(s) encoding the cytokine gene harboring the codon, (b) the triphosphates of the ribo nucleosides comprising one or more unnatural bases necessary for transcription of (i) the mRNA corresponding to the coding sequence of the cytokine and containing the codon comprising one or more unnatural bases, and (ii) the tRNA containing the anticodon comprising one or more unnatural bases, and (c) the unnatural amino acid(s) to be incorporated in to the polypeptide sequence of the cytokine of interest. The host cells are then maintained under conditions which permit expression of the protein of interest.

[0420] The resulting protein comprising the one or more unnatural amino acids, Azk for example, that is expressed may be purified by methods known to those of ordinary skill in the art and may then be allowed to react with an alkyne, such as DBCO comprising a PEG chain having a desired average molecular weight as disclosed herein, under conditions known to those of ordinary skill in the art, to afford the IL-2 conjugates disclosed herein. Other methods are known to those of ordinary skill in the art, such as those disclosed in Zhang et al., Nature 2017, 551(7682): 644-647; WO 2015157555; WO 2015021432; WO 2016115168; WO 2017106767; WO 2017223528; WO 2019014262; WO 2019014267; WO 2019028419; and WO2019/028425.

[0421] Alternatively, a cytokine (e.g., IL-2) polypeptide comprising an unnatural amino acid(s) is prepared by introducing the nucleic acid constructs described herein comprising the tRNA and aminoacyl tRNA synthetase and comprising a nucleic acid sequence of interest with one or more in-frame orthogonal (stop) codons into a host cell. The host cell is cultured in a medium containing appropriate nutrients, is supplemented with (a) the triphosphates of the deoxyribo nucleosides comprising one or more unnatural bases required for replication of the plasmid(s) encoding the cytokine gene harboring the new codon and anticodon, (b) the triphosphates of the ribo nucleosides required for transcription of the mRNA corresponding to (i) the cytokine sequence containing the codon, and (ii) the orthogonal tRNA containing the anticodon, and (c) the unnatural amino acid(s). The host cells are then maintained under conditions which permit expression of the protein of interest. The unnatural amino acid(s) is incorporated into the

polypeptide chain in response to the unnatural codon. For example, one or more unnatural amino acids are incorporated into the cytokine (e.g., IL-2) polypeptide. Alternatively, two or more unnatural amino acids may be incorporated into the cytokine (e.g., IL-2) polypeptide at two or more sites in the protein.

[0422] Once the cytokine (e.g., IL-2) polypeptide incorporating the unnatural amino acid(s) has been produced in the host cell it can be extracted therefrom by a variety of techniques known in the art, including enzymatic, chemical and/or osmotic lysis and physical disruption. The cytokine (e.g., IL-2) polypeptide can be purified by standard techniques known in the art such as preparative ion exchange chromatography, hydrophobic chromatography, affinity chromatography, or any other suitable technique known to those of ordinary skill in the art.

[0423] Suitable host cells may include bacterial cells (e.g., *E. coli*, BL21(DE3)), but most suitably host cells are eukaryotic cells, for example insect cells (e.g. *Drosophila* such as *Drosophila melanogaster*), yeast cells, nematodes (e.g. *C. elegans*), mice (e.g. *Mus musculus*), or mammalian cells (such as Chinese hamster ovary cells (CHO) or COS cells, human 293T cells, HeLa cells, NIH 3T3 cells, and mouse erythroleukemia (MEL) cells) or human cells or other eukaryotic cells. Other suitable host cells are known to those skilled in the art. Suitably, the host cell is a mammalian cell - such as a human cell or an insect cell. In some embodiments, the suitable host cells comprise *E. coli*.

[0424] Other suitable host cells which may be used generally in the embodiments of the disclosure are those mentioned in the examples section. Vector DNA can be introduced into host cells via conventional transformation or transfection techniques. As used herein, the terms "transformation" and "transfection" are intended to refer to a variety of well-recognized techniques for introducing a foreign nucleic acid molecule (e.g., DNA) into a host cell, including calcium phosphate or calcium chloride co-precipitation, DEAE-dextran-mediated transfection, lipofection, or electroporation. Suitable methods for transforming or transfecting host cells are well known in the art.

[0425] When creating cell lines, it is generally preferred that stable cell lines are prepared. For stable transfection of mammalian cells for example, it is known that, depending upon the expression vector and transfection technique used, only a small fraction of cells may integrate the foreign DNA into their genome. In order to identify and select these integrants, a gene that encodes a selectable marker (for example, for resistance to antibiotics) is generally introduced into the host cells along with the gene of interest. Preferred selectable markers include those that confer resistance to drugs, such as G418, hygromycin, or methotrexate. Nucleic acid molecules encoding a selectable marker can be introduced into a host cell on the same vector or can be

introduced on a separate vector. Cells stably transfected with the introduced nucleic acid molecule can be identified by drug selection (for example, cells that have incorporated the selectable marker gene will survive, while the other cells die).

[0426] In one embodiment, the constructs described herein are integrated into the genome of the host cell. An advantage of stable integration is that the uniformity between individual cells or clones is achieved. Another advantage is that selection of the best producers may be carried out. Accordingly, it is desirable to create stable cell lines. In another embodiment, the constructs described herein are transfected into a host cell. An advantage of transfecting the constructs into the host cell is that protein yields may be maximized.

C. Gene Therapy Agent

[0427] In some embodiments, the disclosure provides methods for delivering a gene therapy agent to a cell of an individual, the method comprising a) administering an IL-2 conjugate to the individual, and b) administering a gene therapy agent to the individual. In some embodiments, the disclosure provides methods for treating an individual in need thereof, the method comprising a) administering an IL-2 conjugate to the individual, and b) administering a gene therapy agent to the individual. In some embodiments, the disclosure provides methods for improving gene therapy (e.g., increasing expression of a gene therapy agent) in an individual, the method comprising a) administering an IL-2 conjugate to the individual, and b) administering a gene therapy agent to the individual. In some embodiments, the disclosure provides methods for modulating an immune response to a gene therapy agent, the method comprising a) administering an IL-2 conjugate to the individual, and b) administering a gene therapy agent to the individual. In some embodiments, the disclosure provides methods for suppressing an immune response to a gene therapy agent, the method comprising a) administering an IL-2 conjugate to the individual, and b) administering a gene therapy agent to the individual. In some embodiments, the disclosure provides methods for inducing tolerance to a gene therapy agent, the method comprising a) administering an IL-2 conjugate to the individual, and b) administering a gene therapy agent to the individual. In some embodiments, the disclosure provides methods for preventing immune-related adverse events in an individual, the method comprising a) administering an IL-2 conjugate to the individual, and b) administering a gene therapy agent to the individual. In some embodiments, the IL-2 conjugate reduces binding of the IL-2 conjugate to IL-2R $\beta\gamma$ relative to IL-2R $\alpha\beta\gamma$. In some embodiments, the IL-2 conjugate expands CD4⁺ T regulatory (Treg) cells in the subject. In some embodiments, the IL-2 conjugate suppresses CD8⁺ T cell proliferation in the subject. In some embodiments, the IL-2 conjugate suppresses

effector memory CD8+ T cell proliferation in the subject. In some embodiments, the IL-2 conjugate suppresses vector-specific IFN γ -secreting CD8+ T cells in the subject. In some embodiments, the IL-2 conjugate suppresses transgene-product-specific IFN γ -secreting CD8+ T cells in the subject. In some embodiments, the IL-2 conjugate suppresses production of antibodies against a transgene product. In some embodiments, the gene therapy agent is a viral gene therapy agent (e.g., a viral vector) or a non-viral gene therapy agent (e.g., a lipid nanoparticle comprising a non-viral gene therapy agent). In some embodiments, the gene therapy agent is an adeno-associated virus (AAV) vector, an adenovirus vector, a lentivirus vector, or a herpes simplex virus (HSV) vector. In some embodiments, the IL-2 conjugate suppresses the production of antibodies against a viral vector. In some embodiments, the IL-2 conjugate suppresses the production of antibodies against a viral vector by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 75%, or 100% relative to baseline (e.g., versus negative control). In some embodiments, the IL-2 conjugate suppresses production of antibodies against a capsid protein of a viral vector. In some embodiments, the IL-2 conjugate suppresses the production of antibodies against a capsid protein of a viral vector by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 75%, or 100% relative to baseline (e.g., versus negative control). Suppression of antibodies against a viral vector or a capsid protein of a viral vector may be measured using samples taken at, e.g., about 5-16 weeks after administration of the viral vector, such as about 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, or 16 weeks. In some embodiments, the IL-2 conjugate suppresses immune mediated dorsal root ganglion (DRG) toxicity that can occur after the administration of a viral vector (e.g., an Adeno-associated virus (AAV)) to a subject. In some embodiments, DRG toxicity can be characterized by mononuclear cell infiltration, neuronal degeneration/necrosis, and secondary axonopathy of central and peripheral axons. In some embodiments, the IL-2 conjugate suppresses immune mediated DRG toxicity by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 75%, or 100% relative to baseline (e.g., from negative control). In some embodiments, the IL-2 conjugate suppresses mononuclear cell infiltration by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 75%, or 100% relative to baseline (e.g., from negative control). In some embodiments, the IL-2 conjugate suppresses neuronal degeneration/necrosis by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 75%, or 100% relative to baseline (e.g., from negative control). In some embodiments, the IL-2 conjugate suppresses secondary axonopathy of central and peripheral axons by at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 75%, or 100% relative to baseline (e.g., from negative control).

[0428] In some embodiments of the disclosure, the gene therapy agent (e.g., an AAV particle, an adenovirus particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle) may be administered to a particular tissue of interest, or it may be administered systemically. In some embodiments, an effective amount of the gene therapy agent may be administered to the subject. In some embodiments, an effective amount of the gene therapy agent may be administered parenterally. Parenteral routes of administration may include without limitation intravenous, intraperitoneal, intraosseous, intra-arterial, intracerebral, intramuscular, intrathecal, subcutaneous, intracerebroventricular, intrahepatic, intracranial, intra-cerebrospinal fluid (CSF), intra-dorsal root ganglia (DRG), intraocular, intracisterna magna (ICM), and so forth. In some embodiments, an effective amount of the gene therapy agent may be administered through one route of administration. In some embodiments, an effective amount of the gene therapy agent may be administered through a combination of or multiple routes of administration (e.g., two, three etc.). In some embodiments, an effective amount of the gene therapy agent is administered to one location. In other embodiments, an effective amount of the gene therapy agent may be administered to more than one location.

[0429] An effective amount of gene therapy agent (e.g., an AAV particle, an adenovirus particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle) is administered, depending on the objectives of treatment. For example, where a low percentage of transduction or transfection can achieve the desired therapeutic effect, then the objective of treatment is generally to meet or exceed this level of transduction or transfection. In some embodiments, this level of transduction or transfection can be achieved by transduction or transfection of only about 1 to 5% of the target cells of the desired tissue type, in some embodiments at least about 20% of the cells of the desired tissue type, in some embodiments at least about 50%, in some embodiments at least about 80%, in some embodiments at least about 95%, in some embodiments at least about 99% of the cells of the desired tissue type. The gene therapy agent may be administered by one or more administrations, either during the same procedure or spaced apart by days, weeks, months, or years. One or more of any of the routes of administration described herein may be used. In some embodiments, multiple gene therapy agents may be used to treat the human; for example, an AAV vector and a lentiviral vector.

[0430] Methods to identify cells transduced or transfected by gene therapy agents are known in the art; for example, immunohistochemistry or the use of a marker such as enhanced green fluorescent protein can be used to detect transduction or transfection cells by the gene therapy agent.

[0431] In some embodiments, an effective amount of gene therapy agent (e.g., an AAV particle, an adenovirus particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle) is administered to more than one location simultaneously or sequentially. In other embodiments, an effective amount of the gene therapy agent is administered to a single location more than once (e.g., repeated). In some embodiments, multiple injections of the gene therapy agent are no more than one hour, two hours, three hours, four hours, five hours, six hours, nine hours, twelve hours or 24 hours apart.

[0432] In some embodiments, the methods comprise administering an effective amount of a pharmaceutical composition comprising a gene therapy agent to treat an individual in need of gene therapy treatment. In some embodiments, the viral titer of the viral particles (e.g., rAAV particles) is at least about any of 5×10^{12} , 6×10^{12} , 7×10^{12} , 8×10^{12} , 9×10^{12} , 10×10^{12} , 11×10^{12} , 15×10^{12} , 20×10^{12} , 25×10^{12} , 30×10^{12} , or 50×10^{12} genome copies/mL. In some embodiments, the viral titer of the viral particles (e.g., rAAV particles) is about any of 5×10^{12} to 6×10^{12} , 6×10^{12} to 7×10^{12} , 7×10^{12} to 8×10^{12} , 8×10^{12} to 9×10^{12} , 9×10^{12} to 10×10^{12} , 10×10^{12} to 11×10^{12} , 11×10^{12} to 15×10^{12} , 15×10^{12} to 20×10^{12} , 20×10^{12} to 25×10^{12} , 25×10^{12} to 30×10^{12} , 30×10^{12} to 50×10^{12} , or 50×10^{12} to 100×10^{12} genome copies/mL. In some embodiments, the viral titer of the viral particles (e.g., rAAV particles) is about any of 5×10^{12} to 10×10^{12} , 10×10^{12} to 25×10^{12} , or 25×10^{12} to 50×10^{12} genome copies/mL. In some embodiments, the viral titer of the viral particles (e.g., rAAV particles) is at least about any of 5×10^9 , 6×10^9 , 7×10^9 , 8×10^9 , 9×10^9 , 10×10^9 , 11×10^9 , 15×10^9 , 20×10^9 , 25×10^9 , 30×10^9 , or 50×10^9 transducing units /mL. In some embodiments, the viral titer of the viral particles (e.g., rAAV particles) is about any of 5×10^9 to 6×10^9 , 6×10^9 to 7×10^9 , 7×10^9 to 8×10^9 , 8×10^9 to 9×10^9 , 9×10^9 to 10×10^9 , 10×10^9 to 11×10^9 , 11×10^9 to 15×10^9 , 15×10^9 to 20×10^9 , 20×10^9 to 25×10^9 , 25×10^9 to 30×10^9 , 30×10^9 to 50×10^9 or 50×10^9 to 100×10^9 transducing units/mL. In some embodiments, the viral titer of the viral particles (e.g., rAAV particles) is about any of 5×10^9 to 10×10^9 , 10×10^9 to 15×10^9 , 15×10^9 to 25×10^9 , or 25×10^9 to 50×10^9 transducing units /mL. In some embodiments, the viral titer of the viral particles (e.g., rAAV particles) is at least any of about 5×10^{10} , 6×10^{10} , 7×10^{10} , 8×10^{10} , 9×10^{10} , 10×10^{10} , 11×10^{10} , 15×10^{10} , 20×10^{10} , 25×10^{10} , 30×10^{10} , 40×10^{10} , or 50×10^{10} infectious units/mL. In some embodiments, the viral titer of the viral particles (e.g., rAAV particles) is at least any of about 5×10^{10} to 6×10^{10} , 6×10^{10} to 7×10^{10} , 7×10^{10} to 8×10^{10} , 8×10^{10} to 9×10^{10} , 9×10^{10} to 10×10^{10} , 10×10^{10} to 11×10^{10} , 11×10^{10} to 15×10^{10} , 15×10^{10} to 20×10^{10} , 20×10^{10} to 25×10^{10} , 25×10^{10} to 30×10^{10} , 30×10^{10} to 40×10^{10} , 40×10^{10} to 50×10^{10} , or 50×10^{10} to 100×10^{10} infectious units/mL. In some embodiments, the viral titer of the viral

particles (e.g., rAAV particles) is at least any of about 5×10^{10} to 10×10^{10} , 10×10^{10} to 15×10^{10} , 15×10^{10} to 25×10^{10} , or 25×10^{10} to 50×10^{10} infectious units/mL.

[0433] In some embodiments, the dose of gene therapy agent (e.g., an AAV particle, an adenovirus particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle) administered to the individual is at least about any of 1×10^8 to about 6×10^{13} genome copies/kg of body weight. In some embodiments, the dose of gene therapy agent administered to the individual is about any of 1×10^8 to about 6×10^{13} genome copies/kg of body weight.

[0434] In some embodiments, the total amount of the gene therapy agent (e.g., an AAV particle, an adenovirus particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle) administered to the individual is at least about any of 1×10^9 to about 1×10^{14} genome copies. In some embodiments, the total amount of the gene therapy agent administered to the individual is about any of 1×10^9 to about 1×10^{14} genome copies.

[0435] Compositions of the disclosure comprising the gene therapy (e.g., an AAV particle, an adenovirus particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle) can be used either alone or in combination with one or more additional therapeutic agents in addition to the IL-2 conjugate. The interval between sequential administration can be in terms of at least (or, alternatively, less than) minutes, hours, or days.

[0436] In some embodiments, described herein are gene therapy conjugates. In some embodiments, the gene therapy agent can be delivered to a cell of a subject. In some embodiments, the gene therapy agent can be administered to the subject before an IL-2 conjugate, concurrently with an IL-2 conjugate, or after an IL-2 conjugate. In some embodiments, the gene therapy agent can be administered to the subject concurrently with an IL-2 conjugate.

[0437] In some embodiments, the gene therapy agent is administered to a subject at least about 1 second, about 5 seconds, about 10 seconds, about 15 seconds, about 30 seconds, about 45 seconds, about 1 minute, about 2 minutes, about 5 minutes, about 15 minutes, about 30 minutes, about 1 hour, about 1.5 hours, about 2 hours, about 3 hours, about 4 hours, or about 6 hours before the administration of the IL-2 conjugate. In some embodiments, the gene therapy agent is administered to a subject at least about 12 hours, about 1 day, about 2 days, about 3 days, about 4 days, about 5 days, about 6 days, about 7 days, about 8 days, about 9 days, about 10 days, about 11 days, about 12 days, about 13 days, or about 14 days before the administration of the IL-2 conjugate. In some embodiments, the gene therapy agent is administered to a subject less than about 14 days, about 13 days, about 12 days, about 11 days, 10 days, about 9 days, about 8 days, about 7 days, about 6 days, about 5 days, about 4 days, about 3 days, about 2 days, about 1 day,

about 12 hours, about 6 hours, about 4 hours, about 3 hours, about 2 hours, about 1.5 hours, about 1 hour, about 45 minutes, about 30 minutes, about 15 minutes, about 5 minutes, about 2 minutes, or about 1 minute before the IL-2 conjugate.

[0438] In some embodiments, the gene therapy agent is administered to a subject at least about 1 second, about 5 seconds, about 10 seconds, about 15 seconds, about 30 seconds, about 45 seconds, about 1 minute, about 2 minutes, about 5 minutes, about 15 minutes, about 30 minutes, about 45 minutes, about 1 hour, about 1.5 hours, about 2 hours, about 3 hours, about 4 hours, or about 6 hours after the administration of the IL-2 conjugate. In some embodiments, the gene therapy agent is administered to a subject at least about 12 hours, about 1 day, about 2 days, about 3 days, about 4 days, about 5 days, about 6 days, or about 7 days after the administration of the IL-2 conjugate. In some embodiments, the gene therapy agent is administered to a subject less than about 7 days, about 6 days, about 5 days, about 4 days, about 3 days, about 2 days, about 1 day, about 12 hours, about 6 hours, about 4 hours, about 3 hours, about 2 hours, about 1.5 hours, about 1 hour, about 45 minutes, about 30 minutes, about 15 minutes, about 5 minutes, about 2 minutes, or about 1 minute after the IL-2 conjugate.

[0439] In some embodiments, the disclosure provides methods for using IL-2 conjugates with gene therapy agents for improved gene therapy by inhibiting an adaptive immune response to the gene therapy agent. In some embodiments, the gene therapy agent is a viral particle or a lipid nanoparticle. In some embodiments, the gene therapy agent is an adeno-associated virus (AAV) particle, an adenovirus particle, a lentivirus particle, or a herpes simplex virus (HAV) particle. In some embodiments, the gene therapy agent is a lipid nanoparticle or a liposome. In some embodiments, the immune response to the gene therapy agent is an immune response to the viral particle (e.g., viral capsid proteins, viral envelopes, etc.). In some embodiments, the immune response to the gene therapy agent is an immune response to an LNP (e.g., one or more lipids used to produce the LNP). In some embodiments, the immune response to the gene therapy agent is an immune response to the gene therapy payload; e.g., nucleic acid encoding the therapeutic transgene (a viral genome, a plasmid, a closed ended DNA, an mRNA, an antisense nucleic acid, a siRNA, a shRNA and the like). In some embodiments, the immune response to the gene therapy agent is an immune response to the transgene product (e.g., a therapeutic polypeptide or therapeutic nucleic acid). In some embodiments, the transgene product (e.g., a therapeutic polypeptide or therapeutic nucleic acid) can be expressed and/or synthesized in a subject for at least about 1 week. In some embodiments, the transgene product (e.g., a therapeutic polypeptide or therapeutic nucleic acid) can be expressed and/or synthesized in a subject for at least about 1 week, about 2 weeks, about 3 weeks, about 4 weeks, about 5 weeks, about 6 weeks, about 7

weeks, about 8 weeks, about 9 weeks, about 10 weeks, about 11 weeks, about 12 weeks, about 13 weeks, about 14 weeks, about 15 weeks, about 16 weeks, about 17 weeks, about 18 weeks, about 19 weeks, about 20 weeks, about 22 weeks, about 24 weeks, or about 26 weeks. In some embodiments, a transgene product may, e.g., supply a polypeptide and/or enzymatic activity that is absent or present at a reduced level in a cell or organism. In some embodiments, a transgene product may supply a polypeptide and/or enzymatic activity that indirectly counteracts an imbalance in a cell or organism. In some embodiments, a change in expression of the transgene product comprises changes in expression levels of a therapeutic nucleic acid and/or an endogenous nucleic acid in a cell or organism of at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 75%, or 100% relative to baseline (e.g., from before treatment or from negative control). In some embodiments, a change in expression of the transgene product comprises prolonging the expression of a therapeutic nucleic acid and/or an endogenous nucleic acid in a cell or organism for at least about 1 day, about 2 days, about 3 days, about 4 days, about 5 days, about 6 days, about 1 week, about 2 weeks, about 3 weeks, about 4 weeks, about 5 weeks, about 6 weeks, about 7 weeks, about 8 weeks, about 9 weeks, about 10 weeks, about 11 weeks, about 12 weeks, about 13 weeks, about 14 weeks, about 15 weeks, about 16 weeks, about 17 weeks, about 18 weeks, about 19 weeks, about 20 weeks, about 22 weeks, about 24 weeks, or about 26 weeks relative to baseline (e.g., before treatment or versus negative control). In some embodiments, the negative control is a subject that is administered a gene therapy agent without administration of an IL-2 conjugate. In some embodiments, a change in synthesis of a transgene product comprises changes in synthesis levels of a therapeutic polypeptide encoded by a transgene product and/or an endogenous polypeptide in a cell or organism of at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 75%, or 100% relative to baseline (e.g., from before treatment or from negative control). In some embodiments, a change in synthesis of the transgene product comprises prolonging the synthesis of a therapeutic nucleic acid and/or an endogenous nucleic acid in a cell or organism for at least about 1 day, about 2 days, about 3 days, about 4 days, about 5 days, about 6 days, about 1 week, about 2 weeks, about 3 weeks, about 4 weeks, about 5 weeks, about 6 weeks, about 7 weeks, about 8 weeks, about 9 weeks, about 10 weeks, about 11 weeks, about 12 weeks, about 13 weeks, about 14 weeks, about 15 weeks, about 16 weeks, about 17 weeks, about 18 weeks, about 19 weeks, about 20 weeks, about 22 weeks, about 24 weeks, or about 26 weeks relative to baseline (e.g., from before treatment or versus negative control). In some embodiments, the negative control is a subject that is administered a gene therapy agent without administration of an IL-2 conjugate.

1. Adeno-Associated Virus Vector

[0440] In some embodiments, the gene therapy agent comprises an AAV particle. In an AAV particle for gene therapy, a recombinant AAV (rAAV) genome encoding a heterologous nucleic acid (e.g., a therapeutic transgene) is encapsidated in an AAV capsid. In some embodiments, the viral genome comprises a heterologous nucleic acid and/or one or more of the following components, operatively linked in the direction of transcription, control sequences including transcription initiation and termination sequences, thereby forming an expression cassette.

[0441] In some embodiments, the rAAV genome comprises one or more AAV inverted terminal repeat (ITR) sequences (typically two AAV ITR sequences). For example, an expression cassette may be flanked on the 5' and 3' end by at least one functional AAV ITR sequence. By “functional AAV ITR sequences” it is meant that the ITR sequences function as intended for the rescue, replication and packaging of the AAV virion. See Davidson et al., PNAS, 2000, 97(7):3428-32; Passini et al., J. Virol., 2003, 77(12):7034-40; and Pechan et al., Gene Ther., 2009, 16:10-16, all of which are incorporated herein in their entirety by reference. For practicing some embodiments of the disclosure, the recombinant viral genomes comprise at least all of the sequences of AAV essential for encapsidation into the AAV capsid and the physical structures for infection by the AAV particle. AAV ITRs for use in the vectors of the disclosure need not have a wild-type nucleotide sequence (e.g., as described in Kotin, Hum. Gene Ther., 1994, 5:793-801), and may be altered by the insertion, deletion or substitution of nucleotides or the AAV ITRs may be derived from any of several AAV serotypes. More than 40 serotypes of AAV are currently known, and new serotypes and variants of existing serotypes continue to be identified. See Gao et al., PNAS, 2002, 99(18): 11854-6; Gao et al., PNAS, 2003, 100(10):6081-6; and Bossis et al., J. Virol., 2003, 77(12):6799-810. Use of any AAV serotype is considered within the scope of the present disclosure. In some embodiments, a rAAV vector is a vector derived from an AAV serotype, including without limitation, AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV DJ, AAV DJ8, a goat AAV, bovine AAV, or mouse AAV ITRs or the like. In some embodiments, the AAV nucleic acid (e.g., an rAAV vector) comprises one or more (e.g., in some embodiments two) ITR of AAV1, AAV2, AAV3, AAV4, AAV5, AAV6, AAV7, AAV8, AAVrh8, AAVrh8R, AAV9, AAV10, AAVrh10, AAV11, AAV12, AAV LK03, AAV2R471A, AAV DJ, AAV DJ8, a goat AAV, bovine AAV, or mouse AAV ITRs or the like. In some embodiments, the AAV particle comprises an AAV vector encoding a heterologous transgene flanked by one or more AAV ITRs.

[0442] In some embodiments, the AAV particle comprises a capsid protein selected from an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAVrh32.33 capsid, An AAV-XL32 capsid, an AAV-XL32.1 capsid, an AAV LK03 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV / human bocavirus virus 1), an AAV2HBKO capsid, an AAVPHP.B capsid or an AAVPHP.eB capsid, or a functional variant thereof. By “functional variant” of an AAV capsid, it is meant that the variant capsid is capable of packaging an AAV genome to generate an infectious AAV virion. In further embodiments, a rAAV particle comprises capsid proteins of an AAV serotype from Clades A-F.

[0443] In some embodiments, the disclosure provides AAV particles comprising a recombinant self-complementing genome (e.g., a self-complementary or self-complementing AAV vector). AAV viral particles with self-complementing vector genomes and methods of use of self-complementing rAAV genomes are described in US Patent Nos. 6,596,535; 7,125,717; 7,465,583; 7,785,888; 7,790,154; 7,846,729; 8,093,054; and 8,361,457; and Wang Z., et al., (2003) Gene Ther 10:2105-2111, each of which are incorporated herein by reference in its entirety. An AAV particle comprising a self-complementing genome will quickly form a double stranded DNA molecule by virtue of its partially complementing sequences (e.g., complementing coding and non-coding strands of a heterologous nucleic acid). In some embodiments, the vector comprises a first nucleic acid sequence encoding a heterologous nucleic acid and a second nucleic acid sequence encoding a complement of the nucleic acid, where the first nucleic acid sequence can form intrastrand base pairs with the second nucleic acid sequence along most or all of its length.

[0444] In some embodiments, the first heterologous nucleic acid sequence and a second heterologous nucleic acid sequence are linked by a mutated ITR (e.g., the right ITR). In some embodiments, the ITR comprises the polynucleotide sequence 5’-
 CACTCCCTCTCTGCGCGCTCGCTCGCTCACTGAGGCC
 GGGCGACCAAAGGTCGCCCCACGCCCGGGCTTTGCCCGGGCG – 3’ (SEQ ID NO: 19).
 The mutated ITR comprises a deletion of the D region comprising the terminal resolution sequence. As a result, on replicating a rAAV genome, the rep proteins will not cleave the viral genome at the mutated ITR and as such, a recombinant viral genome comprising the following in

5' to 3' order will be packaged in a viral capsid: an AAV ITR, the first heterologous polynucleotide sequence including regulatory sequences, the mutated AAV ITR, the second heterologous polynucleotide in reverse orientation to the first heterologous polynucleotide and a third AAV ITR.

[0445] Different AAV serotypes are used to optimize transduction of particular target cells or to target specific cell types within a particular target tissue (e.g., a diseased tissue). An AAV particle can comprise viral proteins and viral nucleic acids of the same serotype or a mixed serotype. For example, an AAV particle may contain one or more ITRs and capsid derived from the same AAV serotype, or an AAV particle may contain one or more ITRs derived from a different AAV serotype than capsid of the AAV particle.

[0446] In some embodiments, the AAV capsid comprises a mutation, e.g., the capsid comprises a mutant capsid protein. In some embodiments, the mutation is a tyrosine mutation or a heparin binding mutation. In some embodiments, a mutant capsid protein maintains the ability to form an AAV capsid. In some embodiments, the AAV particle comprises an AAV2 or AAV5 tyrosine mutant capsid (see, e.g., Zhong L. et al., (2008) *Proc Natl Acad Sci U S A* 105(22):7827-7832), such as a mutation in Y444 or Y730 (numbering according to AAV2). In further embodiments, the AAV particle comprises capsid proteins of an AAV serotype from Clades A-F (Gao, et al., *J. Virol.* 2004, 78(12):6381).

[0447] Numerous methods are known in the art for production of AAV particles for gene therapy including transfection stable cell line production and infectious hybrid virus production systems which include adenovirus-AAV hybrids, herpesvirus-AAV hybrids (Conway, JE et al., (1997) *J. Virology* 71(11):8780-8789) and baculovirus-AAV hybrids (Urabe, M. et al., (2002) *Human Gene Therapy* 13(16):1935-1943; Kotin, R. (2011) *Hum Mol Genet.* 20(R1): R2–R6). AAV production cultures for the production of AAV particles all require; 1) suitable host cells, 2) suitable helper virus function, 3) AAV rep and cap genes and gene products; 4) a nucleic acid (such as a therapeutic nucleic acid) flanked by at least one AAV ITR sequences; and 5) suitable media and media components to support AAV production. In some embodiments, the suitable host cell is a primate host cell. In some embodiments, the suitable host cell is a human-derived cell lines such as HeLa, A549, 293, or Perc.6 cells. In some embodiments, the suitable helper virus function is provided by wild-type or mutant adenovirus (such as temperature sensitive adenovirus), herpes virus (HSV), baculovirus, or a plasmid construct providing helper functions. In some embodiments, the AAV rep and cap gene products may be from any AAV serotype. In general, but not obligatory, the AAV rep gene product is of the same serotype as the ITRs of the rAAV genome as long as the rep gene products may function to replicated and package the

rAAV genome. Suitable media known in the art may be used for the production of AAV particles. In some embodiments, the AAV helper functions are provided by adenovirus or HSV. In some embodiments, the AAV helper functions are provided by baculovirus and the host cell is an insect cell (e.g., *Spodoptera frugiperda* (Sf9) cells).

[0448] One method for producing AAV particles is the triple transfection method. Briefly, a plasmid containing a rep gene and a capsid gene, along with a helper adenoviral plasmid, may be transfected (e.g., using the calcium phosphate method) into a cell line (e.g., HEK-293 cells), and virus may be collected and optionally purified. As such, in some embodiments, the AAV particle was produced by triple transfection of a nucleic acid encoding the AAV vector, a nucleic acid encoding AAV rep and cap, and a nucleic acid encoding AAV helper virus functions into a host cell, wherein the transfection of the nucleic acids to the host cells generates a host cell capable of producing AAV particles.

[0449] In some embodiments, AAV particles may be produced by a producer cell line method (see Martin et al., (2013) *Human Gene Therapy Methods* 24:253-269; U.S. PG Pub. No. US2004/0224411; and Liu, X.L. et al. (1999) *Gene Ther.* 6:293-299). Briefly, a cell line (e.g., a HeLa, 293, A549, or Perc.6 cell line) may be stably transfected with a plasmid containing a rep gene, a capsid gene, and a vector genome comprising a promoter-heterologous nucleic acid sequence. Cell lines may be screened to select a lead clone for AAV production, which may then be expanded to a production bioreactor and infected with a helper virus (e.g., an adenovirus or HSV) to initiate AAV production. Virus may subsequently be harvested, adenovirus may be inactivated (e.g., by heat) and/or removed, and the AAV particles may be purified. As such, in some embodiments, the AAV particle was produced by a producer cell line comprising one or more of nucleic acid encoding the rAAV genome, a nucleic acid encoding AAV rep and cap, and a nucleic acid encoding AAV helper virus functions.

[0450] In some embodiments, the nucleic acid encoding AAV rep and cap genes and/or the AAV viral genome are stably maintained in the producer cell line. In some embodiments, nucleic acid encoding AAV rep and cap genes and/or the rAAV genome is introduced on one or more plasmids into a cell line to generate a producer cell line. In some embodiments, the AAV rep, AAV cap, and AAV genome are introduced into a cell on the same plasmid. In other embodiments, the AAV rep, AAV cap, and rAAV genome are introduced into a cell on different plasmids. In some embodiments, a cell line stably transfected with a plasmid maintains the plasmid for multiple passages of the cell line (e.g., 5, 10, 20, 30, 40, 50 or more than 50 passages of the cell). For example, the plasmid(s) may replicate as the cell replicates, or the plasmid(s) may integrate into the cell genome. A variety of sequences that enable a plasmid to replicate

autonomously in a cell (e.g., a human cell) have been identified (see, e.g., Krysan, P.J. et al. (1989) Mol. Cell Biol.9:1026-1033). In some embodiments, the plasmid(s) may contain a selectable marker (e.g., an antibiotic resistance marker) that allows for selection of cells maintaining the plasmid. Selectable markers commonly used in mammalian cells include without limitation blasticidin, G418, hygromycin B, zeocin, puromycin, and derivatives thereof. Methods for introducing nucleic acids into a cell are known in the art and include without limitation viral transduction, cationic transfection (e.g., using a cationic polymer such as DEAE- dextran or a cationic lipid such as lipofectamine), calcium phosphate transfection, microinjection, particle bombardment, electroporation, and nanoparticle transfection (for more details, see e.g., Kim, T.K. and Eberwine, J.H. (2010) Anal. Bioanal. Chem.397:3173-3178).

[0451] In some embodiments, the producer cell line is derived from a primate cell line (e.g., a non-human primate cell line, such as a Vero or FRhL-2 cell line). In some embodiments, the cell line is derived from a human cell line. In some embodiments, the producer cell line is derived from HeLa, 293, A549, or PERC.6® (Crucell) cells. For example, prior to introduction and/or stable maintenance/integration of nucleic acid encoding AAV rep and cap genes and/or the rAAV genome into a cell line to generate a producer cell line, the cell line is a HeLa, 293, A549, or PERC.6® (Crucell) cell line, or a derivative thereof.

[0452] In some embodiments, the producer cell line is adapted for growth in suspension. As is known in the art, anchorage-dependent cells are typically not able to grow in suspension without a substrate, such as microcarrier beads. Adapting a cell line to grow in suspension may include, for example, growing the cell line in a spinner culture with a stirring paddle, using a culture medium that lacks calcium and magnesium ions to prevent clumping (and optionally an antifoaming agent), using a culture vessel coated with a siliconizing compound, and selecting cells in the culture (rather than in large clumps or on the sides of the vessel) at each passage.

[0453] AAV particles of the disclosure may be harvested from AAV production cultures by lysis of the host cells of the production culture or by harvest of the spent media from the production culture, provided the cells are cultured under conditions known in the art to cause release of AAV particles into the media from intact cells, as described more fully in U.S. Patent No.6,566,118). Suitable methods of lysing cells are also known in the art and include for example multiple freeze/thaw cycles, sonication, microfluidization, and treatment with chemicals, such as detergents and/or proteases.

[0454] In a further embodiment, the AAV particles are purified. The term “purified” as used herein includes a preparation of AAV particles devoid of at least some of the other components that may also be present where the AAV particles naturally occur or are initially prepared from.

Thus, for example, isolated AAV particles may be prepared using a purification technique to enrich it from a source mixture, such as a culture lysate or production culture supernatant. Enrichment can be measured in a variety of ways, such as, for example, by the proportion of DNase-resistant particles (DRPs) or genome copies (gc) present in a solution, or by infectivity, or it can be measured in relation to a second, potentially interfering substance present in the source mixture, such as contaminants, including production culture contaminants or in-process contaminants, including helper virus, media components, and the like.

[0455] In some embodiments, the AAV production culture harvest is clarified to remove host cell debris. In some embodiments, the production culture harvest is clarified by filtration through a series of depth filters including for example a grade DOHC Millipore Millistak+ HC Pod Filter, a grade A1HC Millipore Millistak+ HC Pod Filter, and a 0.2 µm Filter Opticap XL1O Millipore Express SHC Hydrophilic Membrane filter. Clarification can also be achieved by a variety of other standard techniques known in the art, such as, centrifugation or filtration through any cellulose acetate filter of 0.2 µm or greater pore size known in the art.

[0456] In some embodiments, the AAV production culture harvest is further treated with Benzonase® to digest any high molecular weight DNA present in the production culture. In some embodiments, the Benzonase® digestion is performed under standard conditions known in the art including, for example, a final concentration of 1-2.5 units/ml of Benzonase® at a temperature ranging from ambient to 37°C for a period of 30 minutes to several hours.

[0457] AAV particles may be isolated or purified using one or more of the following purification steps: equilibrium centrifugation; flow-through anionic exchange filtration; tangential flow filtration (TFF) for concentrating the AAV particles; AAV capture by apatite chromatography; heat inactivation of helper virus; AAV capture by hydrophobic interaction chromatography; buffer exchange by size exclusion chromatography (SEC); nanofiltration; and AAV capture by anionic exchange chromatography, cationic exchange chromatography, or affinity chromatography. These steps may be used alone, in various combinations, or in different orders. In some embodiments, the method comprises all the steps in the order as described below.

Methods to purify AAV particles are found, for example, in Xiao et al., (1998) Journal of Virology 72:2224-2232; US Patent Numbers 6,989,264 and 8,137,948; and WO 2010/148143.

[0458] In some embodiments in which the gene therapy agent comprises an AAV particle, the transgene product (e.g., a therapeutic polypeptide or therapeutic nucleic acid) can be expressed and/or synthesized in a subject for at least about 1 week, about 2 weeks, about 3 weeks, about 4 weeks, about 5 weeks, about 6 weeks, about 7 weeks, about 8 weeks, about 9 weeks, about 10 weeks, about 11 weeks, about 12 weeks, about 13 weeks, about 14 weeks, about 15 weeks, about

16 weeks, about 17 weeks, about 18 weeks, about 19 weeks, about 20 weeks, about 22 weeks, about 24 weeks, or about 26 weeks. In some embodiments, a transgene product may, e.g., supply a polypeptide and/or enzymatic activity that is absent or present at a reduced level in a cell or organism. In some embodiments, a transgene product may supply a polypeptide and/or enzymatic activity that indirectly counteracts an imbalance in a cell or organism. In some embodiments, a change in expression of the transgene product comprises changes in expression levels of a therapeutic nucleic acid and/or an endogenous nucleic acid in a cell or organism of at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 75%, or 100% relative to baseline (e.g., from before treatment or from negative control). In some embodiments, a change in synthesis of a transgene product comprises changes in synthesis levels of a therapeutic polypeptide encoded by a transgene product and/or an endogenous polypeptide in a cell or organism of at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 75%, or 100% relative to baseline (e.g., from before treatment or from negative control).

2. Adenoviral Vector

[0459] In some embodiments, the gene therapy agent comprises an adenovirus particle. Adenoviral vectors for gene therapy are typically adenoviral particles with a recombinant adenovirus (rAd) genome comprising one or more heterologous sequences (i.e., nucleic acid sequence not of adenoviral origin) between two adenoviral ITRs encapsidated into an adenoviral capsid. In some embodiments, the heterologous sequence encodes a therapeutic transgene. In some embodiments, the rAd genome lacks or contains a defective copy of one or more E1 genes, which renders the adenovirus replication- defective. Adenoviruses include a linear, double-stranded DNA genome within a large (~950Å), non-enveloped icosahedral capsid. Adenoviruses have a large genome that can incorporate more than 30kb of heterologous sequence (eg in place of the E1 and/or E3 region) making them uniquely suited for use with larger heterologous genes. They are also known to infect dividing and non-dividing cells and do not naturally integrate into the host genome (although hybrid variants may possess this ability). In some embodiments, the adenoviral vector may be a first generation adenoviral vector with a heterologous sequence in place of E1. In some embodiments, the adenoviral vector may be a second generation adenoviral vector with additional mutations or deletions in E2A, E2B, and/or E4. In some embodiments, the adenoviral vector may be a third generation or gutted adenoviral vector that lacks all viral coding genes, retaining only the ITRs and packaging signal and requiring a helper adenovirus in trans for replication, and packaging. Adenoviral particles have been investigated for use as vectors for transient transfection of mammalian cells as well as gene therapy vectors. For further

description, see, e.g., Danthinne, X. and Imperiale, M.J. (2000) *Gene Ther.*7:1707-14 and Tatsis, N. and Ertl, H.C. (2004) *Mol. Ther.*10:616-29.

[0460] In some embodiments, the adenoviral particle comprises a rAd genome comprising a therapeutic transgene. Use of any adenovirus serotype is considered within the scope of the present disclosure. In some embodiments, the adenoviral particle is derived from an adenovirus serotype, including without limitation, AdHu2, AdHu 3, AdHu4, AdHu5, AdHu7, AdHu11, AdHu24, AdHu26, AdHu34, AdHu35, AdHu36, AdHu37, AdHu41, AdHu48, AdHu49, AdHu50, AdC6, AdC7, AdC69, bovine Ad type 3, canine Ad type 2, ovine Ad, and porcine Ad type 3. The adenoviral particle also comprises capsid proteins. In some embodiments, the adenoviral particle includes one or more foreign viral capsid proteins. Such combinations may be referred to as pseudotyped adenoviral particles. In some embodiments, foreign viral capsid proteins used in pseudotyped adenoviral particles are derived from a foreign virus or from another adenovirus serotype. In some embodiments, the foreign viral capsid proteins are derived from, including without limitation, reovirus type 3. Examples of vector and capsid protein combinations used in pseudotyped adenovirus particles can be found in the following references (Tatsis, N. et al. (2004) *Mol. Ther.*10(4):616-629 and Ahi, Y. et al. (2011) *Curr. Gene Ther.* 11(4):307-320). Different adenovirus serotypes can be used to optimize transduction of particular target cells or to target specific cell types within a particular target tissue (e.g., a diseased tissue). Tissues or cells targeted by specific adenovirus serotypes, include without limitation, lung (e.g. HuAd3), spleen and liver (e.g. HuAd37), smooth muscle, synoviocytes, dendritic cells, cardiovascular cells, tumor cell lines (e.g. HuAd11), and dendritic cells (e.g. HuAd5 pseudotyped with reovirus type 3, HuAd30, or HuAd35). For further description, see Ahi, Y. et al. (2011) *Curr. Gene Ther.* 11(4):307-320, Kay, M. et al. (2001) *Nat. Med.*7(1):33-40, and Tatsis, N. et al. (2004) *Mol. Ther.* 10(4):616-629.

[0461] Numerous methods are known in the art for production of adenoviral particles. For example, for a gutted adenoviral vector, the adenoviral vector genome and a helper adenovirus genome may be transfected into a packaging cell line (e.g., a 293 cell line). In some embodiments, the helper adenovirus genome may contain recombination sites flanking its packaging signal, and both genomes may be transfected into a packaging cell line that expresses a recombinase (e.g., the Cre/loxP system may be used), such that the adenoviral vector of interest is packaged more efficiently than the helper adenovirus (see, e.g., Alba, R. et al. (2005) *Gene Ther.* 12 Suppl 1:S18-27). Adenoviral vectors may be harvested and purified using standard methods, such as those described herein.

3. Lentiviral Vector

[0462] In some embodiments, the gene therapy agent comprises a lentivirus particle. Lentiviral vectors for gene therapy are typically lentiviral particles with a recombinant lentivirus genome comprising one or more heterologous sequences (i.e., nucleic acid sequence not of lentiviral origin) between two long terminal repeats (LTRs). In some embodiments, the heterologous sequence encodes a therapeutic transgene. Lentiviruses are positive-sense, ssRNA retroviruses with a genome of approximately 10 kb. Lentiviruses integrate into the genome of dividing and non-dividing cells. Lentiviral particles may be produced, for example, by transfecting multiple plasmids (typically the lentiviral genome and the genes required for replication and/or packaging are separated to prevent viral replication) into a packaging cell line, which packages the modified lentiviral genome into lentiviral particles. In some embodiments, a lentiviral particle may refer to a first generation vector that lacks the envelope protein. In some embodiments, a lentiviral particle may refer to a second- generation vector that lacks all genes except the gag/pol and tat/rev regions. In some embodiments, a lentiviral particle may refer to a third generation vector that only contains the endogenous rev, gag, and pol genes and has a chimeric LTR for transduction without the tat gene (see Dull, T. et al. (1998) *J. Virol.* 72:8463-71). For further description, see Durand, S. and Cimarelli, A. (2011) *Viruses* 3:132-59.

[0463] Use of any lentiviral vector is considered within the scope of the present disclosure. In some embodiments, the lentiviral vector is derived from a lentivirus including, without limitation, human immunodeficiency virus-1 (HIV-1), human immunodeficiency virus-2 (HIV-2), simian immunodeficiency virus (SIV), feline immunodeficiency virus (FIV), equine infectious anemia virus (EIAV), bovine immunodeficiency virus (BIV), Jembrana disease virus (JDV), visna virus (VV), and caprine arthritis encephalitis virus (CAEV). The lentiviral particle also comprises capsid proteins. In some embodiments, the lentivirus particles include one or more foreign viral capsid proteins. Such combinations may be referred to as pseudotyped lentiviral particles. In some embodiments, foreign viral capsid proteins used in pseudotyped lentiviral particles are derived from a foreign virus. In some embodiments, the foreign viral capsid protein used in pseudotyped lentiviral particles is Vesicular stomatitis virus glycoprotein (VSV-GP). VSV-GP interacts with a ubiquitous cell receptor, providing broad tissue tropism to pseudotyped lentiviral particles. In addition, VSV-GP is thought to provide higher stability to pseudotyped lentiviral particles. In other embodiments, the foreign viral capsid proteins are derived from, including without limitation, Chandipura virus, Rabies virus, Mokola virus, Lymphocytic choriomeningitis virus (LCMV), Ross River virus (RRV), Sindbis virus, Semliki Forest virus (SFV), Venezuelan equine encephalitis virus, Ebola virus Reston, Ebola virus Zaire,

Marburg virus, Lassa virus, Avian leukosis virus (ALV), Jaagsiekte sheep retrovirus (JSRV), Moloney Murine leukemia virus (MLV), Gibbon ape leukemia virus (GALV), Feline endogenous retrovirus (RD114), Human T-lymphotropic virus 1 (HTLV-1), Human foamy virus, Maedi-visna virus (MVV), SARS-CoV, Sendai virus, Respiratory syncytia virus (RSV), Human parainfluenza virus type 3, Hepatitis C virus (HCV), Influenza virus, Fowl plague virus (FPV), or Autographa californica multiple nucleopolyhedro virus (AcMNPV). Examples of vector and capsid protein combinations used in pseudotyped lentivirus particles can be found, for example, in Cronin, J. et al. (2005). *Curr. Gene Ther.*5(4):387-398. Different pseudotyped lentiviral particles can be used to optimize transduction of particular target cells or to target specific cell types within a particular target tissue (e.g., a diseased tissue). For example, tissues targeted by specific pseudotyped lentiviral particles, include without limitation, liver (e.g. pseudotyped with a VSV-G, LCMV, RRV, or SeV F protein), lung (e.g. pseudotyped with an Ebola, Marburg, SeV F and HN, or JSRV protein), pancreatic islet cells (e.g. pseudotyped with an LCMV protein), central nervous system (e.g. pseudotyped with a VSV-G, LCMV, Rabies, or Mokola protein), retina (e.g. pseudotyped with a VSV-G or Mokola protein), monocytes or muscle (e.g. pseudotyped with a Mokola or Ebola protein), hematopoietic system (e.g. pseudotyped with an RD114 or GALV protein), or cancer cells (e.g. pseudotyped with a GALV or LCMV protein). For further description, see Cronin, J. et al. (2005). *Curr. Gene Ther.*5(4):387-398 and Kay, M. et al. (2001) *Nat. Med.*7(1):33-40.

[0464] Numerous methods are known in the art for production of lentiviral particles. For example, for a third-generation lentiviral vector, a vector containing the recombinant lentiviral genome of interest with gag and pol genes may be co-transfected into a packaging cell line (e.g., a 293 cell line) along with a vector containing a rev gene. The recombinant lentiviral genome of interest also contains a chimeric LTR that promotes transcription in the absence of Tat (see Dull, T. et al. (1998) *J. Virol.* 72:8463-71). Lentiviral vectors may be harvested and purified using methods (e.g., Segura MM, et al., (2013) *Expert Opin Biol Ther.*13(7):987-1011) described herein.

4. Herpes Simplex Virus

[0465] In some embodiments, the gene therapy agent comprises an HSV particle. HSV vectors for gene therapy are typically HSV particles with a recombinant HSV genome comprising one or more heterologous sequences (i.e., nucleic acid sequence not of HSV origin) between two terminal repeats (TRs). In some embodiments, the heterologous sequence encodes a therapeutic transgene. HSV is an enveloped, double-stranded DNA virus with a genome of approximately 152 kb. Advantageously, approximately half of its genes are nonessential and may be deleted to

accommodate heterologous sequence. HSV particles infect non-dividing cells. In addition, they naturally establish latency in neurons, travel by retrograde transport, and can be transferred across synapses, making them advantageous for transfection of neurons and/or gene therapy approaches involving the nervous system. In some embodiments, the HSV particle may be replication- defective or replication-competent (e.g., competent for a single replication cycle through inactivation of one or more late genes). For further description, see Manservigi, R. et al. (2010) *Open Virol. J.*4:123-56.

[0466] In some embodiments, the HSV particle comprises a recombinant HSV genome comprising a transgene. Use of any HSV vector is considered within the scope of the present disclosure. In some embodiments, the HSV vector is derived from a HSV serotype, including without limitation, HSV-1 and HSV-2. The HSV particle also comprises capsid proteins. In some embodiments, the HSV particles include one or more foreign viral capsid proteins. Such combinations may be referred to as pseudotyped HSV particles. In some embodiments, foreign viral capsid proteins used in pseudotyped HSV particles are derived from a foreign virus or from another HSV serotype. In some embodiments, the foreign viral capsid protein used in a pseudotyped HSV particle is a Vesicular stomatitis virus glycoprotein (VSV-GP). VSV-GP interacts with a ubiquitous cell receptor, providing broad tissue tropism to pseudotyped HSV particles. In addition, VSV-GP is thought to provide higher stability to pseudotyped HSV particles. In other embodiments, the foreign viral capsid protein may be from a different HSV serotype. For example, an HSV-1 vector may contain one or more HSV-2 capsid proteins. Different HSV serotypes can be used to optimize transduction of particular target cells or to target specific cell types within a particular target tissue (e.g., a diseased tissue). Tissues or cells targeted by specific adenovirus serotypes include without limitation, central nervous system and neurons (e.g. HSV-1). For further description, see Manservigi, R. et al. (2010) *Open Virol J* 4:123-156, Kay, M. et al. (2001) *Nat. Med.*7(1):33-40, and Meignier, B. et al. (1987) *J. Infect. Dis.*155(5):921-930.

[0467] Numerous methods are known in the art for production of HSV particles. HSV vectors may be harvested and purified using standard methods, such as those described herein. For example, for a replication-defective HSV vector, an HSV genome of interest that lacks all of the immediate early (IE) genes may be transfected into a complementing cell line that provides genes required for virus production, such as ICP4, ICP27, and ICP0 (see, e.g., Samaniego, L.A. et al. (1998) *J. Virol.*72:3307-20). HSV vectors may be harvested and purified using methods described (e.g., Goins, WF et al., (2014) *Herpes Simplex Virus Methods in Molecular Biology* 1144:63-79).

5. Non-viral Gene Transfer

[0468] In some embodiments, the gene therapy agent is a non-viral gene therapy agent, e.g., a non-viral vector delivery system. Non-viral vector delivery systems include DNA plasmids, naked nucleic acid, and nucleic acid complexed to a delivery system. For example, the vector may be complexed to a lipid (e.g., a cationic or neutral lipid), a liposome, a polycation, a lipid nanoparticle, or an agent that enhances the cellular uptake of nucleic acid. The nucleic acid may be complexed to an agent suitable for any of the delivery methods described herein. In some embodiments, the nucleic acid encodes a therapeutic transgene.

[0469] Lipid nanoparticles for gene therapy typically comprise a vector genome encapsulated in a lipid particle or a vector genome complexed with a lipid. In some embodiments, the heterologous sequence encodes a therapeutic transgene. In some embodiments, the vector genome is formulated in a lipoplex nanoparticle or liposome. In some embodiments, a lipoplex nanoparticle formulation for the gene therapy agent comprises the synthetic cationic lipid (R)-N,N,N-trimethyl-2,3-dioleoyloxy-1-propanaminium chloride (DOTMA) and the phospholipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE). In some embodiments, the DOTMA/DOPE liposomal component is optimized for delivery and targeting of cells in the individual.

[0470] In some embodiments, nucleic acid comprising the vector genome is mixed with a pharmaceutical composition comprising one or more cationic lipids, including, e.g., (R)-N,N,N-trimethyl-2,3-dioleoyloxy-1-propanaminium chloride (DOTMA) and the phospholipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE). In some embodiments, the pharmaceutical composition comprises at least one lipid. In some embodiments, the pharmaceutical composition comprises at least one cationic lipid. The cationic lipid can be monocationic or polycationic. Any cationic amphiphilic molecule, e.g., a molecule which comprises at least one hydrophilic and lipophilic moiety is a cationic lipid within the meaning of the present disclosure. In some embodiments, the positive charges are contributed by the at least one cationic lipid and the negative charges are contributed by the nucleic acid. In some embodiments, the pharmaceutical composition comprises at least one helper lipid. The helper lipid may be a neutral or an anionic lipid. The helper lipid may be a natural lipid, such as a phospholipid or an analogue of a natural lipid, or a fully synthetic lipid, or lipid-like molecule, with no similarities with natural lipids. In one embodiment, the cationic lipid and/or the helper lipid is a bilayer forming lipid. Examples of helper lipids include, but are not limited to, 1,2-di-(9Z-octadecenoyl)-sn-glycero-3-phosphoethanolamine (DOPE) or analogs or derivatives thereof,

cholesterol (Chol) or analogs or derivatives thereof and/or 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) or analogs or derivatives thereof.

[0471] In some embodiments, the molar ratio of the at least one cationic lipid to the at least one helper lipid is from 10:0 to 3:7, preferably 9:1 to 3:7, 4:1 to 1:2, 4:1 to 2:3, 7:3 to 1:1, or 2:1 to 1:1, preferably about 1:1. In some embodiments, in this ratio, the molar amount of the cationic lipid results from the molar amount of the cationic lipid multiplied by the number of positive charges in the cationic lipid.

[0472] In some embodiments, the lipid is comprised in a vesicle encapsulating the vector genome. The vesicle may be a multilamellar vesicle, an unilamellar vesicle, or a mixture thereof. The vesicle may be a liposome.

6. Vector Genomes

[0473] In some embodiments, the gene therapy agent comprises a therapeutic transgene. In some embodiments, the gene therapy agent comprises a vector genome for delivery and expression of the therapeutic transgene in the desired target in the individual.

[0474] The present disclosure contemplates the use of gene therapy agents for the introduction of one or more nucleic acid sequences encoding a therapeutic polypeptide and/or nucleic acid for packaging into a viral particle (for viral gene therapy agents). The vector genome may include any element to establish the expression of the therapeutic polypeptide and/or nucleic acid, for example, a promoter, an ITR of the present disclosure, a ribosome binding element, terminator, enhancer, selection marker, intron, polyA signal, and/or origin of replication.

[0475] In some embodiments, the therapeutic transgene encodes a therapeutic polypeptide. A therapeutic polypeptide may, e.g., supply a polypeptide and/or enzymatic activity that is absent or present at a reduced level in a cell or organism. Alternatively, a therapeutic polypeptide may supply a polypeptide and/or enzymatic activity that indirectly counteracts an imbalance in a cell or organism. For example, a therapeutic polypeptide for a disorder related to buildup of a metabolite caused by a deficiency in a metabolic enzyme or activity may supply a missing metabolic enzyme or activity, or it may supply an alternate metabolic enzyme or activity that leads to reduction of the metabolite. A therapeutic polypeptide may also be used to reduce the activity of a polypeptide (e.g., one that is overexpressed, activated by a gain-of-function mutation, or whose activity is otherwise misregulated) by acting, e.g., as a dominant-negative polypeptide.

[0476] The vector genomes of the disclosure may encode polypeptides that are intracellular proteins, anchored in the cell membrane, remain within the cell, or are secreted by the cell transduced with the vectors of the disclosure. For polypeptides secreted by the cell that receives

the vector; the polypeptide can be soluble (i.e., not attached to the cell). For example, soluble polypeptides are devoid of a transmembrane region and are secreted from the cell. Techniques to identify and remove nucleic acid sequences which encode transmembrane domains are known in the art.

[0477] In some embodiments, the vector genome of the disclosure encodes polypeptides used to treat a disease or disorder in an individual. Diseases and disorders treated by the gene therapy agent of the disclosure include but are not limited to Huntington disease (HD), progressive supranuclear palsy (PSP), multiple system atrophy (MSA), metachromatic leukodystrophy (MLD), amyotrophic lateral sclerosis (ALS), age-related macular degeneration (AMD), congenital muscular dystrophy (CMD), phenylketonuria (PKU), muscular dystrophy (MD), A1AT deficiency, focal segmental glomerulosclerosis (FSGS), cystinuria, hemophilia A, hemophilia B, Gaucher disease (GBA), Parkinson's disease (PD), and Pompe disease.

[0478] In some embodiments, the therapeutic polypeptide is huntingtin (HTT), tau, amyloid precursor protein, alpha-synuclein, pseudoarylsulfatase (ARSA), superoxide dismutase 1 (SOD1), phenylalanine hydroxylase (PAH), dystrophin, alpha-1-antitrypsin (A1AT), cysteine transporter, Factor VIII (FVIII), Factor IX (FIX), acid beta-glucosidase, glial-derived growth factor (GDNF), brain-derived growth factor (BDNF), tyrosine hydroxylase (TH), GTP-cyclohydrolase (GTPCH), and/or amino acid decarboxylase (AADC), or alpha glucosidase.

[0479] In some embodiments, a heterologous transgene may include without limitation an DNA, mRNA, closed-end DNA (ceDNA), siRNA, an shRNA, an RNAi, a miRNA, an antisense RNA.

[0480] In some embodiments, the heterologous nucleic acid encodes a therapeutic nucleic acid e.g. that can be used to replace, or knock down, one or more defective genes. In some embodiments, a therapeutic nucleic acid may include without limitation an DNA, siRNA, an shRNA, an RNAi, a miRNA, an antisense RNA, a ribozyme or a DNzyme. As such, a therapeutic nucleic acid may encode an RNA that when transcribed from the nucleic acids of the vector can treat a disorder by interfering with translation or transcription of an abnormal or excess protein associated with a disorder of the disclosure. For example, the nucleic acids of the disclosure may encode for an RNA which treats a disorder by highly specific elimination or reduction of mRNA encoding the abnormal and/or excess proteins. Therapeutic RNA sequences include RNAi, small inhibitory RNA (siRNA), micro RNA (miRNA), and/or ribozymes (such as hammerhead and hairpin ribozymes) that can treat disorders by highly specific elimination or reduction of mRNA encoding the abnormal and/or excess proteins.

[0481] In some embodiments, the therapeutic polypeptide or therapeutic nucleic acid is used to treat a disorder of the CNS. Without wishing to be bound to theory, it is thought that a

therapeutic polypeptide or therapeutic nucleic acid may be used to replace a mutated gene with a wild type or improved gene, reduce or eliminate the expression and/or activity of a polypeptide whose gain-of-function has been associated with a disorder, or to enhance the expression and/or activity of a polypeptide to complement a deficiency that has been associated with a disorder (e.g., a mutation in a gene whose expression shows similar or related activity). Non-limiting examples of disorders of the disclosure that may be treated by a therapeutic polypeptide or therapeutic nucleic acid of the disclosure (exemplary genes that may be targeted or supplied are provided in parenthesis for each disorder) include stroke (e.g., *caspase-3*, *Beclin1*, *Ask1*, *PARI*, *HIF1 α* , *PUMA*, and/or any of the genes described in Fukuda, A.M. and Badaut, J. (2013) *Genes* (Basel) 4:435-456), Huntington's disease (mutant HTT), epilepsy (e.g., *SCN1A*, *NMDAR*, *ADK*, and/or any of the genes described in Boison, D. (2010) *Epilepsia* 51:1659-1668), Parkinson's disease (alpha-synuclein), Lou Gehrig's disease (also known as amyotrophic lateral sclerosis; *SOD1*), Alzheimer's disease (tau, amyloid precursor protein), corticobasal degeneration or CBD (tau), corticogasal ganglionic degeneration or CBGD (tau), frontotemporal dementia or FTD (tau), progressive supranuclear palsy or PSP (tau), multiple system atrophy or MSA (alpha-synuclein), cancer of the brain (e.g., a mutant or overexpressed oncogene implicated in brain cancer), and lysosomal storage diseases (LSD). Disorders of the disclosure may include those that involve large areas of the cortex, e.g., more than one functional area of the cortex, more than one lobe of the cortex, and/or the entire cortex. Other non-limiting examples of disorders of the disclosure that may be treated by a therapeutic polypeptide or therapeutic nucleic acid of the disclosure include traumatic brain injury, enzymatic dysfunction disorders, psychiatric disorders (including post-traumatic stress syndrome), neurodegenerative diseases, and cognitive disorders (including dementias, autism, and depression). Enzymatic dysfunction disorders include without limitation leukodystrophies (including Canavan's disease) and any of the lysosomal storage diseases described below.

[0482] In some embodiments, the therapeutic polypeptide or therapeutic nucleic acid is used to treat a lysosomal storage disease. As is commonly known in the art, lysosomal storage diseases are rare, inherited metabolic disorders characterized by defects in lysosomal function. Such disorders are often caused by a deficiency in an enzyme required for proper mucopolysaccharide, glycoprotein, and/or lipid metabolism, leading to a pathological accumulation of lysosomally stored cellular materials. Non-limiting examples of lysosomal storage diseases of the disclosure that may be treated by a therapeutic polypeptide or therapeutic nucleic acid of the disclosure (exemplary genes that may be targeted or supplied are provided in parenthesis for each disorder) include Gaucher disease type 2 or type 3 (acid beta-glucosidase, *GBA*), GM1 gangliosidosis

(beta-galactosidase-1, GLB1), Hunter disease (iduronate 2-sulfatase, IDS), Krabbe disease (galactosylceramidase, GALC), a mannosidosis disease (a mannosidase, such as alpha-D-mannosidase, MAN2B1), β mannosidosis disease (beta-mannosidase, MANBA), metachromatic leukodystrophy disease (pseudoarylsulfatase A, ARSA), mucopolipidosisII/III disease (N-acetylglucosamine-1-phosphotransferase, GNPTAB), Niemann-Pick A disease (acid sphingomyelinase, ASM), Niemann-Pick C disease (Niemann-Pick C protein, NPC1), Pompe disease (acid alpha-1,4-glucosidase, GAA), Sandhoff disease (hexosaminidase beta subunit, HEXB), Sanfilippo A disease (N-sulfoglucosamine sulfohydrolase, MPS3A), Sanfilippo B disease (N-alpha-acetylglucosaminidase, NAGLU), Sanfilippo C disease (heparin acetyl-CoA:alpha-glucosaminidase N-acetyltransferase, MPS3C), Sanfilippo D disease (N-acetylglucosamine-6-sulfatase, GNS), Schindler disease (alpha-N-acetylgalactosaminidase, NAGA), Sly disease (beta-glucuronidase, GUSB), Tay-Sachs disease (hexosaminidase alpha subunit, HEXA), and Wolman disease (lysosomal acid lipase, LIPA).

[0483] In some embodiments, the therapeutic polypeptide encodes Factor VIII, Factor IX, myotubularin, survival motor neuron protein (SMN), retinoid isomerohydrolase (RPE65), NADH-ubiquinone oxidoreductase chain 4, Choroideremia protein (CHM), ornithine transcarbomylase, argininosuccinate synthetase, β -globin, γ -globin, phenylalanine hydroxylase, adrenoleukodystrophy protein (ALD), dystrophin, a truncated dystrophin, an anti-VEGF agent, or a functional variant thereof.

[0484] In some embodiments, the heterologous nucleic acid is operably linked to a promoter. Exemplary promoters include, but are not limited to, the cytomegalovirus (CMV) immediate early promoter, the RSV LTR, the MoMLV LTR, the phosphoglycerate kinase- 1 (PGK) promoter, a simian virus 40 (SV40) promoter and a CK6 promoter, a transthyretin promoter (TTR), a TK promoter, a tetracycline responsive promoter (TRE), an HBV promoter, an hAAT promoter, a LSP promoter, chimeric liver-specific promoters (LSPs), the E2F promoter, the telomerase (hTERT) promoter; the cytomegalovirus enhancer/chicken beta-actin/Rabbit β -globin promoter (CAG promoter; Niwa et al., Gene, 1991, 108(2):193-9) and the elongation factor 1-alpha promoter (EF1-alpha) promoter (Kim et al., Gene, 1990, 91(2):217-23 and Guo et al., Gene Ther., 1996, 3(9):802-10). In some embodiments, the promoter comprises a human β -glucuronidase promoter or a cytomegalovirus enhancer linked to a chicken β -actin (CBA) promoter. The promoter can be a constitutive, inducible or repressible promoter. In some embodiments, the disclosure provides a recombinant vector comprising nucleic acid encoding a heterologous transgene of the present disclosure operably linked to a CBA promoter. Exemplary promoters and descriptions may be found, e.g., in U.S. PG Pub.20140335054.

[0485] Examples of constitutive promoters include, without limitation, the retroviral Rous sarcoma virus (RSV) LTR promoter (optionally with the RSV enhancer), the cytomegalovirus (CMV) promoter (optionally with the CMV enhancer) [see, e.g., Boshart et al., *Cell*, 41:521-530 (1985)], the SV40 promoter, the dihydrofolate reductase promoter, the 13-actin promoter, the phosphoglycerol kinase (PGK) promoter, and the EF1a promoter [Invitrogen].

[0486] Inducible promoters allow regulation of gene expression and can be regulated by exogenously supplied compounds, environmental factors such as temperature, or the presence of a specific physiological state, e.g., acute phase, a particular differentiation state of the cell, or in replicating cells only. Inducible promoters and inducible systems are available from a variety of commercial sources, including, without limitation, Invitrogen, Clontech and Ariad. Many other systems have been described and can be readily selected by one of skill in the art. Examples of inducible promoters regulated by exogenously supplied promoters include the zinc-inducible sheep metallothioneine (MT) promoter, the dexamethasone (Dex)-inducible mouse mammary tumor virus (MMTV) promoter, the T7 polymerase promoter system (WO 98/10088); the ecdysone insect promoter (No et al., *Proc. Natl. Acad. Sci. USA*, 93:3346-3351 (1996)), the tetracycline-repressible system (Gossen et al., *Proc. Natl. Acad. Sci. USA*, 89:5547-5551 (1992)), the tetracycline-inducible system (Gossen et al., *Science*, 268:1766-1769 (1995), see also Harvey et al., *Curr. Opin. Chem. Biol.*, 2:512-518 (1998)), the RU486-inducible system (Wang et al., *Nat. Biotech.*, 15:239-243 (1997) and Wang et al., *Gene Ther.*, 4:432-441 (1997)) and the rapamycin-inducible system (Magari et al., *J. Clin. Invest.*, 100:2865-2872 (1997)). Still other types of inducible promoters which may be useful in this context are those which are regulated by a specific physiological state, e.g., temperature, acute phase, a particular differentiation state of the cell, or in replicating cells only.

[0487] In another embodiment, the native promoter, or fragment thereof, for the transgene will be used. The native promoter can be used when it is desired that expression of the transgene should mimic the native expression. The native promoter may be used when expression of the transgene must be regulated temporally or developmentally, or in a tissue-specific manner, or in response to specific transcriptional stimuli. In a further embodiment, other native expression control elements, such as enhancer elements, polyadenylation sites or Kozak consensus sequences may also be used to mimic the native expression.

[0488] In some embodiments, the regulatory sequences impart tissue-specific gene expression capabilities. In some embodiments, the tissue-specific regulatory sequences bind tissue-specific transcription factors that induce transcription in a tissue specific manner. Such tissue-specific regulatory sequences (e.g., promoters, enhancers, etc.) are well known in the art.

[0489] In some embodiments, the vector comprises an intron. For example, in some embodiments, the intron is a chimeric intron derived from chicken beta-actin and rabbit beta-globin. In some embodiments, the intron is a minute virus of mice (MVM) intron.

[0490] In some embodiments, the vector comprises a polyadenylation (polyA) sequence. Numerous examples of polyadenylation sequences are known in the art, such as a bovine growth hormone (BGH) Poly(A) sequence (see, e.g., accession number EF592533), an SV40 polyadenylation sequence, and an HSV TK pA polyadenylation sequence.

7. Methods for selecting a patient for treatment with a gene therapy agent and an IL-2 conjugate

[0491] In some embodiments, the disclosure provides methods for delivering a nucleic acid to a cell of an individual, the method comprising a) incubating adaptive immune cells from the individual with the gene therapy agent (e.g., an AAV particle, an adenovirus particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle), b) analyzing the adaptive immune cells (e.g., a lymphocyte, a T cell, a CD8+ T cell, an effector T cell, a cytotoxic T cell (i.e., cytotoxic T lymphocyte (CTL)), or an NK cell) for the expression of one or more cytokines wherein expression of a cytokine signature following incubation with the gene therapy agent identifies an individual with adaptive immunity to the gene therapy agent, c) administering an IL-2 conjugate to the individual identified in step b), and d) administering the gene therapy agent to the individual identified in step b). In some embodiments, the adaptive immune cell is a lymphocyte. In some embodiments, the lymphocyte is a B cell, T cell, or NK cell. In some embodiments, the T cell is a CD8+ T cell, an effector T cell, or a cytotoxic T cell (i.e., CTL). In some embodiments, the method further includes the steps of isolating adaptive immune cells from the individual prior to incubating the adaptive immune cells with the gene therapy agent. In some embodiments, the method further includes the steps of isolating T cells from the individual and incubating the T cells in culture media prior to incubating the T cells with the gene therapy agent.

[0492] In some embodiments, the disclosure provides methods for treating an individual in need thereof, the method comprising a) incubating adaptive immune cells from the individual with the gene therapy agent (e.g., an AAV particle, an adenovirus particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle), b) analyzing the adaptive immune cells for the expression of one or more cytokines wherein expression of a cytokine signature following incubation with the gene therapy agent identifies an individual with adaptive immunity to the gene therapy agent, c) administering an IL-2 conjugate to the individual identified in step b), and d) administering the gene therapy agent to the individual identified in step b). In some embodiments, the adaptive

immune cells are T cells or B cells. In some embodiments, the method further includes the steps of isolating adaptive immune cells from the individual prior to incubating the adaptive immune cells with the gene therapy agent. In some embodiments, the method further includes the steps of isolating T cells from the individual and, incubating the T cells in culture media to derive prior to incubating the T cells with the gene therapy agent.

[0493] In some embodiments, the disclosure provides methods for selecting an individual for treatment with a gene therapy agent (e.g., an AAV particle, an adenovirus particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle) and an IL-2 conjugate, the method comprising a) incubating adaptive immune cells from the individual with the gene therapy agent, b) analyzing the adaptive immune cells for the expression of one or more cytokines, wherein expression of a cytokine signature following incubation with the gene therapy agent identifies an individual for treatment with a gene therapy agent and an IL-2 conjugate. c) selecting the individual identified in step b) for treatment with a gene therapy agent and an IL-2 conjugate. In some embodiments, the methods further comprise the steps of d) administering an IL-2 conjugate to the individual identified in step b), and e) administering the gene therapy agent to the individual identified in step b) In some embodiments, the adaptive immune cells are T cells or B cells. In some embodiments, the method further includes the steps of isolating adaptive immune cells from the individual prior to incubating the adaptive immune cells with the gene therapy agent. In some embodiments, the method further includes the steps of isolating T cells from the individual and, incubating the T cells in culture media prior to incubating the T cells with the gene therapy agent.

[0494] In some embodiments, the adaptive immune cells are isolated from peripheral blood mononuclear cells from the individual. In some embodiments, the adaptive immune cell is a T cell. In some embodiments, the T cells are isolated from peripheral blood mononuclear cells from the individual. In some embodiments, the T cells are CD8+ T cells. In some embodiments, the T cells are incubated with the T-cell culture media for about 5 to about 10 days or about 7 to about 8 days. In some embodiments, the T cells are incubated with the T-cell culture media for about any of 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, or more than 12 days. In some embodiments, the T cells are replated prior to the incubation with the gene therapy agent of step c). In some embodiments, the T cells are replated into microwell dishes prior to incubation with the gene therapy agent.

[0495] In some embodiments, the T cells are incubated with the viral gene therapy agent at an MOI of about 1×10^3 to about 1×10^5 or about 1×10^4 . In some embodiments, the T cells are

incubated with the gene therapy agent at an MOI of less than about any of 1×10^3 , 5×10^3 , 1×10^4 , 5×10^4 , 1×10^5 , or 5×10^5 .

[0496] In some embodiments, the T cells are incubated with a non-viral gene therapy agent at a concentration of about 1 ng/mL to about 1 mg/mL. In some embodiments, the T cells are incubated with the non-viral gene therapy agent at a concentration of about 1 ng/mL to about 10 ng/mL, about 10 ng/mL to about 100 ng/mL, about 100 ng/mL to about 1 μ g/mL, about 1 μ g/mL to about 10 μ g/mL, about 10 μ g/mL to about 100 μ g/mL, or about 100 μ g/mL to about 1 mg/mL.

[0497] In some embodiments, the T cells are incubated with the gene therapy agent for about 12 hours to about 36 hours or about 24 hours. In some embodiments, the T cells are incubated with the gene therapy agent for between about 6 hours and about 48 hours, about 6 hours and about 36 hours, about 6 hours and about 24 hours, about 6 hours and about 18 hours, about 6 hours and about 12 hours, about 12 hours and about 48 hours, about 12 hours and about 36 hours, about 12 hours and about 24 hours, about 12 hours and about 18 hours, about 18 hours and about 48 hours, about 18 hours and about 36 hours, about 18 hours and about 24 hours, about 24 hours and about 48 hours, about 24 hours and about 36 hours, or about 36 hours and about 48 hours.

[0498] In some embodiments, a cytokine signature is determined for a gene therapy agent in a particular immune cell (e.g., a T cell or a B cell, etc.) by contacting the particular immune cells from a plurality of individuals with a gene therapy agent and determining changes in expression of one or more cytokines associated with an adaptive immune response, wherein a commonality in changes in expression (e.g., increased or decreased expression) in the one or more cytokines indicates the presence of a cytokine signature. In some embodiments, the cytokines associated with an adaptive immune response are associated with IL-6, TNF- α , TNF- β , IFN- α , IL-10, IL-8, RANTES, GM-CSF, IFN- γ , IP-10, IL-1 β , IL-2, IL-4, or any combination thereof. In some embodiments, the cytokine signature comprises changes in expression in more than any of 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 cytokines. In some embodiments, the change in expression comprises changes in expression levels of cytokines in adaptive immune cells of at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 75%, or 100% relative to baseline (e.g., from before treatment or from negative control). In some embodiments, the change in expression comprises an increase in expression levels of cytokines (i.e., activation biomarkers) in adaptive immune cells of at least about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 75%, or 100% relative to baseline (e.g., from before treatment or from negative control). In certain embodiments, a baseline measurement is a measurement taken in a subject before the gene therapy agent and the IL-2 conjugate is administered to the subject. In certain embodiments, a negative control is a subject that is not administered the gene therapy agent and/or the IL-2

conjugate. In some embodiments, the plurality of individuals comprises more than any of 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 individuals. In some embodiments, the commonality of changes in expression comprises similar changes in expression levels of cytokines in adaptive immune cells in greater than about 25%, 50%, 75% or 90% of the individuals in the plurality of individuals.

[0499] In some embodiments, the cytokine signature comprises increased expression of one or more of IL-6, TNF- α , TNF- β , IFN- α , IL-10, IL-8, RANTES, GM-CSF, IFN- γ , IP-10, IL-1 β , IL-2, IL-4, or any combination thereof. In some embodiments, the cytokine signature comprises increased expression of two or more of IL-6, TNF- α , TNF- β , IFN- α , IL-10, IL-8, RANTES, GM-CSF, IFN- γ , IP-10, IL-1 β , IL-2, IL-4, or any combination thereof (i.e., activation biomarkers). In some embodiments, the cytokine signature comprises increased expression of IL-6, TNF- α , TNF- β , IFN- α , IL-10, IL-8, RANTES, GM-CSF, IFN- γ , IP-10, IL-1 β , IL-2, IL-4, or any combination thereof.

[0500] In some embodiments, expression of the cytokines in the cytokine signature is increased compared to expression of the cytokines in a suitable control. Examples of a suitable control include the cytokine signature from adaptive immune cells that are not incubated with the gene therapy agent and expression of the cytokines in the cytokine signature from the same or similar adaptive immune cells prior to incubation with the gene therapy agent (e.g., wherein the cytokine signature comprises increased expression of one or more of IL-6, TNF- α , TNF- β , IFN- α , IL-10, IL-8, RANTES, GM-CSF, IFN- γ , IP-10, IL-1 β , IL-2, IL-4, or any combination thereof). In some embodiments, the cytokine signature comprises increased expression of two or more of IL-6, TNF- α , TNF- β , IFN- α , IL-10, IL-8, RANTES, GM-CSF, IFN- γ , IP-10, IL-1 β , IL-2, IL-4, or any combination thereof. In some embodiments, the cytokine signature comprises increased expression of IL-6, TNF- α , TNF- β , IFN- α , IL-10, IL-8, RANTES, GM-CSF, IFN- γ , IP-10, IL-1 β , IL-2, IL-4, or any combination thereof. In some embodiments, an increase in expression of any one of IL-6, TNF- α , TNF- β , IFN- α , IL-10, IL-8, RANTES, GM-CSF, IFN- γ , IP-10, IL-1 β , IL-2, IL-4, or any combination thereof by about 10%, about 20%, about 25%, about 50%, about 75%, about 100%, or more than 100% identifies an individual for treatment with a gene therapy agent and an IL-2 conjugate. In some embodiments, the IL-2 conjugate can preferentially expand a T-cell subpopulation, e.g., Treg. In some embodiments, the IL-2 conjugate can preferentially expand only one T-cell subpopulation, e.g., Treg.

[0501] In some embodiments, expression of the cytokines in the cytokine signature is increased compared to expression of the cytokines in the cytokine signature from dendritic cells incubated in the absence of the gene therapy agent or compared to expression of the cytokines in the cytokine signature from T cells prior to incubation with the gene therapy agent, wherein the

cytokine signature comprises increased expression of one or more of IL-6, TNF- α , TNF- β , IFN- α , IL-10, IL-8, RANTES, GM-CSF, IFN- γ , IP-10, IL-1 β , IL-2, IL-4, or any combination thereof. In some embodiments, the cytokine signature comprises increased expression of two or more of IL-6, TNF- α , TNF- β , IFN- α , IL-10, IL-8, RANTES, GM-CSF, IFN- γ , IP-10, IL-1 β , IL-2, IL-4, or any combination thereof. In some embodiments, the cytokine signature comprises increased expression of IL-6, TNF- α , TNF- β , IFN- α , IL-10, IL-8, RANTES, GM-CSF, IFN- γ , IP-10, IL-1 β , IL-2, IL-4, or any combination thereof. In some embodiments, an increase in expression of any one of IL-6, TNF- α , TNF- β , IFN- α , IL-10, IL-8, RANTES, GM-CSF, IFN- γ , IP-10, IL-1 β , IL-2, IL-4, or any combination thereof by about 10%, about 20%, about 25%, about 50%, about 75%, about 100%, or more than 100% identifies an individual for treatment with a gene therapy agent and an IL-2 conjugate.

[0502] Certain techniques and procedures, e.g., for measuring cytokines or gene expression, described or referenced herein are generally well understood and commonly employed using conventional methodology by those skilled in the art, such as, for example, the widely utilized methodologies described in *Molecular Cloning: A Laboratory Manual* (Sambrook et al., 4th ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 2012); *Current Protocols in Molecular Biology* (F.M. Ausubel, et al. eds., 2003); the series *Methods in Enzymology* (Academic Press, Inc.); *PCR 2: A Practical Approach* (M.J. MacPherson, B.D. Hames and G.R. Taylor eds., 1995); *Antibodies, A Laboratory Manual* (Harlow and Lane, eds., 1988); *Culture of Animal Cells: A Manual of Basic Technique and Specialized Applications* (R.I. Freshney, 6th ed., J. Wiley and Sons, 2010); *Oligonucleotide Synthesis* (M.J. Gait, ed., 1984); *Methods in Molecular Biology*, Humana Press; *Cell Biology: A Laboratory Notebook* (J.E. Cellis, ed., Academic Press, 1998); *Introduction to Cell and Tissue Culture* (J.P. Mather and P.E. Roberts, Plenum Press, 1998); *Cell and Tissue Culture: Laboratory Procedures* (A. Doyle, J.B. Griffiths, and D.G. Newell, eds., J. Wiley and Sons, 1993-8); *Handbook of Experimental Immunology* (D.M. Weir and C.C. Blackwell, eds., 1996); *Gene Transfer Vectors for Mammalian Cells* (J.M. Miller and M.P. Calos, eds., 1987); *PCR: The Polymerase Chain Reaction*, (Mullis et al., eds., 1994); *Current Protocols in Immunology* (J.E. Coligan et al., eds., 1991); *Short Protocols in Molecular Biology* (Ausubel et al., eds., J. Wiley and Sons, 2002); *Immunobiology* (C.A. Janeway et al., 2004); *Antibodies* (P. Finch, 1997); *Antibodies: A Practical Approach* (D. Catty., ed., IRL Press, 1988-1989); *Monoclonal Antibodies: A Practical Approach* (P. Shepherd and C. Dean, eds., Oxford University Press, 2000); *Using Antibodies: A Laboratory Manual* (E. Harlow and D. Lane, Cold Spring Harbor Laboratory Press, 1999); *The Antibodies* (M. Zanetti and J. D.

Capra, eds., Harwood Academic Publishers, 1995); and Cancer: Principles and Practice of Oncology (V.T. DeVita et al., eds., J.B. Lippincott Company, 2011).

D. Pharmaceutical Compositions and Formulations

[0503] In some embodiments, the IL-2 conjugate, gene therapy agent, pharmaceutical compositions, and formulations described herein are administered to a subject by multiple administration routes, including but not limited to, parenteral, oral, buccal, rectal, sublingual, or transdermal administration routes. In some embodiments, parenteral administration comprises intravenous, subcutaneous, intramuscular, intracerebral, intranasal, intra-arterial, intra-articular, intradermal, intravitreal, intraosseous infusion, intraperitoneal, intracranial, intra-cerebrospinal fluid (CSF), intra-dorsal root ganglia (DRG), intraocular, intracisterna magna, or intrathecal administration. In some embodiments, the pharmaceutical composition is formulated for local administration. In other instances, the pharmaceutical composition is formulated for systemic administration. In some embodiments, the pharmaceutical composition and formulations described herein are administered to a subject by intravenous, subcutaneous, and intramuscular administration. In some embodiments, the pharmaceutical composition and formulations described herein are administered to a subject by intravenous administration. In some embodiments, the pharmaceutical composition and formulations described herein are administered to a subject by administration. In some embodiments, the pharmaceutical composition and formulations described herein are administered to a subject by intramuscular administration.

[0504] In some embodiments, the pharmaceutical formulations (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) include, but are not limited to, aqueous liquid dispersions, self-emulsifying dispersions, solid solutions, liposomal dispersions, aerosols, solid dosage forms, powders, immediate release formulations, controlled release formulations, fast melt formulations, tablets, capsules, dragees, pills, delayed release formulations, extended release formulations, pulsatile release formulations, multiparticulate formulations (e.g., nanoparticle formulations), and mixed immediate and controlled release formulations.

[0505] In some embodiments, the pharmaceutical formulations (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) include a carrier or carrier materials selected on the basis of compatibility with the composition disclosed herein, and the release profile properties of the desired dosage form. Exemplary carrier materials include, e.g., binders, suspending agents, disintegration agents, filling agents, surfactants, solubilizers, stabilizers, lubricants, wetting agents, diluents, and the like. Pharmaceutically compatible carrier materials

include, but are not limited to, acacia, gelatin, colloidal silicon dioxide, calcium glycerophosphate, calcium lactate, maltodextrin, glycerine, magnesium silicate, polyvinylpyrrolidone (PVP), cholesterol, cholesterol esters, sodium caseinate, soy lecithin, taurocholic acid, phosphatidylcholine, sodium chloride, tricalcium phosphate, dipotassium phosphate, cellulose and cellulose conjugates, sugars sodium stearyl lactylate, carrageenan, monoglyceride, diglyceride, pregelatinized starch, and the like. See, e.g., Remington: The Science and Practice of Pharmacy, Nineteenth Ed (Easton, Pa.: Mack Publishing Company, 1995), Hoover, John E., Remington's Pharmaceutical Sciences, Mack Publishing Co., Easton, Pennsylvania 1975, Liberman, H.A. and Lachman, L., Eds., Pharmaceutical Dosage Forms, Marcel Decker, New York, N.Y., 1980, and Pharmaceutical Dosage Forms and Drug Delivery Systems, Seventh Ed. (Lippincott Williams & Wilkins 1999).

[0506] In some embodiments, the pharmaceutical composition (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) is formulated as an immunoliposome, which comprises an IL-2 conjugate and/or a gene therapy agent or a plurality of IL-2 conjugates and/or a plurality of gene therapy agents bound either directly or indirectly to lipid bilayer of liposomes. Exemplary lipids include, but are not limited to, fatty acids; phospholipids; sterols such as cholesterol; sphingolipids such as sphingomyelin; glycosphingolipids such as gangliosides, globocides, and cerebroside; surfactant amines such as stearyl, oleyl, and linoleyl amines. In some embodiments, the lipid comprises a cationic lipid. In some embodiments, the lipid comprises a phospholipid. Exemplary phospholipids include, but are not limited to, phosphatidic acid ("PA"), phosphatidylcholine ("PC"), phosphatidylglycerol ("PG"), phosphatidylethanolamine ("PE"), phosphatidylinositol ("PI"), and phosphatidylserine ("PS"), sphingomyelin (including brain sphingomyelin), lecithin, lysolecithin, lysophosphatidylethanolamine, cerebroside, diarachidoylphosphatidylcholine ("DAPC"), didecanoyl-L-alpha-phosphatidylcholine ("DDPC"), dielaidoylphosphatidylcholine ("DEPC"), dilauroylphosphatidylcholine ("DLPC"), dilinoleoylphosphatidylcholine, dimyristoylphosphatidylcholine ("DMPC"), dioleoylphosphatidylcholine ("DOPC"), dipalmitoylphosphatidylcholine ("DPPC"), distearoylphosphatidylcholine ("DSPC"), 1-palmitoyl-2-oleoyl-phosphatidylcholine ("POPC"), diarachidoylphosphatidylglycerol ("DAPG"), didecanoyl-L-alpha-phosphatidylglycerol ("DDPG"), dielaidoylphosphatidylglycerol ("DEPG"), dilauroylphosphatidylglycerol ("DLPG"), dilinoleoylphosphatidylglycerol, dimyristoylphosphatidylglycerol ("DMPG"), dioleoylphosphatidylglycerol ("DOPG"), dipalmitoylphosphatidylglycerol ("DPPG"), distearoylphosphatidylglycerol ("DSPG"), 1-palmitoyl-2-oleoyl-phosphatidylglycerol ("POPG"), diarachidoylphosphatidylethanolamine

(“DAPE”), didecanoyl-L-alpha-phosphatidylethanolamine (“DDPE”), dielaidoylphosphatidylethanolamine (“DEPE”), dilauroylphosphatidylethanolamine (“DLPE”), dilinoleoylphosphatidylethanolamine, dimyristoylphosphatidylethanolamine (“DMPE”), dioleoylphosphatidylethanolamine (“DOPE”), dipalmitoylphosphatidylethanolamine (“DPPE”), distearoylphosphatidylethanolamine (“DSPE”), 1-palmitoyl-2-oleoyl-phosphatidylethanolamine (“POPE”), diarachidoylphosphatidylinositol (“DAPI”), didecanoyl-L-alpha-phosphatidylinositol (“DDPI”), dielaidoylphosphatidylinositol (“DEPI”), dilauroylphosphatidylinositol (“DLPI”), dilinoleoylphosphatidylinositol, dimyristoylphosphatidylinositol (“DMPI”), dioleoylphosphatidylinositol (“DOPI”), dipalmitoylphosphatidylinositol (“DPPI”), distearoylphosphatidylinositol (“DSPI”), 1-palmitoyl-2-oleoyl-phosphatidylinositol (“POPI”), diarachidoylphosphatidylserine (“DAPS”), didecanoyl-L-alpha-phosphatidylserine (“DDPS”), dielaidoylphosphatidylserine (“DEPS”), dilauroylphosphatidylserine (“DLPS”), dilinoleoylphosphatidylserine, dimyristoylphosphatidylserine (“DMPS”), dioleoylphosphatidylserine (“DOPS”), dipalmitoylphosphatidylserine (“DPPS”), distearoylphosphatidylserine (“DSPS”), 1-palmitoyl-2-oleoyl-phosphatidylserine (“POPS”), diarachidoyl sphingomyelin, didecanoyl sphingomyelin, dielaidoyl sphingomyelin, dilauroyl sphingomyelin, dilinoleoyl sphingomyelin, dimyristoyl sphingomyelin, sphingomyelin, dioleoyl sphingomyelin, dipalmitoyl sphingomyelin, distearoyl sphingomyelin, and 1-palmitoyl-2-oleoyl-sphingomyelin.

[0507] In some embodiments, the pharmaceutical formulations (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) further include pH adjusting agents or buffering agents which include acids such as acetic, boric, citric, lactic, phosphoric and hydrochloric acids, bases such as sodium hydroxide, sodium phosphate, sodium borate, sodium citrate, sodium acetate, sodium lactate and tris-hydroxymethylaminomethane, and buffers such as citrate/dextrose, sodium bicarbonate and ammonium chloride. Such acids, bases, and buffers are included in an amount required to maintain pH of the composition in an acceptable range.

[0508] In some embodiments, the pharmaceutical formulation includes one or more salts in an amount required to bring osmolality of the composition into an acceptable range. Such salts include those having sodium, potassium or ammonium cations and chloride, citrate, ascorbate, borate, phosphate, bicarbonate, sulfate, thiosulfate or bisulfite anions, suitable salts include sodium chloride, potassium chloride, sodium thiosulfate, sodium bisulfite and ammonium sulfate.

[0509] In some embodiments, the pharmaceutical formulations (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) include, but are not limited to, sugars like

trehalose, sucrose, mannitol, maltose, glucose, or salts like potassium phosphate, sodium citrate, ammonium sulfate and/or other agents such as heparin to increase the solubility and in vivo stability of polypeptides.

[0510] In some embodiments, the pharmaceutical formulations (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) further include diluent that are used to stabilize compounds because they can provide a more stable environment. Salts dissolved in buffered solutions (which also can provide pH control or maintenance) are utilized as diluents in the art, including, but not limited to a phosphate buffered saline solution. In certain instances, diluents increase bulk of the composition to facilitate compression or create sufficient bulk for homogenous blend for capsule filling. Such compounds can include e.g., lactose, starch, mannitol, sorbitol, dextrose, microcrystalline cellulose such as Avicel®, dibasic calcium phosphate, dicalcium phosphate dihydrate, tricalcium phosphate, calcium phosphate, anhydrous lactose, spray-dried lactose, pregelatinized starch, compressible sugar, such as Di-Pac® (Amstar), mannitol, hydroxypropylmethylcellulose, hydroxypropylmethylcellulose acetate stearate, sucrose-based diluents, confectioner's sugar, monobasic calcium sulfate monohydrate, calcium sulfate dihydrate, calcium lactate trihydrate, dextrans, hydrolyzed cereal solids, amylose, powdered cellulose, calcium carbonate, glycine, kaolin, mannitol, sodium chloride, inositol, bentonite, and the like. In some embodiments, the IL-2 conjugates and/or gene therapy agents disclosed herein may be used in pharmaceutical formulations comprising histidine, sorbitol, and polysorbate 80, or any combination that affords a stable formulation and can be administered to subjects in need thereof. In one embodiment, the IL-2 conjugates disclosed herein may be presented as a finished drug product in a suitable container, such as a vial, as follows: IL-2 conjugate (about 2 mg to about 10 mg); L-histidine (about 0.5 mg to about 2 mg); L-histidine hydrochloride (about 1 mg to about 2 mg); sorbitol (about 20 mg to about 80 mg); and polysorbate 80 (about 0.1 mg to about 0.2 mg); with a sufficient quantity of water for injection to provide a liquid formulation suitable for use in the disclosed methods.

[0511] In some embodiments, the pharmaceutical formulations (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) include disintegration agents or disintegrants to facilitate the breakup or disintegration of a substance. The term "disintegrate" include both the dissolution and dispersion of the dosage form when contacted with gastrointestinal fluid. Examples of disintegration agents include a starch, e.g., a natural starch such as corn starch or potato starch, a pregelatinized starch such as National 1551 or Amijel®, or sodium starch glycolate such as Promogel® or Explotab®, a cellulose such as a wood product, methylcellulose, e.g., Avicel®, Avicel® PH101, Avicel® PH102, Avicel® PH105,

Elcema® P100, Emcocel®, Vivacel®, Ming Tia®, and Solka-Floc®, methylcellulose, croscarmellose, or a cross-linked cellulose, such as cross-linked sodium carboxymethylcellulose (Ac-Di-Sol®), cross-linked carboxymethylcellulose, or cross-linked croscarmellose, a cross-linked starch such as sodium starch glycolate, a cross-linked polymer such as crospovidone, a cross-linked polyvinylpyrrolidone, alginate such as alginic acid or a salt of alginic acid such as sodium alginate, a clay such as Veegum® HV (magnesium aluminum silicate), a gum such as agar, guar, locust bean, Karaya, pectin, or tragacanth, sodium starch glycolate, bentonite, a natural sponge, a surfactant, a resin such as a cation-exchange resin, citrus pulp, sodium lauryl sulfate, sodium lauryl sulfate in combination starch, and the like.

[0512] In some embodiments, the pharmaceutical formulations (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) include filling agents such as lactose, calcium carbonate, calcium phosphate, dibasic calcium phosphate, calcium sulfate, microcrystalline cellulose, cellulose powder, dextrose, dextrans, dextrans, starches, pregelatinized starch, sucrose, xylitol, lactitol, mannitol, sorbitol, sodium chloride, polyethylene glycol, and the like.

[0513] Lubricants and glidants are also optionally included in the pharmaceutical formulations described herein (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) for preventing, reducing or inhibiting adhesion or friction of materials. Exemplary lubricants include, e.g., stearic acid, calcium hydroxide, talc, sodium stearyl fumarate, a hydrocarbon such as mineral oil, or hydrogenated vegetable oil such as hydrogenated soybean oil (Sterotex®), higher fatty acids and their alkali-metal and alkaline earth metal salts, such as aluminum, calcium, magnesium, zinc, stearic acid, sodium stearates, glycerol, talc, waxes, Stearowet®, boric acid, sodium benzoate, sodium acetate, sodium chloride, leucine, a polyethylene glycol (e.g., PEG-4000) or a methoxypolyethylene glycol such as Carbowax™, sodium oleate, sodium benzoate, glyceryl behenate, polyethylene glycol, magnesium or sodium lauryl sulfate, colloidal silica such as Syloid™, Cab-O-Sil®, a starch such as corn starch, silicone oil, a surfactant, and the like.

[0514] Plasticizers include compounds used to soften the microencapsulation material or film coatings to make them less brittle. Suitable plasticizers include, e.g., polyethylene glycols such as PEG 300, PEG 400, PEG 600, PEG 1450, PEG 3350, and PEG 800, stearic acid, propylene glycol, oleic acid, triethyl cellulose and triacetin. Plasticizers can also function as dispersing agents or wetting agents.

[0515] Solubilizers include compounds such as triacetin, triethylcitrate, ethyl oleate, ethyl caprylate, sodium lauryl sulfate, sodium docusate, vitamin E TPGS, dimethylacetamide, N-

methylpyrrolidone, N-hydroxyethylpyrrolidone, polyvinylpyrrolidone, hydroxypropylmethyl cellulose, hydroxypropyl cyclodextrins, ethanol, n-butanol, isopropyl alcohol, cholesterol, bile salts, polyethylene glycol 200-600, glycofurol, transcutool, propylene glycol, and dimethyl isosorbide and the like.

[0516] Stabilizers include compounds such as any antioxidation agents, buffers, acids, preservatives and the like. Exemplary stabilizers include L-arginine hydrochloride, tromethamine, albumin (human), citric acid, benzyl alcohol, phenol, disodium biphosphate dehydrate, propylene glycol, metacresol or m-cresol, zinc acetate, polysorbate-20 or Tween® 20, or trometamol.

[0517] Suspending agents include compounds such as polyvinylpyrrolidone, e.g., polyvinylpyrrolidone K12, polyvinylpyrrolidone K17, polyvinylpyrrolidone K25, or polyvinylpyrrolidone K30, vinyl pyrrolidone/vinyl acetate copolymer (S630), polyethylene glycol, e.g., the polyethylene glycol can have a molecular weight of about 300 to about 6000, or about 3350 to about 4000, or about 7000 to about 5400, sodium carboxymethylcellulose, methylcellulose, hydroxypropylmethylcellulose, hydroxymethylcellulose acetate stearate, polysorbate-80, hydroxyethylcellulose, sodium alginate, gums, such as, e.g., gum tragacanth and gum acacia, guar gum, xanthans, including xanthan gum, sugars, cellulosics, such as, e.g., sodium carboxymethylcellulose, methylcellulose, sodium carboxymethylcellulose, hydroxypropylmethylcellulose, hydroxyethylcellulose, polysorbate-80, sodium alginate, polyethoxylated sorbitan monolaurate, polyethoxylated sorbitan monolaurate, povidone and the like.

[0518] Surfactants include compounds such as sodium lauryl sulfate, sodium docusate, Tween 60 or 80, triacetin, vitamin E TPGS, sorbitan monooleate, polyoxyethylene sorbitan monooleate, polysorbates, polaxomers, bile salts, glyceryl monostearate, copolymers of ethylene oxide and propylene oxide, e.g., Pluronic® (BASF), and the like. Additional surfactants include polyoxyethylene fatty acid glycerides and vegetable oils, e.g., polyoxyethylene (60) hydrogenated castor oil, and polyoxyethylene alkylethers and alkylphenyl ethers, e.g., octoxynol 10, octoxynol 40. Sometimes, surfactants is included to enhance physical stability or for other purposes.

[0519] Viscosity enhancing agents include, e.g., methyl cellulose, xanthan gum, carboxymethyl cellulose, hydroxypropyl cellulose, hydroxypropylmethyl cellulose, hydroxypropylmethyl cellulose acetate stearate, hydroxypropylmethyl cellulose phthalate, carbomer, polyvinyl alcohol, alginates, acacia, chitosans and combinations thereof.

[0520] Wetting agents include compounds such as oleic acid, glyceryl monostearate, sorbitan monooleate, sorbitan monolaurate, triethanolamine oleate, polyoxyethylene sorbitan monooleate, polyoxyethylene sorbitan monolaurate, sodium docusate, sodium oleate, sodium lauryl sulfate, sodium docusate, triacetin, Tween 80, vitamin E TPGS, ammonium salts and the like.

[0521] In some embodiments, the disclosure is directed to a pharmaceutical composition comprising the gene therapy agent (e.g., an AAV particle, an adenovirus particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle) and/or the IL-2 conjugate as described herein. The pharmaceutical compositions may be suitable for any mode of administration described herein or known in the art.

[0522] In some embodiments, the pharmaceutical composition (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) comprising a pharmaceutically acceptable excipient. As is well known in the art, pharmaceutically acceptable excipients are relatively inert substances that facilitate administration of a pharmacologically effective substance and can be supplied as liquid solutions or suspensions, as emulsions, or as solid forms suitable for dissolution or suspension in liquid prior to use. For example, an excipient can give form or consistency, or act as a diluent. Suitable excipients include but are not limited to stabilizing agents wetting and emulsifying agents salts for varying osmolarity encapsulating agents, pH buffering substances, and buffers. Such excipients include any pharmaceutical agent suitable for direct delivery to the eye which may be administered without undue toxicity. Pharmaceutically acceptable excipients include, but are not limited to, sorbitol, any of the various TWEEN compounds, and liquids such as water, saline, glycerol and ethanol. Pharmaceutically acceptable salts can be included therein, for example, mineral acid salts such as hydrochlorides, hydrobromides, phosphates, sulfates, and the like; and the salts of organic acids such as acetates, propionates, malonates, benzoates, and the like. A thorough discussion of pharmaceutically acceptable excipients is available in REMINGTON'S PHARMACEUTICAL SCIENCES (Mack Pub. Co., N.J.1991). In some embodiments, the pharmaceutical composition comprising a rAAV particle described herein and a pharmaceutically acceptable carrier is suitable for administration to human. Such carriers are well known in the art (see, e.g., Remington's Pharmaceutical Sciences, 15th Edition, pp. 1035-1038 and 1570-1580).

[0523] Such pharmaceutically acceptable carriers can be sterile liquids, such as water and oil, including those of petroleum, animal, vegetable or synthetic origin, such as peanut oil, soybean oil, mineral oil, and the like. Saline solutions and aqueous dextrose, polyethylene glycol (PEG) and glycerol solutions can also be employed as liquid carriers, particularly for injectable solutions. The pharmaceutical composition (e.g., comprising an IL-2 conjugate and/or gene

therapy agent described herein) may further comprise additional ingredients, for example preservatives, buffers, tonicity agents, antioxidants and stabilizers, nonionic wetting or clarifying agents, viscosity-increasing agents, and the like. The pharmaceutical compositions described herein (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) can be packaged in single unit dosages or in multidosage forms. The compositions are generally formulated as sterile and substantially isotonic solution. Kits and Articles of Manufacture [0524] The gene therapy agent (e.g., an AAV particle, an adenovirus particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle) and/or the IL-2 conjugate as described herein may be contained within a kit or article of manufacture, e.g., designed for use in one of the methods of the disclosure as described herein.

[0525] In some embodiments, the kits or articles of manufacture further include instructions for administration of the IL-2 conjugate and/or gene therapy agent. The kits or articles of manufacture described herein may further include other materials desirable from a commercial and user standpoint including other buffers diluents filters needles syringes and package inserts with instructions for performing any methods described herein. Suitable packaging materials may also be included and may be any packaging materials known in the art, including, for example, vials (such as sealed vials), vessels, ampules, bottles, jars, flexible packaging (e.g., sealed Mylar or plastic bags), and the like. These articles of manufacture may further be sterilized and/or sealed.

[0526] In some embodiments, the kits or articles of manufacture further contain one or more of the buffers and/or pharmaceutically acceptable excipients described herein (e.g., as described in REMINGTON'S PHARMACEUTICAL SCIENCES (Mack Pub. Co., N.J.1991). In some embodiments, the kits or articles of manufacture include one or more pharmaceutically acceptable excipients, carriers, solutions, and/or additional ingredients described herein. The kits or articles of manufacture described herein can be packaged in single unit dosages or in multidosage forms. The contents of the kits or articles of manufacture are generally formulated as sterile and can be lyophilized or provided as a substantially isotonic solution.

E. Therapeutic Regimens

[0527] In some embodiments, the pharmaceutical compositions described herein (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) are administered for therapeutic applications. In some embodiments, the IL-2 conjugate is administered 1 time, 2 times, 3 times, 4 times, 5 times, 6 times, 7 times, 8 times, 9 times, or 10 times. In some embodiments, the gene therapy agent is administered 1 time, 2 times, 3 times, 4 times, 5 times, 6

times, 7 times, 8 times, 9 times, or 10 times. In some embodiments, the IL-2 conjugate and the gene therapy agent is each administered 1 time, 2 times, 3 times, 4 times, 5 times, 6 times, 7 times, 8 times, 9 times, or 10 times. In some embodiments, the IL-2 conjugate is administered the same number of times as the gene therapy agent. In some embodiments, the IL-2 conjugate is administered a different number of as the gene therapy agent. In some embodiments, the IL-2 conjugate is administered 1 time and the gene therapy agent is administered 2 times. In some embodiments, the IL-2 conjugate is administered 2 times and the gene therapy agent is administered 1 time. In some embodiments, the IL-2 conjugate is administered 1 time and the gene therapy agent is administered 3 times. In some embodiments, the IL-2 conjugate is administered 3 times and the gene therapy agent is administered 1 time.

[0528] In some embodiments, the pharmaceutical composition (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) is administered once per day, twice per day, three times per day or more frequently. In some embodiments, pharmaceutical composition (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) is administered daily, every day, every alternate day, five days a week, once a week, every other week, two weeks per month, three weeks per month, once a month, twice a month, three times per month, or more. In some embodiments, the pharmaceutical composition (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) is administered for at least 1 month, 2 months, 3 months, 4 months, 5 months, 6 months, 7 months, 8 months, 9 months, 10 months, 11 months, 12 months, 18 months, 2 years, 3 years, or more.

[0529] In the case wherein the patient's status does improve, upon the doctor's discretion the administration of the composition is given continuously, alternatively, the dose of the composition being administered is temporarily reduced or temporarily suspended for a certain length of time (i.e., a "drug holiday"). In some embodiments, the length of the drug holiday varies between 2 days and 1 year, including by way of example only, 2 days, 3 days, 4 days, 5 days, 6 days, 7 days, 10 days, 12 days, 15 days, 20 days, 28 days, 35 days, 50 days, 70 days, 100 days, 120 days, 150 days, 180 days, 200 days, 250 days, 280 days, 300 days, 320 days, 350 days, or 365 days. The dose reduction during a drug holiday is from 10%-100%, including, by way of example only, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, or 100%.

[0530] In some embodiments, an effective amount of the pharmaceutical composition (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) is administered to a subject in need thereof once per week, once every two weeks, once every three weeks, once every 4 weeks, once every 5 weeks, once every 6 weeks, once every 7 weeks, once every 8

weeks, once every 9 weeks, once every 10 weeks, once every 11 weeks, once every 12 weeks, once every 13 weeks, once every 14 weeks, once every 15 weeks, once every 16 weeks, once every 17 weeks, once every 18 weeks, once every 19 weeks, once every 20 weeks, once every 21 weeks, once every 22 weeks, once every 23 weeks, once every 24 weeks, once every 25 weeks, once every 26 weeks, once every 27 weeks, or once every 28 weeks. In some embodiments, an effective amount of the pharmaceutical composition (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) is administered to a subject in need thereof once per week. In some embodiments, an effective amount of the pharmaceutical composition (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) is administered to a subject in need thereof once every two weeks. In some embodiments, an effective amount of the pharmaceutical composition (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) is administered to a subject in need thereof once every three weeks. In some embodiments, an effective amount of the pharmaceutical composition (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) is administered to a subject in need thereof once every 4 weeks. In some embodiments, an effective amount of the pharmaceutical composition (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) is administered to a subject in need thereof once every 5 weeks. In some embodiments, an effective amount of the pharmaceutical composition (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) is administered to a subject in need thereof once every 6 weeks. In some embodiments, an effective amount of the pharmaceutical composition (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) is administered to a subject in need thereof once every 7 weeks. In some embodiments, an effective amount of the pharmaceutical composition (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) is administered to a subject in need thereof once every 8 weeks. In some embodiments, an effective amount of the pharmaceutical composition (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) is administered to a subject in need thereof once every 9 weeks. In some embodiments, an effective amount of the pharmaceutical composition (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) is administered to a subject in need thereof once every 10 weeks. In some embodiments, an effective amount of the pharmaceutical composition (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) is administered to a subject in need thereof once every 11 weeks. In some embodiments, an effective amount of the pharmaceutical composition (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) is administered to a subject in need thereof once every 12 weeks. In some embodiments, an effective amount of the pharmaceutical

composition (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) is administered to a subject in need thereof once every 13 weeks. In some embodiments, an effective amount of the pharmaceutical composition (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) is administered to a subject in need thereof once every 14 weeks. In some embodiments, an effective amount of the pharmaceutical composition (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) is administered to a subject in need thereof once every 15 weeks. In some embodiments, an effective amount of the pharmaceutical composition (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) is administered to a subject in need thereof once every 16 weeks. In some embodiments, an effective amount of the pharmaceutical composition (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) is administered to a subject in need thereof once every 17 weeks. In some embodiments, an effective amount of the pharmaceutical composition (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) is administered to a subject in need thereof once every 18 weeks. In some embodiments, an effective amount of the pharmaceutical composition (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) is administered to a subject in need thereof once every 19 weeks. In some embodiments, an effective amount of the pharmaceutical composition (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) is administered to a subject in need thereof once every 20 weeks. In some embodiments, an effective amount of the pharmaceutical composition (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) is administered to a subject in need thereof once every 21 weeks. In some embodiments, an effective amount of the pharmaceutical composition (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) is administered to a subject in need thereof once every 22 weeks. In some embodiments, an effective amount of the pharmaceutical composition (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) is administered to a subject in need thereof once every 23 weeks. In some embodiments, an effective amount of the pharmaceutical composition (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) is administered to a subject in need thereof once every 24 weeks.

[0531] Once improvement of the patient's conditions has occurred, a maintenance dose is administered if necessary. Subsequently, the dosage or the frequency of administration, or both, can be reduced, as a function of the symptoms, to a level at which the improved disease, disorder or condition is retained.

[0532] In some embodiments, the amount of a given agent that correspond to such an amount varies depending upon factors such as the particular compound, the severity of the disease, the identity (e.g., weight) of the subject or host in need of treatment, but nevertheless is routinely determined in a manner known in the art according to the particular circumstances surrounding the case, including, e.g., the specific agent being administered, the route of administration, and the subject or host being treated. In some embodiments, the desired dose is conveniently presented in a single dose or as divided doses administered simultaneously (or over a short period of time) or at appropriate intervals, for example as two, three, four or more sub-doses per day.

[0533] In some embodiments, the methods include the dosing of an IL-2 conjugate to a subject in need thereof at a dose in the range from 1 μg of the IL-2 conjugate per kg of the subject's body weight to about 200 μg of the IL-2 conjugate per kg of the subject's body weight, or from about 2 μg of the IL-2 conjugate per kg of the subject's body weight to about 200 μg of the IL-2 conjugate per kg of the subject's body weight, or from about 4 μg of the IL-2 conjugate per kg of the subject's body weight to about 200 μg of the IL-2 conjugate per kg of the subject's body weight, or from about 6 μg of the IL-2 conjugate per kg of the subject's body weight to about 200 μg of the IL-2 conjugate per kg of the subject's body weight, or from about 8 μg of the IL-2 conjugate per kg of the subject's body weight to about 200 μg of the IL-2 conjugate per kg of the subject's body weight, or from about 10 μg of the IL-2 conjugate per kg of the subject's body weight to about 200 μg of the IL-2 conjugate per kg of the subject's body weight, or from about 12 μg of the IL-2 conjugate per kg of the subject's body weight to about 200 μg of the IL-2 conjugate per kg of the subject's body weight, or from about 14 μg of the IL-2 conjugate per kg of the subject's body weight to about 200 μg of the IL-2 conjugate per kg of the subject's body weight, or from about 16 μg of the IL-2 conjugate per kg of the subject's body weight to about 200 μg of the IL-2 conjugate per kg of the subject's body weight, or from about 18 μg of the IL-2 conjugate per kg of the subject's body weight to about 200 μg of the IL-2 conjugate per kg of the subject's body weight, or from about 20 μg of the IL-2 conjugate per kg of the subject's body weight to about 200 μg of the IL-2 conjugate per kg of the subject's body weight, or from about 22 μg of the IL-2 conjugate per kg of the subject's body weight to about 200 μg of the IL-2 conjugate per kg of the subject's body weight, or from about 24 μg of the IL-2 conjugate per kg of the subject's body weight to about 200 μg of the IL-2 conjugate per kg of the subject's body weight, or from about 26 μg of the IL-2 conjugate per kg of the subject's body weight to about 200 μg of the IL-2 conjugate per kg of the subject's body weight, or from about 28 μg of the IL-2 conjugate per kg of the subject's body weight to about 200 μg of the IL-2 conjugate per kg of

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body weight, or from about 160 µg of the IL-2 conjugate per kg of the subject's body weight to about 200 µg of the IL-2 conjugate per kg of the subject's body weight, or from about 170 µg of the IL-2 conjugate per kg of the subject's body weight to about 200 µg of the IL-2 conjugate per kg of the subject's body weight, or from about 180 µg of the IL-2 conjugate per kg of the subject's body weight to about 200 µg of the IL-2 conjugate per kg of the subject's body weight, or from about 190 µg of the IL-2 conjugate per kg of the subject's body weight to about 200 µg of the IL-2 conjugate per kg of the subject's body weight. In some embodiments, the methods include the dosing of an IL-2 conjugate to a subject in need thereof at a dose in the range from 0.001 mg of the IL-2 conjugate per kg of the subject's body weight to about 10 mg of the IL-2 conjugate per kg of the subject's body weight, or from about 0.01 mg of the IL-2 conjugate per kg of the subject's body weight to about 7.5 mg of the IL-2 conjugate per kg of the subject's body weight, or from about 0.02 mg of the IL-2 conjugate per kg of the subject's body weight to about 5 mg of the IL-2 conjugate per kg of the subject's body weight, or from about 0.02 mg of the IL-2 conjugate per kg of the subject's body weight to about 0.5 mg of the IL-2 conjugate per kg of the subject's body weight, or from about 0.02 mg of the IL-2 conjugate per kg of the subject's body weight to about 2.5 mg of the IL-2 conjugate per kg of the subject's body weight, or from about 0.03 mg of the IL-2 conjugate per kg of the subject's body weight to about 2.0 mg of the IL-2 conjugate per kg of the subject's body weight, or from about 0.03 mg of the IL-2 conjugate per kg of the subject's body weight to about 0.4 mg of the IL-2 conjugate per kg of the subject's body weight, or from about 0.04 mg of the IL-2 conjugate per kg of the subject's body weight to about 1.5 mg of the IL-2 conjugate per kg of the subject's body weight, or from about 0.04 mg of the IL-2 conjugate per kg of the subject's body weight to about 0.1 mg of the IL-2 conjugate per kg of the subject's body weight, or from about 0.04 mg of the IL-2 conjugate per kg of the subject's body weight to about 0.08 mg of the IL-2 conjugate per kg of the subject's body weight, or from about 0.05 mg of the IL-2 conjugate per kg of the subject's body weight to about 1.0 mg of the IL-2 conjugate per kg of the subject's body weight, or from about 0.05 mg of the IL-2 conjugate per kg of the subject's body weight to about 0.1 mg of the IL-2 conjugate per kg of the subject's body weight, or from about 0.05 mg of the IL-2 conjugate per kg of the subject's body weight to about 0.08 mg of the IL-2 conjugate per kg of the subject's body weight, or from about 0.06 mg of the IL-2 conjugate per kg of the subject's body weight to about 0.9 mg of the IL-2 conjugate per kg of the subject's body weight, or from about 0.07 mg of the IL-2 conjugate per kg of the subject's body weight to about 0.8 mg of the IL-2 conjugate per kg of the subject's body weight, or from about 0.08 mg of the IL-2 conjugate per kg of the subject's body weight to about 0.7 mg of the IL-2 conjugate per kg of the subject's body weight, or from

[illegible]

of the IL-2 conjugate per kg of the subject's body weight. In some embodiments, the methods include the dosing of an IL-2 conjugate to a subject in need thereof at about 0.08 mg of the IL-2 conjugate per kg of the subject's body weight. In some embodiments, the methods include the dosing of an IL-2 conjugate to a subject in need thereof at about 0.09 mg of the IL-2 conjugate per kg of the subject's body weight. In some embodiments, the methods include the dosing of an IL-2 conjugate to a subject in need thereof at about 0.1 mg of the IL-2 conjugate per kg of the subject's body weight. In some embodiments, the methods include the dosing of an IL-2 conjugate to a subject in need thereof at about 0.3 mg of the IL-2 conjugate per kg of the subject's body weight. The foregoing ranges are merely suggestive, as the number of variables in regard to an individual treatment regime is large, and considerable excursions from these recommended values are not uncommon. Such dosages are altered depending on a number of variables, not limited to the activity of the compound used, the disease or condition to be treated, the mode of administration, the requirements of the individual subject, the severity of the disease or condition being treated, and the judgment of the practitioner. In some embodiments, toxicity and therapeutic efficacy of such therapeutic regimens are determined by standard pharmaceutical procedures in cell cultures or experimental animals, including, but not limited to, the determination of the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between the toxic and therapeutic effects is the therapeutic index and it is expressed as the ratio between LD50 and ED50. Compounds exhibiting high therapeutic indices are preferred. The data obtained from cell culture assays and animal studies are used in formulating a range of dosage for use in a human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with minimal toxicity. The dosage varies within this range depending upon the dosage form employed and the route of administration utilized. Here and throughout, dosing of the IL-2 conjugate is calculated in terms of the mass of the IL-2 component of the conjugate exclusive of the mass of the conjugating moiety (e.g., a water-soluble polymer such as PEG).

[0534] In some embodiments, the methods include the dosing of an IL-2 conjugate to a subject in need thereof at a dose of about 1 µg of the IL-2 conjugate per kg of the subject's body weight, or about 2 µg of the IL-2 conjugate per kg of the subject's body weight, about 4 µg of the IL-2 conjugate per kg of the subject's body weight, about 6 µg of the IL-2 conjugate per kg of the subject's body weight, about 8 µg of the IL-2 conjugate per kg of the subject's body weight, about 10 µg of the IL-2 conjugate per kg of the subject's body weight, about 12 µg of the IL-2 conjugate per kg of the subject's body weight, about 14 µg of the IL-2 conjugate per kg of the subject's body weight, about 16 µg of the IL-2 conjugate per kg of the subject's body weight,

about 18 µg of the IL-2 conjugate per kg of the subject's body weight, about 20 µg of the IL-2 conjugate per kg of the subject's body weight, about 22 µg of the IL-2 conjugate per kg of the subject's body weight, about 24 µg of the IL-2 conjugate per kg of the subject's body weight, about 26 µg of the IL-2 conjugate per kg of the subject's body weight, about 28 µg of the IL-2 conjugate per kg of the subject's body weight, about 30 µg of the IL-2 conjugate per kg of the subject's body weight, about 32 µg of the IL-2 conjugate per kg of the subject's body weight, about 34 µg of the IL-2 conjugate per kg of the subject's body weight, about 36 µg of the IL-2 conjugate per kg of the subject's body weight, about 38 µg of the IL-2 conjugate per kg of the subject's body weight, about 40 µg of the IL-2 conjugate per kg of the subject's body weight, about 42 µg of the IL-2 conjugate per kg of the subject's body weight, about 44 µg of the IL-2 conjugate per kg of the subject's body weight, about 46 µg of the IL-2 conjugate per kg of the subject's body weight, about 48 µg of the IL-2 conjugate per kg of the subject's body weight, about 50 µg of the IL-2 conjugate per kg of the subject's body weight, about 55 µg of the IL-2 conjugate per kg of the subject's body weight, about 60 µg of the IL-2 conjugate per kg of the subject's body weight, about 65 µg of the IL-2 conjugate per kg of the subject's body weight, about 70 µg of the IL-2 conjugate per kg of the subject's body weight, about 75 µg of the IL-2 conjugate per kg of the subject's body weight, about 80 µg of the IL-2 conjugate per kg of the subject's body weight, about 85 µg of the IL-2 conjugate per kg of the subject's body weight, about 90 µg of the IL-2 conjugate per kg of the subject's body weight, about 95 µg of the IL-2 conjugate per kg of the subject's body weight, about 100 µg of the IL-2 conjugate per kg of the subject's body weight, about 110 µg of the IL-2 conjugate per kg of the subject's body weight, about 120 µg of the IL-2 conjugate per kg of the subject's body weight, about 130 µg of the IL-2 conjugate per kg of the subject's body weight, about 140 µg of the IL-2 conjugate per kg of the subject's body weight, about 150 µg of the IL-2 conjugate per kg of the subject's body weight, about 160 µg of the IL-2 conjugate per kg of the subject's body weight, about 170 µg of the IL-2 conjugate per kg of the subject's body weight, about 180 µg of the IL-2 conjugate per kg of the subject's body weight, about 190 µg of the IL-2 conjugate per kg of the subject's body weight, or about 200 µg of the IL-2 conjugate per kg of the subject's body weight. The foregoing ranges are merely suggestive, as the number of variables in regard to an individual treatment regime is large, and considerable excursions from these recommended values are not uncommon. Such dosages are altered depending on a number of variables, not limited to the activity of the compound used, the disease or condition to be treated, the mode of administration, the requirements of the individual subject, the severity of the disease or condition being treated, and the judgment of the practitioner. In some embodiments, toxicity and therapeutic efficacy of such

therapeutic regimens are determined by standard pharmaceutical procedures in cell cultures or experimental animals, including, but not limited to, the determination of the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between the toxic and therapeutic effects is the therapeutic index and it is expressed as the ratio between LD50 and ED50. Compounds exhibiting high therapeutic indices are preferred. The data obtained from cell culture assays and animal studies are used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with minimal toxicity. The dosage varies within this range depending upon the dosage form employed and the route of administration utilized.

[0535] The foregoing ranges are merely suggestive, as the number of variables in regard to an individual treatment regime is large, and considerable excursions from these recommended values are not uncommon. Such dosages are altered depending on a number of variables, not limited to the activity of the compound used, the disease or condition to be treated, the mode of administration, the requirements of the individual subject, the severity of the disease or condition being treated, and the judgment of the practitioner.

[0536] In some embodiments, toxicity and therapeutic efficacy of such therapeutic regimens are determined by standard pharmaceutical procedures in cell cultures or experimental animals, including, but not limited to, the determination of the LD50 (the dose lethal to 50% of the population) and the ED50 (the dose therapeutically effective in 50% of the population). The dose ratio between the toxic and therapeutic effects is the therapeutic index and it is expressed as the ratio between LD50 and ED50. Compounds exhibiting high therapeutic indices are preferred. The data obtained from cell culture assays and animal studies are used in formulating a range of dosage for use in human. The dosage of such compounds lies preferably within a range of circulating concentrations that include the ED50 with minimal toxicity. The dosage varies within this range depending upon the dosage form employed and the route of administration utilized.

[0537] In some embodiments, the IL-2 conjugate is administered before administration of the gene therapy agent (e.g., an AAV particle, an adenovirus particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle). In some embodiments, the IL-2 conjugate is administered to the individual about any of 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 12 hours, 18 hours, 24 hours, 36 hours, 48 hours, 3 days, 4 days, 5 days, 6 days, one week (i.e., 7 days) or more than one week before administration of the gene therapy agent. In some embodiments, the IL-2 conjugate is administered to the individual less than about any of 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 12 hours, 18 hours, 24 hours, 36 hours, 48 hours, 3 days, 4 days, 5 days,

6 days, or one week (i.e., 7 days) before administration of the gene therapy agent. In some embodiments, the IL-2 conjugate and the gene therapy agent are administered at about the same time (e.g., within about one hour). In some embodiments, the IL-2 conjugate is administered after administration of the gene therapy agent. In some embodiments, the IL-2 conjugate is administered to the individual about any of 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 12 hours, 18 hours, 24 hours, 36 hours, 48 hours, 3 days, 4 days, 5 days, 6 days, one week (i.e., 7 days) or more than one week after administration of the gene therapy agent. In some embodiments, the IL-2 conjugate is administered to the individual less than about any of 1 hour, 2 hours, 3 hours, 4 hours, 5 hours, 6 hours, 12 hours, 18 hours, 24 hours, 36 hours, 48 hours, 3 days, 4 days, 5 days, 6 days, or one week (i.e., 7 days) after administration of the gene therapy agent. In some embodiments, the IL-2 conjugate is administered on the same day (e.g., within about 24 hours) as administration of the gene therapy agent.

[0538] In some embodiments, the gene therapy agent (e.g., an AAV particle, an adenovirus particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle) is used in conjunction with the IL-2 conjugate to treat a disease or disorder suitable for treatment by gene therapy. In some embodiments, the disease or disorder is a monogenic disease or disorder.

[0539] In some embodiments, the gene therapy agent (e.g., an AAV particle, an adenovirus particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle) is used in conjunction with the IL-2 conjugate to treat a disorder of the CNS. Non-limiting disorders of the CNS include stroke, Huntington's disease, epilepsy, Parkinson's disease, Lou Gehrig's disease (also known as amyotrophic lateral sclerosis), Alzheimer's disease, corticobasal degeneration or CBD, corticogasal ganglionic degeneration or CBGD, frontotemporal dementia or FTD, progressive supranuclear palsy or PSP, multiple system atrophy or MSA, cancer of the brain, and lysosomal storage diseases (LSD). Other non-limiting examples of disorders of the disclosure that may be treated by a gene therapy in conjunction with an IL-2 conjugate include traumatic brain injury, enzymatic dysfunction disorders, psychiatric disorders (including post-traumatic stress syndrome), neurodegenerative diseases, and cognitive disorders (including dementias, autism, and depression), and enzymatic dysfunction disorders include without limitation leukodystrophies (including Canavan's disease).

[0540] In some embodiments, the gene therapy agent (e.g., an AAV particle, an adenovirus particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle) is used in conjunction with the IL-2 conjugate to treat a lysosomal storage disease. As is commonly known in the art, lysosomal storage disease are rare, inherited metabolic disorders characterized by defects in lysosomal function. Such disorders are often caused by a deficiency in an enzyme required for

proper mucopolysaccharide, glycoprotein, and/or lipid metabolism, leading to a pathological accumulation of lysosomally stored cellular materials. Non-limiting examples of lysosomal storage diseases of the disclosure that may be treated by a therapeutic polypeptide or therapeutic nucleic acid of the disclosure include Gaucher disease type 2 or type 3, GM1 gangliosidosis, Hunter disease, Krabbe disease, a mannosidosis disease, mannosidosis disease, metachromatic leukodystrophy disease, mucopolipidosisII/III disease, Niemann-Pick A disease, Niemann-Pick C disease, Pompe disease, Sandhoff disease, Sanfilippo A disease, Sanfilippo B disease, Sanfilippo C disease, Sanfilippo D disease, Schindler disease, Sly disease, Tay-Sachs disease, and Wolman disease.

[0541] In some embodiments, the gene therapy agent (e.g., an AAV particle, an adenovirus particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle) is used in conjunction with the IL-2 conjugate to treat hemophilia A, hemophilia B, age related macular degeneration, diabetic retinopathy, glaucoma, muscular dystrophy, X-Linked Myotubular Myopathy, spinal muscular atrophy, Leber's congenital amaurosis, choroideremia, Leber hereditary optic neuropathy, ornithine transcarbamylase (OTC) deficiency, citrullinemia type 1, phenylketonuria (PKU), adrenoleukodystrophy, sickle cell disease, muscular dystrophy, or beta thalassemia.

[0542] In some embodiments, the disclosure provides a composition for use in the manufacture of a medicament for delivering nucleic acid to a cell of an individual in need thereof, wherein the composition comprises a gene therapy agent (e.g., an AAV particle, an adenovirus particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle), and wherein the composition is formulated for use in combination with an IL-2 conjugate. In some embodiments, the disclosure provides a composition for use in the manufacture of a medicament for delivering nucleic acid to a cell of an individual in need thereof, wherein the composition comprises an IL-2 conjugate, and wherein the composition is formulated for use in combination with a gene therapy agent (e.g., an AAV particle, an adenovirus particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle).

[0543] In some embodiments, the disclosure provides a composition for use in the manufacture of a medicament for treating an individual in need of gene therapy, wherein the composition comprises a gene therapy agent (e.g., an AAV particle, an adenovirus particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle), and wherein the composition is formulated for use in combination with an IL-2 conjugate. In some embodiments, the disclosure provides a composition for use in the manufacture of a medicament for treating an individual in need of gene therapy, wherein the composition comprises an IL-2 conjugate, and wherein the

composition is formulated for use in combination with a gene therapy agent (e.g., an AAV particle, an adenovirus particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle).

[0544] In some embodiments, the disclosure provides a composition for use in the manufacture of a medicament for modulating an immune response to gene therapy in an individual in need of gene therapy, wherein the composition comprises a gene therapy agent (e.g., an AAV particle, an adenovirus particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle), and wherein the composition is formulated for use in combination with an IL-2 conjugate. In some embodiments, the disclosure provides a composition for use in the manufacture of a medicament for modulating an immune response to gene therapy in an individual, wherein the composition comprises an IL-2 conjugate, and wherein the composition is formulated for use in combination with a gene therapy agent (e.g., an AAV particle, an adenovirus particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle).

[0545] In some embodiments, the disclosure provides a composition for use in the manufacture of a medicament for suppressing an immune response to gene therapy in an individual in need of gene therapy, wherein the composition comprises a gene therapy agent (e.g., an AAV particle, an adenovirus particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle), and wherein the composition is formulated for use in combination with an IL-2 conjugate. In some embodiments, the disclosure provides a composition for use in the manufacture of a medicament for suppressing an immune response to gene therapy in an individual, wherein the composition comprises an IL-2 conjugate, and wherein the composition is formulated for use in combination with a gene therapy agent (e.g., an AAV particle, an adenovirus particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle).

[0546] In some embodiments, the disclosure provides a composition for use in the manufacture of a medicament for improving gene therapy in an individual in need of gene therapy, wherein the composition comprises a gene therapy agent (e.g., an AAV particle, an adenovirus particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle), and wherein the composition is formulated for use in combination with an IL-2 conjugate. In some embodiments, the disclosure provides a composition for use in the manufacture of a medicament for improving gene therapy in an individual, wherein the composition comprises an IL-2 conjugate, and wherein the composition is formulated for use in combination with a gene therapy agent (e.g., an AAV particle, an adenovirus particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle).

[0547] In some embodiments, the disclosure provides a composition for use in the manufacture of a medicament for inducing tolerance to gene therapy in an individual in need of gene therapy, wherein the composition comprises a gene therapy agent (e.g., an AAV particle, an adenovirus

particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle), and wherein the composition is formulated for use in combination with an IL-2 conjugate. In some embodiments, the disclosure provides a composition for use in the manufacture of a medicament for inducing tolerance to gene therapy in an individual, wherein the composition comprises an IL-2 conjugate, and wherein the composition is formulated for use in combination with a gene therapy agent (e.g., an AAV particle, an adenovirus particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle).

[0548] In some embodiments, the disclosure provides a gene therapy agent (e.g., an AAV particle, an adenovirus particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle) for use in delivering nucleic acid to a cell of an individual in need thereof, wherein the gene therapy agent is used in combination with an IL-2 conjugate. In some embodiments, the disclosure provides an IL-2 conjugate for use in delivering nucleic acid to a cell of an individual in need thereof, wherein the IL-2 conjugate is used in combination with a gene therapy agent (e.g., an AAV particle, an adenovirus particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle).

[0549] In some embodiments, the disclosure provides a gene therapy agent (e.g., an AAV particle, an adenovirus particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle) for use in treating an individual in need of gene therapy, wherein the gene therapy agent is used in combination with an IL-2 conjugate. In some embodiments, the disclosure provides an IL-2 conjugate for use in treating an individual in need of gene therapy, wherein the IL-2 conjugate is used in combination with a gene therapy agent (e.g., an AAV particle, an adenovirus particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle).

[0550] In some embodiments, the disclosure provides an IL-2 conjugate for modulating an immune response to gene therapy in an individual in need of gene therapy, wherein the IL-2 conjugate is used in combination with a gene therapy agent (e.g., an AAV particle, an adenovirus particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle).

[0551] In some embodiments, the disclosure provides an IL-2 conjugate for suppressing an immune response to gene therapy in an individual in need of gene therapy, wherein the IL-2 conjugate is used in combination with a gene therapy agent (e.g., an AAV particle, an adenovirus particle, a lentivirus particle, a HSV particle, or a lipid nanoparticle).

III. Kits

[0552] Also provided are kits for use in the methods as described herein. Such kits include a carrier, package, or container that is compartmentalized to receive one or more containers such

as vials, tubes, and the like, each of the container(s) comprising one of the separate elements to be used in a method described herein. Suitable containers include, for example, bottles, vials, syringes, and test tubes. In one embodiment, the containers are formed from a variety of materials such as glass or plastic.

[0553] In some embodiments, the kits comprise articles of manufacture that are useful for developing adoptive cell therapies. In some embodiments, kits comprise one or more of the cytokine (e.g., IL-2) polypeptides or cytokine (e.g., IL-2) conjugates and/or one or more of the gene therapy agents disclosed herein, and optionally one or more pharmaceutical excipients described herein to facilitate the delivery of cytokine (e.g., IL-2) polypeptides or cytokine (e.g., IL-2) conjugates and/or one or more of the gene therapy agents. Such kits might optionally include one or more accessory components comprising inducers of T cell receptor signaling or modulation (e.g., checkpoint antibodies, CD3/CD28 antibodies, major histocompatibility complexes (MHC), and the like), or alternative cytokines or cytokine receptor agonists. Such kits further optionally include an identifying description or label or instructions relating to its use in the methods described herein. In some embodiments, kits comprise one or more polynucleic acid sequences encoding the IL-2 conjugates disclosed herein, an activator of a CD4+ Treg and/or a pharmaceutical composition thereof.

[0554] A kit typically includes labels listing contents and/or instructions for use, and package inserts with instructions for use. A set of instructions will also typically be included.

[0555] In one embodiment, a label is on or associated with the container. In one embodiment, a label is on a container when letters, numbers or other characters forming the label are attached, molded or etched into the container itself, a label is associated with a container when it is present within a receptacle or carrier that also holds the container, e.g., as a package insert. In one embodiment, a label is used to indicate that the contents are to be used for a specific therapeutic application. The label also indicates directions for use of the contents, such as in the methods described herein.

[0556] In certain embodiments, the pharmaceutical compositions (e.g., comprising an IL-2 conjugate and/or gene therapy agent described herein) are presented in a pack or dispenser device which contains one or more unit dosage forms containing a compound provided herein. The pack, for example, contains metal or plastic foil, such as a blister pack. In one embodiment, the pack or dispenser device is accompanied by instructions for administration. In one embodiment, the pack or dispenser is also accompanied with a notice associated with the container in form prescribed by a governmental agency regulating the manufacture, use, or sale of pharmaceuticals, which notice is reflective of approval by the agency of the form of the drug

for human or veterinary administration. Such notice, for example, is the labeling approved by the U.S. Food and Drug Administration for drugs, or the approved product insert. In one embodiment, compositions containing a compound provided herein formulated in a compatible pharmaceutical carrier are also prepared, placed in an appropriate container, and labeled for treatment of an indicated condition.

[0557] All patents, patent applications, websites, other publications or documents, accession numbers and the like cited herein are incorporated by reference in their entirety for all purposes to the same extent as if each individual item were specifically and individually indicated to be so incorporated by reference. If different versions of a sequence are associated with an accession number at different times, the version associated with the accession number at the effective filing date of this application is meant. The effective filing date means the earlier of the actual filing date or filing date of a priority application referring to the accession number, if applicable. Likewise, if different versions of a publication, website or the like are published at different times, the version most recently published at the effective filing date of the application is meant, unless otherwise indicated.

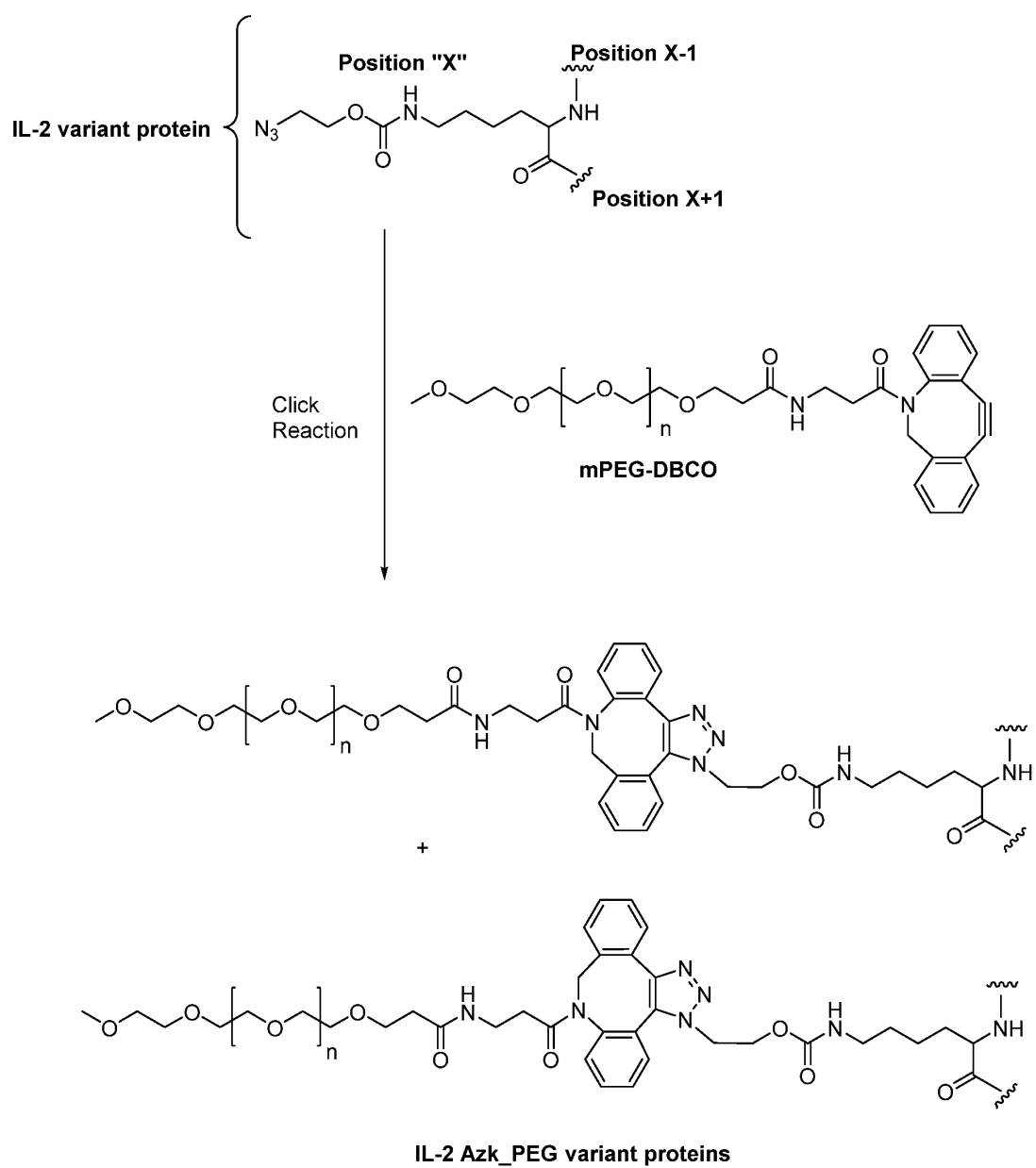
EXAMPLES

[0558] The following examples are provided to illustrate certain disclosed embodiments and are not to be construed as limiting the scope of this disclosure in any way.

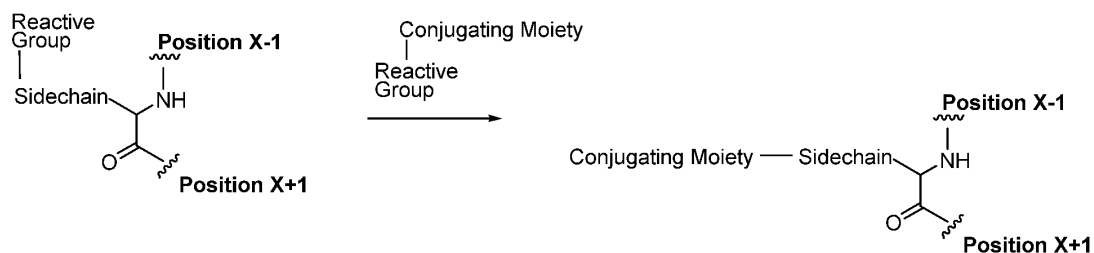
Example 1: Methods

Compound A

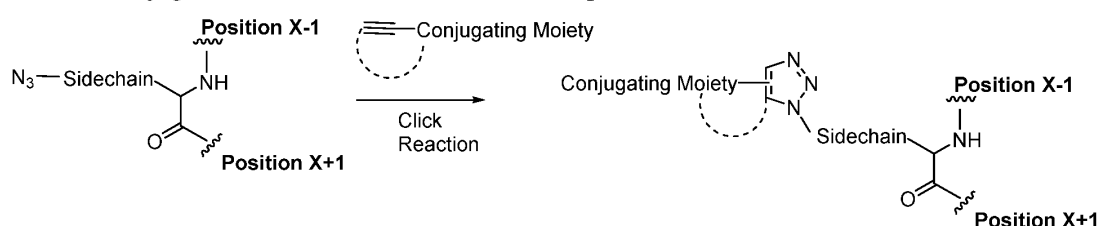
[0559] Scheme 1. Exemplary synthesis of AzK_PEG interleukin variants (wherein n indicates the number of repeating PEG units). Regioisomers formed from the click reaction are shown.



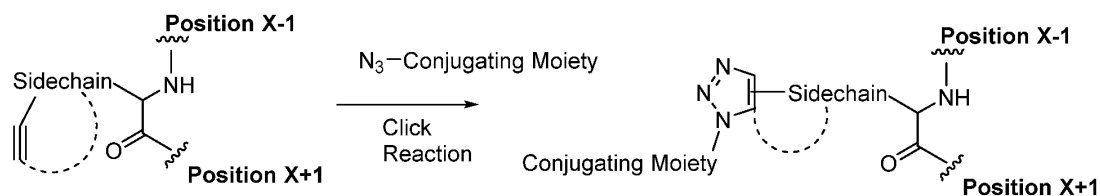
[0560] A conjugation reaction described herein comprises: Exemplary synthesis of X is the



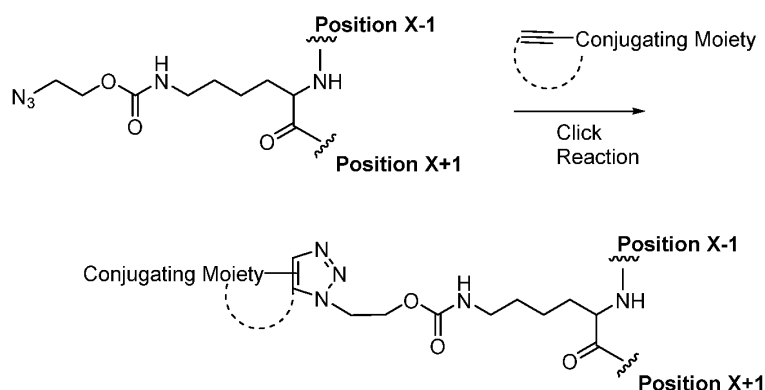
position in the IL-2 conjugate comprising an unnatural amino acid, such as in any one of SEQ ID NOs: 2 to 14 or a sequence having at least 80% sequence identity to SEQ ID NO: 1. The conjugating moiety comprises water soluble polymer. A reactive group comprises an alkyne or azide. A conjugation reaction described herein comprises:



, wherein X is the position in the IL-2 conjugate comprising an unnatural amino acid, such as in any one of SEQ ID NOS: 2 to 14 or a sequence having at least 80% sequence identity to SEQ ID NO: 1. A conjugation reaction described herein comprises:



, wherein X is the position in the IL-2 conjugate comprising an unnatural amino acid, such as in any one of SEQ ID NOS: 2 to 14 or a sequence having at least 80% sequence identity to SEQ ID NO: 1. A conjugation reaction described herein comprises:



, wherein X is the position in the IL-2 conjugate comprising an unnatural amino acid, such as in any one of SEQ ID NOS: 2 to 14. For additional details regarding preparation of IL-2 conjugates, see WO 2021/050554, which is incorporated herein by reference.

PBMC staining for CD4 T_{reg}, CD8 T_{EM}, and LacZ-specific CD8 T cells in mice and rats.

[0561] Blood samples were diluted with 2 ml of Dulbecco's Phosphate Buffered Saline (DBPS) (ThermoFisher, 14190144), and the peripheral blood mononuclear cells (PBMCs) were isolated from the blood samples using SepMateTM-15 (Stem cells technologies, 85420) filled with 5 ml of lymphoprep (Stem cells technologies, 7851). Cells were isolate via centrifugation at 2,000 rpm for 20 minutes, using settings of 10 for acceleration and 4 for deceleration.

[0562] Isolated PBMCs were washed with 200 µl of FACS buffer (Stem cells technologies, 7905) once by centrifugation at 2,000 rpm for 5 minutes. To stain for CD8 T_{EM} and LacZ-specific CD8 T cells, PBMCs were stained with the antibody cocktail described in Table 2 for 30 minutes at 4 °C:

Table 2		
Reagent	Dilution	Source
anti-mouse CD4 PE-Cy7	1:100	Biolegend (Catalog # 100422)
anti-CD8a FITC	1:50	ThermoFisher (Catalog #MA5-16759)
anti-CD62L APC	1:100	Biolegend (Catalog # 104412)
anti-CD44 Pacific Blue	1:100	Biolegend (Catalog # 103020)
Live/Dead Dead Cell Stain Kit	1:100	Invitrogen (Catalog # L34975)
H-2Kb β-galactosidase tetramer	1:20	ThermoFisher (Catalog # NC2097436)

[0563] After the incubation, the cells were washed with FACS buffer 2 times and then fixed with 100 µl of BD Cytfix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences, Franklin Lakes, NJ) for 15 minutes at 4 °C. The cells were washed with the FACS buffer 2 times, and then the samples were run on a flow cytometer for analysis (Novocyte Penteon Flow Cytometer Systems 5 Lasers, Agilent Technology, Santa Clara, CA).

[0564] To stain CD4 T_{reg}, the isolated and washed PBMCs were stained with the antibody cocktail described in Table 3 for 30 minutes at 4 °C:

Table 3		
Target	Dilution	Source
anti-CD8a PerCP-Cy5.5	1:100	BD Biosciences (Catalog # 551162)
anti-CD4 FITC	1:100	BD Biosciences (Catalog #553046)
anti-CD25 PE	1:100	Invitrogen (Catalog # 50-112-9454)
anti-NK1.1 PE-Cy7	1:100	Biolegend (Catalog # 156514)

anti-CD45R/B220 Pacific Blue		Biolegend (Catalog # 103227)
Live/Dead Dead Cell Stain Kit	1:100	Invitrogen (Catalog # L34975)

[0565] After the incubation, the cells were washed with the FACS buffer 2 times and then permeabilized with 100 μ l of Foxp3 Fixation/Permeabilization working solution (Invitrogen, 00-5523-00) for 30-60 minutes at the room temperature. The PBMCs were washed with 1X permeabilization buffer and then stained with the following antibody cocktail (1:100 anti-FOXP3 APC (Invitrogen, Catalog # 17-5773-82), and 1:100 anti-Ki67 BV605 (Biolegend, Catalog # 652413)) for at least 30 minutes at the room temperature. The cells were washed with the FACS buffer 2 times, and then the samples were run on a flow cytometer for analysis (Novocyte Penton Flow Cytometer Systems 5 Lasers, Agilent Technology).

Intracellular cytokine staining

[0566] Splenocytes were harvested from mouse spleens, and 2 million cells were seeded on a 96-well U-bottom plate. The cells were centrifuged at 2,000 rpm for 5 minutes and then resuspended with 100 μ l of the RPMI1640 (ThermoFisher, Catalog # 61870036) containing 10% FBS (ThermoFisher, Catalog # 10082147) and 1X 2-mercaptoethanol (ThermoFisher, Catalog # 21985023). 2 μ g/ml concentration of AAVrh32.33 overlapping peptides (Mimotopes) and LacZ overlapping peptides (Mimotopes) were prepared in the RPMI1640 containing 10% FBS, 1X 2-mercaptoethanol, and 1:750 GolgiStop (BD Biosciences, Catalog # 554715). For the positive control, a 0.10 μ g/ml PMA (Invivogen, tlr-pma) and 2 μ g/ml Ionomycin (invivogen, inh-ion) solution was prepared in the RPIM1640 containing 10% FBS, 1X 2-mercaptoethanol, and 1:750 GolgiStop. Then, 100 μ l of the prepared solution was added to the cells in 100 μ l, resulting in a final concentration of 1 μ g/ml (or 0.05 μ g/ml PMA and 1 μ g/ml Ionomycin).

[0567] Cells were incubated at 37 °C and 5% CO₂ for 5 hours to stimulate the cells. After the stimulation, the cells were centrifuged at 2,000 rpm for 5 minutes, and then washed with the FACS buffer one time.

[0568] Cells were stained with the antibody cocktail described in **Table 4** for 30 minutes at 4 °C:

Table 4		
Target	Dilution	Source
anti-CD4 FITC	1:100	BD Biosciences (Catalog #553046)
anti-CD8a PerCP-Cy5.5	1:100	BD Biosciences (Catalog # 551162)
anti-CD44 Pacific Blue	1:100	Biolegend (Catalog # 103020)
Live/Dead Dead Cell Stain Kit	1:100	Invitrogen (Catalog # L34975)

[0569] Then, the cells were washed 2 times with the FACS buffer. After washing, the cells were permeabilized with 100 μ l of BD Cytfix/Cytoperm Fixation/Permeabilization Solution Kit (BD Biosciences, 554715) for 20 minutes at 4 °C. The cells were washed two times with 1X PermWash and then stained with an antibody cocktail (1:100 anti-IFN γ APC (BD Biosciences, 554413), 1:100 anti-IL-2 PE (Biosciences, 554428), 1:100 anti-TNF α PE-Cy7 (Biosciences, 557644) for 30 minutes at 4 °C. The cells were washed two times with the FACS buffer, and the samples were run on a flow cytometer for analysis (Novocyte Penton Flow Cytometer Systems 5 Lasers, Agilent Technology).

OVA ELISA

[0570] To measure the concentration of OVA protein in mouse and non-human primates (NHPs), chicken OVA ELISA kits (Biomatik, EKF60703-96T) were utilized by following the protocol provided by the manufacturer. Briefly, mouse serum samples were diluted at a 1:25 ratio (a 1:1 ratio for NHP serum samples) using the Sample Dilution Buffer and subsequently underwent two-fold serial dilution. The OVA standard sample was included in the kit, which was diluted in a 1:1 ratio followed by a two-fold serial dilution. The ELISA plates were then incubated at 37 °C for 90 minutes, followed by three wash cycles. Next, 100 μ l of Biotin-labeled antibody was added to each well and incubated at 37 °C for 60 minutes. After four wash cycles, 100 μ l of HRP-Streptavidin conjugate was added to the each well. The plates were again incubated at 37°C for 30 minutes and washed five times. Subsequently, 90 μ l of TMB substrate was added into each well, and the plates were incubated at 37 °C for 15 minutes. Finally, 50 μ l of Stop Solution was added to each well, and the optical density at 450 nm was measured using a microplate reader (Molecular Devices SpectraMax Absorbance Reader, San Jose, CA).

Anti-mouse OVA IgG1 ELISA

[0571] To measure the concentration of mouse anti-OVA IgG1 antibody in mouse serum samples, an anti-OVA IgG1 (mouse) ELISA kit (Cayman Chemical, 500830) was utilized by following the protocol provided by the manufacturer. Briefly, mouse serum samples were diluted at a 1:500 ratio using Assay Buffer and subsequently underwent two-fold serial dilution. The anti-OVA mouse IgG1 standard sample was provided by the kit, which was resuspended in Assay Buffer followed by a two-fold serial dilution. The ELISA plates were incubated at room temperature for 2 hours, followed by four wash cycles. Next, 100 μ l of Goat anti-Mouse IgG1 HRP Detection Antibody was added to each well and incubated at the room temperature for 1

hour. After four wash cycles, 100 µl of TMB Substrate Solution was added to each well of the plate, and the plates were incubated at the room temperature for 30 minutes. After 30-minute TMB incubation, 100 µl of HRP Stop Solution was added to each well, and the optical density at 450 nm was measured using a microplate reader (Molecular Devices SpectraMax Absorbance Reader).

[0572] Mouse anti-OVA IgG1 titers in serum were measured using anti-OVA IgG1 (mouse) ELISA kits from Cayman Chemical. Serum samples were diluted at four levels: 1:500, 1:2500, 1:12,500, and 1:62,500. The ELISA was conducted following the manufacturer's instructions. Anti-OVA IgG1 concentration was determined using a standard curve.

Non-human primate PBMC staining for CD4 T_{regs}

[0573] Cynomolgus macaque blood samples were diluted with 5 ml of Dulbecco's Phosphate Buffered Saline (DPBS) (ThermoFisher, 14190144), and the peripheral blood mononuclear cells (PBMCs) were isolated from the blood samples using SepMateTM-15 (Stem cells technologies, 85420) filled with 3.5 ml of lymphoprep (Stem cells technologies, 7851). For the isolation, the centrifugation was followed at 2,000 rpm for 20 minutes, using settings of 10 for acceleration and 4 for deceleration. The isolated PBMCs were treated with 5 ml of 1X RBC lysis buffer (ThermoFisher, 00-4333-57) for 10 minutes, and the lysis buffer was neutralized with 25 ml of DPBS. The PBMCs were centrifuged at 2,000 rpm for 10 minutes, and the pellet was resuspended with 1 ml of FBS.

[0574] The isolated PBMCs were added to a 96-well U-bottom plate and then washed with 200 µl of FACS buffer (Stem cells technologies, 7905) one time by centrifugating at 2,000 rpm for 5 minutes, and the PBMCs were stained with the following antibody cocktail for CD4 T_{reg} (1:40 anti-CD8a PerCP-Cy5.5 (Biolegend, 301032), 1:40 anti-CD4 FITC (Biolegend, 317408), 1:40 anti-CD25 APC (Biolegend, 302610), 1:40 anti-CD56 PE-Cy7 (Biolegend, 362510) and 1:100 Live/Dead Dead Cell Stain Kit (Invitrogen, L34975)) for 30 minutes at 4 °C. After the incubation, the cells were washed with the FACS buffer 2 times and then permeabilized with 100 µl of Foxp3 Fixation/Permeabilization working solution (Invitrogen, 00-5523-00) for 30-60 minutes at the room temperature. The PBMCs were washed with 1X permeabilization buffer and then stained with the following antibody cocktail (1:40 anti-FOXP3 PE (Biolegend, 320108), 1:40 anti-Ki67 BV421 (BD Biosciences, 562899)) for at least 30 minutes at the room temperature. The cells were washed with the FACS buffer 2 times, and then the samples were

run on a flow cytometer for analysis (Novocyte Penton Flow Cytometer Systems 5 Lasers, Agilent Technology).

Anti-AAV IgG ELISA

[0575] To measure anti-AAV capsid IgG titers in serum, flat-bottom MaxiSorp 96-well plates (ThermoFisher Scientific) were coated with 5E9 GCs/well of AAV in 100 μ L of coating buffer (Bethyl Laboratories, Montgomery, TX) and incubated overnight at 4°C. The plates were washed with wash buffer (R&D Systems, Minneapolis, MN) and blocked with ELISA blocking buffer (Bethyl Laboratories) at 37°C for 1 hour. Serum samples were initially diluted 1:100 with the ELISA blocking buffer and then serially diluted 1:3, reaching up to a 1:218,700 anti-AAV1 IgG dilution. Triplicates of 100 μ L diluted serum samples were added to the blocked plates and incubated at 37°C for 2 hours. After incubation, the plates were washed four times with wash buffer, followed by the addition 100 μ L of anti-mouse IgG HRP secondary antibody diluted 1:5,000 (SouthernBiotech, Birmingham, AL). The plates were incubated at room temperature for 1 hour and washed four times. Subsequently, 100 μ L of 3,3',5,5'-tetramethylbenzidine (TMB) substrate (SeraCare Life Science, LGC Clinical Diagnostics, Milford, MA) was added, and the plates were incubated for 30 minutes at room temperature. The reaction was stopped by adding 100 μ L of TMB stop solution (Seracare Life Science), and absorbance was measured at 450 nm using a microplate reader (Molecular Devices). Mouse anti-AAV1 IgG titers were measured using mouse anti-AAV1 IgG2a (Creative Diagnostics, Shirley, NY) to generate a standard curve for calculating IgG concentrations in mouse serum. For NHP anti-AAV1 IgG titers, the OD450 values from negative controls were averaged and multiplied by 2 to determine a cut-off value. The titer was defined as the highest dilution factor where the cut-off value was detectable.

Non-human primate (NHP) Anti-OVA IgG ELISA

[0576] To measure anti-OVA IgG titers in NHP serum, flat-bottom MaxiSorp 96-well plates (ThermoFisher Scientific) were coated with 10 μ g/mL of OVA (MP Biomedicals, Irvine, CA) in 100 μ L of coating buffer (Bethyl Laboratories) and incubated overnight at 4°C. Plates were then washed with wash buffer (R&D Systems) and blocked with ELISA blocking buffer (Bethyl Laboratories) at 37°C for 1 hour. Serum samples were initially diluted 1:100 in ELISA blocking buffer, then serially diluted 1:3 up to a 1:218,700 dilution. Triplicates of 100 μ L of diluted samples were added to blocked plates and incubated at 37°C for 2 hours. After incubation, the

plates were washed four times with wash buffer, followed by the addition of 100 μ L of anti-monkey IgG HRP secondary antibodies (1:5,000 dilution, SouthernBiotech). The plates were incubated at room temperature for 1 hour and washed four times. Then, 100 μ L of 3,3',5,5'-tetramethylbenzidine (TMB) substrate (SeraCare Life Science) was added and incubated for 30 minutes at room temperature. The reaction was stopped with 100 μ L of TMB stop solution (SeraCare Life Science), and absorbance was measured at 450 nm using a microplate reader (Molecular Devices). To determine anti-OVA IgG titer, the OD450 values from negative controls were averaged and multiplied by 2 to establish a cut-off value, with the titer defined as the highest dilution factor meeting this threshold.

OVA ELISA

[0577] OVA protein levels in serum were measured using an OVA ELISA kit (Biomatik, Kitchener, Ontario, Canada). Serum samples were prepared at four dilution levels: 1:25, 1:125, 1:625, and 1:3,125. The ELISA was performed following the manufacturer's instructions. After the substrate reaction was stopped, absorbance was measured at 450 nm using a microplate reader (Molecular Devices), and OVA protein concentrations were calculated using a standard curve.

Pre-Treatment vs. Co-treatment of Compound A with AAV Gene Therapy

[0578] To evaluate the effect of Compound A treatment on AAV immunogenicity. Male C57BL/6J mice (6-8 weeks old, Jackson Laboratories) mice were administered 2×10^{11} VGs of AAVrh32.33-LacZ with or without Compound A treatments. Compound A was administered at 0.3 mg/kg subcutaneously either 4 days prior-to or at the same time as AAV gene therapy.

[0579] For analysis of immune cells, mice were euthanized and spleens from these mice were processed to generate a single cell suspension of splenocytes. The proportion of effector memory CD8 T cells within the CD8 T-cell population was analyzed by flow cytometry. Recall responses against the AAV capsid or the transgene were tested by restimulation using peptide pools and the analysis was performed using an IFN γ Intracellular Cytokine Staining assay.

[0580] Mouse whole spleens were collected in 1.5 ml tubes containing RPMI1640 (Gibco, Thermo Fisher Scientific, Waltham, MA) supplemented with 10% FBS (Gibco, Thermo Fisher Scientific). The spleens were homogenized in RPMI1640, and the cell suspension was passed through a 40 μ m cell strainer (Thermo Fisher Scientific) into 50 mL conical tubes (Thermo

Fisher Scientific). The cell suspension was centrifuged at 2,000 rpm for 5 minutes at 4°C, and the supernatant was discarded. The pellet was treated with 2 mL of RBC lysis buffer (Thermo Fisher Scientific), briefly vortexed, and incubated at room temperature for 3 minutes. The lysis buffer was then neutralized with at least two washes with RPMI1640. The isolated splenocytes were then resuspended in RPMI1640 supplemented with 10% FBS to the desired volume, and the number of splenocytes was counted with Vi-Cell analyzer (Beckman Coulter, Brea, CA).

[0581] For surface staining, single-cell suspensions isolated from PBMCs and/or spleens were stained using fluorophore conjugated antibodies against surface markers in FACS buffer (DPBS with 2% FBS, Stemcell Technologies, Vancouver, Canada) at 4°C for 30 minutes.

[0582] For intracellular cytokine staining, single-cell suspensions isolated from spleens were first stained with surface marker antibodies in FACS buffer (DPBS with 2% FBS, Stemcell Technologies) at 4°C for 30 minutes. The cells were then permeabilized using either the Foxp3/Transcription Factor Staining Buffer Set (Thermo Fisher Scientific) for Foxp3 and Ki67 staining or the BD Cytofix/Cytoperm Fixation/Permeabilization Kit (BD Biosciences) for intracellular cytokine staining, following the respective manufacturer's protocols.

[0583] All flow cytometry samples were acquired on a Novocyte Penton Flow Cytometer (Agilent Technologies), and the data were analyzed with FlowJo software.

Example 2: Use of Compound A in mice to suppress immune response.

[0584] Mice received 0.3 mg/kg of Compound A subcutaneously or BS. Blood samples were taken at days 0, 4, 7, 14, and 18. As shown in **FIG. 1** and **FIG. 22A**, the CD4 Treg population reached the peak after 4 days from the injection with Compound A, followed by a gradual decline. As shown in **FIG. 22B**, Compound A preferentially expands CD4 Tregs relative to CD8⁺ T cells, CD4⁺ T cells, NK cells, and B cells. N>5/group. For **FIGs. 22A-22B**: Error bars indicate +/-SD; data compiled from 2 studies; statistical analysis by Student's t test with Holm-Šidák correction for multiple comparisons.

[0585] In a separate experiment, mice were administered 2×10^{11} vector genomes (vg) of AAVrh32.33-LacZ in PBMC via intramuscular injection. Samples were taken on days 0, 7, 14, 21, 28, 35, 42, 49, and 62. As shown in **FIGs. 2A and 2B**, Compound A effectively suppresses the expansion of effector memory CD8 T cells and LacZ-specific CD8 T cells stimulated by AAVrh32.33-LacZ administration in PBMC in mice. After 14 days from AAVrh32.33-LacZ intramuscular administration, effector memory CD8 T cells (CD44⁺ CD62L⁻) and LacZ-specific CD8 T cells (H-2K^b DAPIYTNV MHC-Class I tetramer⁺) were

substantially expanded in PBMCs. A single administration of Compound A effectively suppressed the expansion of both effector memory CD8 T cells and LacZ-specific CD8 T cells in PBMC over a long period of time.

[0586] Splenocytes were then harvested from mice after 62 days from the AAV administration and stimulated with either AAVrh32.33 or LacZ overlapping peptides for 5 hours. Intracellular cytokines were stained, and the stained cells were analyzed with a flow cytometer. As shown in **FIGS. 3A and 3B**, Compound A effectively suppresses AAV capsid-specific (**FIG. 3A**) and LacZ-specific (**FIG. 3B**) IFN γ secreting CD8 T cells in the spleen in mice.

[0587] In a separate experiment, mice were administered 2×10^{11} vector genomes (vg) of AAVrh32.33-LacZ in PBMC via intramuscular injection and 0.3 mg/kg of Compound A subcutaneously on the same day. Blood samples were taken on days 0, 7, 14, 21, 28, 35, 42, 49, and 62. As shown in **FIG. 23A**, Compound A effectively suppresses the expansion of effector memory CD8 T cells in mice, measured as a percentage of lymphocytes. A single administration of Compound A effectively suppressed the expansion of effector memory CD8+ T cells for at least 62 days.

[0588] Splenocytes were then harvested from mice after 21 days from the AAV administration and restimulated ex vivo with either AAVrh32.33 or LacZ overlapping peptides for 18 hours. Intracellular cytokines were stained, and the stained cells were analyzed with a flow cytometer. As shown in **FIGs. 23B and 23C**, Compound A effectively suppresses AAV capsid-specific (**FIG. 23B**) and LacZ-specific (**FIG. 23C**) IFN γ secreting CD8 T cells in the spleen in mice. For **FIGs. 23A-23C**: N=5/group; error bars indicate $\pm$ SD; statistical analysis performed by 2way ANOVA with test for multiple comparisons.

[0589] Separately, the kinetics of a transgene delivered by AAVrh32.33 vector were analyzed. To do this, mice were intramuscularly injected with 2×10^{11} vg of AAVrh32.33-OVA with or without a Compound A subcutaneous injection on the same day. Serum was collected from mice every week, and the serum samples were used for ELISA for OVA and anti-OVA IgG1. As shown in **FIGs. 4A, 4B, and 24A**, Compound A enhanced the expression of the OVA gene delivered by AAVrh32.33 vector (**FIGs. 4A and 24A**) and suppressed anti-OVA IgG1 production in mice (**FIG. 4B**). These ELISA results showed that Compound A enhanced the OVA expression while suppressing anti-OVA IgG1 production, indicating that the OVA expression level is conversely correlated with anti-OVA IgG1 production level in serum.

[0590] The effects of transgene delivered by AAVrh32.33 vector were further analyzed. To do this, mice were intramuscularly injected with 5×10^{10} vg of AAVrh32.33-OVA with or without a Compound A (0.3 mg/kg) subcutaneous injection on the same day. Compound A significantly

suppresses effector memory CD8 T-cell expansion (CD8 T_{EM}; **FIG. 24B**) in mice after delivery of the OVA gene by AAVrh32.33 vector. For Fig. 24A: N=3 to 5/group; error bars indicate +/-SD; statistical analysis by Student's t test with Holm-Šidák correction for multiple comparisons. For **FIG. 24B**: N=4 or 5/group; error bars +/-SD; statistical analysis performed by 2way ANOVA with test for multiple comparisons.

[0591] Foxp3+ Tregs are known to suppress immunoglobulin responses. This can occur by direct suppression of B cell activation and immunoglobulin responses or indirectly via suppression of T-cells that assist in B-cell-mediated humoral responses. To test if Compound A treatment mediated expansion of Tregs can suppress formation of antibodies against AAV and/or the transgene, we measured anti-AAV and anti-OVA IgG levels in the sera of mice treated with rAAV1-OVA with or without Compound A treatment. Mice treated with rAAV1-OVA generated AAV specific (**FIG. 27A**) and OVA specific IgG antibodies (**FIG. 27B**) observed starting at 5 weeks post AAV gene therapy. Compound A treated mice showed significantly decreased IgG responses against the AAV and the OVA protein and the suppression persisted for the entire duration of the study. Compound A treated mice showed a significantly decreased IgG responses against the AAV and the OVA protein.

[0592] Peak expansion of regulatory T cells (Tregs) after a single subcutaneous administration of Compound A occurred at 4 days post treatment (**FIGs. 1, 22A, and 22B**). The proportion of expanded Tregs dropped from maximum levels at 7 days post Compound A treatment with Treg levels and was near baseline around 14 days post treatment.

[0593] C57BL/6 mice were treated intramuscularly with the immunogenic capsid AAVrh32.33 expressing LacZ, which encodes for beta-galactosidase, with or without a single subcutaneous dose of Compound A. The single dose of Compound A was administered either 4 days prior to AAV gene therapy (pre-treatment) or co-administered with (i.e., at the same time) the AAV gene therapy (co-treatment). Mice were then sacrificed at 21 days post AAV gene therapy treatment and CD8+ T cell immune responses were measured in the splenocytes by flow cytometry. CD8+ T cell responses were measured either by analyzing the proportion of proliferating CD8+ T cells or the total proportion of effector memory CD8 + T cells (CD8 T_{EM}) (**FIGs. 29A and 29B**). There was a significant increase in proliferating and effector CD8+ T cell proportion in the spleen with AAV gene therapy which was mitigated by Compound A treatment. The mitigation of CD8+ T cell responses were not meaningfully different when Compound A was administered as a pre-treatment or given as a co-treatment. In addition, AAV specific and transgene specific CD8+ T cell responses were analyzed by performing an *ex vivo* restimulation of splenocytes with the AAV peptide pool and the beta-galactosidase peptide pool, respectively. There was a

significant increase in the proportion of IFN γ secreting CD8 $^{+}$ T cells in response to AAV capsid peptides and to transgene specific peptides, which were mitigated by Compound A treatment. There was no meaningful difference in mitigation of AAV and/or transgene specific CD8 $^{+}$ T cell responses when Compound A was given as a pre-treatment or as a co-treatment. Without wishing to be bound by any particular theory, this suggests that administering AAV gene therapy within the timeframe of the expansion of Tregs mediated by Compound A maintains the therapeutic benefit of Compound A of suppressing adverse immune responses to the AAV and/or the transgene.

Example 3: Use of Compound A in rats to suppress immune response.

[0594] Rats were administered 0.3 mg/kg of Compound A subcutaneously 4 days before AAVrh10-EGFP ICM administration (3.3×10^{11} vg/g brain). As shown in **FIG. 5A**, Compound A significantly increased the CD4 Treg population after 7 days from the administration (i.e., Day 3 in **FIG. 5A**), and the expansion lasted for around 2 weeks. The expansion of CD4 Treg cells resulted in significant suppression of CD8 T-cell proliferation (**FIG. 5B**) and effector memory CD8 T-cell (CD44 $^{+}$ CD62L $^{-}$) expansion stimulated by AAVrh10-EGFP after 14 days from the AAV administration (**FIG. 5C**).

[0595] Rats were administered 0.3 mg/kg of Compound A subcutaneously 4 days before AAVrh10-EGFP ICM administration (4.2×10^{11} vg/g brain) (day zero). As shown in **FIG. 25A**, Compound A significantly increased the CD4 Treg population after 7 days from the administration of Compound A (i.e., Day 3 in **FIG. 25A**), and the expansion lasted for around 2 weeks. The expansion of CD4 Treg cells resulted in significant suppression of effector memory CD8 T-cell expansion stimulated by AAVrh10-EGFP for at least 28 days from the AAV administration (**FIG. 25B**) and of proliferating (Ki67 $^{+}$) CD8 T cells (**FIG. 25C**) through at least day 21. For **FIGs. 25A-25C**: N=5/group; error bars +/-SD; statistical analysis performed by 2way ANOVA with test for multiple comparisons.

Example 4: Use of Compound A in non-human primates (NHPs) to suppress immune response.

[0596] Cynomolgus macaques were administered AAVrh32.33-OVA (3×10^{12} vg) with or without 0.05 mg/kg of Compound A subcutaneous administration on the same day. As shown in **FIGs. 6A, 6B, 26A, 26B, and 26C**, subcutaneous Compound A administration significantly increased the CD4 Treg population (**FIGs. 6A and 26A**) and enhanced the expression of the OVA gene delivered by AAVrh32.33 vector in non-human primates (**FIGs. 6B and 26C**) relative to the OVA gene delivered by AACrh32.33 vector in non-human primates without Compound A

(**FIGs. 6A** and **26B**). After a week from the Compound A administration, the CD4 Treg population was substantially increased, and the Treg expansion lasted for around 3 weeks in the monkeys. It was also observed that the Treg expansion was correlated with the enhanced expression level of AAVrh32.33-OVA in serum in the monkeys.

[0597] To test the translatability of mouse model data to higher species, the ability of Compound A treatment to enhance transgene expression was tested in two non-human primate (NHP) models of AAV immunogenicity. In NHPs, rAAV1-OVA treatment generated AAV specific (**FIG. 27C**) and OVA specific IgG antibodies (**FIG. 27D**) observed starting at 5 weeks post AAV gene therapy with peak responses around 7 weeks post AAV treatment. AAV specific and OVA specific IgG antibodies were reduced in NHPs that received Compound A.

[0598] To assess duration of OVA expression, cynomolgus macaques were treated with either AAVrh32.33 -Ova or rAAV1-Ova in conjunction with or without Compound A (up to 0.08 mg/kg). In both studies, there was an increase in CD4+ Tregs. OVA levels in individual animals showed a trend toward increase in expression and enhanced persistence of expression. When data from both studies were compiled, a higher proportion of NHPs that received Compound A treatment in conjunction with AAV had a significantly higher duration of OVA expression (**FIG. 28**), confirming in higher species that Compound A treatment can enhance the efficacy of rAAV based gene therapy.

[0599] Overall, these studies combined suggest a novel approach to repurposing PEGylated IL-2 in the field of gene therapy to suppress adaptive immune responses to AAV and transgene for enhanced transgene expression and hold promise for the clinical success of AAV-based gene therapy. Further, our studies reveal the potential of repurposing other immunomodulatory molecules to enhance the efficacy of gene therapy, which could substantially reduce the cost and time of drug development.

Example 5: Additional IL-2 Conjugates.

[0600] Additional IL-2 conjugates have been characterized as having immunomodulatory properties useful in methods according to the present disclosure, as discussed below. To determine how the differential receptor specificity of exemplary IL-2 conjugates affects activation of primary immune cell subpopulations, concentration-response profiling of lymphocyte activation in human LRS-derived peripheral blood mononuclear cell (PBMC) samples were performed using multi-color flow cytometry. Conjugates of **Table 5** were synthesized by modification of SEQ ID NO: 15.

[0601] Exemplary IL-2 conjugates were subjected to functional analysis are shown in **Table 5**. The IL-2 conjugates were expressed as inclusion bodies in *E. coli*, purified and re-folded using

standard procedures before site-specifically pegylating the IL-2 product using DBCO-mediated copper-free click chemistry to attach stable, covalent mPEG moieties to the AzK (**Scheme 1** of **Example 1**).

[0602] These studies were performed at PrimityBio LLC (Fremont, CA). Primary lymphocytes derived from human LRS samples were treated with dilutions series of exemplary IL-2 compounds and quantified based on pSTAT5 signaling in each lymphocyte cell type using the panel shown in **Table 6**.

[0603] Table 6. Key indicating cell populations

Marker	Cell population
CD3	T cells
CD4	Th cells
CD8	T effector cells
CD45RA	Naïve T cells
CD56	NK cells
CD14/19	Monocyte/ B cells
CD25	Tregs or experienced T cell
CD127	Not Treg
CD62L	Memory T vs effector memory T cell
pSTAT5 (Y694)	Activation marker

[0604] Flow cytometry data were analyzed for activation of different T and NK cell subsets in concentration-response mode, reading pSTAT5 accumulation after treatment with an exemplary IL-2 variant K9_30kDa in reference to the sequence of SEQ ID NO: 15.

[0605] FIG. 7A-FIG. 7B show the dose response curves for pSTAT5 signaling in human LRS primary cell (**FIG. 7A**) and proliferation response in mouse CTLL-2 populations (**FIG. 7B**).

[0606] Table 5 shows the dose response EC50 for pSTAT5 signaling (EC50) in human LRS samples or CTLL-2 proliferation treated with indicated IL-2 variant.

Table 5 Dose response EC50 for pSTAT5 signaling (EC50) in human LRS samples or CTLL-2 proliferation treated with indicated IL-2 variant

Compound	NK cells	CD8+ Tcells	Treg cells	CD8+/Treg ratio	Fold increase in Treg EC50 vs native IL-2	CTLL-2 proliferation
native IL-2	4586	31024	75	414	1	455.8
K9_30kDa	169578	1100679	2217	496	30	504
H16_30kDa	2545257	12070108	34976	345	466	80755
L19_30kDa	6756768	22436430	93205	241	1243	3510
D20_30kDa	2643930	9505217	1129455	8	15059	689939
M23_30kDa	143620	539824	1030	524	14	1102
N26_30kDa	258531	1188859	2459	483	33	2594
N88_30kDa	3298113	11111537	323201	34	4309	66606
E100_30kDa	35088	195823	483	405	6	1676
N119_30kDa	34010	143380	535	268	11	1215
T123_30kDa	33396	152928	269	569	6	255
Q126_30kDa	3676807	19722480	29454	670	393	3584
S127_30kDa	20210	92190	150	615	3	123
T131_30kDa	24207	132922	258	515	3	641
N88R/D109_30kDa	2780819	12503386	175805	71	3663	59577
V91K	20537	102255	142	720	3	99.5
N88R	2312847	15025734	11082	1356	148	363

[0607] The EC50 values (pg/ml) were calculated from dose response curves generated from the MFI plots. *Treg potency change compared to native IL-2 (wild-type IL-2) run in each individual experiment.

[0608] To characterize the pharmacokinetic and pharmacodynamic effects of each IL-2 conjugate in mice, each conjugate described in **Table 7** was administered as a single subcutaneous injection into naïve C57/BL6 mice at approximately 0.9 mg/kg (note – the dose was determined by measuring the mass of the polypeptide protein and did not include the mass of the polyethylene glycol or linker moieties). Samples were collected via terminal bleeding as indicated in **Table 7**, collected at the indicated times, and subjected to PK analysis using ELISA and flow cytometry to quantitate signaling, activation, and proliferation of individual lymphocyte populations. All of the compounds used in this study, except N88_30kD, have the SEQ ID NO: 20 in which the amino acid at the indicated position was substituted with the structure of

Formula (II), Formula (III), or a mixture of Formula (II) and (III) was substituted at the indicated amino acid residue comprising a linear, mPEG group having a molecular weight of 30 kDa. For the N88_30kD variant, the compound has sequence SEQ ID NO: 20 in which the amino acid at the indicated position was substituted with the structure of Formula (IV), Formula (V), or a mixture of Formula (IV) and (V) was substituted at the indicated amino acid residue comprising a linear, mPEG group having a molecular weight of 30 kDa.

Table 7. Mouse PK/PD study details

IL-2 Conjugate	Dose (mg/kg)	Dose Route	Collection time point (Days)	Collection
vehicle	0	subcutaneous	0, 1, 2, 3, 4, 5, 6, 7, 8	Terminal bleed
K9_30kD	0.9	subcutaneous	0, 1, 2, 3, 4, 5, 6, 7, 8	Terminal bleed
L19_30kD	0.9	subcutaneous	0, 1, 2, 3, 4, 5, 6, 7, 8	Terminal bleed
Q126_30kD	0.4	subcutaneous	0, 1, 2, 3, 4, 5, 6, 7, 8	Terminal bleed
E100_30kD	0.9	subcutaneous	0, 1, 2, 3, 4, 5, 6, 7, 8	Terminal bleed
N88R/D109_30kD	0.9	subcutaneous	0, 1, 2, 3, 4, 5, 6, 7, 8	Terminal bleed
T123_30kD	0.8	subcutaneous	0, 1, 2, 3, 4, 5, 6, 7, 8	Terminal bleed
N88_30kD	0.9	subcutaneous	0, 1, 2, 3, 4, 5, 6, 7, 8	Terminal bleed
H16_30kD	0.9	subcutaneous	0, 1, 2, 3, 4, 5, 6, 7, 8	Terminal bleed

[0609] Bioanalysis of plasma samples was performed using a commercially available human IL-2 ELISA assay (Abcam, Cambridge, UK). Concentrations of each IL-2 conjugate dosed and the internal standard in samples derived from plasma were determined following the manufacturer's instructions, and each time point was measured under conditions within the linear range of the standard measurement. The plasma concentration profiles of IL-2 conjugates K9_30kD, L19_30kD, N88R/D109_30kD, H16_30kD, Q126_30kD, and N88_30kD (all dosed at 0.9 mg/kg) are plotted in **FIG. 8**.

[0610] To characterize the pharmacodynamic effects of IL-2 conjugates in mice, each conjugate was dosed as a single subcutaneous injection into naïve C57/B16 mice at a dose of approximately 0.9 mg/kg (note – the dose was determined by measuring the mass of the polypeptide protein and

did not include the mass of the polyethylene glycol or linker moieties). Samples were collected via terminal bleeding as indicated in **Table 7**. Compound formulation, dosing, and sample collection were carried out at Crown Bio (La Jolla, CA). Pharmacodynamic analysis using flow cytometry was carried out by PrimityBio (Fremont, CA) and this analysis was used to quantitate signaling, activation, and proliferation of individual lymphocyte populations.

Table 8. Marker panel for flow cytometry study of IL-2 conjugates in C57/BL6 mice

Flow cytometry marker panel
CD3
CD4
CD8
CD44
CD25
FoxP3
NK1.1
STAT5
Ki-67
ICOS
Helios

[0611] The cells were first gated on singlets using FSC-A by FSC-HOUR to exclude any aggregates or doublets. Within this gate the cells are gated on mid to high forward scatter (FSC-A) and side scatter (SSC-A) to exclude the red blood cells and debris. The T cells are then gated as the CD3+ population. The T cells are then divided into CD4+ T cells and CD8+ T cells. The Tregs are then gated from the CD4+ T cells as the CD25+ FoxP3+ population. The NK cells are identified from the CD3 negative population as the NK1.1 positive population. Statistics and plotting for derivation of EC50 values The Median Fluorescence Intensity (MFI) for each of the cell population, donor, and the IL-2 conjugate dosed was calculated from the signal in the channel detecting phosphorylated STAT5 using CellEngine software. The statistics were analyzed using Spotfire. Within Spotfire, the data was plotted on a log scale for the doses of IL-2 conjugate and a linear scale for the MFI readings. These data were fit using a 4-parameter logistic regression equation. The EC50 was calculated as the inflection point of the curve. For each IL-2 conjugate tested, Treg (CD3+ CD4+CD25 high FoxP3+) cells were quantitated in singlets (total lymphocytes observed). The mean fold change of Treg (% in singlets) from three

independent animals is plotted for each IL-2 conjugate, represented as the maximum percentage of Treg in singlets/ percentage of Treg in singlets in pre-dosed samples. Each bar represents the standard error of the indicated mean. Results showing the maximal fold-change in Treg percentage in singlets from subcutaneous dose of IL-2 conjugates in mice are shown in **FIG. 9**. The following compounds shown in **Table 9** were used in Example 5:

Table 9.

Compound	Structure
H16_30kD	SEQ ID NO: 20 in which the structure of Formula (II), Formula (III), or a mixture of Formula (II) and (III) was substituted at the H16 residue (SEQ ID NO: 21) with a PEG group having a molecular weight of 30 kDa
N88_30kD	SEQ ID NO: 4 in which the structure of Formula (IV), Formula (V), or a mixture of Formula (IV) and (V) was substituted at the N88 residue (SEQ ID NO: 23) with a PEG group having a molecular weight of 30 kDa
L19_30kD	SEQ ID NO: 4 in which the structure of Formula (II), Formula (III), or a mixture of Formula (II) and (III) was substituted at the L19 residue (SEQ ID NO: 22) with a PEG group having a molecular weight of 30 kDa
N88R/D109_30kD	SEQ ID NO: 4 in which N88 is substituted with R and the structure of Formula (IV), Formula (V), or a mixture of Formula (IV) and (V) was substituted at the D109 residue (SEQ ID NO: 24) with a PEG group having a molecular weight of 30 kDa

[0612] Treg percentage in singlets versus time after single subcutaneous dose of IL-2 conjugates in C57/BL6 mice were determined. For each IL-2 conjugate, the samples were subjected to flow cytometry to identify and quantitate the proportion of the Treg (CD3+ CD4+ CD25+ FoxP3+) cell population within the total cell population (singlets). Shown are data for IL-2 K9_30kD, L19_30kD, Q126_30kD, and H16_30kD in **FIG. 10**.

[0613] FIG. 11A shows the proportion of the CD8+ T cell population (CD3+ CD4- CD8+) within the total cell population (singlets) in C57/BL6 mice for the K9_30kD, L19_30kD, Q126_30kD, and H16_30kD IL-2 conjugates. Each point represents the mean of three independent animals, error bars represent the standard error of the mean.

[0614] FIG. 11B shows the CD8+ T cell (CD3+ CD4- CD8+) population within the total cell population (singlets) for IL-2 conjugates E100_30kD, N88R/D109_30kD, T123_30kD,

N88_30kD, and V91_30kD conjugates in C57/BL6 mice. Each point represents the mean of three independent animals, error bars represent the standard error of the mean.

[0615] L19_30kD, H16_30kD, and N88_30kD were dosed subcutaneously into three male Cynomolgus monkeys at doses of 0.37 mg/kg N88_30kD, 0.03 mg/kg L19_30kD, 0.16 mg/kg L19_30kD, 0.12 mg/kg H16_30kD, 0.67 mg/kg H16_30kD, and 0.2 mg/kg H16_30kD. The plasma concentration profiles of IL-2 conjugates L19_30kD, H16_30kD, and N88_30kD are shown in **FIG. 12**.

[0616] The IL-2 conjugates N88_30kD, L19_30kD, H16_30kD, and H16_50kD were dosed subcutaneously into three male Cynomolgus monkeys at doses of 0.37 mg/kg N88_30kD, 0.2 mg/kg H16_30kD, 0.67 mg/kg H16_30kD, 0.03 mg/kg L19_30kD, 0.16 mg/kg L19_30kD, 0.2 mg/kg H16_50kD, and 0.8 mg/kg H16_50kD at Day 0. For each IL-2 conjugate, peripheral blood samples were collected at the indicated timepoints, and the samples were subjected to flow cytometry to identify and quantitate the proportion of the Treg cell population within the total blood cell population (singlets). Each point represents the mean of three independent animals, error bars represent the standard error of the mean. Bioanalysis of plasma samples was performed using a human IL-2 ELISA assay that captures the IL-2 conjugates to the surface using anti-IL-2 and detects the IL-2 conjugate via anti-PEG antibodies. Concentrations of each test article and the internal standard in samples derived from plasma were determined, and each time point was measured under conditions within the linear range of the standard measurement. The results showing the identity and quantity of the proportion of the Treg cell population within the total blood cell population (singlets) in Cynomolgus monkeys following dosing with IL-2 conjugates N88_30kD, H16_30kD, H16_50kD, and L19_30kD are shown in **FIG. 13**.

[0617] The IL-2 conjugates N88_30kD, L19_30kD, H16_30kD, and H16_50kD were dosed subcutaneously into three male Cynomolgus monkeys at doses of 0.37 mg/kg N88_30kD, 0.03 mg/kg L19_30kD, 0.16 mg/kg L19_30kD, 0.12 mg/kg H16_30kD, 0.67 mg/kg H16_30kD, 0.2 mg/kg H16_50kD, and 0.80 mg/kg H16_50kD at Day 0. The results showing the identity and quantity of the proportion of the CD8+ T cell population within the total blood cell population (singlets) in Cynomolgus monkeys following dosing with IL-2 conjugates N88_30kD, H16_30kD, H16_50kD, and L19_30kD are shown in **FIG. 14**.

[0618] The IL-2 conjugates described in **Table 10** below were dosed in Cynomolgus monkeys with 3 male animals in each dosing group (24 total animals). The animals were given a single subcutaneous dose of the indicated IL-2 conjugate at day 0. Each of the conjugates described below, except H16_30kDa and H16_50kDa, had SEQ ID NO: 20 in which the indicated amino acid position is substituted with the structure of Formula (II), Formula (III), or a mixture of

Formula (II) and (III), and they contain a PEG chain of the indicated size. For example, the variant labeled “L19_30kDa” has SEQ ID NO: 20 in which the amino acid at position L19 is replaced by the structure of Formula (II), Formula (III), or a mixture of Formula (II) and (III), and contains a 30 kDa, linear mPEG group. For the H16_30kDa and H16_50kDa variants, the compounds had SEQ ID NO: 20 in which the indicated amino acid position is substituted with the structure of Formula (IV), Formula (V), or a mixture of Formula (IV) and (IV), and a linear, mPEG group having a molecular weight of 30 kDa or 50kDa, respectively.

Table 10.

Group	Test Article	Dose Level (mg/kg)
1	Vehicle	NA
2	N88_30kDa	0.37
3	L19_30kDa	0.03
4	L19_30kDa	0.16
5	H16_30kDa	0.12
6	H16_30kDa	0.67
7	H16_50kDa	0.2
8	H16_50kDa	0.8

[0619] Blood samples were taken from each animal at the following time points: Samples for hematology: Day -1, Day 1 (Predose), 3, 6, 10, 15 and 22. Samples for pharmacokinetics and pharmacodynamics: (Predose), 0.5, 1, 2, 4, 8, 24, 36, 48, 72, 96, 120, 144, 168, and 240, 360, 528 (Day 22) hours post-dose. Cytokine sample collection and analysis: Day -1, and Day 1 (Predose), 8, 24, 72, 120 and 168, 360, and 528 (Day 22) hours post-dose.

[0620] The peak fold-change in white blood cell count (WBC), peak fold-change in lymphocyte count, and the day on which the peak lymphocyte counts were observed for each IL-2 conjugate are indicated in **Table 11**.

Table 11.

Group No.	IL-2 Conjugate	Dose Level (mg/kg)	Peak fold change of WBC	Peak fold change of lymphocytes	Date at the peak of lymphocytes
1	Vehicle	0	1.04	1.05	pre dose

2	N88_30kDa	0.37	1.05	1.19	6 days post
3	L19_30kDa	0.03	1.14	1.28	6 days post
4	L19_30kDa	0.16	2.49	3.21	6 days post
5	H16_30kDa	0.12	1.24	1.31	6 days post
6	H16_30kDa	0.67	2.3	2.76	6 days post
7	H16_50kDa	0.2	1.71	2.25	10 days post
8	H16_50kDa	0.8	2.9	3.38	10 days post

[0621] The pharmacokinetic parameters for the H16_30kD variant in non-human primates at doses of 0.12 mg/kg and 0.67 mg/kg are shown in **Table 12** below and the plots of plasma concentration versus time of the H16_30kD variant at a dose of 0.12 mg/kg and 0.67 mg/kg are shown in **FIG. 15** (the 0.12 mg/kg dose is shown as the lower trace, while the 0.67 mg/kg dose is shown as the upper trace).

Table 12.

Dose of H16_30kD variant	Mean $t_{1/2}$ (hours)	Mean AUC last (h*ng/mL)	Mean C _{max} (ng/mL)
0.12 mg/kg	17.2	18,349	361
0.67 mg/kg	18.0	71,940	1,358

[0622] The plots of plasma concentration of the H16_30kDa variant at a dose of 0.12 mg/kg, and the H16_50kDa variant at a dose of 0.2 mg/kg, are shown in **FIG. 16**, wherein the trace for the 30 kDa variant is shown as the lower trace (squares) and the trace for the 50 kDa variant is shown as the upper trace (triangles). The plot of Treg cells percentage in singlets versus time post-dose in the plasma of non-human primates for the H16_30kDa variant at a dose of 0.12 mg/kg, and the H16_50kDa variant at a dose of 0.2 mg/kg, are shown in **FIG. 17**, wherein the trace for the vehicle is the lower trace (squares), the trace for the 30 kDa variant is shown in the middle trace, and the trace for the 50 kDa variant is shown in the upper trace.

[0623] To study the effects of the IL-2 conjugate (H16_50kD) on delayed-type hypersensitivity (DTH) induced with keyhole limpet hemocyanin (KLH) in mice, DTH was induced in female C57BL/6 mice by KLH sensitization at Day 1 and challenge at Day 7, and the mice were treated with H16_50kD at Days 0 and 3. Details of the study plan are provided in **Table 13** and also in **FIG. 18**.

Table 13. DTH study details

Group	H16_50kD Treatment	Dose Level (mg/kg)	Dose Volume (mL/kg)	Dosing route, frequency	N
1	KLH challenge only+Vehicle	0	0	SC, QD, Day 0 and 3	10
2	KLH + Vehicle	0	0	SC, QD, Day 0 and 3	10
3	KLH + Low Dose	0.03	10	SC, QD, Day 0 and 3	10
4	KLH + Mid Dose	0.1	10	SC, QD, Day 0 and 3	10
5	KLH + High Dose	0.3	10	SC, QD, Day 0 and 3	10
6	KLH + Cyclosporine A (CsA)	60	10	PO, QD, Day 1-9	10

SC: subcutaneous injection; QD: once a day

[0624] Hypersensitivity Induction: On Day 1, Groups 2 to 6 mice received intrascapular subcutaneous (SC) injection with a KLH/CFA/IFA emulsion (2.5 mg/mL) at a dose volume of 0.1 mL (KLH dose of 250 µg). On Day 7, all mice from Groups 1 to 6 received an intradermal (ID) injection of KLH (1 mg/mL PBS) in the right ear (or the left ear if needed) using a Hamilton syringe and dose volume 10 µL (KLH dose level 10 µg).

[0625] The KLH/CFA/IFA emulsion for sensitization was prepared as follows: KLH (Calbiochem; Cat. No. 374807) was prepared in PBS to achieve a 3X solution (7.5 mg/mL). Incomplete Freund's Adjuvant (IFA, Sigma) and Complete Freund's Adjuvant (CFA, Sigma) were placed in ice bath. The KLH solution was provided in a 50 mL conical tube; and an equivalent volume of CFA and IFA (1:1:1) were added to yield a final KLH/CFA/IFA emulsion at the required 1X concentrations of 2.5 mg/mL.

[0626] Administration of vehicle and H16_50kD: Groups 1 to 5 mice were treated subcutaneously (SC in lumbar area) on Day 0 and Day 3. Group 6 mice received the positive control by gavage (PO) from Day 1-9. Administration of Cyclosporine A (CsA, Tokyo Chemical

Industry) (0.5% methyl cellulose (400 cP) in ultrapure water) was done 2 hours prior to KLH injections on Day 1 and Day 7.

[0627] Assessment of ear thickness and blood immunotypes: An 8 mm punch (around the injection site) was taken from the injected ear (and contralateral from Group 1 animals) prior to KLH challenge (on Day 7) and then subsequently on Days 8, 9 and 10. The animals were slightly anesthetized by isoflurane inhalation at approximately 24 h, 48 h and 72 h post KLH challenge. Thickness of the ear flap was measured using an engineering micrometer.

[0628] Whole blood samples (150 μ L) were collected from 5 mice per group for all groups on Day 0, Day 3, Day 7 alternatively via jugular venipuncture (2-3 hours before the H16_50kD dosing and/or KLH injection) and terminally on Day 10. The blood samples were subjected to blood immunophenotyping using flow cytometry analysis (CD45/CD3/CD4/CD25/FoxP3). The relative percentage of CD4+ T cells that are Treg cells (CD25+/FoxP3+) for all timepoints were determined. On Day 10 only, the absolute counts of CD4+ Tregs were determined using lymphocyte counts by the Sysmex system.

[0629] On Day 10, at the end of final assessment, all animals from Group 1-6 were anesthetized (with 1-5% isoflurane) and blood samples (~0.5-1 mL) were collected via abdominal aorta or by intracardiac puncture. Then, animals underwent exsanguination of the abdominal aorta and ear tissues were collected immediately thereafter (both ears from Group 1 and KLH injected ears for Group 2-6) for the ear thickness measurements.

[0630] As shown in **FIG. 19A- FIG. 19B**, dosing with H16_50kD at 0.1 mg/kg and 0.3 mg/kg reduced ear thicknesses as compared to the negative control (vehicle only), indicating reducing the delayed-type hypersensitivity. As shown in Fig. 17C, the relative percentage of CD4+ T cells within CD25+FoxP3+ cell population increased compared to the negative control (vehicle only) and the positive control (Cyclosporine A) by dosing with H16_50kD at 0.03 mg/kg, 0.1 mg/kg and 0.3 mg/kg, respectively.

[0631] As shown in **FIGs. 20A-20C**, the relative percentage of Treg (CD4+CD25+FoxP3+) cells within CD45+ cell population (**FIG. 20A**), within TCR β + cell population (**FIG. 20B**), and within CD4+ cell population (**FIG. 20C**) all increased compared to the negative control (vehicle only) and the positive control (Cyclosporine A) by dosing with H16_50kD at 0.03 mg/kg, 0.1 mg/kg and 0.3 mg/kg, respectively. **FIG. 21** also shows that at the end of Day 10, the absolute counts of Treg (CD4+CD25+FoxP3+) cells were higher in the mice following dosing with H16_50kD at 0.03 mg/kg, 0.1 mg/kg and 0.3 mg/kg, respectively, as compared to the negative control (vehicle only) and the positive control (Cyclosporine A).

[0632] The above data demonstrate that administration of H16_50kD showed therapeutic activity in a disease model (DTH) involving suppression of recall responses to a sensitizing agent by induced Tregs.

CLAIMS

What is claimed is:

1. A method of delivering a gene therapy agent to a cell of a subject, comprising administering an IL-2 conjugate to the subject, wherein the gene therapy agent is administered to the subject before, concurrently with, or after the IL-2 conjugate, wherein the IL-2 conjugate comprises an amino acid sequence having at least 80% sequence identity to SEQ ID NO: 1, in which at least one amino acid residue in the IL-2 conjugate is replaced by an unnatural amino acid linked to a conjugating moiety, and the unnatural amino acid linked to the conjugating moiety is positioned in the amino acid sequence so as to preferentially reduce binding of the IL-2 conjugate to IL-2R $\beta\gamma$ relative to IL-2R $\alpha\beta\gamma$, or is at position P1, T2, S3, S4, S5, T6, K7, K8, Q10, L11, E14, H15, L17, L18, D19, Q21, M22, N25, G26, N28, N29, Y30, K31, K34, T36, M45, P46, K47, A49, T50, E51, L52, K53, H54, Q56, E59, E66, N70, Q73, S74, K75, N76, F77, H78, R80, P81, R82, D83, S86, N87, I88, V90, I91, L93, E94, K96, G97, S98, E99, T100, T101, F102, M103, C104, E105, Y106, A107, D108, E109, T110, A111, T112, E115, N118, R119, T122, F123, S124, Q125, S126, S129, T130, L131, or T132 in reference to the sequence of SEQ ID NO: 1.
2. A method of treating an individual in need thereof with a gene therapy agent, comprising administering an IL-2 conjugate to the subject, wherein the gene therapy agent is administered to the subject before, concurrently with, or after the IL-2 conjugate, and the IL-2 conjugate comprises an amino acid sequence having at least 80% sequence identity to SEQ ID NO: 1, in which at least one amino acid residue in the IL-2 conjugate is replaced by an unnatural amino acid linked to a conjugating moiety, and the unnatural amino acid linked to the conjugating moiety is positioned in the amino acid sequence so as to preferentially reduce binding of the IL-2 conjugate to IL-2R $\beta\gamma$ relative to IL-2R $\alpha\beta\gamma$, or is at position P1, T2, S3, S4, S5, T6, K7, K8, Q10, L11, E14, H15, L17, L18, D19, Q21, M22, N25, G26, N28, N29, Y30, K31, K34, T36, M45, P46, K47, A49, T50, E51, L52, K53, H54, Q56, E59, E66, N70, Q73, S74, K75, N76, F77, H78, R80, P81, R82, D83, S86, N87, I88, V90, I91, L93, E94, K96, G97, S98, E99, T100, T101, F102, M103, C104, E105, Y106, A107, D108, E109, T110, A111, T112, E115, N118, R119, T122, F123, S124, Q125, S126, S129, T130, L131, or T132 in reference to the sequence of SEQ ID NO: 1.

3. A method of increasing expression of a gene therapy agent, comprising:
administering an IL-2 conjugate to a subject;
wherein the gene therapy agent is administered to the subject before, concurrently with,
or after the IL-2 conjugate; and
wherein the IL-2 conjugate comprises an amino acid sequence having at least 80%
sequence identity to SEQ ID NO: 1, in which at least one amino acid residue in the IL-2
conjugate is replaced by an unnatural amino acid linked to a conjugating moiety, and the
unnatural amino acid linked to the conjugating moiety is positioned in the amino acid
sequence so as to preferentially reduce binding of the IL-2 conjugate to IL-2R $\beta\gamma$ relative
to IL-2R $\alpha\beta\gamma$, or is at position P1, T2, S3, S4, S5, T6, K7, K8, Q10, L11, E14, H15, L17,
L18, D19, Q21, M22, N25, G26, N28, N29, Y30, K31, K34, T36, M45, P46, K47, A49,
T50, E51, L52, K53, H54, Q56, E59, E66, N70, Q73, S74, K75, N76, F77, H78, R80,
P81, R82, D83, S86, N87, I88, V90, I91, L93, E94, K96, G97, S98, E99, T100, T101,
F102, M103, C104, E105, Y106, A107, D108, E109, T110, A111, T112, E115, N118,
R119, T122, F123, S124, Q125, S126, S129, T130, L131, or T132 in reference to the
sequence of SEQ ID NO: 1.
4. A method of reducing an immune response to a gene therapy agent, comprising:
administering an IL-2 conjugate to a subject;
wherein the gene therapy agent is administered to the subject before, concurrently with,
or after the IL-2 conjugate; and
wherein the IL-2 conjugate comprises an amino acid sequence having at least 80%
sequence identity to SEQ ID NO: 1, in which at least one amino acid residue in the IL-2
conjugate is replaced by an unnatural amino acid linked to a conjugating moiety, and the
unnatural amino acid linked to the conjugating moiety is positioned in the amino acid
sequence so as to preferentially reduce binding of the IL-2 conjugate to IL-2R $\beta\gamma$ relative
to IL-2R $\alpha\beta\gamma$, or is at position P1, T2, S3, S4, S5, T6, K7, K8, Q10, L11, E14, H15, L17,
L18, D19, Q21, M22, N25, G26, N28, N29, Y30, K31, K34, T36, M45, P46, K47, A49,
T50, E51, L52, K53, H54, Q56, E59, E66, N70, Q73, S74, K75, N76, F77, H78, R80,
P81, R82, D83, S86, N87, I88, V90, I91, L93, E94, K96, G97, S98, E99, T100, T101,
F102, M103, C104, E105, Y106, A107, D108, E109, T110, A111, T112, E115, N118,
R119, T122, F123, S124, Q125, S126, S129, T130, L131, or T132 in reference to the
sequence of SEQ ID NO: 1.

5. A method of preventing immune-related adverse events in a subject, comprising:
administering an IL-2 conjugate to a subject;
wherein a gene therapy agent is administered to the subject before, concurrently with, or after the IL-2 conjugate; and
wherein the IL-2 conjugate comprises an amino acid sequence having at least 80% sequence identity to SEQ ID NO: 1, in which at least one amino acid residue in the IL-2 conjugate is replaced by an unnatural amino acid linked to a conjugating moiety, and the unnatural amino acid linked to the conjugating moiety is positioned in the amino acid sequence so as to preferentially reduce binding of the IL-2 conjugate to IL-2R $\beta\gamma$ relative to IL-2R $\alpha\beta\gamma$, or is at position P1, T2, S3, S4, S5, T6, K7, K8, Q10, L11, E14, H15, L17, L18, D19, Q21, M22, N25, G26, N28, N29, Y30, K31, K34, T36, M45, P46, K47, A49, T50, E51, L52, K53, H54, Q56, E59, E66, N70, Q73, S74, K75, N76, F77, H78, R80, P81, R82, D83, S86, N87, I88, V90, I91, L93, E94, K96, G97, S98, E99, T100, T101, F102, M103, C104, E105, Y106, A107, D108, E109, T110, A111, T112, E115, N118, R119, T122, F123, S124, Q125, S126, S129, T130, L131, or T132 in reference to the sequence of SEQ ID NO: 1.
6. The method of any one of the preceding claims, wherein the method further comprises, before the administration of the gene therapy agent and the IL-2 conjugate to the subject, a) incubating immune cells from the subject with the gene therapy agent and b) analyzing the immune cells for the expression of one or more activation biomarkers or increased expression of one or more activation biomarkers, wherein expression or increased expression of the one or more activation biomarkers following incubation with the gene therapy agent identifies the subject as being in need of the IL-2 conjugate.
7. A method for selecting a subject for treatment with a gene therapy agent and an IL-2 conjugate, the method comprising a) incubating immune cells from the subject with the gene therapy agent, b) analyzing the immune cells for the expression of one or more activation biomarkers or increased expression of one or more activation biomarkers, wherein expression or increased expression of the one or more activation biomarkers following incubation with the gene therapy agent identifies the subject for treatment with the gene therapy agent and the IL-2 conjugate, and c) selecting the subject identified in step b) for treatment with the gene therapy agent and the IL-2 conjugate;
wherein the IL-2 conjugate comprises an amino acid sequence having at least 80%

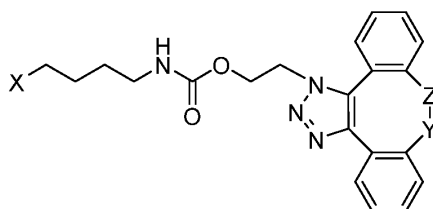
sequence identity to SEQ ID NO: 1, in which at least one amino acid residue in the IL-2 conjugate is replaced by an unnatural amino acid linked to a conjugating moiety, and the unnatural amino acid linked to the conjugating moiety is positioned in the amino acid sequence so as to preferentially reduce binding of the IL-2 conjugate to IL-2R $\beta\gamma$ relative to IL-2R $\alpha\beta\gamma$, or is at position P1, T2, S3, S4, S5, T6, K7, K8, Q10, L11, E14, H15, L17, L18, D19, Q21, M22, N25, G26, N28, N29, Y30, K31, K34, T36, M45, P46, K47, A49, T50, E51, L52, K53, H54, Q56, E59, E66, N70, Q73, S74, K75, N76, F77, H78, R80, P81, R82, D83, S86, N87, I88, V90, I91, L93, E94, K96, G97, S98, E99, T100, T101, F102, M103, C104, E105, Y106, A107, D108, E109, T110, A111, T112, E115, N118, R119, T122, F123, S124, Q125, S126, S129, T130, L131, or T132 in reference to the sequence of SEQ ID NO: 1.

8. The method of the immediately preceding claim, further comprising steps of administering the IL-2 conjugate to the subject identified in step b), and administering the gene therapy agent to the subject identified in step b).
9. The method of any one of claims 6-8, wherein the immune cell is a lymphocyte, a T cell, a CD8+ T cell, an effector T cell, a cytotoxic T cell, or an NK cell.
10. The method of any one of the preceding claims, wherein the unnatural amino acid is linked to the conjugating moiety through a linker.
11. The method of the immediately preceding claim, wherein the linker comprises a homobifunctional linker, a heterobifunctional linker, a cleavable or a non-cleavable dipeptide linker, a maleimide group, a spacer, or a combination thereof.
12. The method of any one of the preceding claims, wherein the unnatural amino acid is a substituted lysine, is a substituted phenylalanine, is a substituted histidine, is a substituted cysteine, comprises an azido group, comprises an alkyne group, comprises an aldehyde group, comprises an aromatic side chain, or comprises a ketone group.
13. The method of any one of the preceding claims, wherein the at least one unnatural amino acid comprises N6-azidoethoxy-L-lysine, N6-((2-azidoethoxy)-carbonyl)-L-lysine, N6-propargylethoxy-L-lysine (PraK), BCN-L-lysine, norbornene lysine, TCO-lysine,

methyltetrazine lysine, allyloxycarbonyllysine, p-acetyl-L-phenylalanine, p-azidomethyl-L-phenylalanine (pAMF), p-iodo-L-phenylalanine, m-acetylphenylalanine, p-propargyloxyphenylalanine, p-propargyl-phenylalanine, 3-methyl-phenylalanine, fluorinated phenylalanine, isopropyl-L-phenylalanine, p-azido-L-phenylalanine, p-acyl-L-phenylalanine, p-benzoyl-L-phenylalanine, p-bromophenylalanine, p-amino-L-phenylalanine, isopropyl-L-phenylalanine, O-allyltyrosine, O-methyl-L-tyrosine, O-4-allyl-L-tyrosine, 4-propyl-L-tyrosine, phosphotyrosine, L-3-(2-naphthyl)alanine, 2-amino-3-((2-((3-(benzyloxy)-3-oxopropyl)amino)ethyl)selanyl)propanoic acid, or 2-amino-3-(phenylselanyl)propanoic acid.

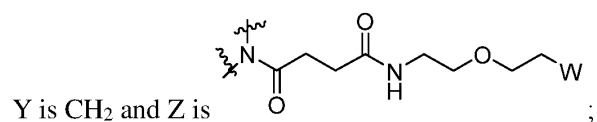
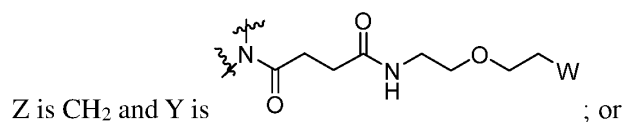
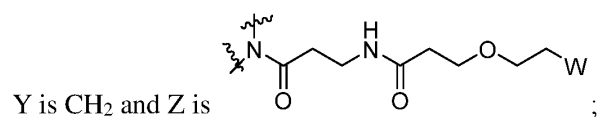
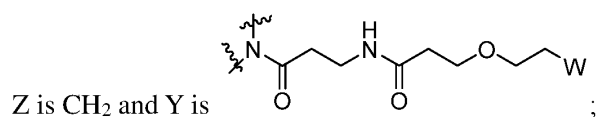
14. The method of any one of the preceding claims, wherein the unnatural amino acid is an azido-substituted lysine.
15. The method of any one of the preceding claims, wherein the unnatural amino acid is *N*6-((2-azidoethoxy)-carbonyl)-L-lysine.
16. The method of any one of the preceding claims, wherein the conjugating moiety comprises a water-soluble polymer.
17. The method of the immediately preceding claim, wherein the water-soluble polymer comprises polyethylene glycol (PEG), poly(propylene glycol) (PPG), copolymers of ethylene glycol and propylene glycol, poly(oxyethylated polyol), poly(olefinic alcohol), poly(vinylpyrrolidone), poly(hydroxyalkylmethacrylamide), poly(hydroxyalkylmethacrylate), poly(saccharides), poly(α -hydroxy acid), poly(vinyl alcohol), polyphosphazene, polyoxazolines (POZ), poly(*N*-acryloylmorpholine), or a combination thereof.
18. The method of the immediately preceding claim, wherein the conjugating moiety comprises PEG.
19. The method of the immediately preceding claim, wherein the conjugating moiety is PEG having a molecular weight of about 10-85 kDa or selected from about 10kDa, 15kDa, 20kDa, 25kDa, 30kDa, 35kDa, 40kDa, 45kDa, 50kDa, 55 kDa, 60kDa, 65kDa, 70kDa, 75 kDa, 80kDa, and 85 kDa.

20. The method of any one of the preceding claims, wherein the conjugating moiety is PEG having a molecular weight of about 20-70 kDa or selected from about 20kDa, 25kDa, 30kDa, 35kDa, 40kDa, 45kDa, 50kDa, 55 kDa, 60kDa, 65kDa, and 70kDa.
21. The method of any one of the preceding claims, wherein the conjugating moiety is PEG having a molecular weight of about 30-60 kDa or selected from about 30kDa, 35kDa, 40kDa, 45kDa, 50kDa, 55 kDa, and 60kDa.
22. The method of any one of the preceding claims, wherein the amino acid linked to the conjugating moiety has the structure of Formula (I):



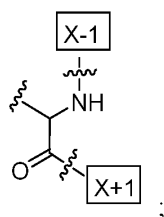
Formula (I);

wherein:



W is a PEG group; and

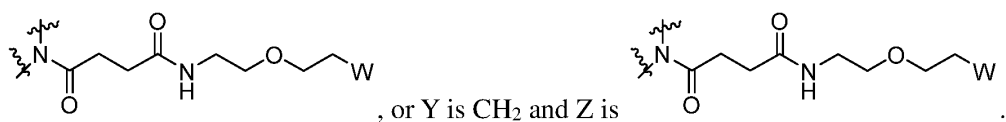
X has the structure:



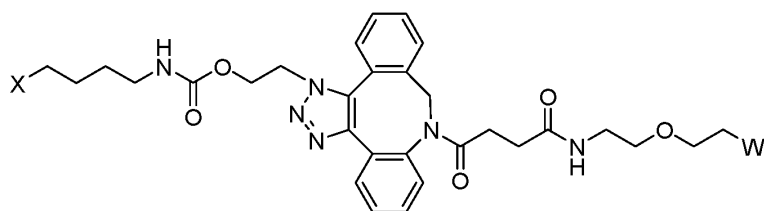
X-1 indicates the point of attachment to the preceding amino acid residue; and

X+1 indicates the point of attachment to the following amino acid residue.

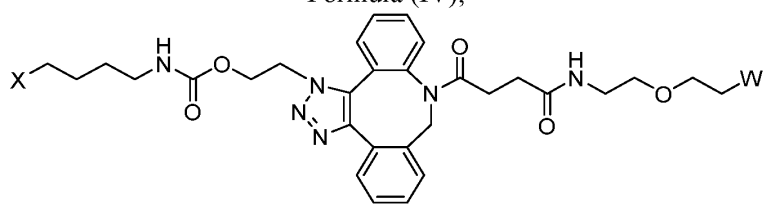
23. The method of the immediately preceding claim, wherein Z is CH₂ and Y is



24. The method of the immediately preceding claim, wherein the structure of Formula (I) has the structure of Formula (IV) or Formula (V):



Formula (IV);



Formula (V);

wherein:

W is a PEG group having a molecular weight of about 5-60 kDa or about 5 kDa, 10 kDa, 15 kDa, 20 kDa, 25 kDa, 30 kDa, 35 kDa, 40 kDa, 45 kDa, 50 kDa, or 60 kDa.

25. The method of any one of the preceding claims, wherein the IL-2 conjugate further comprises an alanine or methionine N-terminal to the first amino acid of the sequence having at least 80% sequence identity to SEQ ID NO: 1.
26. The method of any one of the preceding claims, wherein the IL-2 conjugate comprises an amino acid sequence having at least 85%, 90%, 95%, 96%, 97%, 98%, or 99% identity to SEQ ID NO: 1.
27. The method of any one of the preceding claims, wherein the IL-2 conjugate comprises the amino acid sequence of SEQ ID NO: 1 in which position P1, T2, S3, S4, S5, T6, K7, K8, Q10, L11, E14, H15, L17, L18, D19, Q21, M22, N25, G26, N28, N29, Y30, K31, K34, T36, M45, P46, K47, A49, T50, E51, L52, K53, H54, Q56, E59, E66, N70, Q73, S74, K75, N76, F77, H78, R80, P81, R82, D83, S86, N87, I88, V90, I91, L93, E94, K96, G97, S98, E99, T100, T101, F102, M103, C104, E105, Y106, A107, D108, E109, T110, A111, T112, E115, N118, R119, T122, F123, S124, Q125, S126, S129, T130, L131, or T132 is replaced with the unnatural amino acid.
28. The method of any one of the preceding claims, wherein position K8, L11, E14, H15, L18, D19, M22, N87, E99, or D108 in reference to the sequence of SEQ ID NO: 1 is replaced with the unnatural amino acid.
29. The method of the immediately preceding claim, wherein position L18 in reference to the sequence of SEQ ID NO: 1 is replaced with the unnatural amino acid.
30. The method of claim 28, wherein position H15 in reference to the sequence of SEQ ID NO: 1 is replaced with the unnatural amino acid.
31. The method of any one of the preceding claims, wherein the IL-2 conjugate is capable of expanding CD4⁺ T regulatory (Treg) cells.
32. The method of any one of the preceding claims, wherein the unnatural amino acid and/or the conjugating moiety impairs or blocks the receptor signaling potency of the IL-2 conjugate to IL-2R $\beta\gamma$, or reduces recruitment of IL-2R γ subunit to an IL-2/IL-2R β complex.

33. The method of any one of the preceding claims, wherein the IL-2 conjugate has a receptor signaling potency to IL-2R $\beta\gamma$ that is lower than a receptor signaling potency of wild-type IL-2 to IL-2R $\beta\gamma$.
34. The method of any one of the preceding claims, wherein the IL-2 conjugate has a receptor signaling potency to IL-2R $\alpha\beta\gamma$ that is greater than or equal to a receptor signaling potency of wild-type IL-2 to IL-2R $\alpha\beta\gamma$.
35. The method of any one of the preceding claims, wherein the IL-2 conjugate expands a CD4⁺ Treg population in the subject.
36. The method of any one of the preceding claims, wherein the IL-2 conjugate suppresses CD8⁺ T cell proliferation in the subject.
37. The method of any one of the preceding claims, wherein the IL-2 conjugate suppresses effector memory CD8⁺ T cell proliferation in the subject.
38. The method of any one of the preceding claims, wherein the gene therapy agent comprises a vector and the IL-2 conjugate suppresses vector-specific IFN γ -secreting CD8⁺ T cells in the subject.
39. The method of any one of the preceding claims, wherein the gene therapy agent encodes a transgene product and the IL-2 conjugate suppresses transgene-product-specific IFN γ -secreting CD8⁺ T cells in the subject.
40. The method of any one of the preceding claims, wherein the gene therapy agent encodes a transgene product and the IL-2 conjugate suppresses production of antibodies against the transgene product.
41. The method of any one of the preceding claims, wherein the gene therapy agent encodes a transgene product and the IL-2 conjugate suppresses production of IgG1 antibodies against the transgene product.
42. The method of any one of the preceding claims, wherein the gene therapy agent encodes a transgene product and the IL-2 conjugate prolongs the expression of the transgene

product in the subject relative to a subject that is administered the gene therapy agent and without the IL-2 conjugate.

43. The method of the immediately preceding claim, wherein the prolonged expression of the transgene product is at least about 5 weeks, about 6 weeks, about 8 weeks, about 12 weeks, about 14 weeks, or about 16 weeks.
44. The method of any one of the preceding claims, wherein the gene therapy agent comprises a viral vector.
45. The method of claim 44, wherein the IL-2 conjugate suppresses production of antibodies against the viral vector.
46. The method of claim 44 or claim 45, wherein the IL-2 conjugate suppresses production of antibodies against a capsid protein of the viral vector.
47. The method of any one of claims 44-46, wherein the viral vector is an adeno-associated viral (AAV) particle.
48. The method of the immediately preceding claim, wherein the AAV particle comprises an AAV1 capsid, an AAV2 capsid, an AAV3 capsid, an AAV4 capsid, an AAV5 capsid, an AAV6 capsid, an AAV7 capsid, an AAV8 capsid, an AAVrh8 capsid, an AAV9 capsid, an AAV10 capsid, an AAVrh10 capsid, an AAV11 capsid, an AAV12 capsid, an AAVrh32.33 capsid, an AAV-XL32 capsid, an AAV-XL32.1 capsid, an AAV LK03 capsid, an AAV2R471A capsid, an AAV2/2-7m8 capsid, an AAV DJ capsid, an AAV DJ8 capsid, an AAV2 N587A capsid, an AAV2 E548A capsid, an AAV2 N708A capsid, an AAV V708K capsid, a goat AAV capsid, an AAV1/AAV2 chimeric capsid, a bovine AAV capsid, a mouse AAV capsid rAAV2/HBoV1 (chimeric AAV / human bocavirus virus 1), an AAV2HBKO capsid, an AAVPHP.B capsid or an AAVPHP.eB capsid, or a functional variant thereof.
49. The method of the immediately preceding claim, wherein the AAV capsid comprises a tyrosine mutation, a heparin binding mutation, or an HBKO mutation.

50. The method of any one of claims 47-49, wherein the AAV viral particle comprises an AAV genome comprising one or more inverted terminal repeats (ITRs), wherein the one or more ITRs is an AAV1 ITR, an AAV2 ITR, an AAV3 ITR, an AAV4 ITR, an AAV5 ITR, an AAV6 ITR, an AAV7 ITR, an AAV8 ITR, an AAVrh8 ITR, an AAV9 ITR, an AAV10 ITR, an AAVrh10 ITR, an AAV11 ITR, or an AAV12 ITR.
51. The method of the immediately preceding claim, wherein the one or more ITRs and the capsid of the AAV particle are derived from the same AAV serotype.
52. The method of the immediately preceding claim, wherein the one or more ITRs and the capsid of the AAV particles are derived from different AAV serotypes.
53. The method of any one of claims 44-46, wherein the viral vector is an adenoviral particle.
54. The method of the immediately preceding claim, wherein the adenoviral particle comprises a capsid from Adenovirus serotype 2, 1, 5, 6, 19, 3, 11, 7, 14, 16, 21, 12, 18, 31, 8, 9, 10, 13, 15, 17, 19, 20, 22, 23, 24-30, 37, 40, 41, AdHu2, AdHu 3, AdHu4, , AdHu24, AdHu26, AdHu34, AdHu35, AdHu36, AdHu37, AdHu41, AdHu48, AdHu49, AdHu50, AdC6, AdC7, AdC69, bovine Ad type 3, canine Ad type 2, ovine Ad, or porcine Ad type 3, or a functional variant thereof.
55. The method of any one of claims 44-46, wherein the viral vector is a lentiviral particle.
56. The method of the immediately preceding claim, wherein the lentiviral particle is pseudotyped with vesicular stomatitis virus (VSV), lymphocytic choriomeningitis virus (LCMV), Ross river virus (RRV), Ebola virus, Marburg virus, Mokala virus, Rabies virus, RD114, or a functional variant thereof.
57. The method of any one of claims 44-46, wherein the viral vector is a Herpes simplex virus (HSV) particle.
58. The method of the immediately preceding claim, wherein the HSV particle is an HSV-1 particle or an HSV-2 particle, or a functional variant thereof.
59. The method of any one of claims 1-41, wherein the gene therapy agent comprises a lipid nanoparticle.

60. The method of any one of the preceding claims, wherein the gene therapy agent comprises a nucleic acid encoding a heterologous transgene.
61. The method of the immediately preceding claim, wherein the heterologous transgene is operably linked to a promoter.
62. The method of the immediately preceding claim, wherein the promoter is a constitutive promoter, a tissue- specific promoter, or an inducible promoter.
63. The method of any one of claims 60-62, wherein the nucleic acid comprises closed-end DNA (ceDNA).
64. The method of claim 60, wherein the nucleic acid comprises mRNA.
65. The method of any one of the preceding claims, wherein the gene therapy agent is administered to the subject concurrently with the IL-2 conjugate.
66. The method of any one of claims 1-64, wherein the gene therapy agent is administered to the subject before the IL-2 conjugate.
67. The method of the immediately preceding claim, wherein the gene therapy agent is administered less than 14 days or less than 7 days before the IL-2 conjugate.
68. The method of any one of claims 1-64, wherein the gene therapy agent is administered to the subject after the IL-2 conjugate.
69. The method of the immediately preceding claim, wherein the gene therapy agent is administered less than 7 days, less than 3 days, or less than 1 day after the IL-2 conjugate.
70. The method of any one of claims 1-64, wherein the IL-2 conjugate is administered before, at the same time, or after administration of the gene therapy agent.
71. The method of any one of the preceding claims, wherein the individual has a disease or disorder suitable for treatment by gene therapy.

72. The method of the immediately preceding claim, wherein the disease or disorder is a monogenic disease or disorder.
73. The method of any one of the preceding claims, wherein the gene therapy agent is administered intravenously, intraperitoneally, intra-arterially, intramuscularly, subcutaneously, intracranially, intra-CSF, intra-DRG, intracerebroventricularly, intraocularly, intracisterna magna, or intrahepatically.
74. The method of any one of the preceding claims, wherein the IL-2 conjugate is administered parenterally and/or systemically.
75. The method of any one of the preceding claims, wherein the IL-2 conjugate is administered intravenously, intraperitoneally, intra-arterially, intramuscularly, subcutaneously, intracranially, intra-CSF, intra-DRG, intracerebroventricularly, intraocularly, intracisterna magna, or intrahepatically.
76. The method of any one of the preceding claims, wherein the subject is a mammal.
77. The method of any one of the preceding claims, wherein the subject is a primate.
78. The method of any one of the preceding claims, wherein the subject is a human.
79. The method of any one of the preceding claims, wherein the IL-2 conjugate is administered about 1, 2, 3, 4, 5, 6, or 7 days before the gene therapy agent.
80. The method of any one of claims 1-78, wherein the IL-2 conjugate is administered about 1, 2, 3, or 4 days after the gene therapy agent.
81. The method of any one of claims 1-78, wherein the IL-2 conjugate is administered on the same day as the gene therapy agent.
82. The method of any one of the preceding claims, wherein the IL-2 conjugate is administered at a dose of about 0.02-0.5 mg/kg about 0.03-0.4 mg/kg, about 0.04-0.1 mg/kg, or about 0.05-0.08 mg/kg.

83. The method of the immediately preceding claim, wherein the IL-2 conjugate is administered at a dose of about 0.05 mg/kg.
84. The method of claim 82, wherein the IL-2 conjugate is administered at a dose of about 0.08 mg/kg.
85. The method of claim 82, wherein the IL-2 conjugate is administered at a dose of about 0.3 mg/kg.
86. Use of an IL-2 conjugate for the manufacture of a medicament for use in the method of any one of the preceding claims.
87. An IL-2 conjugate for use in the method of any one of claims 1-85.