

IL-2 MUTANT PROTEIN PROLIFERATING IMMUNE CELLS

Technical field

The present invention relates to the field of protein engineering. In particular, the present invention relates to a novel interleukin-2 (IL-2) mutant and a preparation method thereof. Compared with the wild-type original IL-2 protein, the interleukin-2 (IL-2) mutant possesses a reduced ability to bind to its binding partner IL-2 receptor α subunit, but retains the ability to bind IL-2 receptor β subunit and IL-2 receptor γ subunit and corresponding biological activities, and can better stimulate the proliferation of tumor immune cells, including but not limited to T effector cells and NK cells.

Background

Interleukin-2 (IL-2, Interleukin-2) is a type of cell growth factor in the immune system, which can regulate the cell activity of white blood cells in the immune system, promote the proliferation of Th0 and CTL, and participate in antibody response, hematopoiesis, and tumor surveillance either. IL-2 exerts its effects by binding to IL-2 receptor (IL-2R). IL-2R consists of three chains of α , β and γ , and different combinations thereof can produce receptor forms with different affinities for IL-2: IL-2R γ chain alone cannot bind IL-2, $\beta\gamma$ chain is a medium-affinity IL-2R, and $\alpha\beta\gamma$ chain is a high-affinity IL-2R.

IL-2 is mainly synthesized by activated T cells, especially helper T cells. It stimulates the proliferation and differentiation of T cells, induces the generation of cytotoxic T lymphocytes (CTL) and the differentiation of peripheral blood lymphocytes into cytotoxic cells and lymphokine activated killer (LAK) cells, promotes T cell to express cytokines and cytolytic molecule, promotes the proliferation and differentiation of B cells and the synthesis of immunoglobulin via B cells, and stimulates the production, proliferation and activation of natural killer (NK) cells.

IL-2's ability to expand lymphocyte populations *in vivo* and enhance the effector functions of these cells confers IL-2 anti-tumor effects, thereby making IL-2 immunotherapy an attractive treating option for certain metastatic cancers. Therefore, a high-dosage IL-2 therapy has been approved for patients with metastatic renal cell carcinoma and malignant melanoma. However, IL-2 exhibits a dual function in the immune response, that is, it not only mediates the expansion and activity of effector cells, but is also critically involved in maintaining peripheral immune tolerance.

Side effects resulted from recombinant human IL-2 treatment are related to IL-2 immunotherapy. Patients receiving a high-dosage of IL-2 treatment often experience severe cardiovascular, lung, kidney, liver, gastrointestinal, neurological, skin, blood, and systemic

adverse events, which require close monitoring and inpatient management. Most of these side effects can be explained by the formation of the so-called vascular (or capillary) leak syndrome (VLS), which is a pathological increase in vascular permeability leading to fluid overflow in multiple organs (resulting in, for example, the lungs and skin edema and liver cell damage) and intravascular fluid loss (resulting in a decrease in blood pressure and a compensatory increase in heart rate). Low-dosage IL-2 regimens have been tested in patients to avoid VLS, which is, however, at the cost of reduced treatment outcomes.

Tumor immunity is an effective method for treating tumors in recent years. The killing of tumor cells by T cells and NK cells *in vivo* is achieved by PD-1 or PD-L1 inhibitors, and other similar immunosuppressants, such as CTLA-4, CD-47 antibodies, and the like. IL-2 is a known effective growth-promoting factor for T cells and NK cells, however the application thereof is limited due to the aforementioned side effects.

Cellular immunotherapy is also an effective method for treating tumors or autoimmune diseases in recent years. Immune cells are collected from a human body and cultured *in vitro* to increase the number of the cells and enhance their targeted-killing function. And then the immune cells are returned to the human body to kill pathogens, cancer cells, and mutated cells in blood and tissues, break immune tolerance, activate and enhance the body's immune ability, thereby taking into account the dual effects of treatment and health care. Cellular immunotherapy included Cytokine-induced killer cell (CIK) therapy, dendritic cell (DC) therapy, DC+CIK cell therapy, natural killer cell (NK) therapy, DC-T, CART, CAR-NK cell therapy, and the like. IL-2 is often used in the *in vitro* expansion of immune cells. Wild-type IL-2 also expands non-tumor-killing immune cells. Therefore, IL-2, which can more effectively expand immune cells such as CD8⁺ immune cells, is desired.

Several approaches have been adopted in the art to overcome these problems associated with IL-2 immunotherapy. For example, IL-2 is combined with certain anti-IL-2 monoclonal antibodies to enhance therapeutic effects of IL-2 *in vivo* (Kamimura et al., J Immunol 177, 306-14 (2006); Boyman et al., Science 311, 1924- 27 (2006)). IL-2 can also be mutated in various ways to reduce its toxicity and/or increase its efficacy. For example, Hu et al. (Blood 101, 4853-4861 (2003), US Patent Publication No. 2003/0124678) replaced the arginine residue at position 38 of IL-2 with tryptophan to eliminate the vascular permeability of IL-2 . Shanafelt et al. (Nature Biotechnol 18, 1197-1202 (2000)) mutated asparagine 88 to arginine to enhance the selective expansion of T cells and reduce the expansion of NK cells with toxic side effects. Heaton et al. (Cancer Res 53, 2597-602 (1993); US Patent No. 5,229,109) introduced two mutations, Arg38Ala and Phe42Lys, to reduce the secretion of pro-inflammatory cytokines by NK cells. Gillies et al. (US Patent Publication No. 2007/0036752) reduced VLS by substituting 3 residues (Asp20Thr, Asn88Arg and

Gln126Asp) in IL-2. In Gillies et al. (WO2008/0034473), the interface between mutant IL-2 and CD25 is also changed by amino acid substitutions Arg38Trp and Phe42Lys, thereby reducing the interaction with CD25 and activation of Timf cells to enhance efficacy. For the same purpose, Wittrup et al. (WO2009/061853) obtained IL-2 mutants, which have increased 5 affinities for CD25 while do not activate the receptor, thereby acting as antagonists. The purpose of introducing mutations is to disrupt the interaction with the receptor β subunit and/or γ subunit. However, none of these known IL-2 mutants overcome the above-mentioned problems related to IL-2 immunotherapy, namely, induction of toxicity caused by VLS, induction of tumor tolerance caused by AICD, and immunity inhibition 10 caused by activation of Timf cells. RocheGlicat (CN103492411A) mutated three amino acids at positions of F42A, Y45A and L72G in IL-2, thereby reducing the affinity of IL-2 protein to the high-affinity IL-2 receptor and retaining the affinity of the mutant IL-2 protein to the medium-affinity IL-2 receptor, however, the biological activities of the resulting IL-2 mutant is also reduced. For example, Miami University (CN107074967A) has constructed a fusion 15 protein comprising a first polypeptide of interleukin-2 or a functional variant or fragment thereof and a second polypeptide fused to the open reading frame of the first polypeptide, wherein the second polypeptide comprises the extracellular domain of interleukin-2 receptor α (IL-2R α) or a functional variant or fragment thereof, and wherein the fusion protein has IL-2 activities. The constructed fusion protein eliminates the binding of IL-2 to the 20 high-affinity IL-2R α on the cell surface by self-binding. However, the fusion protein may self-bind or bind to each other between different molecules, which is easy to produce multimers, and not conducive to production and quality control. There is a balance between binding and dissociation in the body for the fusion protein, the binding to the high-affinity IL-2R α on the cell surface cannot be completely eliminated and the fusion protein may still 25 bind to IL-2R α on the cell surface.

Therefore, there is a need in the art to improve the efficacy of IL-2 and stably and conveniently produce enhanced IL-2 polypeptides.

Summary of the invention

30 The purpose of the present invention is to provide a novel IL-2 mutant. Compared with wild-type IL-2, the IL-2 mutant of the present invention can overcome problems related to IL-2 immunotherapy, but can still retain the desired biological activities.

In the first aspect, an IL-2 mutant is provided in the present invention. Compared with wild-type IL-2, the amino acid residues of the IL-2 mutant are mutated, so that the binding ability 35 of IL-2 to its receptor is altered; and the affinity of the IL-2 mutant to the high-affinity IL-2 receptor is eliminated or reduced and the affinity of the IL-2 mutant to the medium-affinity IL-2

receptor is substantially retained.

In a preferred embodiment, the high-affinity IL-2 receptor is a heterotrimeric form of the IL-2 receptor, which is composed of receptor α subunit, receptor β subunit and receptor γ subunit; and the medium-affinity IL-2 receptor contains only the IL-2 receptor β subunit and IL-2 receptor γ subunit without the IL-2 receptor α subunit.

In a preferred embodiment, compared with wild-type IL-2, the binding affinity of the IL-2 mutant to the high-affinity IL-2 receptor is reduced by 55% or more, more preferably 60% or more, 65% or more, 70% or more, 75% or more, 80% or more, 85% or more, 90% or more, 95% or more, 96% or more, 97% or more, 98% or more, 99% or more; and most preferably, the IL-2 mutant does not bind the high-affinity IL-2 receptor;

The binding affinity of the IL-2 mutant to the medium-affinity IL-2 receptor is 50% or more, more preferably 60% or more, 70% or more, 80% or more, 90% or more, 95% or more of the binding affinity of the wild-type IL-2 to the medium-affinity IL-2 receptor; and most preferably, the binding affinity of the IL-2 mutant to the medium-affinity IL-2 receptor is comparable to the binding affinity of the wild-type IL-2 to the medium-affinity IL-2 receptor.

In a preferred embodiment, the IL-2 mutant retains the ability to proliferate activated tumor immune cells, including but not limited to T effector cells and NK cells.

In a specific embodiment, the IL-2 mutant has amino acid residue mutations at one or more of the following positions corresponding to wild-type IL-2: 39, 49, 73, and 109.

In a preferred embodiment, the IL-2 mutant has amino acid residue mutations at any of the following positions corresponding to wild-type IL-2: 39, 49, 73, and 109.

In a preferred embodiment, the IL-2 mutant has an amino acid residue mutation only at position 39 corresponding to wild-type IL-2.

In a preferred embodiment, the IL-2 mutant has amino acid residue mutations at position 39 and optionally at one or more of the following positions corresponding to wild-type IL-2: 49, 73 and 109;

The IL-2 mutant has amino acid residue mutations at position 49 and optionally at one or more of the following positions corresponding to wild-type IL-2: 39, 73 and 109;

The IL-2 mutant has amino acid residue mutations at position 73 and optionally at one or more of the following positions corresponding to wild-type IL-2: 39, 49 and 109;

The IL-2 mutant has amino acid residue mutations at position 109 and optionally at one or more of the following positions corresponding to wild-type IL-2: 39, 49 and 73.

In a preferred embodiment, the IL-2 mutant has mutations at 1, 2, 3, or 4 of positions 39, 49, 73, and 109 corresponding to wild-type IL-2.

In a preferred embodiment, the IL-2 mutant has one or more of the following amino acid residue mutations in wild-type IL-2: M39D, M39E, M39Q, M39N, M39A, K49N, A73T, A73S

and D109N.

In a preferred embodiment, the IL-2 mutant has the following single amino acid residue mutation in wild-type IL-2: M39D, M39E, M39Q, M39N or M39A; preferably M39D, M39E, M39Q or M39N; more preferably M39D, M39E or M39Q; more preferably M39D or M39E; and most preferably M39D.

In a preferred embodiment, the IL-2 mutant eliminates the O glycosylation site.

In a preferred embodiment, the IL-2 mutant is mutated at position 3 corresponding to wild-type IL-2, thereby eliminating the O glycosylation site.

In a preferred embodiment, the IL-2 mutant has the following amino acid residue mutations at position 3 corresponding to the wild-type IL-2 protein: T3A, T3G, T3Q, T3E, T3N, T3D, T3R, T3K, and T3P; and preferably T3A.

In a preferred embodiment, the IL-2 mutant has Cys mutated at position 125: C125L, C125S, C125A; and preferably C125S.

In a second aspect, a fusion protein or conjugate is provided in the present invention, comprising the IL-2 mutant of the first aspect and a non-IL-2 functional moiety.

In a preferred embodiment, the non-IL-2 functional moiety is selected from the following group:

a Fc fragment, including but not limited to: Fc fragment of human IgG1, IgG2, IgG3, IgG4, and Fc fragment mutant with a homology of more than 90%;

a Human serum albumin (HSA);

an anti-HSA antibody or antibody fragment;

a Transferrin;

a Human chorionic gonadotropin β subunit carboxy terminal peptide (CTP);

an Elastin-like peptide (ELP);

an antigen binding portion.

In a preferred embodiment, the antigen binding portion is:

an antibody or an active antibody fragment thereof;

a Fab molecule, scFv molecule and VHH molecule; or

a cell receptor or ligand.

In a preferred embodiment, the IL-2 mutant and the non-IL-2 functional moiety in the fusion protein can be connected directly or through a linker; and the linker can be a repeating sequence of AAA or GS, including but not limited to a repeating sequence of G₃S or a repeating sequence of G₄S; for example, (G₃S)₄.

In a preferred embodiment, the IL-2 mutant or fusion protein can be further modified as follows to form a conjugate:

Polyethylene glycol modification (PEGylation);
Polysialylation modification (PSAization);
Saturated fatty acid modification;
Hyaluronic acid modification (Hyaluronic acid, HA);
5 Polyamino acid modification (proline-alamine-serine polymer, PASation).

In a third aspect, a polynucleotide is provided in the present invention, encoding the IL-2 mutant of the first aspect or the fusion protein or conjugate of the second aspect.

10 In the fourth aspect, an expression vector is provided in the present invention, comprising the polynucleotide of the third aspect.

In a fifth aspect, a host cell is provided in the present invention, comprising the expression vector of the fourth aspect, or having the polynucleotide of the third aspect integrated into the
15 genome of the host cell.

In a preferred embodiment, the host cell is an eukaryotic cell; preferably yeast, insect cell, or animal cell; and it may be a mammalian cell.

In the sixth aspect, a cell-free expression system is provided in the present invention,
20 comprising the expression vector of the fourth aspect.

In a seventh aspect, a pharmaceutical composition is provided in the present invention, comprising the IL-2 mutant protein of the first aspect or the fusion protein or conjugate of the second aspect and a pharmaceutically acceptable excipient.
25

In the eighth aspect, the use of the IL-2 mutant of the first aspect or the fusion protein of the second aspect is provided in the present invention, for preparing a medicament for *in vitro* expansion of T lymphocytes, natural killer NK cells or for the treatment of a disease in an individual.

30 In a preferred embodiment, the disease is a disease in which IL-2 is used for immunotherapy.

In a preferred embodiment, the disease is cancer, immune disease, human immunodeficiency virus HIV infection, hepatitis C virus HCV infection, rheumatoid arthritis, atopic dermatitis and the like.

In a preferred embodiment, the cancer, immune disease, human immunodeficiency virus
35 HIV infection, hepatitis C virus HCV infection, rheumatoid arthritis, atopic dermatitis and the like are treated by stimulating the immune system or proliferating immune cells.

In the ninth aspect, the IL-2 mutant of the first aspect or the fusion protein of the second aspect is provided in the present invention, for use in *in vitro* expansion of T lymphocytes, natural killer NK cells, or treating a disease in an individual.

5 In a preferred embodiment, the disease is a disease in which IL-2 is used for immunotherapy.

In a preferred embodiment, the disease is cancer, immune disease, human immunodeficiency virus HIV infection, hepatitis C virus HCV infection, rheumatoid arthritis, atopic dermatitis and the like.

10 In a preferred embodiment, the cancer is a cancer that is treated by stimulating the immune system or proliferating immune cells.

In a tenth aspect, a treatment method is provided in the present invention, comprising a step of administering the IL-2 mutant protein of the first aspect or the fusion protein or conjugate of the second aspect or the pharmaceutical composition of the sixth aspect to a patient in need of IL-2
15 immunotherapy.

In a preferred embodiment, the treatment method treat cancer or immune diseases, human immunodeficiency virus HIV infection, hepatitis C virus HCV infection, rheumatoid arthritis, atopic dermatitis and the like by stimulating the immune system or proliferating immune cells.

20 It should be understood that within the scope of the present invention, the above technical features of the present invention and the technical features specifically described in the following (such as the embodiments) can be combined with each other to form a new or preferred technical solution, which will not be repeated one by one herein.

25 **Description of the drawings**

Figure 1 shows the binding ability of IL-2 mutants to CD25 detected by enzyme-linked immunosorbent assay;

Figure 2 shows the proliferation of CTLL-2 cells in response to rhIL-2 and mutant interleukin-2 (His tag);

30 Figure 3 shows the proliferation of CTLL-2 cells in response to rhIL-2 and mutant interleukin-2 (Fc tag);

Figure 4 shows the proliferation of NK92 cells in response to rhIL-2 and mutant interleukin-2 (His tag);

35 Figure 5 shows the proliferation of NK92 cells in response to rhIL-2 and mutant interleukin-2 (Fc tag);

Figure 6a-y show the sequence of SEQ ID NO: 1-25, respectively;

Figure 7 a-1 shows the sequence of SEQ ID NO: 26-37, respectively;

Figures 8A and 8B show the affinities of IL-2gm1-HSA, IL-2gm4-HSA, IL-2gm6-HSA, IL-2gm1a-HSA, IL-2gm1b-HSA, IL-2gm1c-HSA, IL-2gm1d-HSA, wild-type IL-2-HSA for human IL-2R α subunit and human recombinant IL-2R $\beta\gamma$ heterodimer, respectively;

5 Figure 9 shows how IL-2 mutants and wild-type IL-2 stimulate the proliferation of NK92 cells;

Figure 10 shows the IFN- γ release induced by IL-2 mutants, in which Figure 10A shows the release of interferon γ from NK92 cells induced by different concentrations of IL-2gm1-HSA, IL-2gm4-HSA and wild-type IL-2-HSA; and Figure 10B shows the release of interferon γ from
10 NK92 cells induced by different concentrations of IL-2gm6-HSA and wild-type IL-2-HSA;

Figure 11 shows the proliferation effects of IL-2gm6-HSA and wild-type IL-2-HSA on NK cells and Treg cells; in which Figure 11A shows the proliferation of NK cells after 6 days of incubation with different concentrations of IL-2gm6-HSA and wild-type IL-2-HSA; and Figure 11B shows the proliferation of Treg cells after 6 days of incubation with different concentrations
15 of IL-2gm6-HSA and wild-type IL-2-HSA; and

Figure 12 shows the effects of IL-2gm1-HSA and wild-type IL-2-HSA on the proliferation of NK cells and CD4⁺ T cells; in which Figure 12A shows that, under the stimulation of the sample concentration of 0-500 nM, IL-2gm1-HSA can significantly increase the proliferation of NK cells as compared with wild-type IL-2-HSA; and Figure 12B shows that, under the stimulation of the
20 concentration of 0-500 nM, the effects of IL-2gm1-HSA on the proliferation of CD4⁺ T cells is significantly weakened, as compared with wild-type IL-2-HSA.

Modes for carrying out the invention

After extensive and in-depth research, the inventors unexpectedly discovered that a new type
25 of IL-2 mutant polypeptide that undergoes glycosylation modification after site-directed mutation of IL-2 polypeptide can eliminate or reduce the affinity of IL-2 protein to high-affinity IL-2 receptor, while retains the affinity of the mutant IL-2 protein to the medium-affinity IL-2 receptor, and retains the biological activities of IL-2. Therefore, the mutant IL-2 protein can better stimulate the proliferation of tumor immune cells, including but not limited to T effector cells and NK cells,
30 thereby achieving the purpose of treatment. The present invention has been completed based on the above findings.

Site-directed mutagenesis

Site-directed mutagenesis is a protein engineering technique that replaces, inserts, or deletes
35 specific nucleotides in a known DNA sequence based on the structure and function of a known protein to produce mutant protein molecules with new properties.

Site-directed mutagenesis technology can change physical and chemical properties of a protein, for example, improving the stability of a protein drug; enhancing the solubility of a protein drug; improving biological properties, including (but not limited to) changing the specificity of an enzyme to substrates, improving enzyme activities, and improving affinities and specificities, etc.

Site-directed mutagenesis technology can increase or eliminate the binding activity between a ligand and receptor, enzyme and substrate by mutating amino acids in the binding domain. Such mutation will lead to changes in the secondary structure or higher structure of a protein and the characteristics of charges. If a mutated amino acid is exactly at the key antigen-antibody interaction site, then the amino acid change is likely to cause changes in the charge and secondary structure or higher structure of the site, and a ligand can not bind to its receptor, or an enzyme can not bind to its substrate, thereby achieving the purpose of mutation. Moreover, the original antibody cannot recognize this site, and a new antigen is formed. This is inevitably possible with this traditional mutation method.

IL-2 mutant of the present invention

In the present invention, site-directed mutagenesis allows changes in the amino acid residues of the IL-2 polypeptide, thereby changing the binding mode of the IL-2 polypeptide and the IL-2R receptor. The affinity of the IL-2 mutant of the present invention to the IL-2 receptor has been altered. In a specific embodiment, the affinity of the IL-2 mutant of the present invention for the high-affinity IL-2 receptor has been eliminated or reduced, while its affinity for the medium-affinity IL-2 receptor is retained, and biological activities of IL-2 is also retained. Therefore, the IL-2 mutant of the present invention can not only better stimulate the proliferation of tumor immune cells, including but not limited to T effector cells and NK cells, its side effects can also be significantly reduced as compared with wild-type IL-2, thereby achieving better treatment purpose.

When there is glycosylation modification to the IL-2 mutant or fusion protein of the present invention, it is preferably expressed in eukaryotic cells and obtained by cell culture. Yeast, insect cells, animal cells can be selected, or transgenic animals can be selected. In a specific embodiment, the host cell is a eukaryotic cell; preferably yeast, insect cells, and animal cells. When there is no glycosylation modification to the IL-2 mutant or fusion protein of the present invention, a cell-free expression, or expression in *E. coli*, yeast, etc., preferably cell-free expression and expression in yeast cells, can be used.

When yeast cells or insect cells are used as host cells, the glycoform of the obtained IL-2 mutant may be of non-human. A skilled person will know that the non-human glycoforms can be further transformed into a human glycoforms.

In other embodiments, it is also possible to obtain IL-2 mutants by using prokaryotic

expression and fermentation or *in vitro* cell-free synthesis, and then correctly glycosylated IL-2 mutants can be obtained through methods such as *in vitro* enzyme catalysis. Both *in vivo* and *in vitro* modifications can achieve the same purpose, that is, site-directed glycosylation of IL-2.

5 **Protein glycosylation modification and mutation**

Protein glycosylation is a complex process of post-translational modification. Glycosylation is performed at a specific site of a protein. The modified site is usually an asparagine residue (N-linked) or a serine/threonine residue (O -Link), N-linked glycosylation usually occurs in Asn-X-Ser/Thr (X is a non-proline amino acid, called N glycosylation site); and O-linked
10 glycosylation usually occurs in Serine (Ser) or threonine (Thr) residue (called O glycosylation site), and an O-glycosidic bond is formed through N-acetylgalactosamine (Gal-NAc) and the hydroxyl of Ser/Thr. Accordingly, the glycosylation site introduced in wild-type IL-2 by site-directed mutagenesis is an artificial glycosylation site.

The inventors artificially introduced glycosylation mutations to increase the
15 three-dimensional structure on the binding surface between the receptor protein and the ligand protein, so that the receptor and the ligand cannot directly contact. And it was also found that mutations at certain sites can also significantly change the binding of a receptor to ligand without glycosylation modification. In the present invention, the number of amino acid mutations is significantly reduced, which is completely different from the site-directed mutation involving
20 multiple amino acids as said above. By changing multiple amino acids, the traditional site-directed mutagenesis will change the hydrophilic or hydrophobic area of the binding surface of a protein, or change the higher structure of the protein, so that two proteins cannot form a stable binding state after contact. However, in the present invention, upon glycosylation mutation, the binding surface is destroyed by the sugar chain, and two proteins cannot get close. When the sites are properly
25 selected, the ability to eliminate binding of the present invention is much higher than that of traditional mutation, or in the present invention, the binding between a ligand and receptor can be reduced or eliminated by only changing one amino acid, such as methionine at position 39.

In particular, the inventors selected mutation sites in the possible binding region of interleukin 2 and interleukin 2 receptor and nearby amino acid sequences, and used sites
30 containing ASN, Thr or Ser in the original amino acid sequence as much as possible. The first two amino acids at Thr or Ser site are preferably selected for mutation, so that the amino acid at the mutation site will be masked by the sugar chain after glycosylation, thereby minimizing the immunogenicity. The mutation of Thr or Ser after Asn position is also a feasible solution.

A single-site mutation at position 39 can change IL2's ability to bind to the receptor
35 regardless of glycosylation. And the potential immunogenicity is extremely low since there is only a single-site mutation.

Traditionally, it is believed that macromolecular amino acids and amino acids containing complex aromatic rings are more immunogenic, and multiple-site mutations will produce more obvious immunogenicity. Due to the large molecular weight of sugar chains, especially N-glycan have complex 2-antenna, 3-antenna to 4-antenna structures (see Jonathan J. Lyons, etc., Glycans instructing immunity: the emerging role of altered glycosylation in clinical immunology. Front. Pediatr., 11 June 2015), therefore mutations near any binding site will affect the binding between two proteins.

According to the teachings of the present invention, a skilled person will know how to introduce fewer mutation sites in wild-type IL-2 through site-directed mutagenesis. In order to reduce the immunogenicity of the obtained IL-2 mutant, for the IL-2 mutant of the present invention, the number of mutation sites shall be reduced as much as possible and existing amino acid residues in natural IL-2 shall be used to generate new glycosylation sites, so that the structure of the obtained IL-2 mutant is similar to that of natural IL-2, thereby avoiding the influence of the mutation on other structural sites of the protein and retaining biological activities. In a specific embodiment, amino acid residue at one or more of the following positions (i.e., 1, 2, 3, or 4 positions) of wild-type IL-2 mutate: 39, 49, 73, and 109. In a specific embodiment, amino acid residue at one or more of the following positions of wild-type IL-2 mutate: M39D, M39E, M39Q, M39N, M39A, K49N, A73T, A73S and D109N; preferably M39D, M39E, M39Q, M39N or M39A single-site mutation; more preferably M39D, M39E, M39Q or M39N single-site mutation; more preferably M39D, M39E or M39Q single-site mutation; more preferably M39D or M39E single-site mutation; and most preferably M39D single-site mutation.

Based on conventional practices in the art, the original O-glycan sites in the IL-2 polypeptide can also be eliminated. The removal of O-glycan won't affect biological activities of IL-2. The structure of O-glycan is complex, the analysis on which is difficult. For reducing the complexity of quality control during the production, genetic engineering can usually be used to eliminate the glycosylation site. Therefore, the IL-2 mutant of the present invention may have the following amino acid residue mutations at position 3 corresponding to the wild-type IL-2 protein: T3A, T3G, T3Q, T3E, T3N, T3D, T3R, T3K and T3P; and preferably T3A. During the purification and renaturation of IL-2 products, activities of IL-2 will be reduced due to mismatching disulfide bonds or the formation of disulfide bonds between molecules. At present, cysteine at the 125th position can be mutated to leucine or serine by site-mutation, so that only one disulfide bond can be formed, thereby ensuring the activity of IL-2 during the refolding process. It is also reported that protein engineering is used to produce a new type of rIL-2, in which cysteine at the 125th position of IL-2 molecule is changed to alanine, and the specific activity of IL-2 is significantly higher than that of natural IL-2. Therefore, the IL-2 mutant of the present invention can have the following amino acid residue mutations at position 125 corresponding to the wild-type IL-2 protein:

C125L, C125A, C125S; and preferably C125S.

The affinity of the IL-2 mutant protein of the present invention to the high-affinity IL-2 receptor is eliminated or reduced, while the affinity of the IL-2 mutant to the medium-affinity IL-2 receptor can be substantially retained. As used herein, “elimination or reduction” refers to a situation where it is changed by more than 50% compared with the original level (for example, compared with the affinity of wild-type IL-2 to the high-affinity IL-2 receptor). Therefore, “the affinity of the IL-2 mutant protein of the present invention to the high-affinity IL-2 receptor is eliminated or reduced” as described herein means that, compared with the wild-type IL-2 protein, the binding affinity of the IL-2 mutant of the present invention to the high-affinity IL-2 receptor is reduced by 50% or more, more preferably 60% or more, 70% or more, 80% or more, 90% or more, 95% or more, and most preferably, the IL-2 mutant protein does not bind the high-affinity IL-2 receptor. Similarly, “the affinity of the IL-2 mutant to the medium-affinity IL-2 receptor can be substantially retained” as described herein means that the binding affinity of the IL-2 mutant to the medium-affinity IL-2 receptor is 50% or more, more preferably 60% or more, 70% or more, 80% or more, 90% or more, 95% or more of the binding affinity of the wild-type IL-2 for the medium-affinity IL-2 receptor. In a preferred embodiment, the binding affinity of the IL-2 mutant to the medium-affinity IL-2 receptor is comparable to the binding affinity of the wild-type IL-2 to the medium-affinity IL-2 receptor.

In addition, due to the large individual differences between subjects, the inventors found that effects of some IL-2 mutant proteins in the prior art are relatively different under different molecular structures or between different experimental batches. For example, there is a big difference between the effects exhibited at low concentration and high concentration. However, the IL-2 mutant of the present invention can exhibit excellent technical effects at both low and high concentrations.

“Corresponding to”

As used herein, the term “corresponding to” has the meaning commonly understood by a skilled person. Specifically, “corresponding to” means that, after two sequences are aligned for homology or sequence identity, one sequence corresponds to a designated position in the other sequence. Therefore, for example, “corresponding to wild-type IL-2” means that a certain amino acid sequence is aligned with the amino acid sequence of wild-type IL-2, and the position on the amino acid sequence that corresponds to wild-type IL-2 is found.

Methods for determining sequence homology or identity well-known to a skilled person include, but not limited to: Computational Molecular Biology, Lesk, AM Ed., Oxford University Press, New York, 1988; Biocomputing: Information Biocomputing: Informatics and Genome Projects (Biocomputing: Informatics and Genome Projects), Smith, DW, Academic Press, New

York, 1993; Computer Analysis of Sequence Data, Part 1, Griffin, AM and Griffin, HG , Humana Press, New Jersey, 1994; Sequence Analysis in Molecular Biology, von Heinje, G., Academic Press, 1987 and Sequence Analysis Primer, Gribskov, M. and Devereux , J. Ed. M Stockton Press, New York, 1991 and Carillo, H. and Lipman, D., SIAM J. Applied Math., 48:1073 (1988). The preferred method for determining identity is to obtain the largest match between tested sequences. The method for determining identity is compiled in a publicly available computer program. Preferred computer program methods for determining the identity between two sequences include but not limited to: GCG package (Devereux, J. et al., 1984), BLASTP, BLASTN and FASTA (Altschul, S, F. et al., 1990). BLASTX program is publically available from NCBI and other sources (BLAST Handbook, Altschul, S. et al., NCBI NLM NIH Bethesda, Md. 20894; Altschul, S. et al., 1990). The well-known Smith Waterman algorithm can also be used to determine identity.

Fusion protein or conjugate of the present invention

Based on the IL-2 mutants of the present invention, a skilled person will know that the IL-2 mutants of the present invention and other non-IL-2 functional moieties can be made into fusion proteins or conjugates. As used herein, a conjugate refers to a water-soluble polymer covalently linked to the residues of the mutant IL-2 polypeptide. In a specific embodiment, the non-IL-2 functional moiety includes, but not limited to: a Fc fragment, Human serum albumin (HSA), an anti-HSA antibody or antibody fragment, Transferrin, Human chorionic gonadotropin β subunit carboxy terminal peptide (CTP), Elastin-like peptide (ELP) and antigen binding portion. In a preferred embodiment, the antigen binding portion can be an antibody or an active antibody fragment thereof, a Fab molecule, scFv molecule and VHH molecule, immunoglobulin molecule, receptor protein molecule or ligand protein molecules; and the immunoglobulin molecule can be an IgG molecule.

Based on conventional operations in the art, a skilled person will know how to obtain a fusion protein or conjugate containing the IL-2 mutant of the present invention. For example, the IL-2 mutant of the present invention can be directly connected to other non-IL-2 functional moieties, or can be connected through a linker. The linker can be a repetitive sequence of AAA or GS, including but not limited to a repetitive sequence of G₃S or a repetitive sequence of G₄S; for example, (G₃S)₄.

Moreover, the IL-2 mutant or fusion protein can also be modified with polyethylene glycol (PEGylation), polysialylated (PSA), saturated fatty acid, and hyaluronic acid (Hyaluronic acid, HA) or polyamino acid (proline-alamine-serine polymer, PAS) to form a conjugate.

Pharmaceutical composition of the present invention and administration method thereof

Based on the IL-2 mutant of the present invention, a pharmaceutical composition is also provided in the present invention. In a specific embodiment, the pharmaceutical composition of the present invention comprises the IL-2 mutant of the present invention or the fusion protein or conjugate according to claim 5 and optionally a pharmaceutically acceptable excipient.

5 Optionally, the composition of the present invention further comprises a pharmaceutically acceptable excipient. If desired, a pharmaceutically acceptable excipient can be added to the IL-2 mutant polypeptide, fusion protein or conjugate of the present invention to form a composition.

Exemplary excipients include, but not limited to, those excipients selected from the group consisting of sugars, inorganic salts, antimicrobial agents, antioxidants, surfactants, buffers, acids,
10 based, amino acids, and combinations thereof. Sugars, such as sugar, derivatized sugar (such as sugar alcohols, aldonic acids, esterified sugars and/or sugar polymers), may be present as excipients. Specific sugar excipients include, for example: monosaccharides, such as fructose, maltose, galactose, glucose, D-mannose, sorbose, etc.; disaccharides, such as lactose, sucrose, trehalose, cellobiose, etc.; polysaccharides, such as raffinose, maltodextrin, dextran, starch, etc.;
15 and sugar alcohols, such as mannitol, xylitol, maltitol, lactitol, xylitol, sorbitol (glucitol), inositol, cyclodextrin, and the like.

Excipients may also include inorganic salts or buffers, such as citric acid, sodium chloride, potassium chloride, sodium sulfate, potassium nitrate, sodium dihydrogen phosphate, disodium hydrogen phosphate, and combinations thereof.

20 The composition may also include an antimicrobial agent for preventing or suppressing the growth of microorganisms. Non-limiting examples of antimicrobial agents suitable for one or more embodiments of the present invention include benzalkonium chloride, benzethonium chloride, benzyl alcohol, phenol, phenethyl alcohol, and combinations thereof.

Antioxidants can also be present in the composition. Antioxidants are used to prevent
25 oxidation, thereby preventing the deterioration of proteins, conjugates, or other components of the formulation. Antioxidants suitable for one or more embodiments of the present invention include, for example, ascorbyl palmitate, butylated hydroxyanisole, butylated hydroxytoluene, hypophosphorous acid, monothioglycerol, and combinations thereof.

Surfactants can be present as excipients. Exemplary surfactants include: polysorbates, such
30 as “Tween 20” and “Tween 80”, and pluronics, such as F68 and F88; sorbitan esters; lipids, such as phospholipids (such as lecithin and other phosphatidyl cholines), fatty acids and fatty esters; steroids such as cholesterol.

Acids or bases can be present in the composition as excipients. Non-limiting examples of acids that can be used include those selected from the group consisting of hydrochloric acid, acetic
35 acid, phosphoric acid, citric acid, malic acid, lactic acid, formic acid, trichloroacetic acid, nitric acid, perchloric acid, phosphoric acid, sulfuric acid, fumaric acid, and combinations thereof.

Examples of suitable bases include, but not limited to, bases selected from the group consisting of sodium hydroxide, sodium acetate, ammonium hydroxide, potassium hydroxide, ammonium acetate, potassium acetate, sodium phosphate, potassium phosphate, sodium citrate, sodium formate, sodium sulfate, potassium sulfate, potassium fumarate, and combinations thereof.

5 Described herein are one or more amino acids that may be present as excipients in the composition. In this regard, exemplary amino acids include arginine, lysine, and glycine.

The amount of the conjugate (i.e., the conjugate formed between the active agent and the polymer agent) in the composition will vary depending on a number of factors, however when the composition is stored in a unit-dosage container (e.g., a vial), a therapeutically effective dosage will be preferred. In addition, the pharmaceutical preparation can be contained in a syringe. The therapeutically effective dosage can be determined experimentally by repeatedly administering increasing amounts of the drug in order to determine which amount will produce a clinically desired endpoint.

15 The amount of any individual excipient in the composition will vary depending on the activity of the excipient and specific needs of the composition. Typically, the optimal amount of any single excipient is determined by routine experimentation, that is, by preparing a composition containing different amounts of excipients (ranging from low to high), detecting stability and other parameters of the composition, and then determining the range for obtaining the best performance without significant adverse effects.

20 However, in general, the excipients in the composition are present in an amount of from about 1% to about 99% by weight, preferably from about 5% to about 98% by weight, more preferably from about 15 to about 95% by weight, and most preferably, less than 30% by weight.

25 These compositions encompass all types of formulations as well as those that are particularly suitable for injection, such as powders or lyophilized formulations and liquid formulations that can be reconstituted. Examples of diluents suitable for reconstituting a solid composition prior to injection include bacteriostatic water for injection, 5% dextrose in water, phosphate buffered saline, sterile water, deionized water, and combinations thereof. For liquid pharmaceutical compositions, solutions and suspensions can be envisaged.

30 The composition of one or more embodiments of the present invention is typically (but not necessarily) administered by injection, and therefore is generally a liquid solution or suspension immediately before administration. Pharmaceutical preparations can also take other forms, such as syrups, creams, ointments, tablets, powders, and the like. Other modes of administration are also included, such as transpulmonary, transrectal, transdermal, transmucosal, oral, intrathecal, intratumoral, peritumoral, intraperitoneal, subcutaneous, intraarterial administration, and so on.

35 The present invention also provides a method for administering a therapeutically effective dosage to a patient. Injection of a medicament (such as intramuscular, subcutaneous and parenteral

administration) are possible. Types of formulations suitable for parenteral administration include, in particular, injection ready-to-use solutions, dry powders combined with a solvent before application, injection ready-to-use suspensions, and dry insoluble compositions combined with a vehicle before application as well as emulsions and liquid concentrates diluted before application.

5 The method of administration can be used to treat any condition that can be treated or prevented by administering the mutant protein, fusion protein, or conjugate. A skilled person will understand which condition can be effectively treated by a particular composition. For example, a patients suffering from a disease selected from the following group can be treated: renal cell carcinoma, metastatic melanoma, hepatitis C virus (HCV), human immunodeficiency virus (HIV),
10 acute myeloid leukemia, non-Hodgkin's lymphoma, cutaneous T-cell lymphoma, juvenile rheumatoid arthritis, atopic dermatitis, breast cancer, and bladder cancer.

 The actual dosage to be administered will vary according to the age, weight and overall condition of a subject, as well as the severity of the condition being treated, and the judgment of a health care professional. The therapeutically effective amount is known to a skill person and/or
15 described in relevant reference texts and documents. In general, the therapeutically effective amount will range from about 0.001 mg to 1000 mg, preferably in a dosage from 0.01 mg per day to 100 mg per day and more preferably in a dosage from 0.10 mg per day to 50 mg per day. A given dosage can be administered periodically until the symptoms such as organophosphate poisoning are alleviated and/or completely eliminated.

20 The unit dosage can be determined based on the judgment from a clinician and the needs from a patient, etc., and can be administered in a variety of administration schedules. The specific dosing schedule will be known to a skilled person or can be determined experimentally using conventional methods. Exemplary dosing schedules include, but not limited to, twice a day, once a day, three times a week, twice a week, once a week, twice a month, once a month, and any
25 combination thereof. Once the clinical endpoint has been achieved, the administration of the composition is suspended.

 It should be understood that the present invention has been described based on the preferred specific embodiments of the present invention, however, the foregoing description and the following examples are intended to illustrate rather than limit the scope of the present invention.
30 Other aspects, advantages, and modifications within the scope of the present invention will be apparent to a skilled person in the field to which the present invention belongs.

Use and method for using the IL-2 mutant of the present invention

 As described above, the IL-2 mutant of the present invention can eliminate or reduce the
35 affinity of the IL-2 protein to the high-affinity IL-2 receptor, while retain the affinity of the mutant IL-2 protein to the medium-affinity IL-2 receptor, and retain the biological activities of IL-2, so as

to better stimulate the proliferation of tumor immune cells, including but not limited to T effector cells and NK cells. Therefore, the IL-2 mutant, fusion protein, conjugate or pharmaceutical composition of the present invention can be prepared into corresponding drugs. The drug can be used to expand T lymphocytes, natural killer NK cells *in vitro* or treat diseases that use IL-2 for immunotherapy. In a specific embodiment, the disease is cancer; for example, a cancer that needs to be treated by stimulating the immune system or by proliferating immune cells. In a specific embodiment, the disease may also be an immune disease, human immunodeficiency virus HIV infection, hepatitis C virus HCV infection, rheumatoid arthritis, atopic dermatitis and the like.

The IL-2 mutant of the present invention can also be used to replace wild-type IL-2 in expanding cells *in vitro* during cell therapy such as CAT-T and CAR-NK.

Advantages of the present invention:

1. The IL-2 mutant protein of the present invention reduces or eliminates the affinity to the high-affinity IL-2 receptor, while substantially retains the affinity to the medium-affinity IL-2 receptor;

2. The structure of the IL-2 mutant of the present invention is similar to that of natural IL-2, thereby avoiding effects of mutation on other structural sites of the protein, and retaining biological activities;

3. Compared with other IL-2 mutants in the prior art, the IL-2 mutant of the present invention has lower immunogenicity;

4. The molecule design for the IL-2 mutant of the present invention is simple and convenient for application on different molecules;

5. The IL-2 mutant of the present invention is convenient for production and quality control, and generally *in vitro* re-modification is not necessary, thereby reducing production steps and improving production efficiency;

6. The IL-2 mutant of the present invention is convenient to form a bifunctional or multifunctional fusion protein or immune composition with other molecules; and

7. The IL-2 mutant of the present invention can be used for immunotherapy, however, it will not cause vascular (or capillary) leak syndrome (VLS) caused by natural IL-2.

The present invention will be further explained below in conjunction with specific embodiments. It should be understood that these embodiments are only used to illustrate the present invention and not to limit the scope of the present invention. The experimental methods without specific conditions in the following examples are usually in accordance with conventional conditions such as the conditions described in Sambrook et al., Molecular Cloning: Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989), or in accordance with the

conditions suggested by the manufacturer.

Example

Example 1. Synthesis of mutant interleukin-2 (IL-2) protein

5 1. Synthesis of Gene

The nucleotide sequence encoding the amino acid sequence of the mutant interleukin-2 (IL-2) protein was obtained by an automated gene synthesis method. In some embodiments, HIS tags were added to the ends of gene fragments to facilitate purification. In some embodiments, IgG1-Fc was added to the ends of gene fragments to facilitate purification. Fc tag was also a common means to extend the half-life of a protein drug. The gene fragment flanks with a single restriction endonuclease cleavage site. All gene synthesis sequences were designed to have a 5' DNA sequence encoding a leader peptide that can target the proteins for secretion in eukaryotic cells.

Number of mutations	Mutations ite	Name of mutants	Protein Tag	Sequence
2	3, 39	IL-2gm1 (T3A, M39N)	HIS	SEQ ID NO: 1
2	3, 39	IL-2gm1 (T3A, M39N)	HSA	SEQ ID NO: 2
2	3, 49	IL-2gm2 (T3A, K49N)	HIS	SEQ ID NO: 3
2	3, 73	IL-2gm3 (T3A, A73T)	HIS	SEQ ID NO: 4
2	3, 73	IL-2gm3 (T3A, A73S)	HIS	SEQ ID NO: 5
2	3, 109	IL-2gm7 (T3A, D109N)	HIS	SEQ ID NO: 6
3	3, 39 and 49	IL-2gm8 (T3A, M39N, K49N)	HIS	SEQ ID NO: 7
3	3, 49 and 73	IL-2gm4 (T3A, K49N, A73T)	HIS	SEQ ID NO: 8
3	3, 49 and 73	IL-2gm4 (T3A, K49N, A73T)	IgG1-Fc	SEQ ID NO: 9
3	3, 39 and 73	IL-2gm9 (T3A, M39N, A73T)	HIS	SEQ ID NO: 10
3	3, 39 and 109	IL-2gm10 (T3A, M39N, D109N)	HIS	SEQ ID NO: 11
3	3, 49 and 109	IL-2gm11 (T3A, K49N, D109N)	HIS	SEQ ID NO: 12
3	3, 73 and 109	IL-2gm12 (T3A, A73T, D109N)	HIS	SEQ ID NO: 13
4	3, 39, 49, 73	IL-2gm5 (T3A, M39N, K49N, A73T)	HIS	SEQ ID NO: 14
4	3, 39, 49, 73	IL-2gm5 (T3A, M39N, K49N, A73T)	IgG1-Fc	SEQ ID NO: 15
4	3, 49, 73, 109	IL-2gm13 (T3A, K49N, A73T, D109N)	HIS	SEQ ID NO: 16
4	3, 39, 73, 109	IL-2gm14 (T3A, M39N, A73T, D109N)	HIS	SEQ ID NO: 17
4	3, 39, 49, 109	IL-2gm15 (T3A, M39N, K49N, D109N)	HIS	SEQ ID NO: 18
5	3, 39, 49, 73, 109	IL-2gm6 (T3A, M39N, K49N, A73T, D109N)	HIS	SEQ ID NO: 19
5	3, 39, 49, 73, 109	IL-2gm6 (T3A, M39N, K49N, A73T, D109N)	IgG1-Fc	SEQ ID NO: 20
5	3, 39, 49, 73, 109	IL-2gm6 (T3A, M39N, K49N, A73T, D109N)	HSA	SEQ ID NO: 21

	73, 109			
4	3, 42, 45, 72	IL-2qm(T3A,F42A,Y45A,L72G)	IgG1-Fc	SEQ ID NO: 22 RocheGlicat (CN103492411B)

2. Construction of Plasmid

The synthesized gene was subcloned into pcDNA3.4 plasmid, and the molecular biology reagents were used in accordance with the manufacturer's instructions.

3. Expression of mutant interleukin-2 (IL-2) protein

5 Expi293F cells (Thermo Fisher Scientific) were used for plasmid transfection. The cells were cultured in a shaker (VWR Scientific) at 37°C and 8% CO₂ and inoculated in Corning Erlenmeyer Flasks the day before transfection. The transfection method was carried out in accordance with the manufacturer's instructions.

10 The cell supernatants on the 2nd day, 4th day, and 5th day were collected for Western blotting to confirm protein expression.

Example 2. Expression of CD25 protein

Gene synthesis

15 The nucleotide sequence encoding the amino acid sequence (SEQ ID NO: 23) of the CD25 protein was obtained by an automated gene synthesis method. SEQ ID NO: 24 (GGGSGGGSGGGSGGGS) is the amino acid sequence of a linker. In some embodiments, gene fragments were co-expressed with IgG1-Fc through a linker to facilitate purification. The gene fragment flanks with a single restriction endonuclease cleavage site. All gene synthesis sequences were designed to have a 5' DNA sequence encoding a leader peptide that can target proteins for

20 secretion in eukaryotic cells. SEQ ID NO: 25 shows an exemplary leader peptide sequence. The synthesized gene was subcloned into pcDNA3.4 plasmid, and the molecular biology reagents were used in accordance with the manufacturer's instructions.

25 Expi293F cells (Thermo Fisher Scientific) were used for plasmid transfection. The cells were cultured in a shaker (VWR Scientific) at 37°C and 8% CO₂ and inoculated in Corning Erlenmeyer Flasks the day before transfection. The transfection method was carried out in accordance with the manufacturer's instructions.

The cell supernatant suspensions on the 2nd day, 4th day, and 5th day were collected for Western blotting to confirm protein expression.

30 Example 3. Experiment to detect the binding affinity to CD25 by using ELISA, Fortebio or biacore

The inventors used enzyme-linked immunoassay to detect the binding ability of IL-2 mutants to CD25.

CD25 (from Example 2) was coated on a 96-well high adsorption microtiter plate (3590, Costar), washed and blocked. The sample to be tested was diluted to an appropriate concentration and added to wells. TMB development was conducted, and a microplate reader (M5, Molerlder Devies) was used at a wavelength of 450/650nm to read the signal value of each well. rhIL-2 is a recombinant human interleukin-2 for injection (Quanqi).

Table 1. Overview of the binding activity of rhIL-2 and IL-2 mutants to CD25

Concentration (ug/ml)	Binding activity (%)		
	1	0.2	0.04
IL-2gm1	0.88%	1.08%	1.66%
IL-2gm2	30.99%	31.26%	16.64%
IL-2gm3	10.33%	5.62%	2.27%
IL-2gm4	14.79%	7.50%	3.18%
IL-2gm5	0.88%	1.08%	1.51%
IL-2gm6	0.88%	1.08%	1.36%
IL-2gm7	8.10%	2.96%	1.82%
IL-2gm8	0.74%	0.99%	1.51%
IL-2gm9	0.88%	1.18%	1.51%
IL-2gm10	0.88%	1.08%	1.51%
IL-2gm11	12.49%	5.03%	2.42%
IL-2gm12	2.77%	1.48%	1.97%
IL-2gm13	2.23%	1.18%	1.51%
IL-2gm14	0.74%	0.99%	1.51%
IL-2gm15	0.68%	0.99%	1.36%
IL-2qm	0.74%	0.99%	1.51%
rhIL-2	100.00%	100.00%	100.00%

Note: The binding activity of IL-2gm at each concentration is based on rhIL-2 (binding activity 100%) as a control.

Results are shown in Figure 1. From the figure, it can be clearly seen that under the experimental concentrations, rhIL-2 can bind CD25, and the dosage-dependent binding activities were: rhIL-2> IL-2gm2> IL-2gm4> IL-2gm11 > IL-2gm3> IL-2gm7> IL-2gm12> IL-2gm13. It can be judged as no binding, when there is no dosage correlation at these concentrations, including IL-2gm1, IL-2gm5, IL-2gm6, IL-2gm8, IL-2gm9, IL-2gm10, IL-2gm14, IL-2gm15, IL-2qm (CN103492411A), demonstrating that the abilities of IL-2gm1, IL-2gm5, IL-2gm6, IL-2gm8,

IL-2gm9, IL-2gm10, IL-2gm14, IL-2gm15 to bind CD25 were completely lost; and the abilities of IL-2gm2, IL-2gm4, IL-2gm11, IL-2gm3, IL-2gm7, IL-2gm12, and IL-2gm13 to bind CD25 were partially lost.

5 **Example 4. Analysis on cell proliferation using CTLL2 cells**

In this example, the inventors used CTLL2 cells to evaluate the activity of rhIL-2 and the mutant interleukin-2 of Example 1 in the cell proliferation analysis.

The same number of CTLL-2 cells (mouse cytotoxic T lymphocyte cell line, which is IL-2 dependent, and highly express CD25 on the cell surface) were inoculated in an experimental plate, and then rhIL-2 and IL-2 mutant were added according to the concentration gradient. After incubating for 48 hours, cell Titer Glo Luminescent buffer was added. Intracellular content of ATP was detected by chemiluminescence (SpectraMaxM5), and the number of cells in each well was detected, so as to detect the effects of different concentrations of rhIL-2 and IL-2 mutants on cell proliferation. The data was analyzed with GraphPad Prism7 software, and the curve was fitted with Nonlinear regression. The EC50 value of cell proliferation (the concentration of the test compound required to exhibit 50% of the maximum response) was obtained from the non-linear regression analysis of the dose-response curve.

Analysis on cell proliferation was used to measure the activity of rhIL-2 and mutant interleukin-2 (His tag), and a summary of the results is shown in Table 2 and FIG. 2. All test articles induced the growth of CTLL-2 cells in a dose-dependent manner. When the cell proliferation folds are comparable, we believe that the larger the EC50, the weaker the activity of stimulating the growth of CTLL2. This change is due to the effects of the mutant protein on the binding to CD25, however the mutant protein retains the ability to activate IL-2R signal transduction through the IL-2R $\beta\gamma$ heterodimer. Therefore, after the concentration was increased, the cells were effectively expanded. Compared with rhIL-2, the highest potency of all IL-2 mutants (His tag) does not exceed 8.314%, demonstrating that IL-2gm (1~15) can eliminate the binding to CD25, and the stimulation effect was weakened since the IL-2R $\alpha\beta\gamma$ heterotrimer was not formed. Preferably, compared with the control sample rhIL-2, the proliferation effects of IL-2gm1, IL-2gm3, IL-2gm5, IL-2gm6, IL-2gm7, IL-2gm8, IL-2gm9, IL-2gm10, IL-2gm12, IL-2gm14 and IL-2gm15 were reduced by more than 50 times (more than 100 times for IL-2gm1, IL-2gm6, and IL-2gm14).

Table 2. Overview of CTLL-2 cell proliferation in response to rhIL-2 and mutant interleukin-2 (His tag)

Tested Sample	EC ₅₀ (nM)	Potency relative to rhIL-2 (%)
rhIL-2	2.90E-03	100%
IL-2gm1-His	8.34E+01	0.003%
IL-2gm2-His	6.38E-02	4.547%

IL-2gm3-His	1.65E-01	1.763%
IL-2gm4-His	9.06E-02	3.199%
IL-2gm5-His	8.221	0.035%
IL-2gm6-His	5.15E+01	0.006%
IL-2gm7-His	3.65E-01	0.794%
IL-2gm8-His	8.838	0.033%
IL-2gm9-His	2.385	0.122%
IL-2gm10-His	1.56E+01	0.019%
IL-2gm11-His	3.49E-02	8.314%
IL-2gm12-His	1.56E-01	1.865%
IL-2gm13-His	1.44E-01	2.012%
IL-2gm14-His	4.79E+01	0.006%
IL-2gm15-His	1.65E+01	0.018%

Analysis on cell proliferation was used to measure the activities of rhIL-2 and mutant interleukin-2 (Fc tag), and a summary of the results is shown in Table 3 and FIG. 3. All test articles induced the growth of CTLL-2 cells in a dose-dependent manner. When the cell proliferation folds are comparable, the larger the EC₅₀, the weaker the activity of stimulating the growth of CTLL2.

5 This change is due to the effects of the mutant protein on the binding to CD25, however the mutant protein retains the activation of IL-2R signaling through the IL-2Rβγ heterodimer. Therefore, after the concentration was increased, the cells were effectively expanded. Compared with rhIL-2, the highest potency of all IL-2 mutants (Fc tag) does not exceed 31.546%. It is proved that the dual form of IL-2gm (4~6) can also eliminate the binding to CD25, and the stimulation effect was
10 weakened since the IL-2Rαβγ heterotrimer was not formed. Preferably, compared with the control sample rhIL-2, the proliferation effects of IL-2qm-Fc, IL-2gm5 and IL-2gm6 on CTLL2 were reduced by more than 50 times, in which IL-2qm-Fc and IL-2gm6-Fc were not observed for significant cell proliferation in the tested concentration range.

Table 3. Overview of CTLL-2 cell proliferation in response to rhIL-2 and mutant
interleukin-2 (Fc tag)

Tested Sample	EC ₅₀ (nM)	Potency relative to rhIL-2 (%)
rhIL-2	2.90E-03	100%
IL-2gm4-Fc	9.19E-03	31.546%
IL-2gm5-Fc	1.73E-01	1.681%
IL-2gm6-Fc	5.95E+00	0.049%
IL-2qm-Fc	2.07E+13	0.000%

Example 5. Analysis on cell proliferation using NK92 cells

The inventors used NK92 cells to evaluate the activities of rhIL-2 and the mutant interleukin-2 of Example 1 in cell proliferation analysis.

20 The same number of NK92 cells (NK-92 cell is a strain of IL-2 dependent NK cell line derived from peripheral blood mononuclear cells of a 50-year-old white male with rapidly progressive non-Hodgkin's lymphoma, and the cell expresses CD25 on the surface) were

inoculated in an experimental plate, and then rhIL-2 and IL-2 mutant were added according to the concentration gradient. After incubating for 72 hours, cell Titer Glo Luminescent buffer was added. Intracellular content of ATP was detected by chemiluminescence (SpectraMaxM5), and the number of cells in each well was detected, so as to detect the effects of different concentrations of rhIL-2 and IL-2 mutants on cell proliferation. The data was analyzed with GraphPad Prism7 software, and the curve was fitted with Nonlinear regression. The EC50 value of cell proliferation (the concentration of the test compound required to exhibit 50% of the maximum response) was obtained from the non-linear regression analysis of the dose-response curve.

Analysis on cell proliferation was used to measure the activity of rhIL-2 and mutant interleukin-2 (His tag), and a summary of the results is shown in Table 4 and FIG. 4. All test articles induced the growth of NK92 cells in a dose-dependent manner. When the cell proliferation folds are comparable, the larger the EC₅₀, the weaker the activity of stimulating the growth of NK92. This change is due to the effects of the mutant protein on the binding to CD25, however the mutant protein retains the ability to activate IL-2R signal transduction through the IL-2Rβγ heterodimer. Therefore, after the concentration was increased, the cells were effectively expanded. Compared with rhIL-2, the highest potency of all IL-2 mutants (His tag) does not exceed 1.721%, demonstrating that for NK-92 cell with CD25 expressed on the surface, IL-2gm (1~15) can eliminate the binding to CD25, and the stimulation effect was weakened since the IL-2Rαβγ heterotrimer was not formed. Among them, IL-2gm1, IL-2gm5 and IL-2gm14 were the most significant, and the proliferation effect was reduced by more than 100 times.

Table 4. Overview of NK92 cell proliferation in response to rhIL-2 and mutant interleukin-2 (His tag)

Tested Sample	EC ₅₀ (nM)	Potency relative to rhIL-2 (%)
rhIL-2	1.93E-03	100
IL-2gm1-His	3.85E+01	0.005%
IL-2gm2-His	1.12E-01	1.721%
IL-2gm3-His	2.21E+00	0.088%
IL-2gm4-His	2.34E-01	0.828%
IL-2gm5-His	20.68	0.009%
IL-2gm6-His	7.08E+00	0.027%
IL-2gm7-His	5.57E-01	0.347%
IL-2gm8-His	8.91	0.022%
IL-2gm9-His	1.067	0.181%
IL-2gm10-His	1.27E+01	0.015%
IL-2gm11-His	2.80E-01	0.690%
IL-2gm12-His	2.96E-01	0.653%
IL-2gm13-His	2.93E-01	0.661%
IL-2gm14-His	2.32E+01	0.008%
IL-2gm15-His	7.76E+00	0.025%

Analysis on cell proliferation was used to measure the activities of rhIL-2 and mutant

interleukin-2 (Fc tag), and a summary of the results is shown in Table 5 and FIG. 5. All test articles induced the growth of NK92 cells in a dose-dependent manner. When the cell proliferation folds are comparable, the larger the EC₅₀, the weaker the activity of stimulating the growth of NK92. This change is due to the effects of the mutant protein on the binding to CD25, however the mutant protein retains the activation of IL-2R signaling through the IL-2Rβγ heterodimer. Therefore, after the concentration was increased, the cells were effectively expanded. Compared with rhIL-2, the highest potency of all IL-2 mutants (Fc tag) does not exceed 34.783%. It is proved that for NK-92 cell with CD25 expressed on the surface, the dual form of IL-2qm-Fc, IL-2gm (4~6) can also reduce the binding to CD25, and the stimulation effect was weakened since the IL-2Rαβγ heterotrimer was not formed. Preferably, compared with rhIL-2, the potency of IL-2qm-Fc and IL-2gm6-Fc are reduced by more than 100 times. However, the amplification folds of IL-2qm on NK92 cells was significantly lower than that of other mutant proteins.

Table 5. Overview of NK92 cell proliferation in response to rhIL-2 and mutant interleukin-2 (Fc tag)

Tested Sample	EC ₅₀ (nM)	Potency relative to rhIL-2 (%)
rhIL-2	5.28E-03	100
IL-2gm4-Fc	1.52E-02	34.783%
IL-2gm5-Fc	7.11E-02	7.425%
IL-2gm6-Fc	8.24E-01	0.641%
IL-2qm-Fc	3.06E-01	1.727%

Example 6. Study on the half-life of the mutant protein of the present invention

The inventors further studied the *in vivo* half-life of the mutant protein obtained by the method of the present invention, and found that the half-life of the mutant protein obtained by the method of the present invention increased to varying degrees.

Example 7. Synthesis of IL-2 mutant protein (HSA tag)

The inventors further synthesized following IL-2 mutants:

Number of mutations	Mutation site	Name of mutants	Protein Tag	Sequence number
2	3, 39	IL-2gm1 (T3A, M39N)	HSA	SEQ ID NO: 26
3	3, 49 and 73	IL-2gm4 (T3A, K49N, A73T)	HSA	SEQ ID NO: 27
5	3, 39, 49, 73, 109	IL-2gm6 (T3A, M39N, K49N, A73T, D109N)	HSA	SEQ ID NO: 28
2	3, 109	IL-2gm7 (T3A, D109N)	HSA	SEQ ID NO: 29
2	3, 39	IL-2gm1a (T3A, M39D)	HSA	SEQ ID NO: 34
2	3, 39	IL-2gm1 b (T3A, M39E)	HSA	SEQ ID NO: 35
2	3, 39	IL-2gm1c (T3A, M39Q)	HSA	SEQ ID NO: 36
2	3, 39	IL-2gm1d (T3A, M39A)	HSA	SEQ ID NO: 37
1	3	IL-2-HSA (T3A) wild type	HSA	SEQ ID NO:30

Encoding sequences for IL-2 mutant molecules IL-2gm1, IL-2gm4, IL-2gm6, IL-2gm7 and

the encoding sequence for HSA were concatenated and constructed into eukaryotic expression vectors by molecular cloning method to prepare expression vectors for IL-2 mutant molecules, respectively. 293E cells cultured in Freestyle medium were used for transient transfection and expression of IL-2 mutant molecules. Twenty-four hours before transfection, 150 ml of 293E cells at 0.5×10^6 cells/ml was inoculated into a 1 L cell culture flask, and cultured in a shaker at 120 rpm in a 37°C 5% CO₂ incubator. During transfection, 150 µl of 293fectin was taken and added into 2.85 ml of OptiMEM, mixed well, and incubated at room temperature for 2 minutes; meanwhile, 150 µg of plasmids used to express IL-2 molecules was diluted in 3 ml of OptiMEM. The above-diluted transfection reagent and plasmid were mixed thoroughly, and incubated at room temperature for 15 minutes. Then all the mixture was added to the cells, mixed, and incubated in a shaker at 120 rpm in a 37°C 5% CO₂ incubator for 7 days. The cell culture supernatant was collected, filtered through a 0.22 micron filter membrane, and then purified on a Q-HP ion exchange chromatography column (GE) by using 20 mM Tris 0-500 mM NaCl, pH 8.0 for linear elution. Samples were collected continuously by volume. Collected components were detected by SDS-PAGE using 4-20% gradient gel (GenScript), and samples were combined according to the electrophoretic purity.

Example 8. Preparation of receptor protein

To study the binding ability of IL-2 mutant molecules to IL-2Rα receptor and IL-2Rβγ heterodimerization receptor, human IL-2Rα receptor and IL-2Rβγ heterodimerization receptor were prepared for Biacore affinity detection.

For designing human IL-2Rα receptor, the encoding sequence for the extracellular domain of IL-2Rα was linked to the encoding sequence for 6×His Tag (SEQ ID NO: 31) and cloned into a eukaryotic expression vector. 293E cells cultured in Freestyle medium were used for transient transfection and expression of IL-2Rα receptor. Twenty-four hours before transfection, 150 ml of 293E cells at 0.5×10^6 cells/ml was inoculated into a 1 L cell culture flask, and cultured in a shaker at 120 rpm in a 37°C 5% CO₂ incubator. During transfection, 150 µl of 293fectin was taken and added into 2.85 ml of OptiMEM, mixed well, and incubated at room temperature for 2 minutes; meanwhile, 150 µg of plasmids used to express IL-2Rα receptor was diluted in 3 ml of OptiMEM. The above-diluted transfection reagent and plasmid were mixed thoroughly, and incubated at room temperature for 15 minutes. Then all the mixture was added to the cells, mixed, and incubated in a shaker at 120 rpm in a 37°C 5% CO₂ incubator for 7 days. The cell culture supernatant was collected, filtered through a 0.22 micron filter membrane, and then purified on a Ni-NTA affinity chromatography column (GE) by using 20 mM PB-0.5 M NaCl-100 mM imidazole for elution. The purified protein was detected by SDS-PAGE using 4-20% gradient gel (GenScript).

The human IL-2Rβγ heterodimerization receptor was designed by using the “Knobs into

Holes” technology to link the encoding sequence for IL-2R β extracellular domain with the encoding sequence for “Knobs” Fc fragment (SEQ ID NO: 32), and cloned into a eukaryotic expression vector. The encoding sequence for IL-2R γ extracellular domain was linked to the encoding sequence for “Holes” Fc fragment (SEQ ID NO: 33), and cloned into a eukaryotic expression vector. 293E cells cultured in Freestyle medium were used for transient transfection and expression of IL-2R $\beta\gamma$ heterodimerization receptor. Twenty-four hours before transfection, 150 ml of 293E cells at 0.5×10^6 cells/ml was inoculated into a 1 L cell culture flask, and cultured in a shaker at 120 rpm in a 37°C 5% CO₂ incubator. During transfection, 150 μ l of 293fectin was taken and added into 2.85 ml of OptiMEM, mixed well, and incubated at room temperature for 2 minutes; meanwhile, 75 μ g of plasmids used to express IL-2R $\beta\gamma$ heterodimerization receptor was diluted in 3 ml of OptiMEM. The above-diluted transfection reagent and plasmid were mixed thoroughly, and incubated at room temperature for 15 minutes. Then all the mixture was added to the cells, mixed, and incubated in a shaker at 120 rpm in a 37°C 5% CO₂ incubator for 7 days. The cell culture supernatant was collected, filtered through a 0.22 micron filter membrane, and then purified on a MabSelect SuRe affinity chromatography column (GE) by using 20 mM citrate-sodium citrate, pH 3.0 for elution. pH was adjusted to neutral with 1 M Tris base. The purified protein was detected by SDS-PAGE using 4-20% gradient gel (GenScript).

Example 9. Experiment to detect binding affinity by Biacore

For studying the affinity of the IL-2 mutant to the receptor compared with the wild-type IL-2, the recombinant monomer IL-2R α subunit was used to determine the affinity of the IL-2 mutant and wild-type IL-2-HSA to human IL-2R α subunit by Biacore 8K (GE) under the following conditions: immobilizing human IL-2R α subunit on a CM5 chip (190RU). The IL-2 mutant and IL-2-HSA were used as analytes in HBS-EP buffer at 25°C. For IL-2R α , the concentration of analyte was reduced from 200 nM to 1.526 nM (1: 2 dilution), and the flow rate was 30 μ l/min (binding time 180 seconds, dissociation time 300 seconds). For IL-2R α , regeneration was performed with 20 mM NaOH, 30 μ l/min for 10 seconds. For IL-2R α , 1: 1 binding was used with RI $\neq$ 0, R_{max} = global fitting data.

Under the following conditions, recombinant IL-2R $\beta\gamma$ heterodimer was used to determine the affinity of IL-2 mutant and the wild-type IL-2-HSA to human IL-2R $\beta\gamma$ heterodimer by Biacore 8K (GE): human hIL-2R β , γ ECD-N-hIgG1Fc was immobilized on a Protein A chip (400RU). IL-2 mutant and IL-2-HSA were used as analytes in HBS-EP buffer at 25°C. For IL-2R $\beta\gamma$, the concentration of analyte was reduced from 200 nM to 1.5625 nM (1: 2 dilution), and the flow rate was 30 μ l/min (binding time 180 seconds, dissociation time 300 seconds). For IL-2R $\beta\gamma$, 10 mM Glycine (pH1.5), 30 μ l/min, 30 seconds was used for regeneration. For IL-2R $\beta\gamma$, 1: 1 binding was used with RI $\neq$ 0, R_{max} = local fitting data.

Results are as follows:

Table 6: Affinities (KD) of IL-2 mutants to the receptor compared with the wild-type IL-2

Tested sample	Human IL-2R α (Steady state)	Human IL-2R α (dynamic state)	Human IL-2R $\beta\gamma$ heterodimer (dynamic state)
IL-2-HSA wild type	5.56E-08M	1.06E-07M	1.53E-08M
IL-2gm1-HSA	no binding	no binding	1.38E-08M
IL-2gm4-HSA	7.26E-08M	3.54E-08M	3.30E-08M
IL-2gm6-HSA	no binding	no binding	2.89E-08M
IL-2gm1a-HSA	no binding	no binding	3.65E-08M
IL-2gm1b-HSA	no binding	no binding	2.83E-08M
IL-2gm1c-HSA	no binding	no binding	3.84E-08M
IL-2gm1d-HSA	6.62E-08M	1.13E-08M	2.30E-08M

Compared with the affinity of wild-type IL-2-HSA to human IL-2R α subunit: there is no binding for IL-2gm1-HSA, IL-2gm6-HSA, IL-2gm1a-HSA, IL-2gm1b-HSA and IL-2gm1c-HSA, and there is binding for IL-2gm4-HSA and IL-2gm1d-HSA;

Compared with the affinity of wild-type IL-2-HSA to human recombinant IL-2R $\beta\gamma$ heterodimer: IL-2gm1-HSA, IL-2gm4-HSA, IL-2gm6-HSA, IL-2gm1a-HSA, IL-2gm1b-HSA, IL-2gm1c-HSA and IL-2gm1d-HSA are comparable to the wild type.

Therefore, IL-2gm1-HSA, IL-2gm1a-HSA, IL-2gm1b-HSA and IL-2gm1c-HSA are preferred target molecules.

Figure 8A shows the affinity of IL-2gm1-HSA, IL-2gm4-HSA, IL-2gm6-HSA, IL-2gm1a-HSA, IL-2gm1b-HSA, IL-2gm1c-HSA, IL-2gm1d-HSA, the wild type IL-2-HSA to human IL-2R α subunit.

Figure 8B shows the affinity of IL-2gm1-HSA, IL-2gm4-HSA, IL-2gm6-HSA, IL-2gm1a-HSA, IL-2gm1b-HSA, IL-2gm1c-HSA, IL-2gm1d-HSA, the wild type IL-2-HSA to human recombinant IL-2R $\beta\gamma$ heterodimer.

Example 10. Analysis on cell proliferation using NK92 cells

NK-92 cell is a strain of IL-2 dependent NK cell line derived from peripheral blood mononuclear cells of a 50-year-old white male with rapidly progressive non-Hodgkin's lymphoma, and the cell expresses CD25 on the surface. The inventors used NK92 cells to evaluate the activities of IL-2gm1-HSA, IL-2gm4-HSA, IL-2gm6-HSA and the wild-type IL-2-HSA in a cell proliferation analysis.

NK92 cells in the logarithmic growth phase were harvested, washed for one time with basal medium MEM- α , and cultured (5000 cells/well) with different concentrations of IL-2gm1-HSA, IL-2gm4-HSA, IL-2gm6-HSA and the wild-type IL-2-HSA in experimental medium (MEM- α medium from Gibco (Cat. No. 32561-037) supplemented with 12.5% fetal bovine serum and 12.5% horse serum) at 37°C and 5% CO₂ incubator for a total of 48 hours. 100 μ l of ATP detection substrate CellTiter-Glo (from promega (Cat. No. G7571)) was added to each well, and the

full-wavelength fluorescence value was detected with a microplate reader (purchased from Molecular Devices (Model I3x)) by endpoint method.

Activities of IL-2gm1-HSA, IL-2gm4-HSA, IL-2gm6-HSA and the wild-type IL-2 molecule IL-2-HSA were measured by an analysis on cell proliferation, and the results are shown in FIG. 9.

- 5 All test articles induced the growth of NK92 cells in a dosage-dependent manner. When the cell proliferation folds are comparable, the larger the EC_{50} , the weaker the activity of stimulating the growth of NK92. This change is due to the effects of the mutant protein on the binding to CD25, however the IL-2gm1-HSA mutant protein retains the activation of IL-2R signaling through the IL-2R $\beta\gamma$ heterodimer. Therefore, after the concentration was increased, the cells were effectively
- 10 expanded. Compared with IL-2-HSA, the specific activity of IL-2gm1-HSA to stimulate the proliferation of NK92 cells was 1.07%, demonstrating that for NK-92 cell with CD25 expressed on the surface, IL-2gm1-HSA can eliminate the binding to CD25, and the stimulation effect was weakened since the IL-2R $\alpha\beta\gamma$ heterotrimer was not formed. Compared with IL-2-HSA, the proliferation effects of IL-2gm1-HSA on NK92 cells are reduced by more than 100 times.
- 15 Compared with IL-2-HSA, the specific activity of IL-2gm4-HSA to stimulate the proliferation of NK92 cells was 1.78%, and compared with IL-2-HSA, the specific activity of IL-2gm6-HSA to stimulate the proliferation of NK92 cells was 0.087%. After the concentration was increased, the cells were effectively expanded and the biological activities are retained.

20 **Example 11. Measuring the release of IFN- γ induced by IL-2 mutant**

- NK92 cells in the logarithmic growth phase were harvested, washed for one time with basal medium MEM- α , and cultured (5000 cells/well) with different concentrations of IL-2gm1-HSA, IL-2gm4-HSA, IL-2gm6-HSA and the wild-type IL-2-HSA in an experimental medium (MEM- α medium from Gibco (Cat. No. 32561-037) supplemented with 12.5% fetal bovine serum and
- 25 12.5% horse serum) at 37°C and 5% CO₂ incubator for a total of 24 hours. The supernatant was harvested and analyzed for the release of IFN- γ using an anti-human IFN- γ ELISA kit from R&D (# SIF50).

Results are shown in Fig. 10A, in which all of IL-2gm1-HSA, IL-2gm4-HSA and the wild-type IL-2-HSA exhibit stimulating effects on interferon gamma.

- 30 Results are shown in Figure 10B, in which both of IL-2gm6-HSA and the wild-type IL-2-HSA exhibit stimulating effects on interferon gamma.

Example 12. Measuring proliferation of PBMC stimulated by IL-2 mutants

- Fresh blood samples from healthy Chinese people (n=2) were collected in sodium heparin
- 35 tubes by the inventors and PBMCs were separated, resuspended in RPMI-1640 medium (containing 10% FBS) and inoculated in a 48-well plate (1×10^6 cells/well). PBMCs were

stimulated with different concentrations of IL-2gm6-HSA and wild-type IL-2-HSA, and cultured in a 37°C, 5% CO₂ incubator for 6 days. Cell surface and intracellular marker antibodies were used for FACS staining to detect different cell populations. All of the samples were obtained by LSRFortessa™ cell analyzer.

5 NK cells are defined as CD3-/CD56+, and Treg cells are defined as CD3+CD4+CD25+Foxp3+.

Results are shown in Figures 11A-B, in which Figure 11A shows that at a concentration of 4 nM, IL-2gm6-HSA exhibits slightly worse stimulating effects on the proliferation of NK cells compared with wild-type IL-2-HSA; and at a concentration of 100 nM, IL-2gm6-HSA
10 significantly stimulates proliferation of NK cells compared with the wild-type IL-2-HSA; and Figure 11B shows that at 4 nM and 100 nM concentrations, stimulating effects of IL-2gm6-HSA on Treg cells are significantly reduced compared with the wild-type IL-2-HSA.

Therefore, in this experiment, IL-2gm6-HSA significantly stimulates the proliferation of NK cells and significantly inhibits the proliferation of Treg cells.

15 Subsequently, the inventors also tested the stimulating effects of IL-2gm1-HSA and the wild-type IL-2-HSA on the proliferation of PBMCs.

NK cells are defined as CD3-/CD56+, and CD4+ cells are defined as CD3+/CD4+.

Results are shown in Figures 12A-B, in which Figure 12A shows that at a stimulating concentration of 0-500 nM, the stimulating effects of IL-2gm1-HSA on the proliferation of NK
20 cells are significantly higher than those of the wild-type IL-2-HSA; and Figure 12B shows that at a stimulating concentration of 0-500 nM, the stimulating effects of IL-2gm1-HSA on the proliferation of CD4+T cells are significantly reduced compared with the wild-type IL-2-HSA.

Therefore, in this experiment, IL-2gm1-HSA significantly stimulates the proliferation of NK cells and significantly inhibits the proliferation of CD4+ T cells.

25 All documents mentioned in the present invention are cited as references in this application, as if each document was individually cited as a reference. In addition, it should be understood that after reading the above teaching content of the present invention, those skilled in the art can make various changes or modifications to the present invention, and these equivalent forms also fall
30 within the scope defined by the appended claims of the present application.

Claims

1. An IL-2 mutant, wherein the IL-2 mutant has a single amino acid residue mutation in wild-type human IL-2: M39D.

5 2. A fusion protein or conjugate, comprising the IL-2 mutant of claim 1 and a non-IL-2 functional moiety.

3. A polynucleotide, encoding the IL-2 mutant of claim 1 or the fusion protein or conjugate of claim 2.

4. An expression vector, comprising the polynucleotide of claim 3.

10 5. A host cell, comprising the expression vector of claim 4, or having the polynucleotide of claim 3 integrated into the genome of the host cell.

6. A pharmaceutical composition, comprising the IL-2 mutant protein of claim 1 or the fusion protein or conjugate of claim 2 and a pharmaceutically acceptable excipient.

15 7. Use of the IL-2 mutant of claim 1 or the fusion protein of claim 2, for preparing a medicament for the treatment of a disease in which IL-2 is used for immunotherapy in an individual.

20