

ORTHOGONAL IL-21 RECEPTOR/CYTOKINE SYSTEMS**CROSS-REFERENCE TO RELATED APPLICATIONS**

[0001] This application claims priority from U.S. Provisional Patent Application No. 63/105,414, filed on October 26, 2020, and U.S. Provisional Patent Application No. 63/212,547, filed on June 18, 2021, each of which is incorporated by reference herein in its entirety.

SEQUENCE LISTING

[0002] A Sequence Listing has been submitted electronically in ASCII format and is hereby incorporated by reference in its entirety. The ASCII copy, created on October 25, 2021, is named Neptune-IL21-PCT_ST25.txt and is 123,571 bytes in size.

BACKGROUND

[0003] Cytokines are potent natural regulators of immune cell proliferation and differentiation. While this potency has made cytokines highly attractive as potential therapeutics, it has also complicated their clinical utility. This has been especially true for cytokines that have multiple cellular targets and, thus, high potential for pleiotropic effects. One example is Interleukin-2 (“IL-2”), a robust T cell mitogen whose anti-cancer activity is offset by unwanted proliferation of regulatory (suppressor) T cells and a painful vascular leak syndrome. In the specific case of IL-2, protein engineering can be used to solve some of the clinical challenges: removing, for example, the cytokine’s capacity to act preferentially on regulatory T cells. An alternative approach involves generating orthogonally constrained forms of cytokines and their receptors. *See* U.S. Patent No. 10,869,887; Sockolosky JT, Trotta E, Parisi G, et al. Selective targeting of engineered T cells using orthogonal IL-2 cytokine-receptor complexes. *Science*. 2018;359(6379):1037-1042. doi:10.1126/science.aar3246, the disclosure of each of which is incorporated by reference herein in its entirety.

[0004] An orthogonal cytokine system is one in which a cytokine and its receptor have been mutated such that they lose compatibility with their native (parental) partners yet retain the capacity to interact productively with one another. Such an orthogonal cytokine:receptor pair can thus be said to demonstrate “privileged” or “private” interactions. The approach of generating orthogonally constrained forms of cytokines and their receptors is of value for cell therapy because it provides a way to limit the scope of a cytokine’s activity solely to the therapeutic (i.e., adoptively transferred) cells – these being the only cells expressing the engineered receptor and, consequently, the only cells capable of responding to the engineered cytokine.

[0005] Interleukin-21 (“IL-21”) is another pleiotropic cytokine with actions in a broad range of lymphoid, myeloid, and epithelial cells. IL-21 regulates both innate and adaptive immune responses; it not only has key roles in antitumor and antiviral responses, but also exerts major effects on inflammatory responses that promote the development of autoimmune diseases and inflammatory disorders. Spolski, R., Leonard, W. Interleukin-21: a double-edged sword with therapeutic potential. *Nat Rev Drug Discov* 13, 379–395 (2014). <https://doi.org/10.1038/nrd4296>. The three-dimensional structure of the natural human IL-21 cytokine:receptor complex is known. *See* Hamming OJ, Kang L, Svensson A, et al. Crystal structure of interleukin-21 receptor (IL-21R) bound to IL-21 reveals that sugar chain interacting with WSXWS motif is integral part of IL-21R. *J Biol Chem.* 2012;287(12):9454-9460. doi:10.1074/jbc.M111.311084, the disclosure of which is incorporated by reference herein in its entirety.

[0006] IL-21 is of particular interest because it enhances cytotoxic T cell responses to viruses and tumors and can act in synergy with other cytokines, such as IL-2 or IL-15. IL-21 does this in part by promoting the persistence of T cells with a stem cell memory phenotype, which has been associated with beneficial outcomes in cell therapy settings. IL-21 is currently undergoing

evaluation as a cancer therapeutic in multiple clinical trials. IL-21 also has significant potential utility in chimeric antigen receptor T (“CAR-T”) cell therapies, where it may help to overcome clinical failures due to poor expansion, anti-tumor efficacy, exhaustion, suppression, and persistence. Thus, an urgent need exists for the ability to modulate the actions of IL-21, particularly by engineering into adoptively transferred cells a desired behavior that is protected from endogenous signaling pathways, that does not affect non-targeted endogenous cells, and that can be controlled once administered to a patient.

SUMMARY

[0007] In one aspect, an orthogonal interleukin-21 receptor alpha chain (an “ortho-IL-21R α ” or “ortho-IL-21R α molecule,” or when referring to a specific ortho-IL-21R α constructed as provided herein, an “RV,” as in “Receptor Variant”) is provided, the ortho-IL-21R α comprising a modified amino acid sequence derived from SEQ ID NO: 4 that binds to an orthogonal interleukin-21 cytokine (an “ortho-IL-21” or “ortho-IL-21 molecule,” or when referring to a specific ortho-IL-21 constructed as provided herein, a “CV,” as in “Cytokine Variant”) but has impaired binding to native IL-21. In one aspect, the ortho-IL-21R α comprises a modified amino acid sequence comprising a substitution of one or more amino acid residues of SEQ ID NO: 4 that contact IL-21, residues in the immediate vicinity of such contact residues, or residues elsewhere in IL-21R α that have an influence on the conformation of the IL-21 binding surface. In one aspect, the ortho-IL-21R α includes an amino acid substitution, numbered relative to SEQ ID NO: 6 (the human IL-21 α ectodomain in mature form lacking the signal peptide), at position: Y10, Q33, Q35, Y36, E38, L39, F67, H68, F69, M70, A71, D72, D73, I74, L94, A96, E97, P126, A127, Y129, M130, K134, S190, Y191, or a combination thereof. In one aspect, the amino acid substitution comprises, consists essentially of, or consists of: D72E/Y129F/D73E; M70I/D73E/Q33H;

D72E/L94V/Y191F; D72E/E38D/M130L; M70G/Y129F; F69L/M70L/D73I; D72K/D73K; E38K; or M70G, or a combination thereof. In one aspect, the amino acid substitution comprises, consists essentially of, or consists of: M70G/Y129F (“RV13,” as in “Receptor Variant 13,” or SEQ ID NO: 19) or M70G (“RV22” or SEQ ID NO: 27).

[0008] In another aspect, an ortho-IL-21 is provided, the ortho-IL-21 comprising a modified amino acid sequence derived from SEQ ID NO: 2 that binds to an ortho-IL-21R α but has impaired binding to native IL-21R α . In one aspect, the ortho-IL-21 comprises a modified amino acid sequence comprising a substitution of one or more amino acid residues of SEQ ID NO: 2 that contact IL-21R α , residues in the immediate vicinity of such contact residues, or residues elsewhere in IL-21 that have an influence on the conformation of the IL-21R α binding surface. In one aspect, the ortho-IL-21 includes an amino acid substitution, numbered relative to SEQ ID NO: 2, at position: R5, H6, I8, R9, M10, Q12, L13, K73, K75, R76, P78, G84, or P104, or a combination thereof. The phrase “numbered relative to SEQ ID NO: 2” means, for numbering purposes, to disregard any epitope tags and signaling peptides. In one aspect, the amino acid substitution comprises, consists essentially of, or consists of: R5Q, R5D, R5E, R5K, R5N, H6L, I8E, R9E, R9D, R9K, R9N, R9Q, M10L, Q12V, L13D, K73V, K73D, K73I, K75D, R76E, R76N, R76A, R5Q/R76E, R5Q/R76A, P78L, G84E, or P104A, or combinations thereof. In one aspect, the amino acid substitution comprises, consists essentially of, or consists of: H6L/R9K/M10L/P78L.

[0009] In an exemplary aspect, an engineered human IL-21 polypeptide is provided that comprises amino acid substitutions, numbered relative to SEQ ID NO: 2: H6L; R9K; M10L; P78L; and optionally comprises G84E or P104A or a combination thereof; and optionally comprises one of K73V or K73I. In one aspect, such an engineered human IL-21 polypeptide may include SEQ

ID NO: 61 (H6L/R9K/M10L/K73V/P78L/G84E) or SEQ ID NO: 62 (H6L/R9K/M10L/K73I/P78L/P104A).

[0010] In another aspect, a system for activating IL-21 signaling in a cell is provided, the system comprising: an ortho-IL-21R α that has impaired binding to native IL-21, the ortho-IL-21R α comprising a modified amino acid sequence derived from SEQ ID NO: 4 comprising a substitution of one or more of the amino acid residues of SEQ ID NO: 4 that contact IL-21, residues in the immediate vicinity of such contact residues, or residues elsewhere in IL-21R α that have an influence on the conformation of the IL-21 binding surface; and an ortho-IL-21 that has impaired binding to native IL-21R α , the ortho-IL-21 comprising a modified amino acid sequence derived from SEQ ID NO: 2 comprising a substitution of one or more amino acid residues of SEQ ID NO: 2 that contact IL-21R α , residues in the immediate vicinity of such contact residues, or residues elsewhere in IL-21 that have an influence on the conformation of the IL-21R α binding surface, wherein the ortho-IL-21R α binds to the ortho-IL-21. In one aspect, the cell is a mammalian cell, an immune cell, a stem cell, or a T cell.

BRIEF DESCRIPTION OF THE FIGURES

[0011] The present invention may be more readily understood by reference to the following figures, wherein:

[0012] **Figure 1** provides a schematic representation of an orthogonal IL-21 system. The cartoon at the extreme left shows the wild-type receptor and cytokine interacting productively with one another, while the adjacent cartoon depicts the impaired interaction between an ortho-IL-21R α and the native cytokine. The cartoon at the extreme right depicts the impaired interaction between ortho-IL-21 and the native (wild-type) receptor, while the adjacent cartoon shows a productive interaction between the two orthogonal molecules (ortho-IL-21R α and ortho-IL-21).

[0013] **Figure 2** provides a schematic representation of pathways for generating an orthogonal IL-21 system.

[0014] **Figure 3** shows the results of a representative assay in which a single (sub-saturating) concentration of IL-21-TLuc16 was tested for binding to a panel of 20 candidate ortho-IL-21R α molecules (all of which had been bound to Streptactin-coated surfaces of wells at saturating concentrations). Eight of the 20 candidate ortho-IL-21R α molecules showed diminished capacity to bind IL-21-TLuc16.

[0015] **Figures 4A-E** show the results of a representative assay in which a range of (sub-saturating) concentrations of IL-21-TLuc16 was tested for binding to a panel of candidate ortho-IL-21R α molecules. The panel included a wild-type receptor as a control and seven of the eight candidate ortho-IL-21R α molecules identified in **Figure 3**. The panel also included 13 additional candidate ortho-IL-21R α molecules that were designed based on results such as those depicted in **Figure 3**; specifically, substitutions present in the eight candidate ortho-IL-21R α molecules were introduced alone or in combination in the additional 13 candidate ortho-IL-21R α molecules. The 21 candidate ortho-IL-21R α molecules were added to Streptactin-coated wells of 96-well plates at saturating concentrations. **Figures 4A-E** show luminometry data for individual plates in which, in each case, the binding of IL-21-TLuc16 to five candidate ortho-IL-21R α molecules and a wild-type control IL-21R α was compared. Eleven of the additional 13 candidate ortho-IL-21R α molecules showed significantly diminished capacity to bind IL-21-TLuc16 relative to the wild-type control (the exceptions being receptors RV23, RV24, and RV28 carrying the single substitutions M70L, F69L, and D73E respectively).

[0016] **Figures 5A** and **5B** show IL-21-induced STAT3 signaling responses by Ba/F3 cells expressing native IL-21R α and the eight candidate ortho-IL-21R α molecules identified as shown

in **Figure 3**. The cells carried a STAT3-regulated *Cypridina noctiluca* luciferase reporter transgene; they were exposed to different concentrations of native IL-21 overnight (approximately 20 hours) before testing the supernatant medium for luciferase activity by luminometry, with Vargulin serving as the enzyme substrate. Cells expressing a form of wild-type IL-21R α lacking its cytoplasmic tail (Δ cyt) were included to show that STAT3 signaling in response to IL-21 depended on the cytoplasmic tail of the receptor, as expected.

[0017] **Figures 6A-6T** show the capacity of native IL-21 and candidate ortho-IL-21 molecules to induce signaling via native IL-21R α and the eight candidate ortho-IL-21R α molecules identified as shown in **Figure 3**. As described with respect to **Figure 5**, luminometry was used to quantify luciferase produced by transfected Ba/F3 cells carrying a STAT3-dependent luciferase reporter transgene resident on the same transposon used to confer expression of native IL-21R α or candidate ortho-IL-21R α molecules. The cells were exposed to increasing concentrations of the candidate ortho-IL-21 molecules overnight (approximately 20 hours) before testing the supernatant culture medium for luciferase activity.

[0018] **Figures 7A-7D** show the capacity of native IL-21 and selected candidate ortho-IL-21 molecules from the collection represented in **Figure 6** to induce signaling via native IL-21R α and selected candidate ortho-IL-21R α molecules. Ba/F3 cells (expressing native IL-21R α or candidate ortho-IL-21 molecules from a transposon that also carried a STAT3-luciferase reporter transgene) were exposed to increasing concentrations of the indicated candidate ortho-IL-21 molecules (for approximately 20 hours) before recovering supernatant medium and testing it for luciferase activity by luminometry as in **Figures 5 and 6**.

[0019] **Figures 8A, 8B, and 8C** show the results of a representative screening experiment in which a collection of 96 cytokines were tested for their capacity to induce STAT3-dependent

signaling responses in Ba/F3 cells expressing wild-type IL-21R α (**Figure 8A**) or the candidate ortho-IL-21R α molecules RV13 (**Figure 8B**) or RV6 (**Figure 8C**) comprising amino acid substitutions M70G/Y129F (SEQ ID NO: 19) or M70I/D73E/Q33H (SEQ ID NO: 12), respectively. As described with respect to **Figures 5** and **6**, luminometry was used to quantify luciferase produced by transfected Ba/F3 cells carrying a STAT3-dependent luciferase reporter transgene resident on the same transposon used to confer expression of native IL-21R α or the candidate ortho-IL-21R α molecule. The cells were exposed to increasing concentrations of the candidate ortho-IL-21 molecules overnight (approximately 20 hours) before testing the supernatant culture medium for luciferase activity. The dotted lines show response curves for the cytokine collection, while the responses caused by five cytokines of interest (wild-type IL-21, negative control CV22, and candidate ortho-IL-21 molecules CV204, CV374, and CV388) are highlighted with solid lines and symbols.

[0020] **Figures 9A** and **9B** show the capacity of native IL-21 and candidate ortho-IL-21 molecules to induce signaling via wild-type IL-21R α (**Figure 9A**) and the candidate ortho-IL-21R α RV13 comprising amino acid substitutions M70G/Y129F (SEQ ID NO: 19) (**Figure 9B**). As described with respect to **Figures 5** and **6**, luminometry was used to quantify luciferase produced by transfected Ba/F3 cells carrying a STAT3-dependent luciferase reporter transgene resident on the same transposon used to confer expression of native IL-21R α or the candidate ortho-IL-21R α molecule. The cells were exposed to increasing concentrations of the candidate ortho-IL-21 molecules overnight (approximately 20 hours) before testing the supernatant culture medium for luciferase activity.

[0021] **Figures 10A, 10B, and 10C** show the capacity of native IL-21 and candidate ortho-IL-21 molecules to induce signaling via wild-type IL-21R α (**Figure 10A**), the candidate ortho-IL-21R α

RV13 comprising amino acid substitutions M70G/Y129F (SEQ ID NO: 19) (**Figure 10B**), and the candidate ortho-IL-21R α RV22 comprising amino acid substitution M70G (SEQ ID NO: 27) (**Figure 10C**). As described with respect to **Figures 5** and **6**, luminometry was used to quantify luciferase produced by transfected Ba/F3 cells carrying a STAT3-dependent luciferase reporter transgene resident on the same transposon used to confer expression of native IL-21R α or the candidate ortho-IL-21R α molecule. The cells were exposed to increasing concentrations of the candidate ortho-IL-21 molecules overnight (approximately 20 hours) before testing the supernatant culture medium for luciferase activity.

DETAILED DESCRIPTION

[0022] Orthogonal IL-21 receptors and orthogonal IL-21 cytokines are provided. The orthogonal IL-21 receptor:cytokine pairs may include an ortho-IL-21R α that has impaired binding to native IL-21 and an ortho-IL-21 that has impaired binding to native IL-21R α , wherein the ortho-IL-21R α binds to ortho-IL-21. The orthogonal IL-21 receptor-cytokine pair may be used to activate a signaling response in cells. The signaling response may be the native one normally downstream of the IL-21 receptor or an alternative one dependent on non-native IL-21 receptors and/or other non-native cellular components. Cells engineered to express the orthogonal IL-21 receptors are also provided, as well as methods for using such cells for treatment of various diseases and disorders.

Definitions

[0023] The terminology as set forth herein is for description only and should not be construed as limiting of the invention. As used in the description and the appended claims, the singular forms “a,” “an,” and “the” are inclusive of their plural forms, unless otherwise expressed.

[0024] “Treat,” “treating,” “treatment,” and the like refer to any action providing a benefit to a subject afflicted with a disease or disorder such as cancer, including improvement in the condition through lessening or suppression of at least one symptom, delay in progression of the disease, and the like.

[0025] The phrases “effective amount” and “therapeutically effective amount” refer to an amount of the composition used in the practice of the invention that is sufficient to provide effective treatment in a subject.

[0026] “Wild type,” “WT,” and “native” are used interchangeably herein and mean an amino acid sequence or a nucleotide sequence that is found in nature, including allelic variations. A WT protein, polypeptide, antibody, immunoglobulin, IgG, and the like has an amino acid sequence or a nucleotide sequence corresponding to that normally found in nature, which has not been intentionally modified.

[0027] The term “polynucleotide” refers to oligonucleotides, nucleotides, or to a fragment of any of these; to DNA or RNA (e.g., mRNA, rRNA, tRNA) of genomic or synthetic origin, which may be single-stranded or double-stranded and may represent a sense or antisense strand; to peptide nucleic acids; or to any DNA-like or RNA-like material, whether natural or synthetic in origin. The term may also encompass nucleic acids, e.g., oligonucleotides, containing known analogs of natural nucleotides, as well as nucleic acid-like structures with synthetic backbones.

[0028] The term “polypeptide” refers to an oligopeptide, peptide, or protein sequence, or to a fragment, portion, or subunit of any of these, and to naturally occurring or synthetic molecules. The term “polypeptide” also includes amino acids joined to each other by peptide bonds or modified peptide bonds, i.e., peptide isosteres, and may contain any type of modified amino acids.

The term “polypeptide” also includes peptides and polypeptide fragments, motifs, and the like. A protein or peptide “chain” refers to a distinct subunit of a larger protein or protein complex.

[0029] By “homologs” is meant that the corresponding proteins (e.g., IL-21R α), such as those from other species, are substantially homologous at the overall protein (i.e., mature protein) level to the human protein, so long as such homologous peptides retain their respective known activities. Various levels of homology, from 35% to 99%, can be present.

[0030] The term “amino acid modification” includes an amino acid substitution, insertion, or deletion in a polypeptide sequence. By “amino acid substitution” or “substitution” is meant the replacement of an amino acid at a particular position in a parent polypeptide sequence with another amino acid. For example, the substitution R94K refers to a modified polypeptide in which the arginine at position 94 is replaced with a lysine. For the preceding example, 94K indicates the substitution of position 94 with a lysine. Multiple substitutions are typically separated by a slash or a comma. For example, R94K/L78V and [R94K, L78V] refer to a double variant comprising the substitutions R94K and L78V. By “amino acid insertion” or “insertion” is meant the addition of an amino acid at a particular position in a parent polypeptide sequence. For example, insert - 94 designates an insertion at position 94. By “amino acid deletion” or “deletion” is meant the removal of an amino acid at a particular position in a parent polypeptide sequence. For example, R94- designates the deletion of arginine at position 94.

[0031] The phrases “conservative modifications” and “conservative sequence modifications” refer to amino acid modifications that do not significantly affect or alter the binding characteristics of the polypeptide containing the amino acid sequence. Such conservative modifications include amino acid substitutions, insertions, and deletions. Modifications can be introduced into a protein by standard techniques known in the art, such as site-directed mutagenesis and PCR-mediated

mutagenesis, or by DNA synthesis. Conservative amino acid substitutions are ones in which the amino acid residue is replaced with an amino acid residue having a similar side chain. Families of amino acid residues having similar side chains have been defined in the art. These families include amino acids with basic side chains (e.g., lysine, arginine, histidine), acidic side chains (e.g., aspartic acid, glutamic acid), uncharged polar side chains (e.g., glycine, asparagine, glutamine, serine, threonine, tyrosine, cysteine, tryptophan), nonpolar side chains (e.g., alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine), beta-branched side chains (e.g., threonine, valine, isoleucine), and aromatic side chains (e.g., tyrosine, phenylalanine, tryptophan, histidine).

[0032] Ordinarily, the conservative substitution variants, homologs, and analogs of the peptides will have an amino acid sequence identity to the disclosed sequences of at least about 35%, at least about 45%, at least about 55%, at least about 65%, at least about 75%, at least about 80%, at least about 85%, at least about 90%, at least about 95%, or at least about 96% to 99%. Rost B. Twilight zone of protein sequence alignments. *Protein Eng.* 1999;12(2):85-94. doi:10.1093/protein/12.2.85.

[0033] Identity or homology with respect to such sequences is defined herein as the percentage of amino acid residues in the candidate sequence that are identical with the known peptides, after aligning the sequences and introducing gaps, if necessary, to achieve the maximum percent homology, and not considering any conservative substitutions as part of the sequence identity. N-terminal, C-terminal, or internal extensions, deletions, or insertions into the peptide sequence shall not be construed as affecting homology.

[0034] A gamma chain (γ c) cytokine means any cytokine where the cognate cytokine receptor complex includes the common cytokine receptor gamma chain (γ c). γ c cytokines include IL-2, IL-4, IL-7, IL-9, IL-15 and IL-21.

[0035] An orthogonal cytokine:receptor pair refers to variants of a natural cytokine:receptor pair that interact effectively with one another (i.e., such that they can be used to initiate physiologically consequential signaling responses in cells) but are significantly impaired in their capacity to interact with their natural counterparts. The orthogonal cytokine can either be a mutated or otherwise modified natural cytokine (a “mutein”) or a completely synthetic protein that acts as an agonist on the orthogonal cytokine receptor (sometimes referred to as a “synthekine”). The orthogonal cytokine (whether a mutein or a synthekine) shows no, or only attenuated, agonist activity toward the wild-type cytokine receptor.

[0036] Thus, the orthogonal cytokine:receptor pair may comprise a genetically engineered pair of proteins that are modified by amino acid changes to: (a) lack or reduce binding to the native cytokine or cognate receptor; and (b) specifically bind to the counterpart engineered (orthogonal) ligand or receptor. Upon binding of the orthogonal cytokine, the orthogonal receptor activates signaling that is transduced through native cellular elements to provide for a biological activity that mimics the native response, but that is specific to an engineered cell expressing the orthogonal receptor. Non-native receptor or cellular elements (e.g., non-native cytoplasmic domains in the receptor) can be involved to modify the signaling response. The orthogonal receptor does not bind to any endogenous cytokine or only does so with attenuated avidness, including the native counterpart of the orthogonal cytokine, while the orthogonal cytokine does not bind to any endogenous receptor or only does so with attenuated avidness, including the native counterpart of the orthogonal receptor. In some aspects, the affinity of the orthogonal cytokine for the orthogonal

receptor is comparable to the affinity of the native cytokine for the native receptor, e.g., having an affinity that is at least about 1% of the native cytokine receptor pair affinity, at least about 5%, at least about 10%, at least about 25%, at least about 50%, at least about 75%, at least about 100%, and may be higher, e.g. 2×, 3×, 4×, 5×, 10× or more of the affinity of the native cytokine for the native receptor.

[0037] The term “candidate” when used in qualification of an orthogonal protein (IL-21 or IL-21R α) infers a prototypic or developmental status. Analytical procedures may remove the qualification if a candidate protein is found to have the desired degree of binding privilege and orthogonal functionality.

[0038] As used herein, the phrases “does/do not bind” and “incapable of binding” refer to no detectable binding, or an insignificant binding, i.e., having a binding affinity much lower than that of the natural ligand. “Impaired binding” refers to binding that is lower than the normal level of binding between the corresponding wild-type components (e.g., cytokine and receptor).

[0039] The present invention includes an orthogonal cytokine system based on IL-21, which is a member of the γ c family of cytokines. IL-21 is structurally related to IL-2 and signals via a dimeric receptor comprised of IL-21R α and γ c. Whereas the IL-2 receptor promotes a STAT5-dominated signaling response, STAT3 dominates the IL-21 response. IL-21 promotes a form of T cell differentiation that correlates with good outcomes in adoptive cellular therapy (“ACT”), in particular ACT using CAR T cells, and it shows enhanced anti-tumor properties compared to IL-2 in various tumor model systems. As a result, IL-21 may prove to be a better adjunct for ACT than IL-2. Accordingly, in one aspect, an IL-21 orthogonal system is provided that comprises: 1) an ortho-IL-21 with impaired binding to native IL-21R α ; and 2) an ortho-IL-21R α capable of

binding the ortho-IL-21 while exhibiting impaired binding to native IL-21 (**Figure 1; Figure 2**, path A).

Orthogonal Interleukin-21 Receptors

[0040] In one aspect, an orthogonal interleukin-21 receptor is provided. The orthogonal interleukin-21 receptor can include modification of either of the chains making up the overall protein. In some aspects, an ortho-IL-21R α is provided. The ortho-IL-21R α includes a modified amino acid sequence derived from wild-type human IL-21R α (SEQ ID NO: 4 – human IL-21R α in mature form lacking the signal peptide). The ortho-IL-21R α binds to an ortho-IL-21 but has impaired binding to native IL-21. Human IL-21R α is represented by SEQ ID NO: 3.

[0041] In some aspects, the modified amino acid sequence comprises a substitution of one or more of the amino acid residues of SEQ ID NO: 4 that contact IL-21, residues in the immediate vicinity of such contact residues, or residues elsewhere in IL-21R α that have an influence on the conformation of the IL-21 binding surface. Amino acids within the immediate vicinity are those within 1, 2, 3, 4, or 5 amino acids of the contact residues in the primary protein sequence, or amino acid residues that are similarly nearby in the tertiary protein structure. In some aspects, the ortho-IL-21R α includes an amino acid substitution, numbered relative to SEQ ID NO: 6, at position: Y10, Q33, Q35, Y36, E38, L39, F67, H68, F69, M70, A71, D72, D73, I74, L94, A96, E97, P126, A127, Y129, M130, K134, S190, or Y191, or a combination thereof.

[0042] One strategy for creating an orthogonal version of IL-21 involves first mutating IL-21R α such that it suffers reduced binding capability to IL-21. Twenty IL-21R α amino acids make significant direct contacts with IL-21 within a binding surface of 990 Å². Mutating any of these 20 residues may impair the capacity of the receptor to bind normally to IL-21. For example, the methionine residue at position 70 of IL-21R α has been identified as a major contributor to the

binding interaction. The large hydrophobic side chain of this residue fits into an IL-21 pocket comprised of mostly hydrophilic residues (Arginine-9, Glutamine-12, Arginine-76, Lysine-73, and Isoleucine-16), repositioning some of them for improved contact with IL-21R α (notably, Arginine-9 and Arginine-76 of IL-21 contact Aspartic acid-72 and Aspartic acid-73 of IL-21R α , respectively). Changing Methionine-70 to a different residue could adjust this repositioning such that some of these contacts are weakened or lost. Changing Aspartic acid-72 or -73 of IL-21R α would also likely decrease the binding free energy of the interaction such that compensatory changes in IL-21 (*e.g.*, at positions 9 and 76) would be required to restore it.

[0043] Helix C of IL-21 exists in two interchangeable states (one disordered, the other α -helical) in the free structure of the cytokine. The α -helical form is stabilized in the complex of IL-21 with IL-21R α , as is the first part of the CD loop. Helix C of IL-21 contains the above-mentioned Lysine-73 and Arginine-76, which are proximal to Methionine-70 of IL-21R α . The CD loop of IL-21 includes Lysine-77, Proline-79, and Serine-80, which collectively form a pocket for Tyrosine-36 of IL-21R α ; Lysine-77 also makes an ionic contact with Glutamic acid-38 of IL-21R α . Changing the CD loop amino acid sequence to the analogous sequence found in IL-4 results in a ten-fold enhancement of IL-21 potency measured using a cellular assay. This observation suggests that significant binding energy is expended in stabilizing helix C of IL-21. Moreover, it suggests that changes to Glutamic acid-38 and Tyrosine-36 of IL-21R α could significantly impact IL-21 binding in a manner that might be reversible by compensatory changes to IL-21 in helix C or the CD loop.

[0044] Discrete avian sequence motifs can be used in the design of an orthogonal IL-21 system. Of 44 available avian sequences of IL-21, 39 have significantly (six residues) shortened CD loops compared to humans and mice. Together with the absence of Tyrosine-36 in all of the 74 available

avian IL-21R α sequences, this suggests helix C of avian IL-21 likely engages its receptor in a distinct fashion to that of human IL-21. This also provides an additional basis for modifications to the binding residues mentioned above for constructing an orthogonal IL-21 system.

[0045] Based on structural considerations, candidate amino acids may be identified that can be modified to change IL-21R α such that its binding to native IL-21 is impaired. These changes must, however, be of a character that will not be incompatible with compensatory changes in IL-21 (i.e., changes in IL-21 that would restore binding to ortho-IL-21R α). Changes to IL-21R α resulting in large alterations to its conformation are not desirable because the number of compensatory changes required to restore binding may either present too great an engineering challenge or be incompatible with cytokine function (e.g., because γ c binding is lost, or because the cytokine or cytokine receptor becomes unstable, difficult to express, immunogenic, or pharmacologically problematic).

[0046] At least two kinds of assays can be used to screen ortho-IL-21R α for a loss of binding to IL-21. One is a direct binding assay, which can be carried out using purified proteins and a sensitive biophysical analytical procedure such as surface plasmon resonance (“SPR”) or biolayer interferometry (“BLI”). Another assay is a functional assay involving an appropriate cell line. Ba/F3 cells have been used successfully for this purpose because they possess a number of useful traits: (i) they do not express endogenous IL-21R α ; (ii) they express mouse γ c, which substitutes effectively for human γ c in signaling with human IL-21R α and human IL-21; (iii) they respond to IL-21R signaling by phosphorylating STAT3 and proliferating; and (iv) they permit use of a STAT3 reporter transgene (such as one expressing luciferase) as a facile and attractive means for monitoring IL-21 signaling. Jurkat or Molt-3 cells are alternative choices, both of which show

minimal or absent expression of endogenous IL-21R α but express γc and are IL-21R signaling-competent. HeLa cells transfected to express γc may also be used.

[0047] The strategy for isolating candidate ortho-IL-21R α involves expressing the variants individually in Ba/F3 cells (or one of the alternatives just mentioned) by transfection. Flow cytometry is used to confirm the presence of the candidate ortho-IL-21R α molecules on the cell surface and the presence of epitopes recognized by available antibodies. In one aspect, the candidate ortho-IL-21R α includes an amino acid substitution at position: Y10, Q33, Q35, Y36, E38, L39, F67, H68, F69, M70, A71, D72, D73, I74, L94, A96, E97, P126, A127, Y129, M130, K134, S190, or Y191, or combinations thereof (where the numbers refer to the residue position in the mature IL-21R α ectodomain (SEQ ID NO: 6), and letters refer to amino acid identity using the single letter code, the first letter being the wild-type residue and the second, if present, the substitute residue). In one aspect, the amino acid substitution comprises, consists essentially of, or consists of: M70G, M70A, M70I, M70L, Y129F, Y129A, Y129S, Y129H, M130F, M130S, Y36H, Y36F, FMAD(69-73) to LLADI, FYM(128-130) to FHF, D72K, D73K, D72E, D73E, D73I, E38K, E38S, E38D, F67L, F67Y, F69L, S190Y, Y191F, Q35L, H68Q, I74V, L94I, L94V, E97D, A127T, Q33N, Q33H, K134R, M130I, M130L, S190T, or combinations thereof. In one aspect, the amino acid substitution comprises, consists essentially of, or consists of: D72E/Y129F/D73E; M70I/D73E/Q33H; D72E/L94V/Y191F; D72E/E38D/M130L; M70G/Y129F; F69L/M70L/D73I; D72K/D73K; E38K; or M70G, or combinations thereof. In one aspect, the amino acid substitution comprises, consists essentially of, or consists of: [H68Q,E38D,Q33H], [Y129F,H68Q,Y191F], [Y36F,H68Q,D73E], [Y129F,F67Y,M130L], [L94V,Q33H,M130L], [Y36F,F67Y,E38D], [D72E,E38D,M130L], [Y36F,M70I,L94V], [M70I,F67Y,Y191F], [Y129H, M130F], [Y129F, M130S], [M70G, D73E], [M70G, D73E,

Q33H], [M70I, D73E], [M70I, E38K], and [M70I, E38K, D73E]. In one aspect, the amino acid substitution comprises, consists essentially of, or consists of: M70G/Y129F or M70G.

[0048] Ba/F3 cells expressing candidate ortho-IL-21R α may be incubated with native human IL-21 before analysis for a signaling response. In some experiments, a time course may be used to improve assay sensitivity and resolution. Other experiments involve comparisons of dose-response curves. IL-21 responsiveness is detected by monitoring proliferation of the Ba/F3 cells, tyrosine phosphorylation of STAT3 by flow cytometry or immunoblotting, or expression of a reporter (such as luciferase or secreted alkaline phosphatase) from a STAT3-dependent reporter transgene present in the cells. Whereas native IL-21R α allows a robust response to IL-21 using any of these analytical techniques, the desired ortho- form of the receptor will have a reduced or no response. Ortho-IL-21R α molecules demonstrating this nonresponsive property are candidates for orthogonally restricted IL-21 cytokine-receptor systems.

Orthogonal Interleukin-21 Cytokines

[0049] Another aspect provides an ortho-IL-21 having a modified amino acid sequence derived from wild-type human IL-21 (SEQ ID NO: 2 – human IL-21 in mature form lacking the signal peptide) that binds to an ortho-IL-21R α but has impaired binding to native IL-21R α . Human IL-21 is represented by SEQ ID NO: 1.

[0050] In some aspects, the ortho-IL-21 binds to an ortho-IL-21R α but has impaired binding to native IL-21R α . In further aspects, the modified amino acid sequence comprises a substitution of one or more amino acid residues of SEQ ID NO: 2 that contact IL-21R α , residues in the immediate vicinity of such contact residues, or residues elsewhere in IL-21R that have an influence on the conformation of the IL-21 α binding surface.

[0051] Methods are also available to identify orthogonal forms of IL-21. Ten residues of IL-21 participate in polar interactions with IL-21R α : Arginine-5, Arginine-9, Arginine-11, Glutamine-12, Aspartic acid-15, Serine-70, Lysine-73, Arginine-76, Lysine-77, and Serine-80. Among these, Arginine-5, Arginine-9, Arginine-11, Glutamine-12, Lysine-73, Arginine-76, and Lysine-77 also form significant van der Waals contacts with IL-21R α . Isoleucine-8, Isoleucine-16, Glutamine-19, Tyrosine-23, Isoleucine-66, Valine-69, and Proline-79 make additional van der Waals contacts. Substitutions can be made to any of these residues to overcome the changes present in candidate ortho-IL-21R α molecules and restore binding.

[0052] The crystal structure of IL-21 bound by IL-21R α can be used to identify IL-21 residues proximal to changes engineered into IL-21R α . If, for example, changing Methionine-70 of IL-21R α is sufficient to abrogate IL-21 binding, then it is possible that compensatory changes to any of the following Methionine-70 contact residues in IL-21 may restore binding: Arginine-9, Glutamine-12, Isoleucine-16, Lysine-73, and Arginine-76. However, in addition to Methionine-70, these five IL-21 residues contact the following IL-21R α residues: Tyrosine-10, Leucine-39, Glutamic acid-38, Phenylalanine-67, Alanine-71, Aspartic acid-72, Aspartic acid-73, Tyrosine-129, Methionine-130, and Tyrosine-191. In turn, the ten IL-21R α residues just mentioned mediate additional contacts with IL-21 residues Arginine-5, Isoleucine-8, Glutamine-19, Serine-70, and Lysine-77. Therefore, just considering direct contacts present in the native IL-21:IL-21R α crystal structure, a single substitution at position Methionine-70 in IL-21R α could impact interactions mediated by ten IL-21 residues (Arginine-5, Isoleucine-8, Arginine-9, Glutamine-12, Isoleucine-16, Glutamine-19, Serine-70, Lysine-73, Arginine-76, and Lysine-77). This infers that compensation for the substitution (i.e., restoring IL-21 binding) may be accomplished by changes to one or more of these ten residues. It is also possible that changes to residues that do not make

direct contacts with IL-21R α can result in conformational adjustments that work at a distance to allow for binding to be restored to the Methionine-70-substituted variant of IL-21R α .

[0053] Phage display or yeast display technology can permit the screening of large mutation spaces. This is typically accomplished by creating highly diverse libraries of variants wherein small numbers of residues in a protein are changed in a random (or semi-random) combinatorial fashion. The library is then screened for the desired properties. For the present invention, screening can include identifying members of a library of IL-21 variants that are capable of binding to a candidate ortho-IL-21R α . If, as in the example just described, a substitution at Methionine-70 is sufficient to abrogate binding of IL-21R α to native IL-21, then an IL-21 library can be constructed that has randomized combinatorial mutations in the ten potentially impacted contact residues (Arginine-5, Isoleucine-8, Arginine-9, Glutamine-12, Isoleucine-16, Glutamine-19, Serine-70, Lysine-73, Arginine-76, and Lysine-77). The theoretical complexity of such a library could exceed 10^{13} . It is typically challenging and impractical to generate libraries comprised of more than 10^8 - 10^9 variants. Screening of such a library could involve a selection process in which phage or yeast displaying variant forms of IL-21 attached to their surfaces are separated based on their capacity to adhere to a matrix coated with a candidate ortho-IL-21R α (and not to a matrix coated with wild-type IL-21R α). Repeated cycles of such selection are performed to enrich for the desired properties in IL-21. Analytical screening procedures (including SPR or BLI) are used to characterize the products of the selection in detail and identify those with optimal properties.

[0054] In one aspect, the ortho-IL-21 comprises a modified amino acid sequence comprising a substitution of one or more amino acid residues of SEQ ID NO: 2 that contact IL-21R α , residues in the immediate vicinity of such contact residues, or residues elsewhere in IL-21 that have an influence on the conformation of the IL-21R α binding surface. In one aspect, the ortho-IL-21

includes an amino acid substitution, numbered relative to SEQ ID NO: 2, at position: R5, H6, I8, R9, M10, Q12, L13, K73, K75, R76, P78, G84, or P104, or a combination thereof. In one aspect, the amino acid substitution comprises, consists essentially of, or consists of: R5Q, R5D, R5E, R5K, R5N, H6L, I8E, R9E, R9D, R9K, R9N, R9Q, M10L, Q12V, L13D, K73V, K73D, K73I, K75D, R76E, R76N, R76A, R5Q/R76E, R5Q/R76A, P78L, G84E, or P104A, or combinations thereof. In one aspect, the amino acid substitution comprises, consists essentially of, or consists of: H6L/R9K/M10L/P78L.

Additional Method for Identifying Orthogonal IL-21 receptor-cytokine pairs

[0055] An alternative approach to the identification of ortho-IL-21 molecules involves iterative cycles of mutagenesis, again focused on small numbers of residues selected from those that make direct intermolecular contacts in the IL-21:IL-21R α structure. This approach may also include residues that are near to contact points and/or residues in potentially relevant structural features. A broad version of this approach can involve any of the residues that are proximal to the entire area of contact with IL-21R α and additional semi-randomly selected residues in proximal structural features. A more focused version involves residues – such as the ten potentially relevant for the Methionine-70 substitution – that might be directly impacted by the specific substitution(s) present in the candidate ortho-IL-21R α . An alternative broad approach is one in which substitutions are made with minimal, if any, consideration of the structure of IL-21, but instead with a partial emphasis on substitutions that occur in IL-21 orthologues from other species. This approach involves single substitutions in individual residues throughout the IL-21 primary sequence.

[0056] The iterative process begins with a large number (e.g., 10-100) of candidate ortho-IL-21 forms. In some versions of the approach, double or triple mutants are included in the initial

collection, but in other versions, only single point mutations of IL-21 are evaluated. These candidate ortho-IL-21 molecules are tested for activity using the cellular assay described above (employing, for example, Ba/F3 cells carrying a STAT3-dependent luciferase reporter transgene). A minimum of two kinds of cells are used in the assay: cells expressing a candidate ortho-IL-21R α and, as a counter-screen, cells expressing wild-type IL-21R α .

[0057] The likelihood of the alternative approach succeeding corresponds to the number of candidate ortho-IL-21R α molecules examined. Expanding this number reduces the likelihood of inadvertently selecting a candidate ortho-IL-21R α that does not readily allow for IL-21 binding to be restored (even partially) with small numbers (*e.g.*, less than three) of substitutions. Expanding this number also increases the likelihood of being able to isolate parallel mutually orthogonal systems that do not demonstrate crosstalk with each other or with wild-type IL-21 or IL-21R α .

[0058] The data from the initial screening round may be deconvoluted and analyzed focusing on identifying substitutions in IL-21 that in isolation promote improved binding to candidate ortho-IL-21R α molecules and diminished binding to native IL-21R α . A second round of screening may be performed in which positively scoring substitutions from the first round are combined in new candidate ortho-IL-21 molecules. These candidate ortho-IL-21 molecules (and, if considered desirable, additional variations in which conservative or nonconservative substitutions are made at the positively scoring positions) are tested again for improved binding to candidate ortho-IL-21R α molecules and impaired binding to native IL-21R α .

[0059] Additional rounds of screening may be performed involving further combinations of substitutions until at least one candidate ortho-IL-21 has been isolated with the desired properties (absence of activity with native IL-21R α and near-normal activity with at least one candidate ortho-IL-21R α).

[0060] The alternative screening approach may, in some circumstances, be facilitated using candidate ortho-IL-21R α molecules that retain reduced – but not entirely absent – binding to wild-type IL-21. Such reduced-binding candidate ortho-IL-21R α molecules may prove more permissive than non-binding candidate ortho-IL-21R α molecules (i.e., candidate ortho-IL-21R α molecules lacking any binding to wild-type IL-21) to a restoration of some IL-21 binding activity by small numbers (e.g., less than three) of discrete substitutions in IL-21. Once candidate ortho-IL-21 molecules have been isolated by the screening procedure outlined above, additional screening steps may be performed involving new candidate ortho-IL-21R α molecules in which additional substitutions are compounded with the ones already present. In some aspects, these additional mutations may entirely eliminate binding to wild-type IL-21 while retaining the capacity to bind the ortho-IL-21. Multiple rounds of this receptor mutagenesis may be performed along with subsequent refining cytokine mutagenesis rounds.

[0061] The binding properties of the products of the screening approach may be analyzed using purified proteins and BLI or SPR. The products that most closely resemble wild-type IL-21 and IL-21R α in their binding kinetics may be chosen as candidate orthogonal IL-21 systems.

[0062] In one aspect, candidate ortho-IL-21 molecules may be engineered according to the process described in one or more of U.S. Patent Nos. 8,005,620, 8,635,029, and 8,412,461, as well as Govindarajan S, Mannervik B, Silverman JA, et al. Mapping of amino acid substitutions conferring herbicide resistance in wheat glutathione transferase. *ACS Synth Biol.* 2015;4(3):221-227. doi:10.1021/sb500242x; Musdal Y, Govindarajan S, Mannervik B. Exploring sequence-function space of a poplar glutathione transferase using designed information-rich gene variants. *Protein Eng Des Sel.* 2017;30(8):543-549. doi:10.1093/protein/gzx045; and Liao J, Warmuth MK, Govindarajan S, et al. Engineering proteinase K using machine learning and

synthetic genes. *BMC Biotechnol.* 2007;7:16. Published 2007 Mar 26. doi:10.1186/1472-6750-7-16, each of which is incorporated by reference herein in its entirety.

[0063] In one aspect, candidate ortho-IL-21 molecules are expressed as fusion proteins between a modified amino acid sequence derived from SEQ ID NO: 2 as described above and a second amino acid sequence that facilitates purification, increases stability and half-life of the ortho-IL-21 molecules in vivo, or improves drug properties that are critical for successful dosing of ortho-IL-21 molecules in patients. Suitable second amino acid sequences are known in the art, and include, but are not limited to, serum albumin, Fc fragments of IgG, single-chain Fc antibody fragments, ABD035, and the like. Fc fragments can be modified, for example, with electrostatic steering mutations, to prevent, or at least significantly limit, the formation of homodimers.

Orthogonal IL-21 receptor-cytokine systems

[0064] Another aspect provides a system for activating IL-21 signaling in a cell. The system includes a candidate ortho-IL-21R α that has impaired binding to native IL-21, the candidate ortho-IL-21R α comprising a modified amino acid sequence derived from SEQ ID NO: 4 comprising a substitution of one or more of the amino acid residues of SEQ ID NO: 4 that contact IL-21, residues in the immediate vicinity of such contact residues, or residues elsewhere in IL-21R α that have an influence on the conformation of the IL-21 binding surface; and an ortho-IL-21 that has impaired binding to native IL-21R α , the ortho-IL-21 comprising a modified amino acid sequence derived from SEQ ID NO: 2 comprising a substitution of one or more amino acid residues of SEQ ID NO: 2 that contact IL-21R α , residues in the immediate vicinity of such contact residues, or residues elsewhere in IL-21 that have an influence on the conformation of the IL-21R α binding surface wherein the ortho-IL-21R α binds to the ortho-IL-21.

[0065] In some aspects, the cell is a T cell. In further aspects, the cell is a CAR T cell. The orthogonal cytokine and orthogonal receptor can be any of the candidate ortho-IL-21 and candidate ortho-IL-21R α molecules described herein. For example, in one aspect, the ortho-IL-21R α includes an amino acid substitution, numbered relative to SEQ ID NO: 6, comprising, consisting essentially of, or consisting of: M70G/Y129F or M70G; and the ortho-IL-21 includes an amino acid substitution, numbered relative to SEQ ID NO: 2, comprising, consisting essentially of, or consisting of one of: (1) H6L/R9K/M10L/P78L; (2) H6L/R9K/M10L/P78L/K73V; (3) H6L/R9K/M10L/P78L/K73I; (4) H6L/R9K/M10L/P78L/K73V/G84E; (5) H6L/R9K/M10L/P78L/K73I/G84E; (6) H6L/R9K/M10L/P78L/K73V/P104A; and (7) H6L/R9K/M10L/P78L/K73I/P104A.

Expression Vectors for Orthogonal Cytokines and Receptors

[0066] Another aspect provides expression vectors comprising a nucleic acid encoding any of the candidate orthogonal IL-21 proteins (e.g., receptors or cytokines) described herein. Orthogonal proteins, such as ortho-IL-21 or ortho-IL-21R α , may be produced by recombinant methods. Ortho-IL-21R α may be introduced on an expression vector into a cell to be engineered. DNA encoding an orthogonal protein may be obtained from various sources as designed during the engineering process.

[0067] Amino acid sequence variants may be prepared by introducing appropriate nucleotide changes into the nucleic acid coding sequence encoding the protein. The nucleic acid codons that encode amino acids are known to those skilled in the art. The specific codons selected may be chosen to optimize expression in the host cells being used. The amino acid variants may represent insertions, substitutions, and specified deletions of residues as described herein. Any combination of insertions, substitutions, and specified deletions may be made to arrive at the final construct,

provided that the final construct possesses the desired biological activity as defined herein. In some aspects, the nucleic acid encodes an ortho-IL-21R α as described herein.

[0068] Nucleic acids are “operably linked” when placed into a functional relationship with another nucleic acid sequence. For example, DNA for a signal sequence is operably linked to DNA for a polypeptide if the DNA for a signal sequence is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if the promoter or enhancer affects the transcription of the sequence; or a ribosome binding site is operably linked to a coding sequence if the ribosome binding site is positioned so as to facilitate translation. Generally, “operably linked” means that the DNA sequences being linked are contiguous, and, in the case of a secretory leader, contiguous and in reading phase. However, some sequences, such as enhancers, do not have to be contiguous to be effective.

[0069] The nucleic acid encoding the ortho-IL-21 or ortho-IL-21R α may be inserted into a replicable vector for expression. Many such vectors are available. The vector components generally include, but are not limited to, one or more of the following: an origin of replication, one or more marker genes, an enhancer element, a promoter, and a transcription termination sequence. Vectors may include viral vectors, plasmid vectors, integrating vectors, transposons, and the like. For example, a suitable transposon/transposase-based polynucleotide vector system is described in U.S. Patent No. 10,041,077, which is incorporated by reference herein in its entirety.

[0070] The ortho-IL-21 or ortho-IL-21R α may be recombinantly produced without modification or as a fusion polypeptide with a heterologous polypeptide, e.g., a signal sequence or other polypeptide having a specific cleavage site at the N-terminus of the mature protein or polypeptide. In general, the signal sequence may be a component of the vector, or it may be a part of the coding

sequence that is inserted into the vector. The heterologous signal sequence selected may be one that is recognized and processed (i.e., cleaved by a signal peptidase) by the host cell. In mammalian cell expression, the native signal sequence may be used, or other mammalian signal sequences may be suitable, such as signal sequences from secreted polypeptides of the same or related species, as well as viral secretory leaders, for example, the herpes simplex gD signal.

[0071] Expression vectors typically contain a selection gene, also termed a selectable marker. The selection gene encodes a protein necessary for the survival or growth of transformed host cells grown in a selective culture medium. Host cells not transformed with the vector containing the selection gene will not survive in the culture medium. Typical selection genes encode proteins that: (a) confer resistance to antibiotics or other toxins, e.g., ampicillin, neomycin, methotrexate, or tetracycline, (b) complement auxotrophic deficiencies, or (c) supply critical nutrients not available from complex media.

[0072] Expression vectors may contain a promoter that may be recognized by the host organism and may be operably linked to an orthogonal protein coding sequence. Promoters may be untranslated sequences located upstream (5') to the start codon of a structural gene (generally within about 100 to 1000 bp) that control the transcription and translation of the particular nucleic acid sequence to which they are operably linked. Such promoters typically fall into two classes: inducible and constitutive. Inducible promoters are promoters that initiate increased levels of transcription from DNA under their control in response to some change in culture conditions, e.g., the presence or absence of a nutrient or a change in temperature.

[0073] Transcription from vectors in mammalian host cells may be controlled, for example, by promoters obtained from the genomes of viruses such as polyoma virus, fowlpox virus, adenovirus (such as Adenovirus 2), bovine papilloma virus, avian sarcoma virus, cytomegalovirus, a retrovirus

(such as murine stem cell virus), hepatitis-B virus, and Simian Virus 40 ("SV40"), from heterologous mammalian promoters, e.g., the actin promoter, phosphoglycerate kinase ("PGK"), or an immunoglobulin promoter, from heat-shock promoters, provided such promoters are compatible with the host cell systems. The early and late promoters of SV40 are conveniently obtained as an SV40 restriction fragment that also contains the SV40 viral origin of replication.

[0074] The expression vector may also include an enhancer sequence. Enhancers are cis-acting elements of DNA, usually about from 10 to 300 bp, which act on a promoter to increase its transcription. Enhancers are relatively orientation and position independent, having been found 5' and 3' to the transcription unit, within an intron, as well as within the coding sequence itself. Many enhancer sequences are known from mammalian genes (e.g., globin, elastase, albumin, α -fetoprotein, and insulin). Typically, however, one will use an enhancer from a eukaryotic cell virus. Examples may include the SV40 enhancer on the late side of the replication origin, the cytomegalovirus early promoter enhancer, the polyoma enhancer on the late side of the replication origin, and adenovirus enhancers. The enhancer may be spliced into the expression vector at a position 5' or 3' to the coding sequence but is preferably located at a site 5' from the promoter.

[0075] Expression vectors used in eukaryotic host cells will also contain sequences necessary for the termination of transcription and for stabilizing the mRNA. Such sequences are commonly available from the 5' and 3' untranslated regions of eukaryotic or viral DNAs or cDNAs.

[0076] Expression vectors might also be comprised of inducible regulatory elements for the purpose of controlling expression of a transgene (encoding, for example, ortho-IL-21 or ortho-IL-21R α) with small molecules or other stimulatory agents. Examples of regulatory elements include, but are not limited to, promoters containing tetracycline operators that render them sensitive to regulation by tetracycline or derivatives thereof (such as doxycycline). Promoters may also be

inducibly regulated by CRISPRa (clustered regularly interspaced short palindromic repeats-activation) using fusions of transcriptional effectors and catalytically dead Cas9. Such promoters may in turn be downstream of other control systems such as those involving dimerizers (of an antibody-based and/or chemical nature) or components based on the Notch receptor.

Engineered Cells

[0077] One aspect of the invention provides cells that have been engineered to express an ortho-IL-21R α . The cells may be genetically engineered to include any suitable expression vector described herein. In some aspects, the expression vector comprises a coding sequence that encodes the orthogonal receptor, the coding sequence being operably linked to a promoter active in the desired cell. Various vectors may be used for this purpose, e.g., transposons, viral vectors, plasmid vectors, and minicircle vectors, which can be integrated into the target cell genome or can be episomally maintained. In one aspect, the expression vector is a synthetic transposon that can be integrated into the genome by means of a transposase enzyme. Examples of transposon/transposase systems include Sleeping Beauty, PiggyBac, LeapIn® from ATUM Bio, and derivatives thereof.

[0078] The engineered cell may be a host cell for preparing recombinant protein in vitro. Suitable host cells for recombinant expression of orthogonal proteins include prokaryotes, yeast, and higher eukaryote cells, such as various mammalian host cell lines.

[0079] In some aspects, the engineered cell is further modified beyond the expression of an ortho-IL-21R α . Modifications suitable for use in engineered cells are known in the art and include expression of a CAR, a T cell Receptor ("TCR"), or other receptor or receptor derivatives that recognize specific antigens on antigen presenting cells.

[0080] In some aspects, the engineered cell is a cell intended for therapeutic use. Examples of therapeutic engineered cells may include stem cells, e.g., a hematopoietic stem cell, a natural killer (“NK”) cell, or a T cell. In some aspects, the engineered cell is a T cell. The term “T cells” refers to mammalian immune effector cells that may be characterized by expression of a CD3 and/or a T cell antigen receptor, which cells may be engineered to express an ortho-IL-21R α . In some aspects, the T cells are selected from naïve, activated, or post-activation CD8⁺ T cells; cytotoxic CD8⁺ T cells; naïve, activated, or post-activation CD4⁺ T cells; helper T cells, e.g., TH1, TH2, TH9, TH11, TH22, and TFH; regulatory T cells, e.g., TR1, natural TReg, and inducible TReg; and memory T cells, e.g., central memory T cells, effector memory T cells, NKT cells, and $\gamma\delta$ T cells.

[0081] Ortho-IL-21 may be used as an adjunct to ACT. T cells may be engineered to express the ortho-IL-21R α by gene (cDNA, minigene, or other nucleic acid construct) transfection, transduction, or transposition. Patients receiving the ACT may be treated (and/or pretreated) with the ortho-IL-21 and dosed repeatedly as needed to augment and sustain a desirable T cell presence and responses.

[0082] Therapeutic cells may also be engineered to express ortho-IL-21. This could be accomplished using any of the methods appropriate for ectopic expression of ortho-IL-21R α . The ortho-IL-21 could be expressed in the same or different cells as those that express ortho-IL-21R α , allowing for autocrine or paracrine action, respectively. An example of a paracrine arrangement could be CD4⁺ T cells expressing the ortho-IL-21 and CD8⁺ T cells expressing the matched ortho-IL-21R α .

[0083] In some aspects, ortho-IL-21 may be expressed in a membrane-tethered form. This has previously been accomplished with natural IL-21 by fusing the cytokine to the amino-terminus of an IgG4 CH₂-CH₃ moiety that was itself fused to a CD4 transmembrane domain. Related strategies

have been employed to tether other cytokines to the membranes of cells. Such membrane tethering limits the diffusion of the cytokine and restricts its action to the immediate vicinity of the cells expressing the membrane-bound cytokine. In vivo, this approach could be exploited to ensure ortho-IL-21R α -expressing cells only encounter the ortho-IL-21 when they are proximal to a specific type of cell and/or location in the body. In vitro, the approach may facilitate certain kinds of selective differentiation protocols (e.g., the differentiation of NK cells from stem cells in the presence of K562 [or other] feeder cells expressing membrane-bound IL-21 and CD137L).

[0084] Engineered cells may be provided in pharmaceutical compositions suitable for therapeutic use, e.g., for human treatment. Therapeutic formulations comprising such cells can be frozen or prepared for administration with physiologically acceptable carriers, excipients, or stabilizers (Remington's Pharmaceutical Sciences 16th edition, Osol, A. Ed. (1980)) in the form of aqueous solutions. The cells may be formulated, dosed, and administered in a fashion consistent with good medical practice.

Methods of Treatment

[0085] A cell such as a T cell engineered to express one of the ortho-IL-21R α molecules described herein may be used to treat a broad range of conditions. Engineered properties in this therapy may allow for beneficial T cell differentiation, resistance to exhaustion, capacity for long-term persistence, anamnestic responses, and in-built safety features allowing for responses to be halted when they become pathogenic.

[0086] Methods are provided for enhancing cellular responses by engineering cells from a recipient or donor by introduction of an ortho-IL-21R α of the invention and stimulating the ortho-IL-21R α by contacting the engineered cell with ortho-IL-21. The subject methods may include a step of obtaining the targeted cells, e.g., T cells, hematopoietic stem cells, etc., which may be

isolated from a biological sample or may be derived in vitro from a source of progenitor cells. The cells may be transduced or transfected with an expression vector comprising a sequence encoding the ortho-IL-21R α , which step may be performed in any suitable culture medium.

[0087] In some aspects, the engineered T cells may be contacted with the ortho-IL-21 in vivo, i.e., where the engineered T cells are transferred to a recipient, and an effective dose of the ortho-IL-21 is injected into the recipient and allowed to contact the engineered T cells in their native environment, e.g., in lymph nodes, etc. In other aspects, the contacting is performed in vitro. In such in vitro aspects, the contacting may be accomplished using soluble ortho-IL-21 comprised, or not, of a fusion to another protein moiety such as an immunoglobulin Fc domain. In further such in vitro aspects, the contacting could be accomplished by encounter with other cells expressing secreted or membrane-tethered ortho-IL-21.

[0088] Another aspect provides a method for treating a subject in need thereof, including introducing an engineered cell expressing an ortho-IL-21R α to the subject and activating the cell by contacting it with an effective amount of an ortho-IL-21. In some aspects, the cell is a T cell, while in further aspects the cell is a CAR-T cell. In another aspect, the cell is a T cell expressing a native or modified TCR. In another aspect, the cell is an NK cell. In another aspect, the cell is a macrophage or other myeloid cell or a leukocyte.

[0089] In some aspects, the ortho-IL-21 is delivered as a fusion protein with a heterologous polypeptide. Suitable heterologous polypeptides are known in the art and include serum albumin, Fc fragments of IgG, single-chain Fc antibody fragments, ABD035, and the like. Fc fragments may be modified, for example, with electrostatic steering or other mutations, to prevent, or at least significantly limit, the formation of homodimers.

[0090] Another aspect provides a method for treating a subject in need thereof, including introducing an engineered cell expressing an ortho-IL-21R α to the subject and introducing a second engineered cell expressing an ortho-IL-21. In some aspects, the cell is a T cell, while in further aspects the cell is a CAR-T cell.

[0091] A “subject,” can be any mammal and may also be referred to as a “patient.” Examples of mammalian subjects include research animals (e.g., a mouse or rat), domesticated farm animals (e.g., cow, horse, pig), pets (e.g., dog, cat), and humans. In some aspects, the subject is a human.

[0092] In some aspects, the subject being treated has been diagnosed as having cancer. “Cancer” and “malignancy” are used as synonymous terms and refer to any of a number of diseases that are characterized by uncontrolled, abnormal proliferation of cells, the ability of affected cells to spread locally or through the bloodstream and lymphatic system to other parts of the body (i.e., metastasize), as well as any of a number of characteristic structural and molecular features. A “cancer cell” refers to a cell undergoing early, intermediate, or advanced stages of multi-step neoplastic progression. The features of early, intermediate, and advanced stages of neoplastic progression have been described using microscopy. Cancer cells at each of the three stages of neoplastic progression generally have abnormal karyotypes, including translocations, inversion, deletions, isochromosomes, monosomies, and extra chromosomes. Cancer cells include “hyperplastic cells,” that is, cells in the early stages of malignant progression, “dysplastic cells,” that is, cells in the intermediate stages of neoplastic progression, and “neoplastic cells,” that is, cells in the advanced stages of neoplastic progression. Examples of cancers are sarcoma, breast, lung, brain, bone, liver, kidney, colon, and prostate cancer. In some aspects, the engineered cells are used to treat cancer selected from the group consisting of colon cancer, brain cancer, breast cancer, fibrosarcoma, and squamous carcinoma. In some aspects, the cancer is selected from the

group consisting of melanoma, breast cancer, colon cancer, lung cancer, and ovarian cancer. In some aspects, the cancer being treated is metastatic cancer.

[0093] In case of cancer treatment, the method of treatment may further include the step of ablating the cancer. Ablating the cancer may be accomplished using a method selected from the group consisting of cryoablation, thermal ablation, radiotherapy, chemotherapy, radiofrequency ablation, electroporation, alcohol ablation, high intensity focused ultrasound, photodynamic therapy, administration of monoclonal antibodies, and administration of immunotoxins.

[0094] In some aspects, the subject being treated has been diagnosed as having an infection. As used herein, the term “infection” refers to infection of one or more cells of a subject by an infectious agent. Infectious agents include, but are not limited to, bacteria, viruses, protozoans, and fungi. Intracellular pathogens are of particular interest. Infectious diseases are disorders caused by infectious agents. Some infectious agents cause no recognizable symptoms or disease under certain conditions but have the potential to cause symptoms or disease under changed conditions. The subject methods may be used in the treatment of chronic pathogen infections, including but not limited to viral infections, e.g., retrovirus, lentivirus, hepadnavirus, herpes viruses, pox viruses, and human papilloma viruses; intracellular bacterial infections, e.g., *Mycobacterium*, *Chlamydia*, *Ehrlichia*, *Rickettsia*, *Brucella*, *Legionella*, *Francisella*, *Listeria*, *Coxiella*, *Neisseria*, *Salmonella*, *Yersinia* sp, and *Helicobacter pylori*; and intracellular protozoan pathogens, e.g., *Plasmodium* sp, *Trypanosoma* sp, *Giardia* sp, *Toxoplasma* sp, and *Leishmania* sp.

[0095] In some aspects, the subject being treated has been diagnosed as having an autoimmune disease. Autoimmune diseases are characterized by T and B lymphocytes that aberrantly target self-proteins, -polypeptides, -peptides, or other self-molecules, causing injury and/or malfunction of an organ, tissue, or cell-type within the body. Autoimmune diseases include diseases such as

rheumatoid arthritis, systemic lupus erythematosus, multiple sclerosis, autoimmune hepatitis, insulin dependent diabetes mellitus, and degenerative diseases such as osteoarthritis, Alzheimer's disease, and macular degeneration.

[0096] To effect treatment, one or more cells of the subject may be contacted with ortho-IL-21. Where the cells are contacted with the ortho-IL-21 in vitro, the ortho-IL-21 may be added to the engineered cells in a dose and for a period of time sufficient to activate signaling from the ortho-IL-21R α , which may utilize the native cellular machinery, e.g., accessory proteins, co-receptors, and the like. Any suitable culture medium may be used. The cells thus activated may be used for any desired purpose, including experimental purposes relating to determination of antigen specificity, cytokine profiling, and the like, and for delivery in vivo.

[0097] Where the contacting is performed in vivo, an effective dose of engineered cells expressing ortho-IL-21R α are infused to the recipient, in combination with or prior to administration of the ortho-IL-21. Dosage and frequency may vary depending on the agent, mode of administration, and the like. The dosage may also be varied for localized administration, e.g., intranasal, inhalation, and the like, or for systemic administration, e.g., i.m., i.p., i.v., and the like. Generally, at least about 10^4 engineered cells/kg are administered, at least about 10^5 engineered cells/kg, at least about 10^6 engineered cells/kg, at least about 10^7 engineered cells/kg, or more.

EXAMPLES

Example 1: Binding Assay for Identification of Candidate Ortho-IL-21R α Molecules with Impaired Binding to Native IL-21.

[0098] A direct interaction assay (quantifying the capacity of IL-21R α to bind native or mutant forms of IL-21) provides an alternative to a cell-based assay for the identification of variants of IL-21R α with compromised IL-21 binding activity (i.e., candidate orthogonal variants). The

feasibility of exploiting such an assay is enhanced by the fact that the native interaction (IL-21R α :IL-21) is avid (KD~70pM). Of many possible forms of a binding assay, one involving luciferase fusion proteins is appealing because of its simplicity and the potential for it to be adapted to a medium- or high-throughput format.

[0099] One version of an IL-21R α :IL-21 binding assay involves attaching the receptor ectodomain to a surface, bathing the coated surface in a solution of an IL-21-luciferase fusion protein, followed by quantitation of bound IL-21 based on luminescence when the relevant luciferase substrate is added. Alternatively, IL-21 may be immobilized, and an IL-21R α -luciferase fusion protein may be used in solution.

[0100] In either form of the binding assay, a desirable orientation of the immobilized receptor or cytokine can be accomplished through use of an affinity tag such as Twin-Strep-Tag II, which is a high affinity peptide ligand for the Streptactin protein. The IL-21R α ectodomain bearing a carboxy-terminal Twin-Strep-Tag II peptide can be efficiently and selectively immobilized on the surfaces of wells of 96-well plates that have been pre-coated with Streptactin protein. In this manner, the immobilized IL-21R α should be oriented with its cytokine-binding domain distal from the plate surface. Similarly, IL-21 may be immobilized in a related fashion if it, too, bears an amino- or carboxy-terminal Twin-Strep-Tag II peptide tag.

[0101] An assay was established using plate-immobilized human IL-21R α ectodomain (via a carboxy-terminal Twin-Strep-Tag II) and a fusion protein (SEQ ID NO: 5) ("IL-21-TLuc16" in **Figure 3**) in which the 16KD Turbo-luciferase polypeptide (ThermoFisher) was linked (via a Glycine-Serine-containing peptide) to the carboxy-terminus of human IL-21. Bound IL-21 was detected using the TurboLuc Luciferase One-Step Glow Assay Kit (ThermoFisher) and standard

luminometry. This assay could also be established with luciferase fused at the amino-terminus of IL-21 or with an alternative form of luciferase (e.g., NanoLuc; ThermoFisher).

[0102] Twenty candidate ortho-IL-21R α molecules were tested for their capacity to bind IL-21-TLuc16. The wild-type human IL-21R α ectodomain (mature form lacking the signal peptide) (V0 (SEQ ID NO: 6)) and the 20 candidate ortho-IL-21R α molecules shown in Table 1 (SEQ ID NOs: 7-25 & 63) (substitutions shown in the headers and by underline within the sequences) were expressed in HEK 293 cells as secreted proteins.

Table 1

SEQ ID NO: 6	Human IL-21R α ectodomain (mature form lacking the signal peptide) CPDLVCYTDYLQTVICILEMWNLHPSTLTLTWQDQYEELKDEATSC SLHRSAHNATHATYTCHMDVFHFMADDIFSVNITDQSGNYSQECGS FLLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFYMLKGKLQYEL QYRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSYELQVRAGP MPGSSYQGTWSEWSDPVIFQTQSEELKE
SEQ ID NO: 7	>RV1 H68Q, E38D, Q33H ectodomain CPDLVCYTDYLQTVICILEMWNLHPSTLTLTWHDQYEDLKDEATSC SLHRSAHNATHATYTCHMDVFQFMADDIFSVNITDQSGNYSQECGS FLLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFYMLKGKLQYEL QYRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSYELQVRAGP MPGSSYQGTWSEWSDPVIFQTQSEELKE
SEQ ID NO: 8	>RV2 D72E, Y129F, D73E ectodomain CPDLVCYTDYLQTVICILEMWNLHPSTLTLTWQDQYEELKDEATSC SLHRSAHNATHATYTCHMDVFHFM ~~AE~~ IFSVNITDQSGNYSQECGS FLLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFFMLKGKLQYEL QYRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSYELQVRAGP MPGSSYQGTWSEWSDPVIFQTQSEELKE
SEQ ID NO: 9	>RV3 Y129F, H68Q, Y191F ectodomain CPDLVCYTDYLQTVICILEMWNLHPSTLTLTWQDQYEELKDEATSC SLHRSAHNATHATYTCHMDVFQFMADDIFSVNITDQSGNYSQECGS FLLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFFMLKGKLQYEL QYRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSYELQVRAGP MPGSS ~~F~~ QGTWSEWSDPVIFQTQSEELKE
SEQ ID NO: 10	>RV4 Y36F, H68Q, D73E ectodomain

	CPDLVCYTDYLQTVICILEMWNLHPSTLTLTWQDQFEELKDEATSC SLHRSAHNATHATYTCHMDVFQFMADEIFSVNITDQSGNYSQECGS FLLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFYMLKGKLQYEL QYRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSYELQVRAGP MPGSSYQGTWSEWSDPVIFQTQSEELKE
SEQ ID NO: 11	>RV5 Y129F, F67Y, M130L ectodomain CPDLVCYTDYLQTVICILEMWNLHPSTLTLTWQDQYEELKDEATSC SLHRSAHNATHATYTCHMDVYHFMADDIFSVNITDQSGNYSQECG SFLLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFFLLKGKLQYEL QYRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSYELQVRAGP MPGSSYQGTWSEWSDPVIFQTQSEELKE
SEQ ID NO: 12	>RV6 M70I, D73E, Q33H ectodomain CPDLVCYTDYLQTVICILEMWNLHPSTLTLTWHDQYEELKDEATSC SLHRSAHNATHATYTCHMDVFHFIADEIFSVNITDQSGNYSQECGSF LLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFYMLKGKLQYELQ YRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSYELQVRAGPM PGSSYQGTWSEWSDPVIFQTQSEELKE
SEQ ID NO: 13	>RV7 D72E, L94V, Y191F ectodomain CPDLVCYTDYLQTVICILEMWNLHPSTLTLTWQDQYEELKDEATSC SLHRSAHNATHATYTCHMDVFHFMAEDIFSVNITDQSGNYSQECGS FVLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFYMLKGKLQYEL QYRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSYELQVRAGP MPGSSFQGTWSEWSDPVIFQTQSEELKE
SEQ ID NO: 14	>RV8 L94V, Q33H, M130L ectodomain CPDLVCYTDYLQTVICILEMWNLHPSTLTLTWHDQYEELKDEATSC SLHRSAHNATHATYTCHMDVFHFMAADDIFSVNITDQSGNYSQECGS FVLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFYLLKGKLQYEL QYRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSYELQVRAGP MPGSSYQGTWSEWSDPVIFQTQSEELKE
SEQ ID NO: 15	>RV9 Y36F, F67Y, E38D ectodomain CPDLVCYTDYLQTVICILEMWNLHPSTLTLTWQDQFEDLKDEATSC SLHRSAHNATHATYTCHMDVYHFMADDIFSVNITDQSGNYSQECG SFLLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFYMLKGKLQYE LQYRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSYELQVRAGP MPGSSYQGTWSEWSDPVIFQTQSEELKE
SEQ ID NO: 16	>RV10 D72E, E38D, M130L ectodomain CPDLVCYTDYLQTVICILEMWNLHPSTLTLTWQDQYEDLKDEATSC SLHRSAHNATHATYTCHMDVFHFMAEDIFSVNITDQSGNYSQECGS FLLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFYLLKGKLQYEL

	QYRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSYELQVRAGP MPGSSYQGTWSEWSDPVIFQTQSEELKE
SEQ ID NO: 17	>RV11 Y36F, M70I, L94V ectodomain CPDLVCYTDYLQTVICILEMWNLHPSTLTLTWQDQFEELKDEATSC SLHRSAHNATHATYTCHMDVFHFIADDIFSVNITDQSGNYSQECGSF YLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFYMLKGKLQYEL QYRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSYELQVRAGP MPGSSYQGTWSEWSDPVIFQTQSEELKE
SEQ ID NO: 18	>RV12 M70I, F67Y, Y191F ectodomain CPDLVCYTDYLQTVICILEMWNLHPSTLTLTWQDQYEELKDEATSC SLHRSAHNATHATYTCHMDVYHFIADDIFSVNITDQSGNYSQECGS FLLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFYMLKGKLQYEL QYRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSYELQVRAGP MPGSSYQGTWSEWSDPVIFQTQSEELKE
SEQ ID NO: 19	>RV13 M70G, Y129F ectodomain CPDLVCYTDYLQTVICILEMWNLHPSTLTLTWQDQYEELKDEATSC SLHRSAHNATHATYTCHMDVFHFGADDIFSVNITDQSGNYSQECGS FLLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFEMMLKGKLQYEL QYRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSYELQVRAGP MPGSSYQGTWSEWSDPVIFQTQSEELKE
SEQ ID NO: 20	>RV14 Y36H ectodomain CPDLVCYTDYLQTVICILEMWNLHPSTLTLTWQDQHEELKDEATSC SLHRSAHNATHATYTCHMDVFHFMMADDIFSVNITDQSGNYSQECGS FLLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFYMLKGKLQYEL QYRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSYELQVRAGP MPGSSYQGTWSEWSDPVIFQTQSEELKE
SEQ ID NO: 21	>RV15 F69L M70L D73I ectodomain CPDLVCYTDYLQTVICILEMWNLHPSTLTLTWQDQYEELKDEATSC SLHRSAHNATHATYTCHMDVFHLLADIIFSVNITDQSGNYSQECGSF LLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFYMLKGKLQYELQ YRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSYELQVRAGPM PGSSYQGTWSEWSDPVIFQTQSEELKE
SEQ ID NO: 22	>RV16 Y129H M130F ectodomain CPDLVCYTDYLQTVICILEMWNLHPSTLTLTWQDQYEELKDEATSC SLHRSAHNATHATYTCHMDVFHFMMADDIFSVNITDQSGNYSQECGS FLLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFHFLKGKLQYEL QYRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSYELQVRAGP MPGSSYQGTWSEWSDPVIFQTQSEELKE
SEQ ID NO: 23	>RV17 Y129F, M130S ectodomain

	CPDLVCYTDYLQTVICILEMWNLHPSTLTLTWQDQYEELKDEATSC SLHRSAHNATHATYTCHMDVFHFMADDIFSVNITDQSGNYSQECGS FLLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFFSLKGKLQYEL QYRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSEYELQVRAGP MPGSSYQGTWSEWSDPVIFQTQSEELKE
SEQ ID NO: 24	>RV18 D72K, D73K ectodomain CPDLVCYTDYLQTVICILEMWNLHPSTLTLTWQDQYEELKDEATSC SLHRSAHNATHATYTCHMDVFHFM ~~AKK~~ IFSVNITDQSGNYSQECGS FLLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFYMLKGKLQYEL QYRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSEYELQVRAGP MPGSSYQGTWSEWSDPVIFQTQSEELKE
SEQ ID NO: 25	>RV19 E38K ectodomain CPDLVCYTDYLQTVICILEMWNLHPSTLTLTWQDQYE ~~KL~~ KDEATSC SLHRSAHNATHATYTCHMDVFHFMADDIFSVNITDQSGNYSQECGS FLLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFYMLKGKLQYEL QYRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSEYELQVRAGP MPGSSYQGTWSEWSDPVIFQTQSEELKE
SEQ ID NO: 63	>RV20 F67L ectodomain CPDLVCYTDYLQTVICILEMWNLHPSTLTLTWQDQYEELKDEATSC SLHRSAHNATHATYTCHMDV ~~L~~ HFMADDIFSVNITDQSGNYSQECGS FLLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFYMLKGKLQYEL QYRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSEYELQVRAGP MPGSSYQGTWSEWSDPVIFQTQSEELKE

[0103] Clarified supernatant fluids from the transiently transfected cells were tested for the presence of IL-21R α with an Enzyme-Linked Immunosorbent Assay (“ELISA”) comprised of Streptactin-coated plates, dilutions of the supernatant fluids, and detection using the combination of a mouse monoclonal antibody specific for the human IL-21R α , a horseradish peroxidase-conjugated rat antibody specific for mouse IgG, and a chromogenic substrate for the peroxidase. This ELISA established the dilutions required to ensure saturation of the Streptactin-coated wells with each of the candidate ortho-IL-21R α molecules. Candidate ortho-IL-21R α saturated wells were incubated with a solution of IL-21-TLuc16 for 1 h (or longer in some experiments) at 4 °C

(or room temperature in some experiments). The wells were washed before addition of the luciferase substrate (Coelenterazine) solution and luminometry.

[0104] **Figure 3** shows the results of a representative assay in which a single (sub-saturating) concentration of IL-21-TLuc16 was tested for binding to the panel of 20 candidate ortho-IL-21R α molecules (SEQ ID NOs: 7-25 & 63) (all of which had been bound to the Streptactin-coated surfaces of the wells at saturating concentrations). Eight of the candidate ortho-IL-21R α molecules showed diminished capacity to bind IL-21-TLuc16. Repeat experiments (involving titrations of the IL-21-TLuc16) confirmed the results.

[0105] The eight candidate IL-21R α molecules (the “Receptor Variants” or “RV”s) that showed impaired binding to IL-21-TLuc16 (i.e., RV2, RV6, RV7, RV10, RV13, RV15, RV18, and RV19) may be tested for their capacity to bind candidate ortho-IL-21 with the eventual goal of isolating forms of the cytokine(s) that bind productively (with respect to signaling and immunologic activity) to candidate ortho-IL-21R α but not to native IL-21R α .

[0106] An additional 13 candidate ortho-IL-21R α molecules carrying alternative combinations of the amino acid substitutions present in the eight candidate ortho-IL-21R α molecules were similarly tested for their capacity to bind IL-21-TLuc16. The 13 candidate ortho-IL-21R α molecules shown in **Table 2** (SEQ ID NOs: 26-38) (substitutions shown in the headers and by underline within the sequences) were expressed in HEK 293 cells as secreted proteins.

Table 2

SEQ ID NO: 26	>RV21 D72E ectodomain CPDLVCYTDYLQTVICILEMWNLHPSTLTLTWQDQYEELKDEATSC SLHRSAHNATHATYTCHMDVFHFMAEDIFSVNITDQSGNYSQECGS FLLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFYMLKGKLQYEL QYRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSYELQVRAGP MPGSSYQGTWSEWSDPVIFQTQSEELKE
SEQ ID NO: 27	>RV22 M70G ectodomain

	CPDLVCYTDYLQTVICILEMWNLHPSTLTLTWQDQYEELKDEATSC SLHRSAHNATHATYTCHMDVFHFGADDIFSVNITDQSGNYSQECGS FLLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFYMLKGKLQYEL QYRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSYELQVRAGP MPGSSYQGTWSEWSDPVIFQTQSEELKE
SEQ ID NO: 28	>RV23 M70L ectodomain CPDLVCYTDYLQTVICILEMWNLHPSTLTLTWQDQYEELKDEATSC SLHRSAHNATHATYTCHMDVFHFLADDIFSVNITDQSGNYSQECGS FLLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFYMLKGKLQYEL QYRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSYELQVRAGP MPGSSYQGTWSEWSDPVIFQTQSEELKE
SEQ ID NO: 29	>RV24 F69L ectodomain CPDLVCYTDYLQTVICILEMWNLHPSTLTLTWQDQYEELKDEATSC SLHRSAHNATHATYTCHMDVFHLMADDIFSVNITDQSGNYSQECGS FLLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFYMLKGKLQYEL QYRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSYELQVRAGP MPGSSYQGTWSEWSDPVIFQTQSEELKE
SEQ ID NO: 30	>RV25 D73I ectodomain CPDLVCYTDYLQTVICILEMWNLHPSTLTLTWQDQYEELKDEATSC SLHRSAHNATHATYTCHMDVFHFMA~~DI~~IFSVNITDQSGNYSQECGS FLLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFYMLKGKLQYEL QYRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSYELQVRAGP MPGSSYQGTWSEWSDPVIFQTQSEELKE
SEQ ID NO: 31	>RV26 D72K ectodomain CPDLVCYTDYLQTVICILEMWNLHPSTLTLTWQDQYEELKDEATSC SLHRSAHNATHATYTCHMDVFHFMAK~~D~~IFSVNITDQSGNYSQECGS FLLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFYMLKGKLQYEL QYRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSYELQVRAGP MPGSSYQGTWSEWSDPVIFQTQSEELKE
SEQ ID NO: 32	>RV27 D73K ectodomain CPDLVCYTDYLQTVICILEMWNLHPSTLTLTWQDQYEELKDEATSC SLHRSAHNATHATYTCHMDVFHFMA~~D~~KIFSVNITDQSGNYSQECGS FLLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFYMLKGKLQYEL QYRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSYELQVRAGP MPGSSYQGTWSEWSDPVIFQTQSEELKE
SEQ ID NO: 33	>RV28 D73E ectodomain CPDLVCYTDYLQTVICILEMWNLHPSTLTLTWQDQYEELKDEATSC SLHRSAHNATHATYTCHMDVFHFMA~~D~~EIFSVNITDQSGNYSQECGS FLLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFYMLKGKLQYEL

	QYRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSYELQVRAGP MPGSSYQGTWSEWSDPVIFQTQSEELKE
SEQ ID NO: 34	>RV29 M70G, D73E ectodomain CPDLVCYTDYLQTVICILEMWNLHPSTLTLTWQDQYEELKDEATSC SLHRSAHNATHATYTCHMDVFHF ~~G~~ ADEIFSVNITDQSGNYSQECGS FLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFYMLKGKLQYEL QYRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSYELQVRAGP MPGSSYQGTWSEWSDPVIFQTQSEELKE
SEQ ID NO: 35	>RV30 M70G, D73E, Q33H ectodomain CPDLVCYTDYLQTVICILEMWNLHPSTLTLTW ~~H~~ DQYEELKDEATSC SLHRSAHNATHATYTCHMDVFHF ~~G~~ ADEIFSVNITDQSGNYSQECGS FLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFYMLKGKLQYEL QYRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSYELQVRAGP MPGSSYQGTWSEWSDPVIFQTQSEELKE
SEQ ID NO: 36	>RV31 M70I, D73E ectodomain CPDLVCYTDYLQTVICILEMWNLHPSTLTLTWQDQYEELKDEATSC SLHRSAHNATHATYTCHMDVFHF ~~I~~ ADEIFSVNITDQSGNYSQECGSF LLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFYMLKGKLQYELQ YRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSYELQVRAGPM PGSSYQGTWSEWSDPVIFQTQSEELKE
SEQ ID NO: 37	>RV32 M70I, E38K ectodomain CPDLVCYTDYLQTVICILEMWNLHPSTLTLTWQDQYE ~~K~~ LKDEATSC SLHRSAHNATHATYTCHMDVFHF ~~I~~ ADDIFSVNITDQSGNYSQECGSF LLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFYMLKGKLQYELQ YRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSYELQVRAGPM PGSSYQGTWSEWSDPVIFQTQSEELKE
SEQ ID NO: 38	>RV33 M70I, E38K, D73E ectodomain CPDLVCYTDYLQTVICILEMWNLHPSTLTLTWQDQYE ~~K~~ LKDEATSC SLHRSAHNATHATYTCHMDVFHF ~~I~~ ADEIFSVNITDQSGNYSQECGSF LLAESIKPAPPFNVTVTFSGQYNISWRSDYEDPAFYMLKGKLQYELQ YRNRGDPWAVSPRRKLISVDSRSVSLLPLEFRKDSSYELQVRAGPM PGSSYQGTWSEWSDPVIFQTQSEELKE

[0107] As shown in **Figures 4A-E**, 11 of the candidate ortho-IL-21R α molecules showed diminished capacity to bind IL-21-TLuc16 (i.e., ortho-IL-21R α molecules RV23, RV24, and RV28 showed binding that was equivalent or nearly equivalent to that of the wild-type receptor, whereas all the other ortho-IL-21R α molecules showed significantly more impaired binding).

Example 2: Attenuated IL-21-Mediated Signaling Mediated by Candidate Ortho-IL-21R α Molecules Revealed Using a Ba/F3 Cellular Assay and a STAT3-Responsive Reporter Construct.

[0108] To test further the extent to which the candidate ortho-IL-21R α candidates were compromised in their capacity to bind IL-21, the eight candidates from Example 1 and **Figure 3** were expressed in a lymphoid cell line, namely Ba/F3 cells (a mouse pre-B cell lymphoma line). Ba/F3 cells are dependent on the cytokine IL-3 for growth but will also proliferate robustly in response to IL-21 if first rendered positive for expression of IL-21R α .

[0109] Ba/F3 cells were electroporated with LeapIn Transposase® mRNA and transposons encoding wild-type or candidate ortho-IL-21R α (RV2: D72E/Y129F/D73E; RV6: M70I/D73E/Q33H; RV7: D72E/L94V/Y191F; RV10: D72E/E38D/M130L; RV13: M70G/Y129F; RV15: F69L/M70L/D73I; RV18: D72K/D73K; RV19: E38K; and Δ cyt, which is a form of IL-21R α lacking almost all of its cytoplasmic tail). The transposons also carried a STAT-3-regulated gene encoding the secreted *Cypridina noctiluca* luciferase, a constitutively expressed cytosolic click beetle luciferase, and a constitutively expressed gene encoding puromycin N-acetyl transferase.

[0110] As an example, the RV13-expression construct was prepared by oligonucleotide-dependent DNA synthesis by ATUM (www.atum.bio). This plasmid is over 13Kb in size and comprises four independent genes within a piece of DNA that is flanked first by genomic insulator sequences (from the human *D4Z4* locus on one side and from the chicken locus encoding β -globin on the other), then by transposon inverted terminal repeat sequences. The insulator sequences are intended to protect the genes within the transposon from position effects (i.e., effects dependent on the site of transposon integration in the genome) that might reduce or variegate expression. The inverted terminal repeat sequences are recognized by ATUM's proprietary LeapIn® transposase

enzymes that mediate integration of the transposon into genomic DNA. The four genes present inside the transposon are described in **Table 3** (in the order they occur within the transposon). The genes and the transposon that contains them were designed according to standard molecular biology principles compatible with the construct assembly methodology used routinely by ATUM. Variants of this vector encoding wild-type or other candidate ortho-IL-21R α molecules were generated by making appropriate changes in the fourth gene listed in **Table 3**.

Table 3

	Promoter	Open Reading Frame	Terminator	Relative orientation
PGK-puro	Human PGK	<i>Streptomyces</i> Puromycin-N-actely transferase	Human beta-globin	Antisense
STAT3-cLuc	Minimal promoter from the cytomegalovirus immediate early gene 1, preceded by six STAT3 binding sites	<i>Cypridina noctiluca</i> luciferase	Human alpha-globin	Sense
EEF2-eLuc	Mouse EEF2	<i>Pyrearinus termitilluminans</i> luciferase	<i>Bos taurus</i> growth hormone	Sense
HSV-IL-21R-RV13	Attenuated promoter from herpes simplex virus 1	Human IL-21R α RV13 (M70G/Y129F)	<i>Oryctolagus cuniculus</i> beta-globin	Sense

[0111] The transposon vector encoding wild-type IL-21R α or any of the candidate ortho-IL-21R α molecules was co-transfected into Ba/F3 cells together with in vitro-transcribed mRNA encoding the relevant LeapIn® transposase using either the MaxCyte ATx or ThermoFisher Neon instruments according to the manufacturer's instructions. Puromycin selection (1 μ g/ml or higher) was imposed at 48 hours after transfection and continued for at least a week after all the cells in

an untransfected control culture had died. Flow cytometry was used to confirm that the puromycin-selected cells showed uniform expression of IL-21R α .

[0112] To assay IL-21 responses, the Ba/F3 cells were first incubated for 20-24 hours in RPMI-1640 medium lacking serum and exogenous cytokines. After washing, they were stimulated with IL-21 (in the presence of 0.4% [vol/vol] serum) for a further 20-24 hours with varying concentrations of IL-21 (or candidate ortho-IL-21) in round-bottom 96-well plates (~100,000 cells per well) before assaying secreted luciferase (from the STAT3-cLuc gene in the transposon), intracellular luciferase (from the constitutively expressed EEF2-eLuc gene in the transposon), or ATP accumulation. The secreted luciferase assay was used to inform on STAT3-dependent signaling in the cells, as occurs when IL-21 engages its receptor. The other two assays (monitoring cytoplasmic eLuc or ATP accumulation) were used to inform on cell number (i.e., proliferation).

[0113] As expected, Ba/F3 cells failed to proliferate if they were not provided IL-3. When expressing the wild-type form of IL-21R α , they showed IL-21-dose-dependent proliferation (by ATP or eLuc assay as mentioned above). There was also IL-21-dose-dependent accumulation of Cypridina luciferase (from the STAT3-regulated reporter gene) in the supernatant fluids from treated cultures (**Figures 5A-B**). The secretion of Cypridina luciferase depended on the IL-21R α cytoplasmic domain (because a tailless form of IL-21R α [Δ cyt] did not induce proliferation in response to IL-21; **Figure 5A**); the secretion was also significantly impaired when the tyrosine residues in the cytoplasmic domain were replaced with phenylalanine residues (not shown).

[0114] *Cypridina noctiluca* luciferase activity was readily detected by adding the relevant luciferase substrate (Vargulin) to samples of supernatant fluids from the cells and measuring light emission using a luminometer. **Figures 5A** and **5B** show luciferase activity detected as light emission (relative light units or RLU) following admixture of 20 μ L of the supernatant fluid from

each of the wells with 50 μ L of VLAR-2 reagent buffer (Targeting Systems) containing Vargulin at the manufacturer's recommended concentration.

[0115] The results confirmed the findings of the binding assays described and shown in Example 1 and **Figure 3** with respect to impaired IL-21-dependent signaling mediated by the eight candidate ortho-IL-21R α molecules.

Example 3: Screening of Candidate Ortho-IL-21 Molecules: Testing for STAT3-Dependent Signaling Responses in Cells Expressing Candidate Ortho-IL-21R α Molecules or Wild-Type IL-21R α .

[0116] Wild-type or candidate ortho-IL-21 molecules were produced from transiently transfected HEK-293 cells according to procedures that are routinely used at ATUM (www.atum.bio). Expression vectors for this purpose carried the IL-21 open reading frame downstream of an optimized cytomegalovirus Immediate Early Gene 1 promoter. A signal peptide from the human IL-2 gene was used in place of the native one. Epitope tags for detection, quantitation, immobilization, or purification were fused to the amino- or carboxy-termini of the IL-21 coding sequence. In one series of vectors (including those encoding the forms of IL-21 specified as SEQ ID NOs: 39-60 in **Table 4**), the element fused to the amino terminus was a Twin-Strep-Tag followed by three copies of a Glycine-Glycine-Glycine-Glycine-Serine linker moiety, while the element fused to the carboxy terminus comprised two copies of the same Glycine-Glycine-Glycine-Glycine-Serine linker followed by an N-Myc epitope tag (recognized by the 9E10 monoclonal antibody). A second series of vectors (including those encoding the forms of IL-21 specified as SEQ ID NOs: 61-62 in **Table 4**) featured no tags at the carboxy terminus but had the following element at the amino-terminus of IL-21: Twin-Strep-Tag followed immediately by the

N-Myc epitope tag then three copies of the Glycine-Glycine-Glycine-Glycine-Serine linker moiety.

[0117] Cultures (10 mL) of transfected cells were maintained for approximately five days (until viability dropped below about 50%). The supernatant fluids were harvested, filtered, and aliquoted. Quantitation of the IL-21 present in the supernatant fluids was accomplished using ELISAs (and in some cases Biolayer interferometry assays) directed against the various epitope tags or specific for the IL-21 moiety.

[0118] Selected candidate ortho-IL-21 molecules (mature protein sequences, including epitope tags but absent the signal peptide) are shown in **Table 4** (SEQ ID NOs: 39-62) (substitutions shown in the headers and by underline within the sequences).

Table 4

SEQ ID NO: 39	>IL21_CV1_R5Q APWSHPQFEKGGGSGGGSGGSSAWSHQPFEKGGGGSGGGGSGGGG SQGQDQHMRMRQLIDIVDQLKNYVNDLVPEFLPAPEDVETNCEWS AFSCFQKAQLKSANTGNNERIINVSIIKKLKRKPPSTNAGRQKHRLT CPSCDSYEKKPPKEFLERFKSLLQKMIHQHLSSRTHGSEDSGGGGSG GGGSEQKLISEEDL*
SEQ ID NO: 40	>IL21_CV2_R5D APWSHPQFEKGGGSGGGSGGSSAWSHQPFEKGGGGSGGGGSGGGG SQGQDDHMIRMRQLIDIVDQLKNYVNDLVPEFLPAPEDVETNCEWS AFSCFQKAQLKSANTGNNERIINVSIIKKLKRKPPSTNAGRQKHRLT CPSCDSYEKKPPKEFLERFKSLLQKMIHQHLSSRTHGSEDSGGGGSG GGGSEQKLISEEDL*
SEQ ID NO: 41	>IL21_CV3_R5E APWSHPQFEKGGGSGGGSGGSSAWSHQPFEKGGGGSGGGGSGGGG SQGQDEHMIRMRQLIDIVDQLKNYVNDLVPEFLPAPEDVETNCEWS AFSCFQKAQLKSANTGNNERIINVSIIKKLKRKPPSTNAGRQKHRLT CPSCDSYEKKPPKEFLERFKSLLQKMIHQHLSSRTHGSEDSGGGGSG GGGSEQKLISEEDL*
SEQ ID NO: 42	>IL21_CV4_R5K APWSHPQFEKGGGSGGGSGGSSAWSHQPFEKGGGGSGGGGSGGGG

	SQGQDKHMIRMRLIDIVDQLKNYVNDLVPEFLPAPEDVETNCEWS AFSCFQKAQLKSANTGNNERIINVSIKKLKRKPPSTNAGRQKHRLT CPSCDSYEKKPPKEFLERFKSLLQKMIHQHLSRTHGSEDSGGGGSG GGGSEQKLISEEDL*
SEQ ID NO: 43	>IL21_CV5_R5N APWSHPQFEKGGGSGGGSGGSSAWSHQPFEKGGGGSGGGSGGGG SQGQDNHMIRMRLIDIVDQLKNYVNDLVPEFLPAPEDVETNCEWS AFSCFQKAQLKSANTGNNERIINVSIKKLKRKPPSTNAGRQKHRLT CPSCDSYEKKPPKEFLERFKSLLQKMIHQHLSRTHGSEDSGGGGSG GGGSEQKLISEEDL*
SEQ ID NO: 44	>IL21_CV6_I8E APWSHPQFEKGGGSGGGSGGSSAWSHQPFEKGGGGSGGGSGGGG SQGQDRHMERMRQLIDIVDQLKNYVNDLVPEFLPAPEDVETNCEW SAFSCFQKAQLKSANTGNNERIINVSIKKLKRKPPSTNAGRQKHRL TCPSCDSYEKKPPKEFLERFKSLLQKMIHQHLSRTHGSEDSGGGGSG GGGGSEQKLISEEDL*
SEQ ID NO: 45	>IL21_CV7_R9E APWSHPQFEKGGGSGGGSGGSSAWSHQPFEKGGGGSGGGSGGGG SQGQDRHMIEMRQLIDIVDQLKNYVNDLVPEFLPAPEDVETNCEWS AFSCFQKAQLKSANTGNNERIINVSIKKLKRKPPSTNAGRQKHRLT CPSCDSYEKKPPKEFLERFKSLLQKMIHQHLSRTHGSEDSGGGGSG GGGSEQKLISEEDL*
SEQ ID NO: 46	>IL21_CV8_R9D APWSHPQFEKGGGSGGGSGGSSAWSHQPFEKGGGGSGGGSGGGG SQGQDRHMI ~~D~~ MRQLIDIVDQLKNYVNDLVPEFLPAPEDVETNCEWS AFSCFQKAQLKSANTGNNERIINVSIKKLKRKPPSTNAGRQKHRLT CPSCDSYEKKPPKEFLERFKSLLQKMIHQHLSRTHGSEDSGGGGSG GGGSEQKLISEEDL*
SEQ ID NO: 47	>IL21_CV9_R9K APWSHPQFEKGGGSGGGSGGSSAWSHQPFEKGGGGSGGGSGGGG SQGQDRHMI ~~K~~ MRQLIDIVDQLKNYVNDLVPEFLPAPEDVETNCEWS AFSCFQKAQLKSANTGNNERIINVSIKKLKRKPPSTNAGRQKHRLT CPSCDSYEKKPPKEFLERFKSLLQKMIHQHLSRTHGSEDSGGGGSG GGGSEQKLISEEDL*
SEQ ID NO: 48	>IL21_CV10_R9N APWSHPQFEKGGGSGGGSGGSSAWSHQPFEKGGGGSGGGSGGGG SQGQDRHMINMRQLIDIVDQLKNYVNDLVPEFLPAPEDVETNCEWS AFSCFQKAQLKSANTGNNERIINVSIKKLKRKPPSTNAGRQKHRLT CPSCDSYEKKPPKEFLERFKSLLQKMIHQHLSRTHGSEDSGGGGSG GGGSEQKLISEEDL*

SEQ ID NO: 49	>IL21_CV11_R9Q APWSHPQFEKGGGSGGGSGGSSAWSHQPFEKGGGSGGGSGGGG SQGQDRHMIQMRQLIDIVDQLKNYVNDLVPEFLPAPEDVETNCEWS AFSCFQKAQLKSANTGNNERIINVSIKKLKRKPPSTNAGRQKHRLT CPSCDSYEKKPPKEFLERFKSLLQKMIHQHLSSRTHGSEDSGGGGSG GGGSEQKLISEEDL*
SEQ ID NO: 50	>IL21_CV12_Q12V APWSHPQFEKGGGSGGGSGGSSAWSHQPFEKGGGSGGGSGGGG SQGQDRHMIRMRLIDIVDQLKNYVNDLVPEFLPAPEDVETNCEWS AFSCFQKAQLKSANTGNNERIINVSIKKLKRKPPSTNAGRQKHRLT CPSCDSYEKKPPKEFLERFKSLLQKMIHQHLSSRTHGSEDSGGGGSG GGGSEQKLISEEDL*
SEQ ID NO: 51	>IL21_CV13_L13D APWSHPQFEKGGGSGGGSGGSSAWSHQPFEKGGGSGGGSGGGG SQGQDRHMIRMRLIDIVDQLKNYVNDLVPEFLPAPEDVETNCEWS AFSCFQKAQLKSANTGNNERIINVSIKKLKRKPPSTNAGRQKHRLT CPSCDSYEKKPPKEFLERFKSLLQKMIHQHLSSRTHGSEDSGGGGSG GGGSEQKLISEEDL*
SEQ ID NO: 52	>IL21_CV14_K73V APWSHPQFEKGGGSGGGSGGSSAWSHQPFEKGGGSGGGSGGGG SQGQDRHMIRMRLIDIVDQLKNYVNDLVPEFLPAPEDVETNCEWS AFSCFQKAQLKSANTGNNERIINVSIKVLKRKPPSTNAGRQKHRLT CPSCDSYEKKPPKEFLERFKSLLQKMIHQHLSSRTHGSEDSGGGGSG GGGSEQKLISEEDL*
SEQ ID NO: 53	>IL21_CV15_K73D APWSHPQFEKGGGSGGGSGGSSAWSHQPFEKGGGSGGGSGGGG SQGQDRHMIRMRLIDIVDQLKNYVNDLVPEFLPAPEDVETNCEWS AFSCFQKAQLKSANTGNNERIINVSIKDLKRKPPSTNAGRQKHRLT CPSCDSYEKKPPKEFLERFKSLLQKMIHQHLSSRTHGSEDSGGGGSG GGGSEQKLISEEDL*
SEQ ID NO: 54	>IL21_CV16_K75D APWSHPQFEKGGGSGGGSGGSSAWSHQPFEKGGGSGGGSGGGG SQGQDRHMIRMRLIDIVDQLKNYVNDLVPEFLPAPEDVETNCEWS AFSCFQKAQLKSANTGNNERIINVSIKKLDRKPPSTNAGRQKHRLT CPSCDSYEKKPPKEFLERFKSLLQKMIHQHLSSRTHGSEDSGGGGSG GGGSEQKLISEEDL*
SEQ ID NO: 55	>IL21_CV17_R76E APWSHPQFEKGGGSGGGSGGSSAWSHQPFEKGGGSGGGSGGGG SQGQDRHMIRMRLIDIVDQLKNYVNDLVPEFLPAPEDVETNCEWS

	AFSCFQKAQLKSANTGNNERIINVSIKKLKEKPPSTNAGRQKHRLT CPSCDSYEKKPPKEFLERFKSLLQKMIHQHLSRTHGSEDSGGGGSG GGGSEQKLISEEDL*
SEQ ID NO: 56	>IL21_CV18_R76N APWSHPQFEKGGGSGGGSGGSSAWSHQPFEKGGGGSGGGSGGGG SQGQDRHMIRMRLIDIVDQLKNYVNDLVPEFLPAPEDVETNCEWS AFSCFQKAQLKSANTGNNERIINVSIKKLK_NKPPSTNAGRQKHRLT CPSCDSYEKKPPKEFLERFKSLLQKMIHQHLSRTHGSEDSGGGGSG GGGSEQKLISEEDL*
SEQ ID NO: 57	>IL21_CV19_R76A APWSHPQFEKGGGSGGGSGGSSAWSHQPFEKGGGGSGGGSGGGG SQGQDRHMIRMRLIDIVDQLKNYVNDLVPEFLPAPEDVETNCEWS AFSCFQKAQLKSANTGNNERIINVSIKKLK_AKPPSTNAGRQKHRLT CPSCDSYEKKPPKEFLERFKSLLQKMIHQHLSRTHGSEDSGGGGSG GGGSEQKLISEEDL*
SEQ ID NO: 58	>IL21_CV21_R5Q_R76E APWSHPQFEKGGGSGGGSGGSSAWSHQPFEKGGGGSGGGSGGGG SQGQDQHMIRMRLIDIVDQLKNYVNDLVPEFLPAPEDVETNCEWS AFSCFQKAQLKSANTGNNERIINVSIKKLKEKPPSTNAGRQKHRLT CPSCDSYEKKPPKEFLERFKSLLQKMIHQHLSRTHGSEDSGGGGSG GGGSEQKLISEEDL*
SEQ ID NO: 59	>IL21_CV22_R5Q_R76A APWSHPQFEKGGGSGGGSGGSSAWSHQPFEKGGGGSGGGSGGGG SQGQDQHMIRMRLIDIVDQLKNYVNDLVPEFLPAPEDVETNCEWS AFSCFQKAQLKSANTGNNERIINVSIKKLK_AKPPSTNAGRQKHRLT CPSCDSYEKKPPKEFLERFKSLLQKMIHQHLSRTHGSEDSGGGGSG GGGSEQKLISEEDL*
SEQ ID NO: 60	>IL21_CV204_H6L_M10L_K73V_P78L_P104A APWSHPQFEKGGGSGGGSGGSSAWSHQPFEKGGGGSGGGSGGGG SQGQDRLMIRLRQLIDIVDQLKNYVNDLVPEFLPAPEDVETNCEWS AFSCFQKAQLKSANTGNNERIINVSIKVLKRKL_PSTNAGRQKHRLT CPSCDSYEKKPAKEFLERFKSLLQKMIHQHLSRTHGSEDSGGGGSG GGGSEQKLISEEDL*
SEQ ID NO: 61	>IL21_CV374_H6L_R9K_M10L_K73V_P78L_G84E APWSHPQFEKGGGSGGGSGGSSAWSHQPFEKEQKLISEEDLGGGG GGGGSGGGGSQGQDRLMIKLRQLIDIVDQLKNYVNDLVPEFLPAPE DVETNCEWSAFSCFQKAQLKSANTGNNERIINVSIKVLKRKL_PSTNA ERRQKHRLTCPSCDSYEKKPPKEFLERFKSLLQKMIHQHLSRTHGS EDS*
SEQ ID NO: 62	>IL21_CV388_H6L_R9K_M10L_K73I_P78L_P104A

	APWSHPQFEKGGGSGGGSGGSSAWSHHPQFEKEQKLISEEDLGGGGS GGGGSGGGGSQGGQDR ~~L~~ MI ~~K~~ L ~~R~~ QLIDIVDQLKNYVNDLVPEFLPAPE DVETNCEWSAFSCFQKAQLKSANTGNNERIINVSILK ~~R~~ K ~~L~~ PSTNA GRRQKHRLTCPSCDSEYK ~~K~~ PA ~~K~~ EFLERFKSLLQKMIHQHLSRTHGS EDS*
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[0119] Ba/F3 cells expressing wild-type IL-21R α or the eight candidate ortho-IL-21R α molecules from Example 1 and **Figure 3** were stimulated with candidate ortho-IL-21 molecules CV1-CV22 (SEQ ID NOs: 39-59). The signaling responses of the Ba/F3 cells to the ortho-IL-21 molecules were monitored as above using the STAT3-luciferase assay.

[0120] Ba/F3 cells were exposed to four concentrations (100, 50, 25, and 12.5 ng/mL) of the indicated candidate ortho-IL-21 molecules (**Figures 6A-6J**: candidate ortho-IL-21 molecules containing R5Q, R5D, R5E, R5K, R5N, I8E, R9E, R9D, R9K, R9N, R9Q, and wild-type IL-21; **Figures 6K-6T**: candidate ortho-IL-21 molecules containing Q12V, L13D, K73V, K73D, K75D, R76E, R76N, R76A, R5Q/R76E, R5Q/R76A, and wild-type IL-21). The Ba/F3 cells differed in the form of ortho-IL-21R α molecule they expressed (RV2: D72E/Y129F/D73E; RV6: M70I/D73E/Q33H; RV7: D72E/L94V/Y191F; RV10: D72E/E38D/M130L; RV13: M70G/Y129F; RV15: F69L/M70L/D73I; RV18: D72K/D73K; RV19: E38K; and wild-type IL-21R α). The cells were placed in serum-free medium for 24 hours before a subsequent overnight incubation (~20 hours) with the candidate ortho-IL-21 molecules in round-bottom 96-well plates (~100,000 cells per well). As described above, the transposon conferring expression of WT and candidate ortho-IL-21R α molecules also carried a STAT3-regulated gene encoding the secreted *Cypridina noctiluca* luciferase. **Figures 6A-6T** show activity of this luciferase detected as light emission (relative light units) following admixture of 20 μ L of the supernatant fluid from each of

the wells with 50 μ L of VLAR-2 reagent buffer (Targeting Systems) containing the *Cypridina noctiluca* luciferase substrate (Vargulin) at the manufacturer's recommended concentration.

[0121] As shown in **Figures 6A-6T**, the following cells expressing ortho-IL-21R α molecules made measurable responses to ortho-IL-21 molecules:

Table 5

Ortho-IL-21 (cytokine no.)	Ortho-IL-21R α (receptor no.)	Figure
R5K (CV4)	M70I/D73E/Q33H (RV6)	6C
R5K (CV4)	E38K (RV19)	6I
R5Q (CV1)	E38K (RV19)	6I
R9K (CV9)	M70G/Y129F (RV13)	6J
Q12V (CV12)	M70I/D73E/Q33H (RV6)	6M
K73V (CV14)	M70I/D73E/Q33H (RV6)	6M
K75D (CV16)	M70I/D73E/Q33H (RV6)	6M
R76A (CV19)	F69L/M70L/D73I (RV15)	6P or 6Q
Q12V (CV12)	E38K (RV19)	6S
K75D (CV16)	E38K (RV19)	6S
R76N (CV18)	E38K (RV19)	6S
Q12V (CV12)	M70G/Y129F (RV13)	6T
L13D (CV13)	M70G/Y129F (RV13)	6T
K73V (CV14)	M70G/Y129F (RV13)	6T
K75D (CV16)	M70G/Y129F (RV13)	6T

[0122] Cells expressing IL-21R α (the wild-type receptor) made measurable responses to the majority of variant cytokines, though importantly, the design of the experiment (featuring only four relatively high concentrations of the variant cytokines) precluded the possibility of quantifying the extent of their signaling activity relative to wild-type IL-21.

[0123] Selected candidate ortho-IL-21 molecules from the collection represented in **Figure 6A-T** and **Table 4** (namely R5K (CV4), Q12V (CV12), R76A (CV19), and K73V (CV14)) were re-tested with Ba/F3 cells expressing wild-type IL-21R α or candidate ortho-IL-21R α molecules RV6, RV10, RV13, or RV15 (*i.e.*, M70I/D73E/Q33H, D72E/E38D/M130L, M70G/Y129F, or F69L/M70L/D73I respectively). The results of this experiment are shown in **Figures 7A-D**. As expected, when exposed to wild-type IL-21, cells expressing the candidate ortho-IL-21R α molecules made diminished signaling responses compared to those expressing wild-type IL-21R α (solid lines with filled symbols in each graph). Furthermore, as predicted by the results in **Figures 6A-T**, in three cases (for cells expressing candidate ortho-IL-21R α molecules RV6, RV19, or RV13) the cells made stronger responses when they were exposed to the candidate ortho-IL-21 molecules (CV12, CV19, or CV14, respectively) than to wild-type IL-21 (open versus closed diamond symbols in **Figures 7A, C, and D**, respectively). All four candidate ortho-IL-21 molecules were impaired to varying extents in their capacity to cause responses via wild-type IL-21R α , with candidate ortho-IL-21 molecule CV19 being the most severely compromised in this respect (open versus closed circle symbols in **Figure 7C**).

[0124] The results of these experiments identified IL-21 substitutions associated with impaired capacity to signal via wild-type IL-21R α with – in some cases – low-level capacity to induce signaling via specific candidate ortho-IL-21R α molecules.

[0125] Infolog variants of IL-21 were generated according to the principles described in Govindarajan S, Mannervik B, Silverman JA, et al. Mapping of amino acid substitutions conferring herbicide resistance in wheat glutathione transferase. *ACS Synth Biol.* 2015;4(3):221-227. doi:10.1021/sb500242x and Musdal Y, Govindarajan S, Mannervik B. Exploring sequence-function space of a poplar glutathione transferase using designed information-rich gene variants. *Protein Eng Des Sel.* 2017;30(8):543-549. doi:10.1093/protein/gzx045. These Infolog variants of IL-21 were screened for their capacity to induce signaling in Ba/F3 cells expressing wild-type IL-21R α or candidate ortho-IL-21R α molecules as described. Representative data from one such screening experiment are provided in **Figures 8A-8C**. The experimental results depicted in **Figures 8A-8C** derived from the analysis of 96 cytokines, one of which comprised the wild-type form of IL-21, another comprised a negative control variant (CV22, which bears two disabling substitutions [R5Q/R76A]; SEQ ID NO:59), and 94 Infolog variants, each of which was a candidate ortho-IL-21 molecule. **Figure 8A** shows the STAT3 responses elicited in cells expressing wild-type IL-21R α exposed to the cytokine collection, whereas **Figures 8B** and **8C** show responses made by cells expressing the candidate ortho-IL-21R α molecules RV13 and RV6, respectively. RV13 is comprised of amino acid substitutions M70G and Y129F (SEQ ID NO:19), whereas RV6 is comprised of amino acid substitutions M70I, D73E, and Q33H (SEQ ID NO:12). The highlighted curves in the three figures show responses made by the three kinds of cells to five selected cytokines, namely, wild-type IL-21, CV22, CV204 (SEQ ID NO: 60, comprised of H6L/M10L/K73V/P78L/P104A), CV374 (SEQ ID NO: 61, comprised of H6L/R9K/M10L/K73V/P78L/G84E) and CV388 (SEQ ID NO: 62, comprised of H6L/R9K/M10L/K73I/P78L/P104A).

[0126] The highlighted cytokines were subsequently retested in an independent focused experiment of a similar design (involving Ba/F3 cells expressing wild-type IL-21R α (**Figure 9A**) or the candidate ortho-IL-21R α RV13 (**Figure 9B**)). As in the screening experiment (**Figures 8A-8C**), CV22, the negative control cytokine, failed to elicit a signaling response in cells expressing either form of IL-21R α . By contrast, wild-type IL-21 induced a strong response in cells expressing the wild-type form of IL-21R α , but a much weaker response in the RV13-expressing cells. CV204 elicited a response in cells expressing wild-type IL-21R α (**Figures 8A and 9A**) or either of the candidate ortho-IL-21R α molecules (**Figures 8B, 8C, and 9B**).

[0127] Strikingly, CV374 and CV388 were significantly compromised in their capacity to induce signaling in cells expressing wild-type IL-21R α (**Figures 8A and 9A**) but, like CV204, demonstrated good activity with cells expressing RV13 (**Figures 8B and 9B**). In this Ba/F3 assay, therefore, ortho-IL-21 molecules CV374 and CV388 showed the kind of signaling selectivity expected of cytokines that have orthogonal functionality to native IL-21.

[0128] Candidate ortho-IL-21R α RV13 (SEQ ID NO:19) carries two substitutions relative to wild-type IL-21R α , namely M70G and Y129F, whereas candidate ortho-IL-21R α RV22 (SEQ ID NO:27) carries just M70G. These two variant receptors appear to be equivalently compromised in their capacity to bind native IL-21 (**Figure 4B**). They also accounted for a similar pattern of reactivity to the collection of IL-21 molecules used in **Figures 9A and 9B**. Specifically, like RV13 (**Figure 10B**), RV22 mediated significantly impaired signaling responses to wild-type IL-21 (and the negative control molecule CV22) but conferred good responses to CV204, CV374, and CV388 (**Figure 10C**). As in **Figure 9A**, both CV374 and CV388 showed impaired responses with cells expressing wild-type IL-21R α , while CV204 behaved similarly to wild-type IL-21 (**Figure 10A**). These results replicate the key observations made from the data in **Figures 9A and 9B**, while also

showing that RV22 may be interchangeable with RV13. Since RV22 carries one fewer substitution than RV13, RV22 may be favored for therapeutic purposes.

[0129] In most settings, in vitro assays will have only very limited predictive value of the effects of a therapeutic in vivo: many therapeutic targets are expressed in multiple cell types (often having opposing effects on the response in vivo), or the therapeutic effect of a given target is dependent on other auxiliary cells. In such situations, an in vitro model, which by its very nature is simplistic, is not a particularly good proxy for the much more complicated situation in vivo. This is not the case in the instant application: the target receptor is synthetic and will only be expressed in cells specifically engineered to do so. Given the specificity of the orthogonal cytokine-receptor system, this significantly reduces the complexity, giving an in vitro assay a better predictive value.

[0130] The complete disclosure of all patents, patent applications, and publications, and electronically available materials cited herein are incorporated by reference. The foregoing detailed description and examples have been given for clarity of understanding only. No unnecessary limitations are to be understood therefrom. While theories may be presented describing possible mechanisms through which the compounds are effective, the inventors are not bound by theories described herein. The invention is not limited to the exact details shown and described, for variations obvious to one skilled in the art will be included within the invention defined by the claims.

CLAIMS

What is claimed is:

1. An engineered human IL-21 polypeptide that comprises amino acid substitutions, numbered relative to SEQ ID NO: 2:

H6L;

R9K;

M10L;

P78L;

and optionally comprises G84E or P104A or a combination thereof;

and optionally comprises one of K73V or K73I.

2. The engineered human IL-21 polypeptide of claim 1, comprising amino acid substitution K73V.

3. The engineered human IL-21 polypeptide of claim 1, comprising amino acid substitution K73I.

4. The engineered human IL-21 polypeptide of claim 1, comprising amino acid substitution G84E.

5. The engineered human IL-21 polypeptide of claim 1, comprising amino acid substitution P104A.

6. The engineered human IL-21 polypeptide of claim 1, wherein the polypeptide binds to an engineered human CD360 protein ectodomain.

7. The engineered human IL-21 polypeptide of claim 6, wherein the engineered human CD360 protein ectodomain is modified at one or more residues selected from Y10, Q33, Q35,

Y36, E38, L39, F67, H68, F69, M70, A71, D72, D73, I74, L94, A96, E97, P126, A127, Y129, M130, K134, S190, and Y191.

8. The engineered human IL-21 polypeptide of claim 6, wherein the engineered human CD360 protein ectodomain is modified at M70.

9. The engineered human IL-21 polypeptide of claim 6, wherein the engineered human CD360 protein ectodomain is modified at M70 and Y129.

10. The engineered human IL-21 polypeptide of claim 8, wherein the engineered human CD360 protein ectodomain comprises amino acid substitution M70G.

11. The engineered human IL-21 polypeptide of claim 9, wherein the engineered human CD360 protein ectodomain comprises amino acid substitutions M70G and Y129F.

12. The engineered human IL-21 polypeptide of claim 1, comprising amino acid substitutions K73V and G84E.

13. The engineered human IL-21 polypeptide of claim 1, comprising amino acid substitutions K73I and P104A.

14. An engineered human IL-21 polypeptide that comprises a set of amino acid substitutions, numbered relative to SEQ ID NO: 2: H6L, R9K, M10L, K73V, P78L, and G84E.

15. An engineered human IL-21 polypeptide that comprises a set of amino acid substitutions, numbered relative to SEQ ID NO: 2: H6L, R9K, M10L, K73I, P78L, and P104A.

16. A system for selective activation of a receptor in a cell, the system comprising: (a) an engineered receptor comprising the human CD360 protein ectodomain including an amino acid substitution at M70G; and (b) the engineered human IL-21 polypeptide of claim 1.

17. A system for selective activation of a receptor in a cell, the system comprising: (a) an engineered receptor comprising the human CD360 protein ectodomain including amino acid substitutions at M70G and Y129F; and (b) the engineered human IL-21 polypeptide of claim 1.
18. The system of claim 16, wherein the engineered human CD360 receptor is expressed by a mammalian cell.
19. The system of claim 17, wherein the engineered human CD360 receptor is expressed by a mammalian cell.
20. The system of claim 18, wherein the mammalian cell is an immune cell or a stem cell.
21. The system of claim 19, wherein the mammalian cell is an immune cell or a stem cell.
22. The system of claim 20, wherein the mammalian cell is an immune cell, and the immune cell is a T cell.
23. The system of claim 21, wherein the mammalian cell is an immune cell, and the immune cell is a T cell.
24. A pharmaceutical composition comprising the engineered human IL-21 polypeptide of claim 1; and a pharmaceutically acceptable excipient.
25. A kit comprising the system of claim 16.
26. A kit comprising the system of claim 17.
27. The engineered human IL-21 polypeptide of claim 1, wherein the engineered human IL-21 polypeptide is fused to an Fc domain of IgG or to albumin.