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(54) **COMPOSITIONS AND METHODS OF  
TREATING INFLAMMATORY AND  
AUTOIMMUNE DISEASES**

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**ABSTRACT**

Described herein are immunosuppressive molecules including immunosuppressive variants of IL-2, and use of such molecules to treat inflammatory and autoimmune disorders.

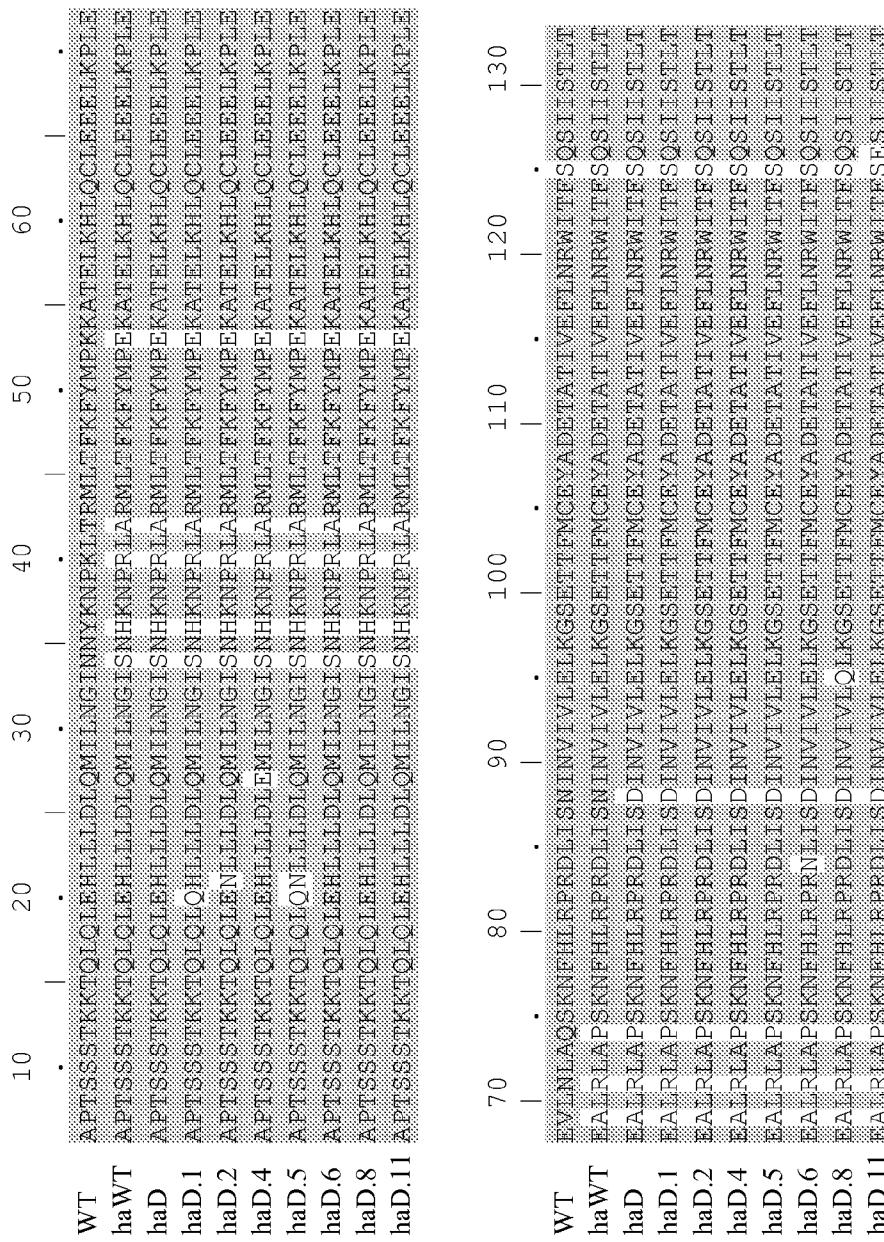


FIG. 1

FIG. 2A

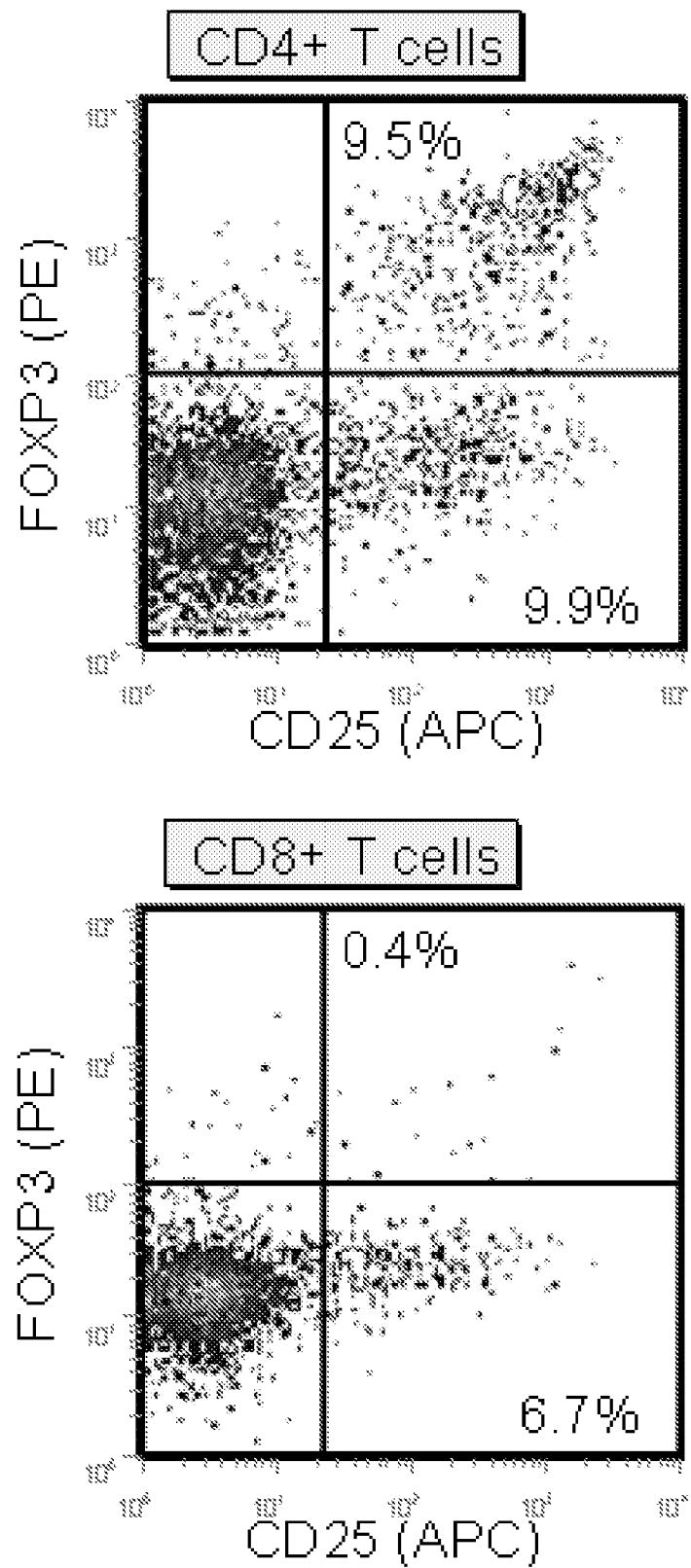
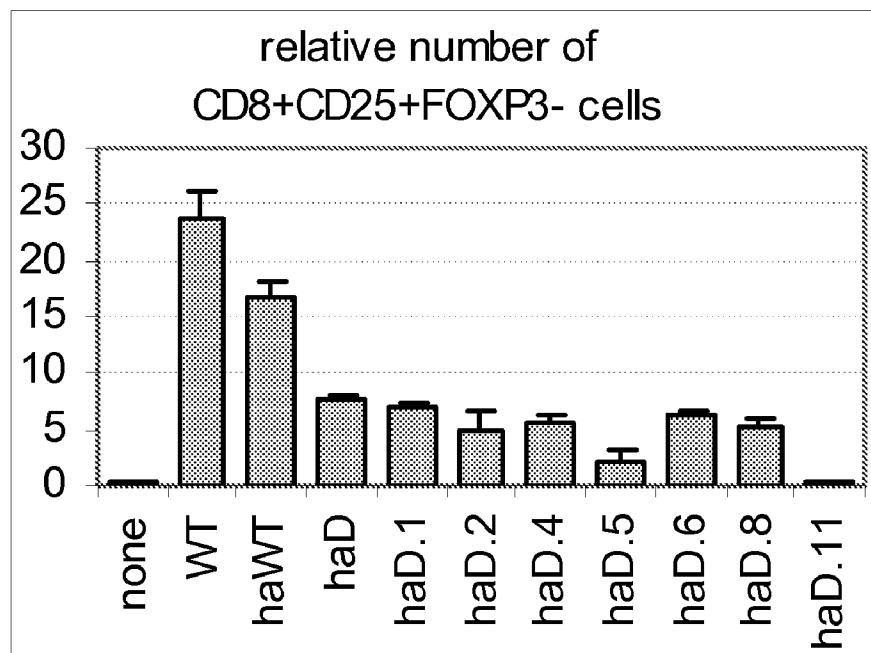


FIG. 2B

10 nM IL-2 variant:



100 pM IL-2 variant:

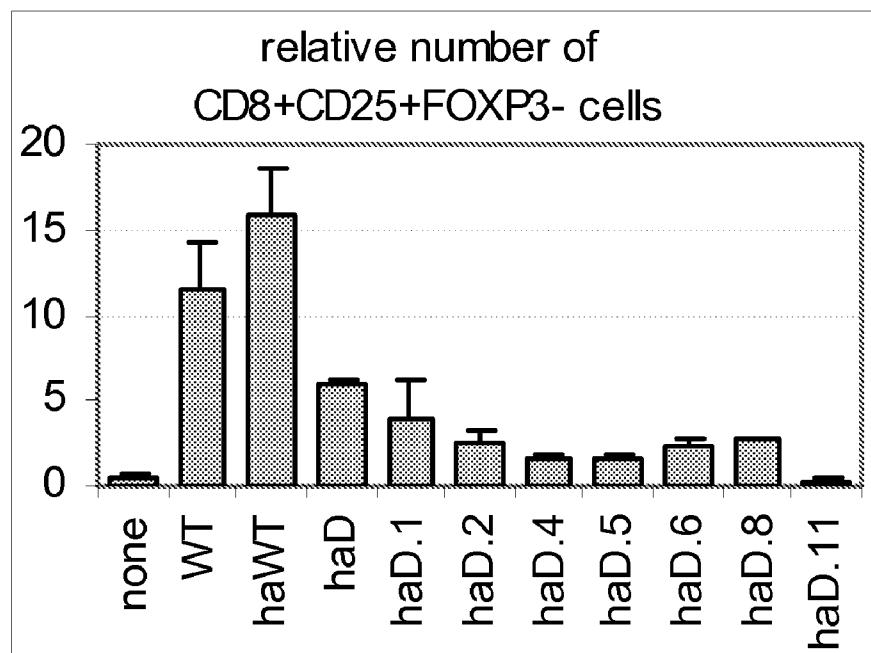
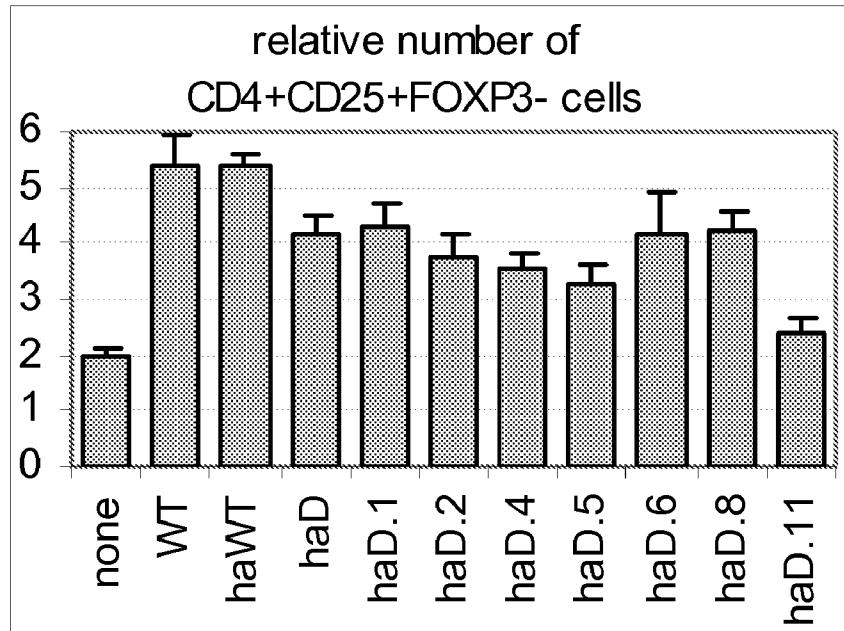


FIG. 2C

10 nM IL-2 variant:



100 pM IL-2 variant:

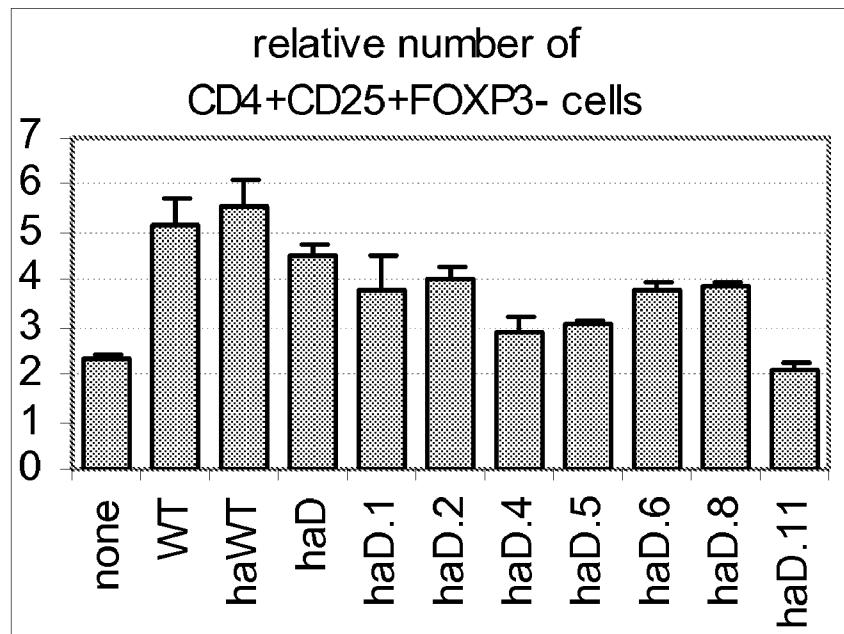
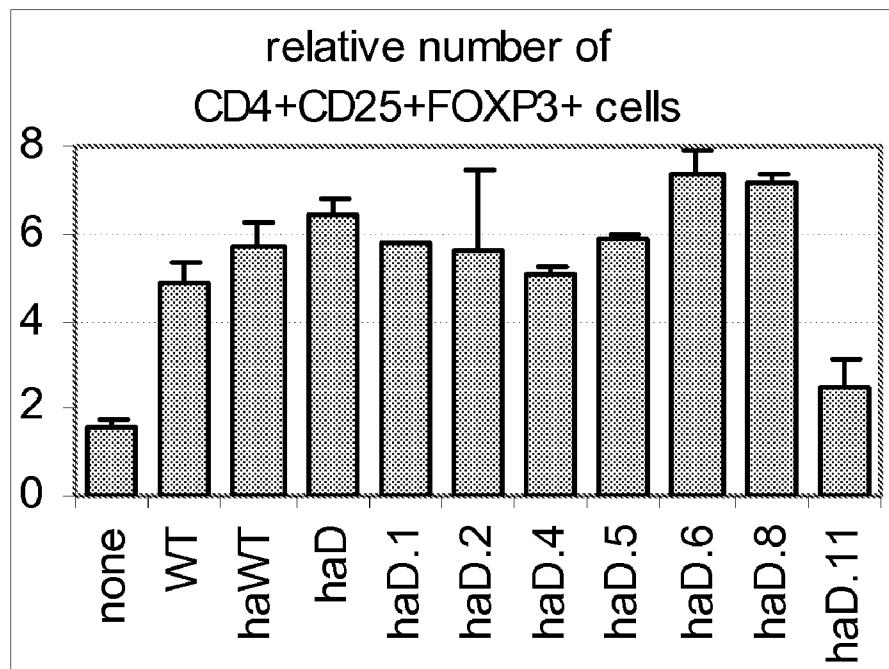


FIG. 2D

10 nM IL-2 variant:



100 pM IL-2 variant:

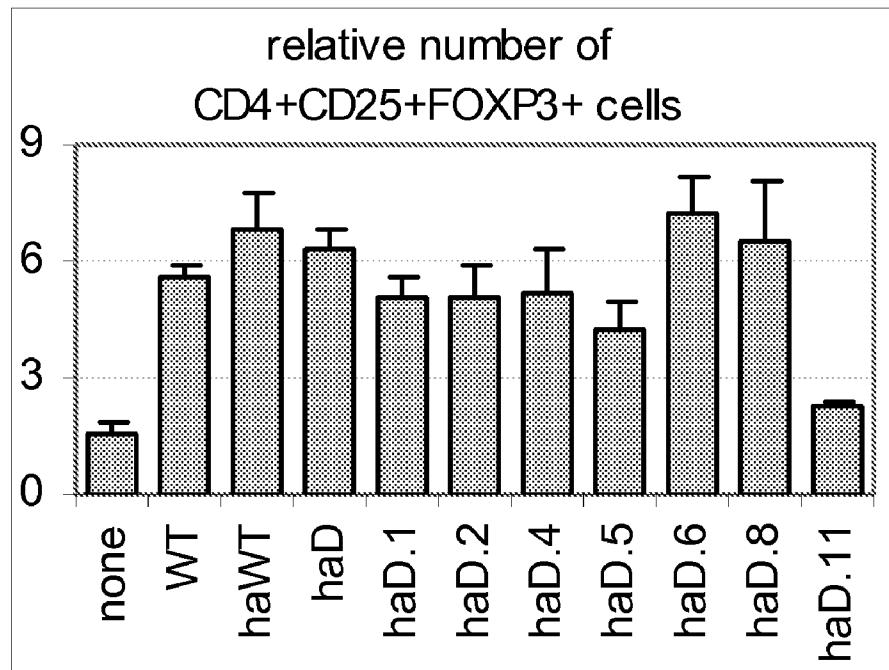
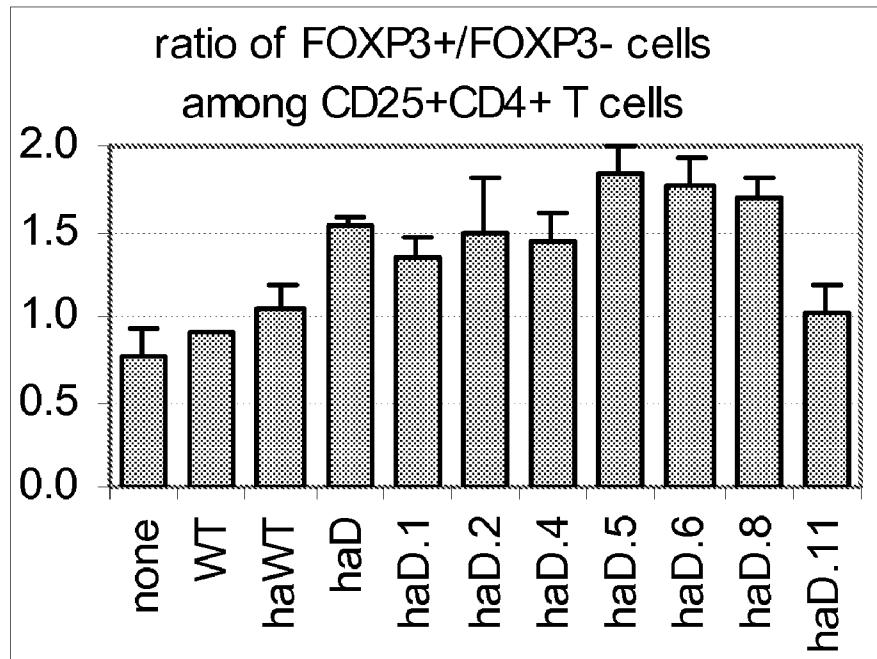


FIG. 2E

10 nM IL-2 variant:



100 pM IL-2 variant:

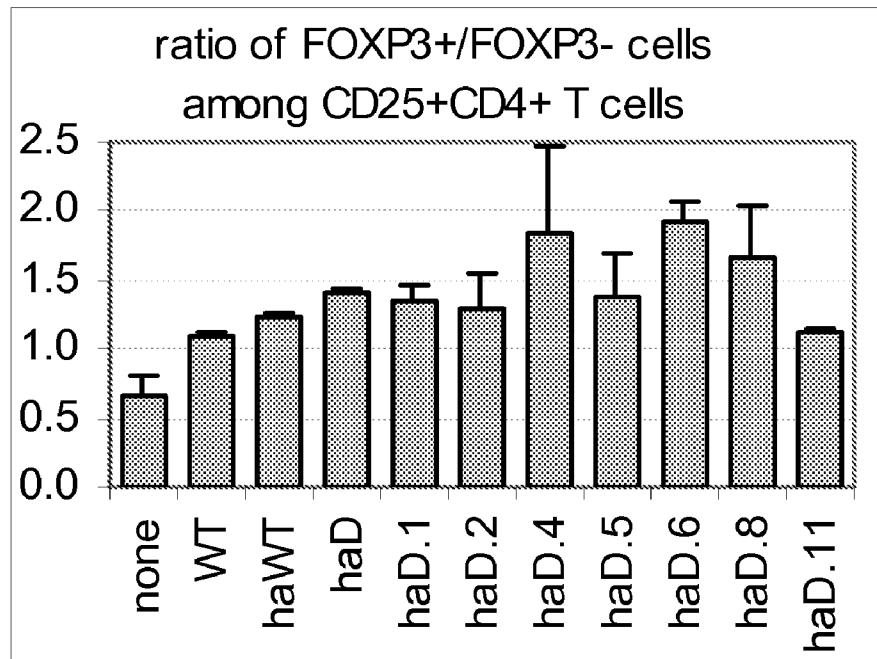
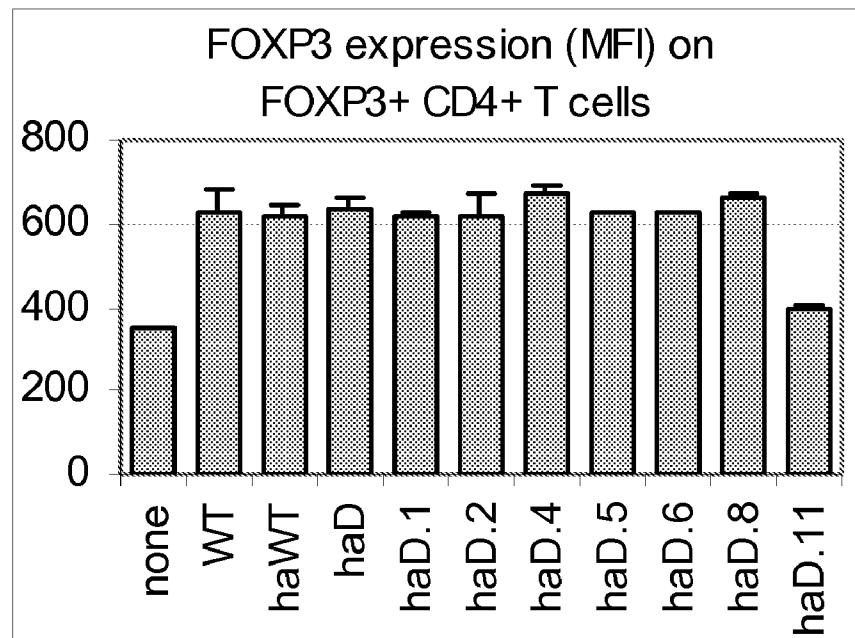
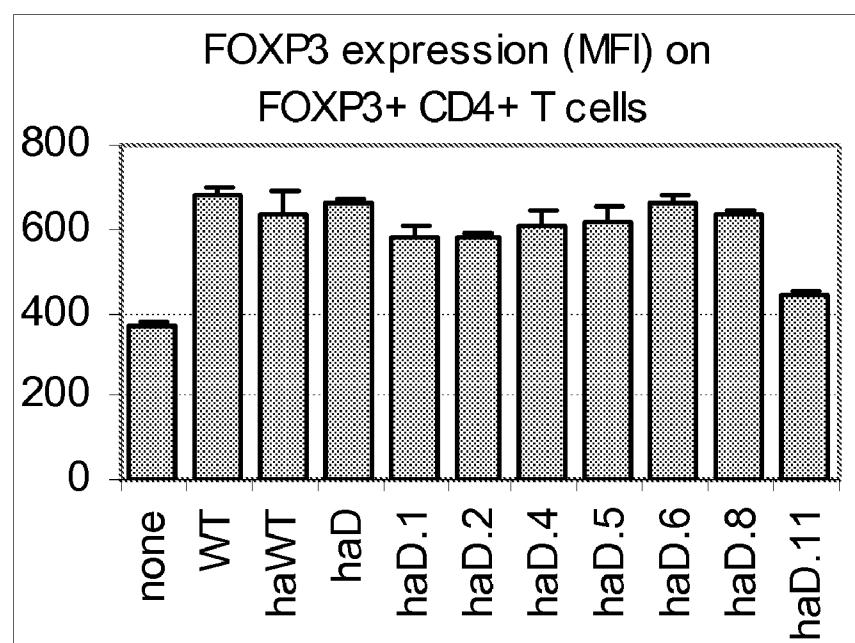


FIG. 2F

10 nM IL-2 variant:



100 pM IL-2 variant:



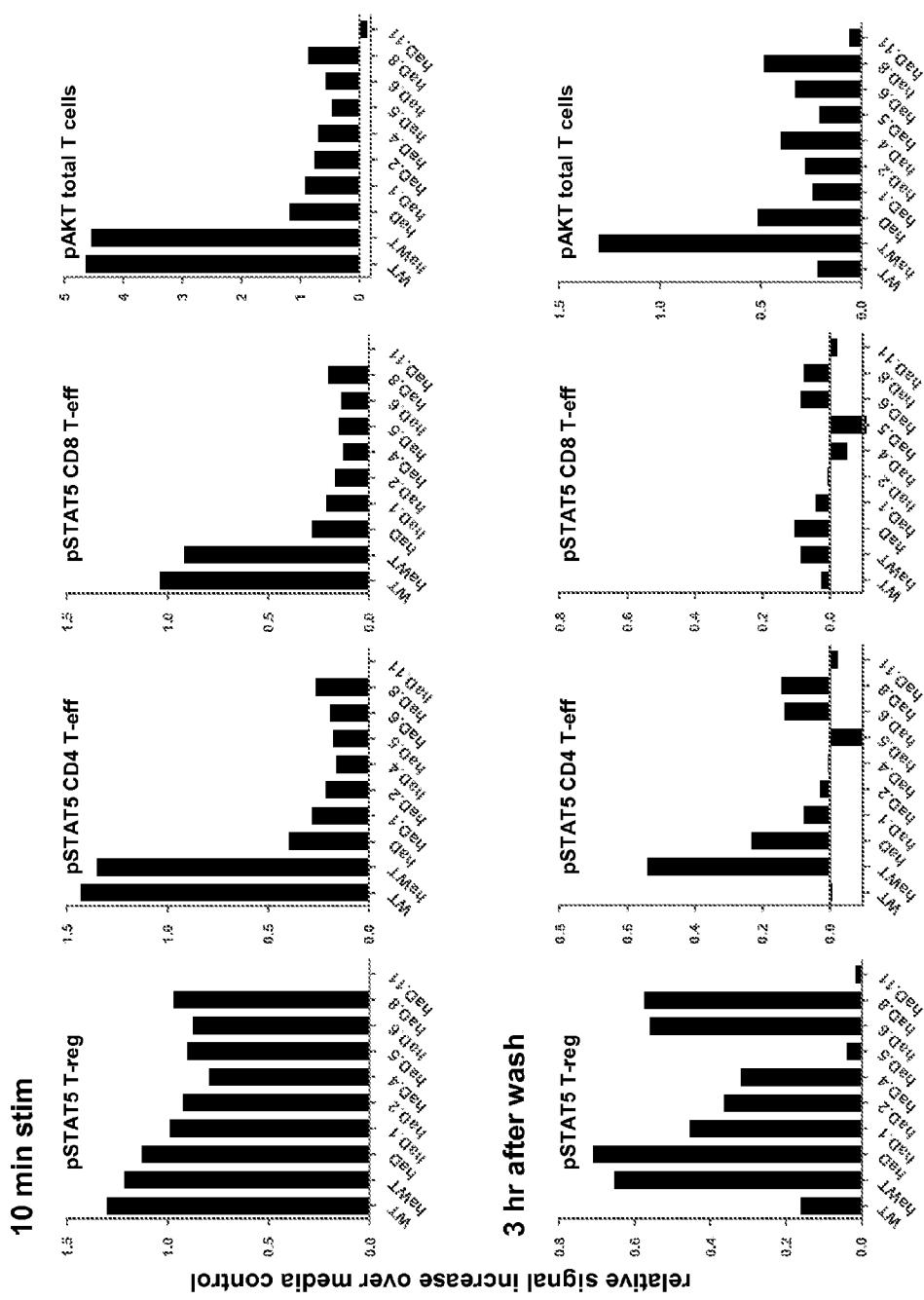


FIG. 3

## COMPOSITIONS AND METHODS OF TREATING INFLAMMATORY AND AUTOIMMUNE DISEASES

[0001] This application claims priority to U.S. Provisional Patent Application No. 61/146,111, filed Jan. 21, 2009, which is incorporated herein by reference in its entirety.

### BACKGROUND

[0002] IL-2 binds three transmembrane receptor subunits: IL-2R $\beta$  and IL-2R $\gamma$  which together activate intracellular signaling events upon IL-2 binding, and CD25 (IL-2R $\alpha$ ) which serves to present IL-2 to the other 2 receptor subunits. The signals delivered by IL-2R $\beta\gamma$  include those of the PI3-kinase, Ras-MAP-kinase, and STAT5 pathways.

[0003] T cells require expression of CD25 to respond to the low concentrations of IL-2 that typically exist in tissues. T cells that express CD25 include both CD4 $^{+}$  FOXP3 $^{+}$  regulatory T cells (T-reg cells)—which are essential for suppressing autoimmune inflammation—and FOXP3 $^{-}$  T cells that have been activated to express CD25. FOXP3 $^{-}$ CD25 $^{+}$  T effector cells (T-eff) may be either CD4 $^{+}$  or CD8 $^{+}$  cells, both of which can be pro-inflammatory and may contribute to autoimmunity, organ graft rejection or graft-versus-host disease. IL-2-stimulated STAT5 signaling is crucial for normal T-reg cell growth and survival and for high FOXP3 expression.

[0004] Because of the low affinity IL-2 possesses for each of the three IL-2R chains, a further reduction in affinity for IL-2R $\beta$  and/or IL-2R $\gamma$  could be offset by an increased affinity for CD25. Mutational variants of IL-2 have been generated that exhibit up to 170-fold higher affinity for CD25 (US Patent Application Publication No. 2005/0142106; Rao et al., *Biochemistry* 44, 10696-701 (2005)). These variants were reported to associate for several days with cell surface CD25 and to chronically promote growth of an IL-2-dependent cell line. The authors report that the mutants stimulate persistent T cell growth and, thus, may be useful in methods of viral immunotherapy and in treating cancer or other hyperproliferative disorders. High doses of IL-2 (Prolleukin) are administered to cancer patients to induce anti-tumor immunity, a treatment that is often associated with undesirable toxicity. U.S. Pat. No. 6,955,807 describes IL-2 variants that are said to have reduced toxicity. The patent attributes the toxicity to IL-2-induced stimulation of natural killer (NK) cells, which only express IL-2R $\beta$  and IL-2R $\gamma$ . The IL-2 variants described therein were said to have reduced toxicity because they selectively activate CD25 $^{+}$  T cells over NK cells. Again the IL-2 variants were said to be useful in therapeutic methods wherein it is beneficial to generally stimulate the immune system, e.g., the treatment of cancer or infectious diseases.

### SUMMARY

[0005] Provided herein are immunosuppressive mutational variants of IL-2 that preferentially promote the growth/survival of FOXP3 $^{+}$  regulatory T cells (T-reg cells) over the growth/survival of potentially proinflammatory FOXP3 $^{-}$ CD25 $^{+}$  T cells. By increasing the ratio of T-reg to other T cells and/or by increasing FOXP3 expression in T-reg without activating FOXP3 $^{-}$ CD25 $^{+}$  T cells, these variants should suppress undesirable inflammation.

[0006] The unique properties of these IL-2 variants stem from two sets of mutations. One set of mutations results in a

reduced affinity for the signaling chains of the IL-2 receptor (IL-2R $\beta$ /CD122 and/or IL-2R $\gamma$ /CD132) and/or a reduced capacity to induce a signaling event from one or both subunits of the IL-2 receptor. The second set of mutations confers higher affinity for CD25 (IL-2R $\alpha$ ) and may include mutations described by Rao et al. (US Patent Application Publication No. 2005/0142106).

[0007] As described herein, certain IL-2 variants induce signaling events that preferentially induce survival, proliferation, activation and/or function of T-reg cells. In certain embodiments, the IL-2 variant retains the capacity to stimulate, in T-reg cells, STAT5 phosphorylation and/or phosphorylation of one or more of signaling molecules downstream of the IL-2R, e.g., p38, ERK, SYK and LCK. In other embodiments, the IL-2 variant retains the capacity to stimulate, in T-reg cells, transcription or protein expression of genes or proteins, such as FOXP3 or IL-10, that are important for T-reg cell survival, proliferation, activation and/or function. In other embodiments, the IL-2 variant exhibits a reduced capacity to stimulate endocytosis of IL-2/IL-2R complexes on the surface of CD25 $^{+}$  T cells. In other embodiments, the IL-2 variant demonstrates inefficient, reduced, or absence of stimulation of PI3-kinase signaling, such as inefficient, reduced or absent phosphorylation of AKT and/or mTOR (mammalian target of rapamycin). In yet other embodiments, the IL-2 variant retains the ability of wt IL-2 to stimulate STAT5 phosphorylation and/or phosphorylation of one or more of signaling molecules downstream of the IL-2R in T-reg cells, yet demonstrates inefficient, reduced, or absent phosphorylation of STAT5, AKT and/or mTOR or other signaling molecules downstream of the IL-2R in FOXP3 $^{-}$ CD4 $^{+}$  or CD8 $^{+}$  T cells or NK cells. In other embodiments, the IL-2 variant is inefficient or incapable of stimulating survival, growth, activation and/or function of FOXP3 $^{-}$ CD4 $^{+}$  or CD8 $^{+}$  T cells or NK cells.

[0008] Provided are methods of treating an inflammatory or autoimmune disorder. The methods comprise administering a therapeutically effective amount of one or more immunosuppressive IL-2 variants to a subject.

[0009] Further provided are methods of promoting proliferation, survival, growth, or activation of regulatory T cells. The methods comprising contacting a FOXP3-positive (FOXP3 $^{+}$ ) T cell with an immunosuppressive IL-2 variant.

[0010] Also provided is the use of an immunosuppressive IL-2 variant in the preparation of a medicament for the treatment of an inflammatory or autoimmune disorder.

[0011] Also provided are compositions of IL-2 variants conjugated to additional protein sequences or other chemical that prolong the stability and/or half-life of the therapeutic *in vivo*.

### BRIEF EXPLANATION OF THE DRAWINGS

[0012] FIG. 1. Sequences of IL-2 variants. Sequences that vary from germline human IL-2 are not shaded in gray.

[0013] FIG. 2A-2F. FIG. 2A An example of flow cytometric data and gating strategy. Representative data is shown in FIG. 1A where 9.5% of CD4 $^{+}$  cells are CD25 $^{+}$ FOXP3 $^{+}$ , 9.9% of CD4 $^{+}$  cells are CD25 $^{+}$ FOXP3 $^{-}$  and 6.7% of CD8 $^{+}$  cells are CD25 $^{+}$ FOXP3 $^{-}$ . FOXP3 $^{+}$ CD8 $^{+}$  T cells are typically very infrequent. FIG. 2B. Relative number of CD8 $^{+}$ CD25 $^{+}$ FOXP3 $^{-}$  T cells. FIG. 2C. Relative number of CD4 $^{+}$ CD25 $^{+}$ FOXP3 $^{-}$  T cells. FIG. 2D. Relative number of CD4 $^{+}$ CD25 $^{+}$ FOXP3 $^{+}$  T cells. FIG. 2E. Ratio of FOXP3 $^{+}$ /FOXP3 $^{-}$  cells

among CD25<sup>+</sup>CD4<sup>+</sup> T cells. FIG. 2F. IL-2-mediated FOXP3 upregulation in FOXP3<sup>+</sup>CD4<sup>+</sup> T cells.

[0014] FIG. 3. IL-2 muteins stimulate phospho-STAT5 in T-reg but are inefficient at stimulating signals in other T cells. T cells were stimulated with IL-2 as described in Example 3. Phospho-STAT5 was measured by flow cytometry and phospho-AKT was measured by ELISA (MesoScale Discovery). Abbreviations are as follows: T-reg, FOXP3<sup>+</sup>CD4<sup>+</sup> T cells; CD4 T-eff, CD4<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>-</sup> "effector" T cells; CD8 T-eff, CD8<sup>+</sup>CD25<sup>+</sup>FOXP3<sup>-</sup> "effector" T cells.

#### DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

[0015] FOXP3<sup>+</sup> regulatory T cells (T-reg cells) are essential for maintaining normal immune homeostasis and immune tolerance to self tissues, as well as for suppressing undesirable inflammation. T-reg cells exert their suppressor and regulatory functions through multiple mechanisms which are likely to be regulated by temporal and environmental factors. Current immunosuppressive therapeutics generally target individual proinflammatory pathways and as such often exhibit partial efficacy or are applicable to specific diseases. An alternative immunosuppressive modality might involve the elevation of the numbers and activation state of natural suppressor cells to better enable them to deliver appropriate suppressor molecules/activities at sites of inflammation.

[0016] Described herein are therapeutic agents that selectively promote T-reg cell proliferation, survival, activation and/or function. By "selectively promote," it is meant the therapeutic agent promotes the activity in T-reg cells but has limited or lacks the ability to promote the activity in non-regulatory T cells. Further described herein are assays to screen for agents that selectively promote T-reg cell proliferation, survival, activation and/or function. Agents that may be screened include, but are not limited to, small molecules, peptides, polypeptides, proteins including antibodies, e.g., monoclonal, humanized, human, monovalent, bivalent, and multivalent antibodies.

[0017] In certain embodiments, the agent is an IL-2 variant. In particular, the IL-2 variant promotes these activities of T-reg cell growth/survival but have a reduced ability, as compared to wild-type IL-2, to promote non-regulatory T-cell (FOXP3<sup>-</sup>CD25<sup>+</sup>) and NK cell proliferation, survival, activation and/or function. In certain embodiments, such IL-2 variants function through a combination of elevated affinity for the IL-2R subunit IL-2R $\alpha$  (CD25) and a reduced affinity for the signaling subunits IL-2R $\beta$  and/or IL-2R $\gamma$ . Whereas IL-2 and variants thereof have been used in the art as immunostimulatory agents, e.g., in methods of treating cancer or infectious diseases, the IL-2 variants described herein are particularly useful as immunosuppressive agents, e.g., in methods of treating inflammatory disorders.

[0018] IL-2 variants comprise a sequence of amino acids at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93% at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identical to wild-type IL-2. IL-2 variants further include a sequence of amino acids at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, at least 91%, at least 92%, at least 93% at least 94%, at least 95%, at least 96%, at least 97%, at least 98%, at least 99% identical to a functional fragment of wild-type IL-2. As used herein, "wild-type IL-2" shall mean the polypeptide having the following amino acid sequence:

APTS SSKKTQLQLEHLLDLQMLNGINNYKNPKLTRMLTFKPYMP

KKATELKHLQCLEEELKPLEEVNLNAQSKNFHLRPRDLISNINVIVL

ELKGSETTFMCEYADETATIVEFLNWRITFXQSIISTLT

[0019] wherein X is C, S, A or V (SEQ ID NO:1).

[0020] Variants may contain one or more substitutions, deletions, or insertions within the wild-type IL-2 amino acid sequence. Residues are designated herein by the one letter amino acid code followed by the IL-2 amino acid position, e.g., K35 is the lysine residue at position 35 of SEQ ID NO:1. Substitutions are designated herein by the one letter amino acid code followed by the IL-2 amino acid position followed by the substituting one letter amino acid code, e.g., K35A is a substitution of the lysine residue at position 35 of SEQ ID NO:1 with an alanine residue.

[0021] In one aspect, the invention provides immunosuppressive IL-2 variants that have a higher affinity for IL-2R $\alpha$  than wild-type IL-2. U.S. Published Patent Application No. 2005/0142106 (incorporated herein by reference in its entirety) describes IL-2 variants that have higher affinity for IL-2R $\alpha$  than does wild-type IL-2 and methods of making and screening for such variants. Preferred IL-2 variants contain one or more mutations in positions of the IL-2 sequence that either contact IL-2R $\alpha$  or alter the orientation of other positions contacting IL-2R $\alpha$ , resulting in higher affinity for IL-2R $\alpha$ . The mutations may be in or near areas known to be in close proximity to IL-2R $\alpha$  based on published crystal structures (Xinquan Wang, Mathias Ricker, K. Christopher Garcia. *Science* 310:1159 2005). IL-2 residues believed to contact IL-2R $\alpha$  include K35, R38, F42, K43, F44, Y45, E61, E62, K64, P65, E68, V69, L72, and Y107.

[0022] IL-2 variants having greater affinity for IL-2R $\alpha$  can include a change in N29, N30, Y31, K35, T37, K48, E68, V69, N71, Q74, S75, or K76. Preferred variants include those having one or more of the following mutations: N29S, N30S, N30D, Y31H, Y31S, K35R, T37A, K48E, V69A, N71R, and Q74P.

[0023] Immunosuppressive IL-2 variants also include variants that demonstrate altered signaling through certain pathways activated by wild-type IL-2 via the IL-2R and result in preferential proliferation/survival/activation of T-reg. Molecules known to be phosphorylated upon activation of the IL-2R include STAT5, p38, ERK, SYK, LCK, AKT and mTOR. Compared to wild-type IL-2, the immunosuppressive IL-2 variant can possess a reduced PI3K signaling ability in FOXP3<sup>-</sup> T cells, which can be measured by a reduction in the phosphorylation of AKT and/or mTOR as compared to wild-type IL-2. Such variants may include mutations in positions that either contact IL-2R $\beta$  or IL-2R $\gamma$  or alter the orientation of other positions contacting IL-2R $\beta$  or IL-2R $\gamma$ . IL-2 residues believed to contact IL-2R $\beta$  include L12, Q13, H16, L19, D20, M23, R81, D84, S87, N88, V91, I92, and E95. IL-2 residues believed to contact IL-2R $\gamma$  include Q11, L18, Q22, E110, N119, T123, Q126, S127, I129, S130, and T133. In certain embodiments, the IL-2 variant comprises a mutation at E15, H16, Q22, D84, N88, or E95. Examples of such mutations include E15Q, H16N, Q22E, D84N, N88D, and E95Q.

[0024] In certain embodiments, the IL-2 variant comprises a combination of mutations. Examples of IL-2 variants having a combination of mutations are provided in FIG. 1 and include haWT (SEQ ID NO:2), haD, (SEQ ID NO:3), haD.1 (SEQ ID NO:4), haD.2 (SEQ ID NO:5), haD.4 (SEQ ID

NO:6), haD.5 (SEQ ID NO:7), haD.6 (SEQ ID NO:8), haD.8 (SEQ ID NO:9), and haD.11 (SEQ ID NO:10)d. In preferred embodiments, the IL-2 variant stimulates STAT5 phosphorylation in FOXP3-positive regulatory T cells but has reduced ability to induce STAT5 and AKT phosphorylation in FOXP3-negative T cells as compared to wild-type IL-2. Preferred variants having such properties include haD, haD.1, haD.2, haD.4, haD.5, haD.6, and haD.8.

[0025] The IL-2 variants may further comprise one or more mutations as compared to the wild-type IL-2 sequence that do not have an effect on the affinity for IL-2R $\beta$  or IL-2R $\gamma$ , provided the IL-2 variant promotes the preferential proliferation, survival, activation or function of FOXP3 $^{+}$  T-reg over that of other T cells that do not express FOXP3. In preferred embodiments, such mutations are conservative mutations.

[0026] The IL-2 variant may comprise one or more compounds to increase the serum-half-life of the IL-2 variant when administered to a patient. Such half-life extending molecules include water soluble polymers (e.g., polyethylene glycol (PEG)), low- and high-density lipoproteins, antibody Fc (monomer or dimer), transthyretin (TTR), and TGF- $\beta$  latency associated peptide (LAP). Also contemplated are IL-2 variants comprising a combination of serum half-life extending molecules, such as PEGylated TTR (US Pat. Appl. Publ. No. 2003/0195154).

[0027] Methods of Making an Immunosuppressive IL-2 Variant

[0028] The immunosuppressive IL-2 variants can be produced using any suitable method known in the art, including those described in U.S. Pat. No. 6,955,807 for producing immunostimulatory IL-2 variants (incorporated herein by reference). Such methods include constructing a DNA sequence encoding the IL-2 variant and expressing those sequences in a suitably transformed host. This method will produce the recombinant variant of this invention. However, the variants may also be produced by chemical synthesis or a combination of chemical synthesis and recombinant DNA technology. Batch-wise production or perfusion production methods are known in the art. See Freshey, R. I. (ed), "Animal Cell Culture: A Practical Approach," 2nd ed., 1992, IRL Press. Oxford, England; Mather, J. P. "Laboratory Scaleup of Cell Cultures (0.5-50 liters)," Methods Cell Biol 57: 219-527 (1998); Hu, W. S., and Aunins, J. G., "Large-scale Mammalian Cell Culture," Curr Opin Biotechnol 8: 148-153 (1997); Konstantinov, K. B., Tsai, Y., Moles, D., Matanguhan, R., "Control of long-term perfusion Chinese hamster ovary cell culture by glucose auxostat," Biotechnol Prog 12:100-109 (1996).

[0029] In one embodiment of a recombinant method for producing a variant, a DNA sequence is constructed by isolating or synthesizing a DNA sequence encoding the wild type IL-2 and then changing one or more codons by site-specific mutagenesis. This technique is well known. See, e.g., Mark et. al., "Site-specific Mutagenesis Of The Human Fibroblast Interferon Gene", Proc. Natl. Acad. Sci. USA 81, pp. 5662-66 (1984); and U.S. Pat. No. 4,588,585, incorporated herein by reference.

[0030] Another method of constructing a DNA sequence encoding the IL-2 variant would be chemical synthesis. This for example includes direct synthesis of a peptide by chemical means of the protein sequence encoding for an IL-2 variant exhibiting the properties described herein. This method may incorporate both natural and unnatural amino acids at positions that affect the interactions of IL-2 with the IL2R $\alpha$ ,

IL-2R $\beta$ , or IL-2R $\gamma$ . Alternatively, a gene which encodes the desired IL-2 variant may be synthesized by chemical means using an oligonucleotide synthesizer. Such oligonucleotides are designed based on the amino acid sequence of the desired IL-2 variant, and preferably selecting those codons that are favored in the host cell in which the recombinant variant will be produced. In this regard, it is well recognized that the genetic code is degenerate—that an amino acid may be coded for by more than one codon. For example, Phe (F) is coded for by two codons, TTC or TTT, Tyr (Y) is coded for by TAC or TAT and his (H) is coded for by CAC or CAT. Trp (W) is coded for by a single codon, TGG. Accordingly, it will be appreciated that for a given DNA sequence encoding a particular IL-2 variant, there will be many DNA degenerate sequences that will code for that IL-2 variant.

[0031] The DNA sequence encoding the IL-2 variant, whether prepared by site directed mutagenesis, chemical synthesis or other methods, may or may not also include DNA sequences that encode a signal sequence. Such signal sequence, if present, should be one recognized by the cell chosen for expression of the IL-2 variant. It may be prokaryotic, eukaryotic or a combination of the two. It may also be the signal sequence of native IL-2. The inclusion of a signal sequence depends on whether it is desired to secrete the IL-2 variant from the recombinant cells in which it is made. If the chosen cells are prokaryotic, it generally is preferred that the DNA sequence not encode a signal sequence. If the chosen cells are eukaryotic, it generally is preferred that a signal sequence be encoded and most preferably that the wild-type IL-2 signal sequence be used.

[0032] Standard methods may be applied to synthesize a gene encoding an IL-2 variant. For example, the complete amino acid sequence may be used to construct a back-translated gene. A DNA oligomer containing a nucleotide sequence coding for an IL-2 variant may be synthesized. For example, several small oligonucleotides coding for portions of the desired polypeptide may be synthesized and then ligated. The individual oligonucleotides typically contain 5' or 3' overhangs for complementary assembly.

[0033] Once assembled (by synthesis, site-directed mutagenesis or another method), the DNA sequences encoding an IL-2 variant will be inserted into an expression vector and operatively linked to an expression control sequence appropriate for expression of the IL-2 variant in the desired transformed host. Proper assembly may be confirmed by nucleotide sequencing, restriction mapping, and expression of a biologically active polypeptide in a suitable host. As is well known in the art, in order to obtain high expression levels of a transfected gene in a host, the gene must be operatively linked to transcriptional and translational expression control sequences that are functional in the chosen expression host. The choice of expression control sequence and expression vector will depend upon the choice of host. A wide variety of expression host/vector combinations may be employed.

[0034] Any suitable host may be used to produce the IL-2 variant, including bacteria, fungi (including yeasts), plant, insect, mammal, or other appropriate animal cells or cell lines, as well as transgenic animals or plants. More particularly, these hosts may include well known eukaryotic and prokaryotic hosts, such as strains of *E. coli*, *Pseudomonas*, *Bacillus*, *Streptomyces*, fungi, yeast, insect cells such as *Spodoptera frugiperda* (Sf9), animal cells such as Chinese hamster ovary (CHO) and mouse cells such as NS/O, African green monkey cells such as COS 1, COS 7, BSC 1, BSC 40,

and BNT 10, and human cells, as well as plant cells in tissue culture. For animal cell expression, CHO cells and COS 7 cells in cultures and particularly the CHO cell line CHO (DHFR-) or the HKB line are preferred.

**[0035]** It should of course be understood that not all vectors and expression control sequences will function equally well to express the DNA sequences described herein. Neither will all hosts function equally well with the same expression system. However, one of skill in the art may make a selection among these vectors, expression control sequences and hosts without undue experimentation. For example, in selecting a vector, the host must be considered because the vector must replicate in it. The vectors copy number, the ability to control that copy number, and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered. For example, preferred vectors for use in this invention include those that allow the DNA encoding the IL-2 variants to be amplified in copy number. Such amplifiable vectors are well known in the art. They include, for example, vectors able to be amplified by DHFR amplification (see, e.g., Kaufman, U.S. Pat. No. 4,470,461, Kaufman and Sharp, "Construction Of A Modular Dihydrafolate Reductase cDNA Gene: Analysis Of Signals Utilized For Efficient Expression", Mol. Cell. Biol., 2, pp. 1304-19 (1982)) or glutamine synthetase ("GS") amplification (see, e.g., U.S. Pat. No. 5,122,464 and European published application 338,841).

**[0036]** The IL-2 variants may be glycosylated or unglycosylated depending on the host organism used to produce the variant. If bacteria are chosen as the host, then the IL-2 variant produced will be unglycosylated. Eukaryotic cells, on the other hand, will glycosylate the IL-2 variant, although perhaps not in the same way as native IL-2 is glycosylated. The IL-2 variant produced by the transformed host can be purified according to any suitable method. Various methods are known for purifying IL-2. See, e.g. Current Protocols in Protein Science, Vol 2. Eds: John E. Coligan, Ben M. Dunn, Hidde L. Ploehg, David W. Speicher, Paul T. Wingfield, Unit 6.5 (Copyright 1997, John Wiley and Sons, Inc).

**[0037]** The biological activity of the IL-2 variants can be assayed by any suitable method known in the art. Such assays include those described in the Examples below.

#### **[0038] Indications**

**[0039]** Diseases, disorders, or conditions may be amenable to treatment with or may be prevented by administration of a T-reg-selective IL-2 variant to a subject. Such diseases, disorders, and conditions include, but are not limited to, inflammation, autoimmune disease, paraneoplastic autoimmune diseases, cartilage inflammation, fibrotic disease and/or bone degradation, arthritis, rheumatoid arthritis, juvenile arthritis, juvenile rheumatoid arthritis, pauciarticular juvenile rheumatoid arthritis, polyarticular juvenile rheumatoid arthritis, systemic onset juvenile rheumatoid arthritis, juvenile ankylosing spondylitis, juvenile enteropathic arthritis, juvenile reactive arthritis, juvenile Reter's Syndrome, SEA Syndrome (Seronegativity, Enthesopathy, Arthropathy Syndrome), juvenile dermatomyositis, juvenile psoriatic arthritis, juvenile scleroderma, juvenile systemic lupus erythematosus, juvenile vasculitis, pauciarticular rheumatoid arthritis, polyarticular rheumatoid arthritis, systemic onset rheumatoid arthritis, ankylosing spondylitis, enteropathic arthritis, reactive arthritis, Reter's Syndrome, SEA Syndrome (Seronegativity, Enthesopathy, Arthropathy Syndrome), dermatomyositis, psoriatic arthritis, scleroderma, systemic lupus erythematosus, vasculitis, myolitis, polymyolitis, dermatomyolitis,

osteoarthritis, polyarteritis nodosa, Wegener's granulomatosis, arteritis, polymyalgia rheumatica, sarcoidosis, scleroderma, sclerosis, primary biliary sclerosis, sclerosing cholangitis, Sjogren's syndrome, psoriasis, plaque psoriasis, guttate psoriasis, inverse psoriasis, pustular psoriasis, erythrodermic psoriasis, dermatitis, atopic dermatitis, atherosclerosis, lupus, Still's disease, Systemic Lupus Erythematosus (SLE), myasthenia gravis, inflammatory bowel disease (IBD), Crohn's disease, ulcerative colitis, celiac disease, multiple sclerosis (MS), asthma, COPD, Guillain-Barre disease, Type I diabetes mellitus, thyroiditis (e.g., Graves' disease), Addison's disease, Raynaud's phenomenon, autoimmune hepatitis, GVHD, transplantation rejection, and the like. In specific embodiments, pharmaceutical compositions comprising a therapeutically effective amount of a T-reg-selective IL-2 variant are provided.

**[0040]** The term "treatment" encompasses alleviation or prevention of at least one symptom or other aspect of a disorder, or reduction of disease severity, and the like. A T-reg-selective IL-2 variant need not effect a complete cure, or eradicate every symptom or manifestation of a disease, to constitute a viable therapeutic agent. As is recognized in the pertinent field, drugs employed as therapeutic agents may reduce the severity of a given disease state, but need not abolish every manifestation of the disease to be regarded as useful therapeutic agents. Similarly, a prophylactically administered treatment need not be completely effective in preventing the onset of a condition in order to constitute a viable prophylactic agent. Simply reducing the impact of a disease (for example, by reducing the number or severity of its symptoms, or by increasing the effectiveness of another treatment, or by producing another beneficial effect), or reducing the likelihood that the disease will occur or worsen in a subject, is sufficient. One embodiment of the invention is directed to a method comprising administering to a patient A T-reg-selective IL-2 variant in an amount and for a time sufficient to induce a sustained improvement over baseline of an indicator that reflects the severity of the particular disorder.

#### **[0041] Pharmaceutical Compositions**

**[0042]** In some embodiments, the invention provides pharmaceutical compositions comprising a therapeutically effective amount of one or a plurality of T-reg-selective IL-2 variants of the invention together with a pharmaceutically acceptable diluent, carrier, solubilizer, emulsifier, preservative, and/or adjuvant. In addition, the invention provides methods of treating a patient by administering such pharmaceutical composition. The term "patient" includes human and animal subjects.

**[0043]** In certain embodiments, acceptable formulation materials preferably are nontoxic to recipients at the dosages and concentrations employed. In certain embodiments, the pharmaceutical composition may contain formulation materials for modifying, maintaining or preserving, for example, the pH, osmolality, viscosity, clarity, color, isotonicity, odor, sterility, stability, rate of dissolution or release, adsorption or penetration of the composition. In such embodiments, suitable formulation materials include, but are not limited to, amino acids (such as glycine, glutamine, asparagine, arginine or lysine); antimicrobials; antioxidants (such as ascorbic acid, sodium sulfite or sodium hydrogen-sulfite); buffers (such as borate, bicarbonate, Tris-HCl, citrates, phosphates or other organic acids); bulking agents (such as mannitol or glycine); chelating agents (such as ethylenediamine tetraacetic acid (EDTA)); complexing agents (such as caffeine, poly-

vinylpyrrolidone, beta-cyclodextrin or hydroxypropyl-beta-cyclodextrin); fillers; monosaccharides; disaccharides; and other carbohydrates (such as glucose, sucrose, mannose or dextrans); proteins (such as serum albumin, gelatin or immunoglobulins); coloring, flavoring and diluting agents; emulsifying agents; hydrophilic polymers (such as polyvinylpyrrolidone); low molecular weight polypeptides; salt-forming counterions (such as sodium); preservatives (such as benzalkonium chloride, benzoic acid, salicylic acid, thimerosal, phenethyl alcohol, methylparaben, propylparaben, chlorhexidine, sorbic acid or hydrogen peroxide); solvents (such as glycerin, propylene glycol or polyethylene glycol); sugar alcohols (such as mannitol or sorbitol); suspending agents; surfactants or wetting agents (such as pluronics, PEG, sorbitan esters, polysorbates such as polysorbate 20, polysorbate, triton, tromethamine, lecithin, cholesterol, tyloxapal); stability enhancing agents (such as sucrose or sorbitol); tonicity enhancing agents (such as alkali metal halides, preferably sodium or potassium chloride, mannitol sorbitol); delivery vehicles; diluents; excipients and/or pharmaceutical adjuvants. See, REMINGTON'S PHARMACEUTICAL SCIENCES, 18<sup>th</sup> Edition, (A. R. Genmo, ed.), 1990, Mack Publishing Company.

[0044] The therapeutically effective amount of T-reg-selective IL-2 variant-containing pharmaceutical composition to be employed will depend, for example, upon the therapeutic context and objectives. One skilled in the art will appreciate that the appropriate dosage levels for treatment will vary depending, in part, upon the molecule delivered, the indication for which the T-reg-selective IL-2 variant is being used, the route of administration, and the size (body weight, body surface or organ size) and/or condition (the age and general health) of the patient.

[0045] In certain embodiments, the clinician may titer the dosage and modify the route of administration to obtain the optimal therapeutic effect. A typical dosage may range from about 0.1  $\mu$ g/kg to up to about 30 mg/kg or more, depending on the factors mentioned above. In specific embodiments, the dosage may range from 0.1  $\mu$ g/kg up to about 30 mg/kg, optionally from 1  $\mu$ g/kg up to about 30 mg/kg or from 10  $\mu$ g/kg up to about 5 mg/kg.

[0046] Dosing frequency will depend upon the pharmacokinetic parameters of the particular T-reg-selective IL-2 variant in the formulation used. Typically, a clinician administers the composition until a dosage is reached that achieves the desired effect. The composition may therefore be administered as a single dose, or as two or more doses (which may or may not contain the same amount of the desired molecule) over time, or as a continuous infusion via an implantation device or catheter. Further refinement of the appropriate dosage is routinely made by those of ordinary skill in the art and is within the ambit of tasks routinely performed by them.

[0047] The route of administration of the pharmaceutical composition is in accord with known methods, e.g., orally, through injection by intravenous, intraperitoneal, intracerebral (intra-parenchymal), intracerebroventricular, intramuscular, intra-ocular, intraarterial, intraportal, or intralesional routes; by sustained release systems or by implantation devices. In certain embodiments, the compositions may be administered by bolus injection or continuously by infusion, or by implantation device.

[0048] Combination Therapies

[0049] In further embodiments, T-reg-selective IL-2 variant is administered in combination with other agents useful

for treating the condition with which the patient is afflicted. Examples of such agents include both proteinaceous and non-proteinaceous drugs. When multiple therapeutics are co-administered, dosages may be adjusted accordingly, as is recognized in the pertinent art. "Co-administration" and combination therapy are not limited to simultaneous administration, but also include treatment regimens in which a T-reg-selective IL-2 variant is administered at least once during a course of treatment that involves administering at least one other therapeutic agent to the patient.

[0050] In certain embodiments, a T-reg-selective IL-2 variant is administered in combination with an inhibitor of the PI3-K/AKT/mTOR pathway, e.g., rapamycin (rapamune, sirolimus). Inhibitors of this pathway in combination with IL-2 favor T-reg enrichment.

[0051] The invention having been described, the following examples are offered by way of illustration, and not limitation.

## EXAMPLES

### Example 1

#### Panel of IL-2 Mutants

[0052] To examine the potential for generating IL-2 variants with reduced capacity to stimulate FOXP3<sup>+</sup>CD25<sup>+</sup> "effector" T cells (T-eff) but not T-reg, a series of IL-2 mutants was generated in which amino acids predicted to interact with the IL-2R $\beta$  and/or IL-2R $\gamma$  chain were altered. These variants also contained a set of previously described mutations that conferred high affinity for CD25 (variant "2-4" in Rao et al., *Biochemistry* 44, 10696-701 (2005)). This series of variants is shown in FIG. 1. Variant haWT contained only the mutations that contributed to the high affinity for CD25. Variants haD, haD.1, haD.2, etc., also contained mutations predicted to alter interactions with IL-2R $\beta$  and/or IL-2R $\gamma$ . In all assays, variant haD.11 was not capable of inducing any signal or altering any cellular phenotype and, as such, served as a control for CD25 binding without IL-2R signaling. All the IL-2 variants contained the C125S mutation for improved manufacturability and terminated with FLAG and HIS-tag sequences (DYKDDDDKGSSHHHHH) (SEQ ID NO:11)

[0053] Several assays were used to assess the ability of the IL-2 variants to induce signaling events and T cell growth. These included assays to detect:

[0054] 1. Growth and survival of T cell subsets and measurement of FOXP3 expression.

[0055] 2. Cell signaling (e.g. detection of phosphorylated STAT5 and AKT using flow cytometric and ELISA-based methods).

### Example 2

#### Enrichment of FOXP3<sup>+</sup> Cells and Retention of FOXP3 Upregulation During Long Term T Cell Culture

[0056] Total PBMC were activated in 24-well plates at  $4 \times 10^6$  cells per well with 100 ng/ml anti-CD3 (OKT3). On day 3 of culture, cells were washed 3 times and rested in fresh media for 3 days. Cells were then washed and seeded in 96 well flat-bottom plates with IL-2 variants at either 10 nM or 100 pM. Three days later cells were counted and analyzed by flow cytometry. (FIG. 2A)

[0057] As expected, CD8<sup>+</sup>CD25<sup>+</sup> T cells were especially responsive to WT IL-2 and variant haWT, however, all vari-

ants that contained mutated IL-2R $\beta$  and/or  $\gamma$  contact residues were very inefficient at promoting accumulation of activated CD8 $^{+}$ CD25 $^{+}$  T cells (FIG. 2B). A similar trend was observed for CD4 $^{+}$ CD25 $^{+}$ FOXP3 $^{-}$  T cells (FIG. 2C). In contrast, the growth/survival of FOXP3 $^{+}$ CD4 $^{+}$  T cells was stimulated by several IL-2 variants to a degree similar to that of WT IL-2 (FIG. 2D). As a result, the ratio of FOXP3 $^{+}$  to FOXP3 $^{-}$  T cells among CD4 $^{+}$  CD25 $^{+}$  T cells was increased by several IL-2 variants with IL-2R $\beta$  $\gamma$ -contact residue mutations (FIG. 2E). Furthermore, the mutations did not impair IL-2-stimulated FOXP3 upregulation in T-reg (FIG. 2F).

### Example 3

#### Mutations that Reduce Signaling in FOXP3 $^{-}$ T Cells but Stimulate STAT5 Signaling in T-Reg

**[0058]** The IL-2 variants were screened for their ability to stimulate AKT and STAT5 phosphorylation in T cell subsets. Several IL-2 variants were as potent, or nearly as potent, as wt IL-2 at stimulating STAT5 in FOXP3 $^{+}$  T cells 10 min after stimulation. Three hours after washing IL-2 from the media, some IL-2 variants (haD, haD.1, haD.2, haD.4, haD.6, and haD.8) continued to stimulate sustained STAT5 signaling at levels higher than that seen with wt IL-2. In contrast, for

FOXP3 $^{-}$  T cells, STAT5 and AKT responses to the haD variants after 10 min stimulation were not nearly as high as those stimulated by wt IL-2 or haWT. After 3 hrs, weak STAT5 and AKT signals similar to those seen with wt IL-2 were observed in T-eff, however, at this late timepoint wt IL-2 signaling had diminished greatly. In FOXP3 $^{+}$  T cells, AKT signaling is not normally stimulated by IL-2 (Zeiser R, et al, 2008 *Blood* 111:453) thus the phospho-AKT signal observed in total T cell lysates can be attributed to T-eff.

**[0059]** Methods: Previously activated (with anti-CD3 for 2-3 days) and rested (in fresh culture medium for 2-5 days) T cells were exposed to 1 nM wt or mutant IL-2 for 10 min at 37° C. Cells were then stained (10 min timepoint) or washed and cultured for an additional 3 hrs (3 hr timepoint). To measure phospho-AKT by ELISA, a 50  $\mu$ l culture was stopped by adding an equal volume of 2x lysis buffer and lysates were measured for phospho-AKT with multiplex ELISA plates according to the manufacturer's protocols (MesoScale Discovery, Gaithersburg, Md.). To measure phospho-STAT5 by flow cytometry, a 50  $\mu$ l culture was stopped by adding 1 ml of FOXP3 Fix/Perm Buffer (BioLegend, San Diego, Calif.), incubation at 25° C. for 20 min, and staining for cell surface markers, FOXP3 and phospho-STAT5 according the BioLegend FOXP3 staining protocol.

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#### SEQUENCE LISTING

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Asn Pro Lys Leu Thr Arg Met Leu Thr Phe Lys Phe Tyr Met Pro Lys
35 40 45

Lys Ala Thr Glu Leu Lys His Leu Gln Cys Leu Glu Glu Leu Lys
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Pro Leu Glu Glu Val Leu Asn Leu Ala Gln Ser Lys Asn Phe His Leu
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Arg Pro Arg Asp Leu Ile Ser Asn Ile Asn Val Ile Val Leu Glu Leu
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Lys Gly Ser Glu Thr Thr Phe Met Cys Glu Tyr Ala Asp Glu Thr Ala
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Asn Pro Arg Leu Ala Arg Met Leu Thr Phe Lys Phe Tyr Met Pro Glu
35 40 45

Lys Ala Thr Glu Leu Lys His Leu Gln Cys Leu Glu Glu Glu Leu Lys
50 55 60

Pro Leu Glu Glu Ala Leu Arg Leu Ala Pro Ser Lys Asn Phe His Leu
65 70 75 80

Arg Pro Arg Asp Leu Ile Ser Asn Ile Asn Val Ile Val Leu Glu Leu
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Lys Gly Ser Glu Thr Thr Phe Met Cys Glu Tyr Ala Asp Glu Thr Ala
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Ile Ser Thr Leu Thr
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Leu Leu Leu Asp Leu Gln Met Ile Leu Asn Gly Ile Ser Asn His Lys
20 25 30

Asn Pro Arg Leu Ala Arg Met Leu Thr Phe Lys Phe Tyr Met Pro Glu
35 40 45

Lys Ala Thr Glu Leu Lys His Leu Gln Cys Leu Glu Glu Glu Leu Lys
50 55 60

Pro Leu Glu Glu Ala Leu Arg Leu Ala Pro Ser Lys Asn Phe His Leu
65 70 75 80

Arg Pro Arg Asp Leu Ile Ser Asp Ile Asn Val Ile Val Leu Glu Leu
85 90 95

Lys Gly Ser Glu Thr Thr Phe Met Cys Glu Tyr Ala Asp Glu Thr Ala
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Thr Ile Val Glu Phe Leu Asn Arg Trp Ile Thr Phe Ser Gln Ser Ile
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Ile Ser Thr Leu Thr
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 Asn Pro Arg Leu Ala Arg Met Leu Thr Phe Lys Phe Tyr Met Pro Glu  
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 Lys Ala Thr Glu Leu Lys His Leu Gln Cys Leu Glu Glu Glu Leu Lys  
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 Asn Pro Arg Leu Ala Arg Met Leu Thr Phe Lys Phe Tyr Met Pro Glu  
 35                   40                   45  
  
 Lys Ala Thr Glu Leu Lys His Leu Gln Cys Leu Glu Glu Glu Leu Lys  
 50                   55                   60  
  
 Pro Leu Glu Glu Ala Leu Arg Leu Ala Pro Ser Lys Asn Phe His Leu  
 65                   70                   75                   80  
  
 Arg Pro Arg Asp Leu Ile Ser Asp Ile Asn Val Ile Val Leu Glu Leu  
 85                   90                   95  
  
 Lys Gly Ser Glu Thr Thr Phe Met Cys Glu Tyr Ala Asp Glu Thr Ala  
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Asn Pro Arg Leu Ala Arg Met Leu Thr Phe Lys Phe Tyr Met Pro Glu  
35 40 45

Lys Ala Thr Glu Leu Lys His Leu Gln Cys Leu Glu Glu Glu Leu Lys  
50 55 60

Pro Leu Glu Glu Ala Leu Arg Leu Ala Pro Ser Lys Asn Phe His Leu  
65 70 75 80

Arg Pro Arg Asp Leu Ile Ser Asp Ile Asn Val Ile Val Leu Glu Leu  
85 90 95

Lys Gly Ser Glu Thr Thr Phe Met Cys Glu Tyr Ala Asp Glu Thr Ala  
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Ile Ser Thr Leu Thr  
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20 25 30

Asn Pro Arg Leu Ala Arg Met Leu Thr Phe Lys Phe Tyr Met Pro Glu  
35 40 45

Lys Ala Thr Glu Leu Lys His Leu Gln Cys Leu Glu Glu Glu Leu Lys  
50 55 60

Pro Leu Glu Glu Ala Leu Arg Leu Ala Pro Ser Lys Asn Phe His Leu  
65 70 75 80

Arg Pro Arg Asp Leu Ile Ser Asp Ile Asn Val Ile Val Leu Glu Leu  
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Lys Gly Ser Glu Thr Thr Phe Met Cys Glu Tyr Ala Asp Glu Thr Ala  
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Thr Ile Val Glu Phe Leu Asn Arg Trp Ile Thr Phe Ser Gln Ser Ile  
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Asn Pro Arg Leu Ala Arg Met Leu Thr Phe Lys Phe Tyr Met Pro Glu
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Lys Ala Thr Glu Leu Lys His Leu Gln Cys Leu Glu Glu Glu Leu Lys
50           55           60

Pro Leu Glu Glu Ala Leu Arg Leu Ala Pro Ser Lys Asn Phe His Leu
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Arg Pro Arg Asn Leu Ile Ser Asp Ile Asn Val Ile Val Leu Glu Leu
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Lys Gly Ser Glu Thr Thr Phe Met Cys Glu Tyr Ala Asp Glu Thr Ala
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Ile Ser Thr Leu Thr
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Leu Leu Leu Asp Leu Gln Met Ile Leu Asn Gly Ile Ser Asn His Lys
20          25           30

Asn Pro Arg Leu Ala Arg Met Leu Thr Phe Lys Phe Tyr Met Pro Glu
35           40           45

Lys Ala Thr Glu Leu Lys His Leu Gln Cys Leu Glu Glu Glu Leu Lys
50           55           60

Pro Leu Glu Glu Ala Leu Arg Leu Ala Pro Ser Lys Asn Phe His Leu
65           70           75           80

Arg Pro Arg Asp Leu Ile Ser Asp Ile Asn Val Ile Val Leu Gln Leu
85           90           95

Lys Gly Ser Glu Thr Thr Phe Met Cys Glu Tyr Ala Asp Glu Thr Ala
100          105          110

Thr Ile Val Glu Phe Leu Asn Arg Trp Ile Thr Phe Ser Gln Ser Ile
115          120          125

Ile Ser Thr Leu Thr
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Leu Leu Leu Asp Leu Gln Met Ile Leu Asn Gly Ile Ser Asn His Lys  
 20 25 30

Asn Pro Arg Leu Ala Arg Met Leu Thr Phe Lys Phe Tyr Met Pro Glu  
 35 40 45

Lys Ala Thr Glu Leu Lys His Leu Gln Cys Leu Glu Glu Leu Lys  
 50 55 60

Pro Leu Glu Glu Ala Leu Arg Leu Ala Pro Ser Lys Asn Phe His Leu  
 65 70 75 80

Arg Pro Arg Asp Leu Ile Ser Asp Ile Asn Val Ile Val Leu Glu Leu  
 85 90 95

Lys Gly Ser Glu Thr Thr Phe Met Cys Glu Tyr Ala Asp Glu Thr Ala  
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His

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**1.** A method of treating an inflammatory disorder in a subject, said method comprising administering to a subject in need thereof a therapeutically effective amount of an IL-2 variant, wherein said IL-2 variant

- (a) comprises a sequence of amino acids at least 80% identical to SEQ ID NO:1;
- (b) stimulates STAT5 phosphorylation in FOXP3-positive regulatory T cells; and
- (c) has a reduced ability compared to the polypeptide set forth as SEQ ID NO:1 to induce phosphorylation of STAT5 in FOXP3-negative T cells.

**2.** The method of claim 1, wherein the inflammatory disorder is selected from the group consisting of asthma, diabetes, arthritis, allergy, organ graft rejection and graft-versus-host disease.

**3.** The method of claim 1, wherein said IL-2 variant comprises a sequence of amino acids at least 90% identical to SEQ ID NO:1.

**4.** The method of claim 1, wherein said IL-2 variant comprises a sequence of amino acids at least 95% identical to SEQ ID NO:1.

**5.** The method of claim 1, wherein the IL-2 variant has a higher affinity for IL-2R $\alpha$  than does the polypeptide set forth as SEQ ID NO:1;

**6.** The method of claim 1, wherein the IL-2 variant promotes FOXP3-positive regulatory T cell growth or survival in vitro.

**7.** The method of claim 1, wherein the IL-2 variant comprises a mutation in the polypeptide sequence set forth in SEQ ID NO:1 at a position selected from the group consisting of amino acid 30, amino acid 31, amino acid 35, amino acid 69, and amino acid 74.

**8.** The method of claim 7, wherein the mutation at position 30 is N30S.

**9.** The method of claim 7, wherein the mutation at position 31 is Y31H.

**10.** The method of claim 7, wherein the mutation at position 35 is K35R.

**11.** The method of claim 7, wherein the mutation at position 69 is V69A.

**12.** The method of claim 7, wherein the mutation at position 74 is Q74P.

**13.** The method of claim 1, wherein the IL-2 variant induces STAT5 phosphorylation in ex vivo FOXP3-positive T cells comprising a functional IL-2 receptor complex but has a reduced ability to induce phosphorylation of STAT5.

**14.** The method of claim 13, wherein the IL-2 variant comprises a mutation in the polypeptide sequence set forth in SEQ ID NO:1 at position 88.

**15.** The method of claim 14, wherein the mutation at position 88 is N88D.

**16.** The method of claim **1**, wherein the IL-2 variant is conjugated to a chemical or polypeptide that extends the serum half-life of said IL-2 variant in vivo.

**17.** A method of promoting FOXP3-positive regulatory T cell growth or survival, said method comprising contacting a FOXP3-positive regulatory T cell with an IL-2 variant, wherein said IL-2 variant

- (a) comprises a sequence of amino acids at least 80% identical to SEQ ID NO:1;
- (b) stimulates STAT5 phosphorylation in said FOXP3-positive regulatory T cells; and
- (c) has a reduced ability compared to the polypeptide set forth as SEQ ID NO:1 to induce phosphorylation of STAT5 in FOXP3-negative T cells.

**18.** The method of claim **16**, wherein the FOXP3-positive regulatory T cell is contacted in vitro.

**19.** The method of claim **18**, wherein the IL-2 variant is conjugated to a chemical or polypeptide that extends the serum half-life of said IL-2 variant in vivo.

**20.** Use of an IL-2 variant in the preparation of a medicament for the treatment of an inflammatory disease, wherein said IL-2 variant

- (a) comprises a sequence of amino acids at least 80% identical to SEQ ID NO:1;
- (b) stimulates STAT5 phosphorylation in FOXP3-positive regulatory T cells; and
- (c) has a reduced ability compared to the polypeptide set forth as SEQ ID NO:1 to induce phosphorylation of STAT5 in FOXP3-negative T cells.

**21.** The method of claim **20**, wherein the IL-2 variant is conjugated to a chemical or polypeptide that extends the serum half-life of said IL-2 variant in vivo.

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