

INTERLEUKIN-2 DERIVATIVE

TECHNICAL FIELD

The present invention belongs to the field of molecular biology, and particularly relates to interleukin-2 derivatives and complexes thereof.

BACKGROUND ART

Interleukin-2 (IL-2), discovered in 1976, was called T cell growth factor (TCGF) at the time. IL-2 is a globular glycoprotein that plays an important role in maintaining the normal functions of T lymphocytes and NK cells. Natural IL-2 is a polypeptide composed of 133 amino acid residues, which has a molecular weight of about 15kD and three cysteine residues, located at positions 58, 105 and 125, respectively. Post-translational modifications include Thr glycosylation at position 3, disulfide bonds formed by cysteine residues at positions 58 and 105, and the formation of high-level structures mainly composed of 4 α helices and some linking sequences (loops), which are essential for IL-2's function (Bazan et al., Science 257, 410-413 (1992)).

IL-2 is produced mainly by activated T cells and is capable of: promoting the proliferation and differentiation of T cells to maintain their activity; stimulating the production, proliferation and activation of natural killer (NK) cells; inducing the generation of cytotoxic T lymphocytes (CTLs); inducing and activating lymphokine-activated cell killer (LAK) cells and tumor infiltrating lymphocytes; promoting the expression of cytokines and cytolytic molecules by T cells; and promoting the proliferation of B cells (Waldmann et al., Nat. Rev. Immunol. 6,595-601 (2009)). All of these cells directly or indirectly have the effect of killing cells infected by the foreign microorganisms and cancerous cells. Therefore, IL-2 has good antiviral and anticancer effects and wide clinical application potential.

IL-2 mediates its action by binding to IL-2 receptor (IL-2R), which consists of 3 subunits, namely α (CD25), β (CD122) and γ (CD132) receptor subunits, wherein α receptors are mainly expressed on the surface of T suppressor cells (Treg) and some endothelial cells, while β and γ receptor subunits are highly expressed on effector T cells (Teff) and NK cells. IL-2 differs in its

affinity to different complexes of the receptor subunits. IL-2 has high affinity to the complex composed of α , β and γ receptor subunits and intermediate affinity (approximately 100-fold lower) to the complex composed of β and γ receptor subunits. Both of the complexes are able to transmit signal upon IL-2 binding (Minami et al., *Annu. Rev. Immunol.* 11, 245-268 (1993)). However, clinically, under the condition of low dose of IL-2, it will preferentially bind to the high-affinity receptors on the surface of Treg cells, which will cause immunosuppression and fail to achieve the therapeutic effect. A high dose of IL-2 will neutralize the immune suppression caused by Treg activation by activating a large number of effector T cells. At the same time, there will be more toxic side effects and cell apoptosis.

Due to the anti-tumor effect of IL-2, a high dose of IL-2 (Aldesleukin) was approved by FDA in 1992 for the clinical treatment of melanoma and renal cell carcinoma. However, patients receiving high-dose IL-2 treatment often experience severe side effects, including cardiovascular, pulmonary edematous, hepatic, gastrointestinal, neurological, and hematological events. Most of these side effects can be explained by Vascular (or Capillary) Leak Syndrome (VLS), which is also an indicator for clinical and animal experiments to evaluate the side effects of IL-2 treatment. VLS is caused by the expression of high-affinity receptors (α , β and γ subunits) of IL-2 on endothelial cells (Krieg et al., *Proc. Nat. Acad. Sci. USA* 107, 11906-11 (2010)). Therefore, weakening or elimination of the binding to α receptor will help to weaken the function of IL-2 of promoting the inhibition of T cell proliferation activity, meanwhile which will also reduce the binding to α receptor of endothelial cell, thereby reducing or eliminating the side effects caused by IL-2 treatment. The binding sites of IL-2 and α receptor subunit are mainly at positions 37, 38, 41, 42, 43, 44, 45, 61, 62, 65, 68 and 72 (Rickert. M. et al., *Science* 308 :1477-1480 (2005)). Merck, Roche or other scientific research institutions have made some mutations around these amino acids on the surface of IL-2 that bind to α receptor subunit. For example, the interaction between the mutant from Merck (R38W, F42K, WO2008003473A2) and α receptor subunit is reduced to activate effector T cells to enhance efficacy. The IL-2 mutants from Roche (F42A, Y45A and L72G, US 2016/0208017A1) do not bind to α receptor, but can normally bind to β and γ receptor subunit complexes, which can exert effects and are currently in clinical practice.

Therefore, the reduction or elimination of the interaction between IL-2 and α receptor subunit may be an important aspect to improve the effectiveness of treatment and to reduce the side effects of treatment in tumor patients.

SUMMARY OF THE INVENTION

In view of the defects in the prior art, the present invention provides an IL-2 derivative and a complex thereof.

In the first aspect, the present invention provides an IL-2 derivative. In a specific embodiment, the IL-2 derivative is introduced with at least one cysteine residue based on wild-type IL-2, and the binding plane of the IL-2 derivative and α receptor subunit is partially or completely blocked, while the affinity of the IL-2 derivative to the complex of β and γ receptor subunits is basically retained. The amino acid sequence of the wild-type IL-2 is shown in SEQ ID NO: 1.

Further, the at least one cysteine residue introduced based on wild-type IL-2 is capable of:

- a) forming an intramolecular disulfide bond of the IL-2 derivative; or
- b) enabling the IL-2 derivative to bind to a blocking module through a intermolecular disulfide bond.

Optionally, the at least one cysteine residue is introduced by means of point mutation.

Optionally, in one embodiment, a first cysteine residue and a second cysteine residue are introduced by means of point mutation based on wild-type IL-2; and one or both of the first cysteine residue and the second cysteine residue are amino acids related to the binding plane of the wild-type IL-2 and α receptor subunit, or amino acids in the vicinity thereof. Further, the amino acid positions related to the binding plane of wild-type IL-2 and α receptor subunit are position 37, position 38, position 41, position 42, position 43, position 44, position 45, position 61, position 62, position 65, position 68 and position 72.

Further, the first cysteine residue is an amino acid at position 37, position 38, position 41, position 42, position 43, position 44, position 45, position 61 or position 62 of the wild-type IL-2, or an amino acid in the vicinity thereof; and the second cysteine residue is an amino acid at position 61, position 62, position 65, position 68 or position 72 of the wild-type IL-2, or an amino acid in the vicinity thereof. Optionally, the term “vicinity” refers to: 1) 1 to 4 amino acids that are adjacent in the primary structure; and/or 2) amino acids that are adjacent in the tertiary structure.

Further, the first cysteine residue is an amino acid point mutant selected from the group consisting of: K35C, L36C, R38C, M39C, L40C, T41C, F42C, K43C, F44C and E61C.

Further, the second cysteine residue is an amino acid point mutant selected from the group consisting of: V69C, E62C, P65C, T111C, Y107C, A112C, T113C, I114C, L72C and A73C.

Optionally, a combination of the first cysteine residue and the second cysteine residue that form the intramolecular disulfide bond is a combination of amino acid point mutants selected from the group consisting of: M39C and V69C; F44C and E62C; F44C and P65C; F42C and V69C; E61C and Y107C; F42C and P65C; F42C and T111C; F42C and A112C; F42C and T113C; T41C and A112C; L40C and A112C; T113C and T113C; L40C and I114C; M39C and L72C; M39C and A73C; R38C and V69C; R38C and L72C; L36C and V69C; L36C and L72C; L36C and A73C; K35C and V69C; and K43C and A112C.

Optionally, the center-of-mass vector distance between the first cysteine residue and the second cysteine residue is less than 6Å.

Optionally, in a second embodiment, a third cysteine residue is introduced by means of point mutation based on wild-type IL-2; and the third cysteine residue is an amino acid related to the binding plane of wild-type IL-2 and α receptor subunit, or an amino acid in the vicinity thereof.

Further, the third cysteine residue is an amino acid at position 37, position 38, position 41, position 42, position 43, position 44, position 45, position 61, position 62, position 65, position 68 or position 72 of the wild-type IL-2, or an amino acid in the vicinity thereof. Optionally, the term “vicinity” refers to: 1) 1 to 4 amino acids that are adjacent in the primary structure; and/or 2) amino acids that are adjacent in the tertiary structure.

Further, the third cysteine residue is an amino acid point mutant selected from the group consisting of: P34C, K35C, T37C, R38C, T41C, K43C, F44C, Y45C, E61C, E62C, K64C, P65C, E68C and L72C.

Further, the blocking module has or is introduced with a fourth cysteine residue; and the third cysteine residue on the IL-2 derivative and the fourth cysteine residue on the blocking module

are capable of forming an intermolecular disulfide bond.

Optionally, the center-of-mass vector distance between the third cysteine residue and the fourth cysteine residue is less than 6Å.

Optionally, the blocking module is an extracellular segment of the α receptor subunit.

Further, the amino acid sequence of the extracellular segment of the α receptor subunit is shown in SEQ ID NO: 24.

Further, the fourth cysteine residue on the extracellular segment of the α receptor subunit is an amino acid point mutant selected from the group consisting of: D4C, D5C, M25C, N27C, R35C, R36C, K38C, S39C, G40C, S41C, L42C, I118C, Y119C and H120C.

Optionally, a combination of the third cysteine residue and the fourth cysteine residue that form the intramolecular disulfide bond is a combination of amino acid point mutants selected from the group consisting of: T41C and N27C; P34C and D4C; E68C and L42C; Y45C and R35C; R38C and H120C; L72C and M25C; E61C and S39C; T41C and I118C; K35C and D4C; T37C and D4C; R38C and D4C; R38C and D5C; T41C and L42C; T41C and Y119C; K43C and R35C; K43C and R36C; F44C and L42C; K43C and L42C; E61C and K38C; E62C and K38C; K64C and S39C; K64C and G40C; K64C and S41C; and P65C and K38C.

In either the first embodiment or the second embodiment, optionally, the cysteine residue at position 125 of the wild-type IL-2 is converted into another amino acid residue by means of point mutation.

Further optionally, the point mutant at position 125 of the wild-type IL-2 is C125A.

Optionally, the amino acid sequence of the IL-2 derivative is shown in SEQ ID NO: 3 to SEQ ID NO: 24, or in SEQ ID NO: 26 to SEQ ID NO: 40.

In the second aspect, the present invention provides a complex. In a specific embodiment, the complex comprises:

1) an IL-2 derivative, which is introduced with a third cysteine residue based on wild-type IL-2;

and

2) a blocking module, which has or is introduced with a fourth cysteine residue;

wherein the third cysteine residue on the IL-2 derivative and the fourth cysteine residue on the blocking module are capable of forming an intermolecular disulfide bond, thereby forming a complex of the IL-2 derivative and the blocking module; and wherein the binding plane of the IL-2 derivative and α receptor subunit is partially or completely blocked, while the affinity of the IL-2 derivative to the complex of β and γ receptor subunits is basically retained.

Optionally, the at least one cysteine residue is introduced by means of point mutation.

Further, the third cysteine residue is an amino acid related to the binding plane of the wild-type IL-2 and α receptor subunit, or an amino acid in the vicinity thereof.

Further, the third cysteine residue is an amino acid at position 37, position 38, position 41, position 42, position 43, position 44, position 45, position 61, position 62, position 65, position 68 or position 72 of the wild-type IL-2, or an amino acid in the vicinity thereof. Optionally, the term “vicinity” refers to: 1) 1 to 4 amino acids that are adjacent in the primary structure; and/or 2) amino acids that are adjacent in the tertiary structure.

Optionally, the center-of-mass vector distance between the third cysteine residue and the fourth cysteine residue is less than 6Å.

Further, the third cysteine residue is an amino acid point mutant selected from the group consisting of: P34C, K35C, T37C, R38C, T41C, K43C, F44C, Y45C, E61C, E62C, K64C, P65C, E68C and L72C.

Optionally, the blocking module is an extracellular segment of the α receptor subunit, the amino acid sequence of which is shown in SEQ ID NO: 25.

Further, the fourth cysteine residue on the extracellular segment of the α receptor subunit is an amino acid point mutant selected from the group consisting of: D4C, D5C, M25C, N27C, R35C, R36C, K38C, S39C, G40C, S41C, L42C, I118C, Y119C and H120C.

Optionally, a combination of the third cysteine residue and the fourth cysteine residue that form

the intramolecular disulfide bond is a combination of amino acid point mutants selected from the group consisting of: T41C and N27C; P34C and D4C; E68C and L42C; Y45C and R35C; R38C and H120C; L72C and M25C; E61C and S39C; T41C and I118C; K35C and D4C; T37C and D4C; R38C and D4C; R38C and D5C; T41C and L42C; T41C and Y119C; K43C and R35C; K43C and R36C; F44C and L42C; K43C and L42C; E61C and K38C; E62C and K38C; K64C and S39C; K64C and G40C; K64C and S41C; and P65C and K38C.

Optionally, the point mutant at position 125 of the wild-type IL-2 is C125A.

Optionally, a combination of the amino acid sequence of the IL-2 derivative and the sequence of the extracellular segment of the α receptor subunit is a combination selected from the group consisting of: SEQ ID NO: 26 and SEQ ID NO: 50; SEQ ID NO: 27 and SEQ ID NO: 51; SEQ ID NO: 28 and SEQ ID NO: 52; SEQ ID NO: 29 and SEQ ID NO: 53; SEQ ID NO: 30 and SEQ ID NO: 54; SEQ ID NO: 31 and SEQ ID NO: 55; SEQ ID NO: 32 and SEQ ID NO: 56; SEQ ID NO: 33 and SEQ ID NO: 57; SEQ ID NO: 34 and SEQ ID NO: 58; SEQ ID NO: 35 and SEQ ID NO: 59; SEQ ID NO: 36 and SEQ ID NO: 60; SEQ ID NO: 37 and SEQ ID NO: 61; SEQ ID NO: 38 and SEQ ID NO: 62; SEQ ID NO: 39 and SEQ ID NO: 63; SEQ ID NO: 40 and SEQ ID NO: 64; SEQ ID NO: 41 and SEQ ID NO: 65; SEQ ID NO: 42 and SEQ ID NO: 66; SEQ ID NO: 43 and SEQ ID NO: 67; SEQ ID NO: 44 and SEQ ID NO: 68; SEQ ID NO: 45 and SEQ ID NO: 69; SEQ ID NO: 46 and SEQ ID NO: 70; SEQ ID NO: 47 and SEQ ID NO: 71; SEQ ID NO: 48 and SEQ ID NO: 72; and SEQ ID NO: 49 and SEQ ID NO: 73.

In the third aspect, the present invention provides an isolated polynucleotide. In a specific embodiment, the isolated polynucleotide encodes the IL-2 derivative as described above or the complex as described above.

In the fourth aspect, the present invention provides an expression vector. In a specific embodiment, the expression vector comprises the isolated polynucleotide as described above.

In the fifth aspect, the present invention provides a host cell. In a specific embodiment, the host cell comprises the isolated polynucleotide as described above.

In the sixth aspect, the present invention provides a composition. In a specific embodiment, the composition comprises the IL-2 derivative as described above or the complex as described

above, and a pharmaceutically acceptable carrier.

In the seventh aspect, the present invention provides the use of the IL-2 derivative as described above or the complex as described above for preparing drugs or preparations for treating diseases.

In the eighth aspect, the present invention provides the use of the IL-2 derivative as described above or the complex as described above for preparing a composition for stimulating the immune system of an individual.

In the ninth aspect, the present invention provides a method for producing the IL-2 derivative. In a specific embodiment, the method includes culturing the host cell as described above under conditions suitable for expressing the IL-2 derivative.

In the tenth aspect, the present invention provides a method for producing the complex. In a specific embodiment, the method includes culturing the host cell as described above under conditions suitable for expressing the complex.

In the IL-2 derivative or complex according to specific embodiments of the present invention, by forming intra-molecular or intermolecular disulfide bonds, the binding sites where the IL-2 derivative binds to α receptor are blocked, thereby the structure for binding to the α receptor is precluded.

The IL-2 derivative or complex according to the present invention provides a new direction for reducing VLS, or reducing or eliminating the toxic and side effects caused by IL-2 treatment.

DESCRIPTION OF THE DRAWINGS

Fig. 1 shows SDS-PAGE electrophoretograms of the purified IL-2 derivatives according to one embodiment of the present invention; wherein the “reduced” means that the reducing agent β -ME was added to the loading buffer, and the “non-reduced” means that no reducing agent was added.

Fig. 2 shows signal graphs of binding affinities of the IL-2 derivatives to IL2R α tested by Fortebio according to one embodiment of the present invention, wherein the concentration is

100 nM. The irrelevant protein control used is HER2. Among them, Fig. 2A is related to IL-2 wt C125A; Fig. 2B is related to IL-2 mutant 1; Fig. 2C is related to IL-2 mutant 2; Fig. 2D is related to IL-2 mutant 3; Fig. 2E is related to IL-2 mutant 4; Fig. 2F is related to IL-2 complex 1; Fig. 2G is related to IL-2 complex 2; Fig. 2H is related to IL-2 complex 3; and Fig. 2I is related to IL-2 complex 4.

Fig. 3 shows signal graphs of binding affinities of the IL-2 derivatives to IL2R $\beta\gamma$ tested by Fortebio and Ka, Kd, KD and R2 thereof according to one embodiment of the present invention, wherein the concentration is 1.25 nM to 40 nM. Among them, Fig. 3A is related to IL-2 wt C125A; Fig. 3B is related to IL-2 mutant 1; Fig. 3C is related to IL-2 mutant 2; Fig. 3D is related to IL-2 mutant 3; Fig. 3E is related to IL-2 mutant 4; Fig. 3F is related to IL-2 complex 1; Fig. 3G is related to IL-2 complex 2; Fig. 3H is related to IL-2 complex 3; and Fig. 3I is related to IL-2 complex 4.

Fig. 4 is a graph illustrating the CTLL-2 (T cell) proliferation experiment according to one embodiment of the present invention.

DETAILED DESCRIPTION

Hereinafter, the present invention will be further described in conjunction with examples. It should be understood that these examples are used for illustrative purposes only and are not intended to limit the protection scope of the present invention.

In the following examples, the experimental methods without special instructions were usually carried out in accordance with conventional conditions or in accordance with the conditions recommended by the manufacturer. See, for example, Sambrook et al, Molecular Cloning: A Laboratory Manual (New York: Cold Spring Harbor Laboratory Press, 1989). Unless otherwise specified, the reagents used are commercially available or publicly available reagents.

Herein, the relative amino acid positions of the IL-2 derivatives and wild-type IL-2 were determined based on the amino acid sequence of wild-type IL-2 (e.g., SEQ ID NO: 1).

The amino acid sequence of Wild type IL-2 (IL-2 wt, SEQ ID NO: 1) is as follows:

APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKATELKHLQCL

EEELKPLEEVNLNLAQSKNFHLRPRDLISNINVIVLELKGSETTFMCEYADETATIVEFLNR
WITFCQSIISTLT.

The nucleotide sequence of wild-type IL-2 is shown in SEQ ID NO: 146.

Herein, the relative amino acid positions of CD25-ECD were determined based on the amino acid sequence as shown in SEQ ID NO: 25.

Herein, the terms “first”, “second”, “third”, etc., are only used for distinguishing purposes, and are not intended to limit the order. For example, the derivative may only have a “third” cysteine residue, without “first” and “second” cysteine residues.

Herein, the term “IL-2 derivative” encompasses IL-2 mutant, and complex formed by IL-2 mutant with other molecule. The term “IL-2 mutant” refers to a molecule formed through mutations (e.g., point mutations or insertion mutations) based on the wild-type IL-2.

Herein, the term “IL2R α ” refers to interleukin-2 receptor α , also referred to as “ α receptor subunit”; “IL2R β ” refers to interleukin-2 receptor β , also referred to as “ β receptor subunit”; IL2R γ ” refers to interleukin-2 receptor γ , also referred to as “ γ receptor subunit”; and “IL2R $\beta\gamma$ ” refers to the complex formed by interleukin-2 receptor β and receptor γ , also referred to as “complex of β and γ receptor subunits”.

In the prior art, in order to reduce or eliminate the binding of IL-2 and α receptors, simple mutations are made to the amino acids on the binding plane of IL-2 to α receptor.

In specific embodiments of the present invention, the strategy adopted was to introduce additional cysteine residues to form new disulfide bonds within the IL-2 molecule, or to form complexes of IL-2 and other blocking modules through intermolecular disulfide bonds, which partially or completely blocks the binding sites on IL-2 for α receptor, while the affinity to the complex of β and γ receptor subunits is not affected.

In the first specific embodiment, a first cysteine residue and a second cysteine residue are introduced to form an IL-2 intramolecular disulfide bond, which makes the IL-2 derivative more stable in structure and can further form a barrier to destroy the binding plane for α

receptor's binding.

The first cysteine residue and the second cysteine residue are introduced by making appropriate point mutations based on wild-type IL-2. The positions of the first cysteine residue and the second cysteine residue to be introduced are mainly determined by the following methods:

- 1) The positions of the first and the second cysteine residues were the amino acid residues on the binding plane of IL-2 and α receptor or amino acid residues in the vicinity thereof, wherein the amino acid residues on the binding plane of IL-2 and α receptors were the amino acids at positions 37, 38, 41, 42, 43, 44, 45, 61, 62, 65, 68 and 72;
- 2) The structure of IL-2 and the distance between atoms should be fully considered; and
- 3) Through design according to bioinformatics and protein engineering, two suitable sites around the above amino acid residues were found on the basis of the center-of-mass vector distance of the residues, where an intramolecular disulfide bond can be formed, but an intermolecular disulfide bond was difficult or impossible to be formed.

After the suitable sites are determined, the original amino acid residues are mutated into the first cysteine residue and the second cysteine residue by mutation. Then after a normal process of transcription and translation, the mutated IL-2 derivative (IL-2 mutant) was produced with the intramolecular disulfide bond.

In some embodiments, the original free cysteine at position 125 of IL-2 was mutated to prevent its interference to the formation of the intermolecular disulfide bond.

In some embodiments, the amino acid sequences of IL-2 mutants obtained through the design are shown in Table 1.

Table 1. The amino acid sequence of IL-2 mutants obtained through the design

Mutant	Mutation site	Amino acid sequence	SEQ ID NO:	Nucleotide sequence (SEQ ID NO:)
IL-2 wt (C125)	IL-2 wt (C125A)	APTSSSTKKTQLQLEHLLDLQMILNGI NNYKNPKLTRMLTFKFYMPKKATELK	2	74

A)		HLQCLEEEELKPLEEVLNLAQSKNFHLR PRDLISNINVIVLELKGSETTFMCEYAD ETATIVEFLNRWITFAQSIISTLT		
IL-2 Mutant 1	IL-2 (M39C, V69C, C125A)	APTSSSTKKTQLQLEHLLLDLQMILNGI NNYKNPKLTRCLTFKFYMPKKATELK HLQCLEEEELKPLEECLNLAQSKNFHLR PRDLISNINVIVLELKGSETTFMCEYAD ETATIVEFLNRWITFAQSIISTLT	3	75
IL-2 Mutant 2	IL-2 (F44C, E62C, C125A)	APTSSSTKKTQLQLEHLLLDLQMILNGI NNYKNPKLTRMLTFKCYMPKKATELK HLQCLEEELKPLEEVLNLAQSKNFHLR PRDLISNINVIVLELKGSETTFMCEYAD ETATIVEFLNRWITFAQSIISTLT	4	76
IL-2 Mutant 3	IL-2 (F44C, P65C, C125A)	APTSSSTKKTQLQLEHLLLDLQMILNGI NNYKNPKLTRMLTFKCYMPKKATELK HLQCLEEEELKCLEEVLNLAQSKNFHLR PRDLISNINVIVLELKGSETTFMCEYAD ETATIVEFLNRWITFAQSIISTLT	5	77
IL-2 Mutant 4	IL-2 (F42C, V69C, C125A)	APTSSSTKKTQLQLEHLLLDLQMILNGI NNYKNPKLTRMLTCKFYMPKKATELK HLQCLEEEELKPLEECLNLAQSKNFHLR PRDLISNINVIVLELKGSETTFMCEYAD ETATIVEFLNRWITFAQSIISTLT	6	78
IL-2 Mutant 5	IL-2 (E61C, Y107C, C125A)	APTSSSTKKTQLQLEHLLLDLQMILNGI NNYKNPKLTRMLTFKFYMPKKATELK HLQCLECELKPLEEVLNLAQSKNFHLR PRDLISNINVIVLELKGSETTFMCECAD ETATIVEFLNRWITFAQSIISTLT	7	79
IL-2 Mutant 6	IL-2 (F42C, P65C, C125A)	APTSSSTKKTQLQLEHLLLDLQMILNGI NNYKNPKLTRMLTCKFYMPKKATELK HLQCLEEEELKCLEEVLNLAQSKNFHLR PRDLISNINVIVLELKGSETTFMCEYAD ETATIVEFLNRWITFAQSIISTLT	8	80
IL-2 Mutant 7	IL-2 (F42C, T111C, C125A)	APTSSSTKKTQLQLEHLLLDLQMILNGI NNYKNPKLTRMLTCKFYMPKKATELK HLQCLEEEELKPLEEVLNLAQSKNFHLR PRDLISNINVIVLELKGSETTFMCEYAD ECATIVEFLNRWITFAQSIISTLT	9	81
IL-2 Mutant 8	IL-2 (F42C, A112C, C125A)	APTSSSTKKTQLQLEHLLLDLQMILNGI NNYKNPKLTRMLTCKFYMPKKATELK HLQCLEEEELKPLEEVLNLAQSKNFHLR PRDLISNINVIVLELKGSETTFMCEYAD ETCTIVEFLNRWITFAQSIISTLT	10	82
IL-2 Mutant 9	IL-2 (F42C, T113C, C125A)	APTSSSTKKTQLQLEHLLLDLQMILNGI NNYKNPKLTRMLTCKFYMPKKATELK HLQCLEEEELKPLEEVLNLAQSKNFHLR PRDLISNINVIVLELKGSETTFMCEYAD ETACIVEFLNRWITFAQSIISTLT	11	83
IL-2 Mutant	IL-2 (T41C,	APTSSSTKKTQLQLEHLLLDLQMILNGI NNYKNPKLTRMLCFKFYMPKKATELK	12	84

10	A112C, C125A)	HLQCLEEELKPLEEVLNLAQSKNFHLR PRDLISNINVIVLELKGSETTFMCEYAD ETCTIVEFLNRWITFAQSIISTLT		
IL-2 Mutant 11	IL-2 (L40C, A112C, C125A)	APTSSSTKKTQLQLEHLLLDLQMILNGI NNYKNPKLTRMCTFKFYMPKKATELK HLQCLEEELKPLEEVLNLAQSKNFHLR PRDLISNINVIVLELKGSETTFMCEYAD ETCTIVEFLNRWITFAQSIISTLT	13	85
IL-2 Mutant 12	IL-2 (L40C, T113C, C125A)	APTSSSTKKTQLQLEHLLLDLQMILNGI NNYKNPKLTRMCTFKFYMPKKATELK HLQCLEEELKPLEEVLNLAQSKNFHLR PRDLISNINVIVLELKGSETTFMCEYAD ETACIVEFLNRWITFAQSIISTLT	14	86
IL-2 Mutant 13	IL-2 (L40C, I114C, C125A)	APTSSSTKKTQLQLEHLLLDLQMILNGI NNYKNPKLTRMCTFKFYMPKKATELK HLQCLEEELKPLEEVLNLAQSKNFHLR PRDLISNINVIVLELKGSETTFMCEYAD ETATCVEFLNRWITFAQSIISTLT	15	87
IL-2 Mutant 14	IL-2 (M39C, L72C, C125A)	APTSSSTKKTQLQLEHLLLDLQMILNGI NNYKNPKLTRCLTFKFYMPKKATELK HLQCLEEELKPLEEVLNCAQSKNFHLR PRDLISNINVIVLELKGSETTFMCEYAD ETATIVEFLNRWITFAQSIISTLT	16	88
IL-2 Mutant 15	IL-2 (M39C, A73C, C125A)	APTSSSTKKTQLQLEHLLLDLQMILNGI NNYKNPKLTRCLTFKFYMPKKATELK HLQCLEEELKPLEEVLNLCQSKNFHLR PRDLISNINVIVLELKGSETTFMCEYAD ETATIVEFLNRWITFAQSIISTLT	17	89
IL-2 Mutant 16	IL-2 (R38C, V69C, C125A)	APTSSSTKKTQLQLEHLLLDLQMILNGI NNYKNPKLTCMLTFKFYMPKKATELK HLQCLEEELKPLEECLNLAQSKNFHLR PRDLISNINVIVLELKGSETTFMCEYAD ETATIVEFLNRWITFAQSIISTLT	18	90
IL-2 Mutant 17	IL-2 (R38C, L72C, C125A)	APTSSSTKKTQLQLEHLLLDLQMILNGI NNYKNPKLTCMLTFKFYMPKKATELK HLQCLEEELKPLEEVLNCAQSKNFHLR PRDLISNINVIVLELKGSETTFMCEYAD ETATIVEFLNRWITFAQSIISTLT	19	91
IL-2 Mutant 18	IL-2 (L36C, V69C, C125A)	APTSSSTKKTQLQLEHLLLDLQMILNGI NNYKNPKCTRMLTFKFYMPKKATELK HLQCLEEELKPLEECLNLAQSKNFHLR PRDLISNINVIVLELKGSETTFMCEYAD ETATIVEFLNRWITFAQSIISTLT	20	92
IL-2 Mutant 19	IL-2 (L36C, L72C, C125A)	APTSSSTKKTQLQLEHLLLDLQMILNGI NNYKNPKCTRMLTFKFYMPKKATELK HLQCLEEELKPLEEVLNCAQSKNFHLR PRDLISNINVIVLELKGSETTFMCEYAD ETATIVEFLNRWITFAQSIISTLT	21	93
IL-2 Mutant	IL-2 (L36C,	APTSSSTKKTQLQLEHLLLDLQMILNGI NNYKNPKCTRMLTFKFYMPKKATELK	22	94

20	A73C, C125A)	HLQCLEEELKPLEEVLNLCQSKNFHLR PRDLISNINVIVLELKGSETTFMCEYAD ETATIVEFLNRWITFAQSIISTLT		
IL-2 Mutant 21	IL-2 (K35C, V69C, C125A)	APTSSSTKKTQLQLEHLLDLQMILNGI NNYKNPCLTRMLTFKFYMPKKATELK HLQCLEEELKPLEECLNLAQSKNFHLR PRDLISNINVIVLELKGSETTFMCEYAD ETATIVEFLNRWITFAQSIISTLT	23	95
IL-2 Mutant 22	IL-2 (K43C, A112C, C125A)	APTSSSTKKTQLQLEHLLDLQMILNGI NNYKNPKLTRMLTFCFYMPKKATELK HLQCLEEELKPLEEVLNLAQSKNFHLR PRDLISNINVIVLELKGSETTFMCEYAD ETCTIVEFLNRWITFAQSIISTLT	24	96

The expression hosts can be *E. coli* or mammalian cells.

In the second specific embodiment, IL-2 forms a complex with a blocking module through an intermolecular disulfide bond, which completely or partially blocks the binding plane of IL-2 and α receptor, thereby blocking the binding of IL-2 to endogenous α receptor. The disulfide bond between IL-2 and the blocking module was formed by the third cysteine residue introduced into the wild-type IL-2 and the fourth cysteine residue present on or introduced into the blocking module.

In one embodiment, the blocking module was the extracellular segment of α receptor. The amino acid sequence of the extracellular segment of the wild-type α receptor was shown in SEQ ID NO: 25. In the natural state, the binding of α receptor to IL-2 was not stable (having a high dissociation coefficient K_d), and a stable heterodimer cannot be formed. The α receptor must be combined with β and γ receptor subunits to form a high-affinity receptor for IL-2. Therefore, it was impossible to form a stable complex by co-expression of two wild-type molecules. However, through the disulfide bond formed between the third cysteine residue which was introduced into the wild-type IL-2 and the fourth cysteine residue which was introduced into the extracellular segment of the α receptor (CD25-ECD), the IL-2 derivative and the extracellular segment of the α receptor (CD25-ECD) can form a complex to block the binding of IL-2 to the endogenous α receptor.

The amino acid sequence of the extracellular segment of the wild-type α receptor is shown in SEQ ID NO: 25:

ELCDDDPPEIPHATFKAMAYKEGTMLNCECKRGFRRIKSGSLYMLCTGNSSHSSWDNQ

CQCTSSATRNTTKQVTPQPPEEQKERKTTEMQSPMQPVDQASLPGHCREPPPWENEATE
RIYHFVVGQMVYYQCVQGYRALHRGPAESVCKMTHGKTRWTQPQLICTG.

The nucleotide sequence of the extracellular segment of the wild-type α receptor is shown in SEQ ID NO: 145.

The positions of the third cysteine residue and the fourth cysteine residue to be introduced were mainly determined by the following methods:

- 1) The positions of the third cysteine residue were the amino acid residues on the binding plane of IL-2 and α receptor or amino acid residues in the vicinity thereof, wherein the amino acid residues on the binding plane of IL-2 and α receptors were the amino acids at positions 37, 38, 41, 42, 43, 44, 45, 61, 62, 65, 68 and 72; and
- 2) Through design according to bioinformatics and protein engineering, suitable binding plane sites in IL-2 and α receptor extracellular domain (CD25-ECD) were found respectively on the basis of the center-of-mass vector distance of the residues, where the mutation did not affect the structure of the protein.

After the suitable sites are determined, the original amino acid residue on IL-2 was mutated to the third cysteine residue by mutation, and the original amino acid residue on the extracellular domain of the α receptor (CD25-ECD) was mutated to the fourth cysteine residue. After the co-expression of the mutants of IL-2 and extracellular domain of α receptor, disulfide bond can be formed between IL-2 and the extracellular domain of α receptor through transcription and translation, to form a new molecule, IL-2/CD25-ECD heterodimer. The complex cannot bind to the endogenous α receptor *in vivo*, but can bind to the complex of β and γ receptor subunits, so as to achieve the purpose of not activating Treg.

In one embodiment, the original free cysteine at position 125 in IL-2 was mutated to prevent the formation of additional disulfide bond on the extracellular segment of the α receptor with cysteine mutation or IL-2 with cysteine mutation, which affects the formation of the dimer of the α receptor and the IL-2.

In some embodiments, the amino acid sequences of the IL-2 mutants and CD25-ECD mutants

obtained through the design are shown in Table 2.

Table 2. The amino acid sequences of IL-2 mutants and CD25-ECD mutants obtained through the design

Complex	Mutant and mutation site	Amino acid sequence	SEQ ID NO:	Nucleotide sequence (SEQ ID NO:)
IL-2 Complex 1	IL-2 (C125A, T41C) Pair 1	APTSSSTKKTQLQLEHLLLDLQMILNGIN NYKNPKLTRMLCFKFYMPKKATELKHLQ CLEEELKPLEEVLNLAQSKNFHLRPRDLIS NINVIVLELKGSETTFMCEYADETATIVEF LNRWITFAQSIISTLT	26	97
	CD25-ECD (N27C) Pair 1	ELCDDDPPEIPHATFKAMAYKEGTMLCC ECKRGFRRIKSGSLYMLCTGNSSHSSWD NQCQCTSSATRNTTKQVTPQPEEQKERK TTEMQSPMQPVDQASLPGHCREPPPWEN EATERIYHFVVGQMVYYQCVQGYRALH RGPAESVCKMTHGKTRWTQPQLICTG	50	121
IL-2 Complex 2	IL-2 (C125A, P34C) Pair 2	APTSSSTKKTQLQLEHLLLDLQMILNGIN NYKNCKLTRMLTFKFYMPKKATELKHLQ CLEEELKPLEEVLNLAQSKNFHLRPRDLIS NINVIVLELKGSETTFMCEYADETATIVEF LNRWITFAQSIISTLT	27	98
	CD25-ECD (D4C) Pair 2	ELCCDDPPEIPHATFKAMAYKEGTMLNC ECKRGFRRIKSGSLYMLCTGNSSHSSWD NQCQCTSSATRNTTKQVTPQPEEQKERK TTEMQSPMQPVDQASLPGHCREPPPWEN EATERIYHFVVGQMVYYQCVQGYRALH RGPAESVCKMTHGKTRWTQPQLICTG	51	122
IL-2 Complex 3	IL-2 (C125A, E68C) Pair 3	APTSSSTKKTQLQLEHLLLDLQMILNGIN NYKNPKLTRMLTFKFYMPKKATELKHLQ CLEEELKPLECVLNLAQSKNFHLRPRDLIS NINVIVLELKGSETTFMCEYADETATIVEF LNRWITFAQSIISTLT	28	99
	CD25-ECD (L42C) Pair 3	ELCDDDPPEIPHATFKAMAYKEGTMLNC ECKRGFRRIKSGSCYMLCTGNSSHSSWD NQCQCTSSATRNTTKQVTPQPEEQKERK TTEMQSPMQPVDQASLPGHCREPPPWEN EATERIYHFVVGQMVYYQCVQGYRALH RGPAESVCKMTHGKTRWTQPQLICTG	52	123
IL-2 Complex 4	IL-2 (C125A, Y45C) Pair 4	APTSSSTKKTQLQLEHLLLDLQMILNGIN NYKNPKLTRMLTFKFCMPKKATELKHLQ CLEEELKPLEEVLNLAQSKNFHLRPRDLIS NINVIVLELKGSETTFMCEYADETATIVEF LNRWITFAQSIISTLT	29	100

	CD25-EC D (R35C) Pair 4	ELCDDDPPEIPHATFKAMAYKEGTMLNC ECKRGFCRIKSGSLYMLCTGNSSHSSWD NQCQCTSSATRNTTKQVTPQPREEQKERK TTEMQSPMQPVDQASLPGHCREPPPWEN EATERIYHFVVGQMVYYQCVQGYRALH RGPAESVCKMTHGKTRWTQPQLICTG	53	124
IL-2 Complex 5	IL-2 (C125A, R38C) Pair 5	APTSSSTKKTQLQLEHLLLDLQMILNGIN NYKNPKLTCMLTFKFYMPKKATELKHLQ CLEEELKPLEEVLNLAQSKNFHLRPRDLIS NINVIVLELKGSETTFMCEYADETATIVEF LNRWITFAQSIISTLT	30	101
	CD25-EC D (H120C) Pair 5	ELCDDDPPEIPHATFKAMAYKEGTMLNC ECKRGFRRIKSGSLYMLCTGNSSHSSWD NQCQCTSSATRNTTKQVTPQPREEQKERK TTEMQSPMQPVDQASLPGHCREPPPWEN EATERIYCFVVGQMVYYQCVQGYRALH RGPAESVCKMTHGKTRWTQPQLICTG	54	125
IL-2 Complex 6	IL-2 (C125A, L72C) Pair 6	APTSSSTKKTQLQLEHLLLDLQMILNGIN NYKNPKLTCMLTFKFYMPKKATELKHLQ CLEEELKPLEEVLNCAQSKNFHLRPRDLIS NINVIVLELKGSETTFMCEYADETATIVEF LNRWITFAQSIISTLT	31	102
	CD25-EC D (M25C) Pair 6	ELCDDDPPEIPHATFKAMAYKEGTCLNCE CKRGFRRIKSGSLYMLCTGNSSHSSWDN QCQCTSSATRNTTKQVTPQPREEQKERKTT EMQSPMQPVDQASLPGHCREPPPWENEA TERIYHFVVGQMVYYQCVQGYRALHRG PAESVCKMTHGKTRWTQPQLICTG	55	126
IL-2 Complex 7	IL-2 (C125A, E61C) Pair 7	APTSSSTKKTQLQLEHLLLDLQMILNGIN NYKNPKLTRMLTFKFYMPKKATELKHLQ CLECELKPLEEVLNLAQSKNFHLRPRDLIS NINVIVLELKGSETTFMCEYADETATIVEF LNRWITFAQSIISTLT	32	103
	CD25-EC D (S39C) Pair 7	ELCDDDPPEIPHATFKAMAYKEGTMLNC ECKRGFRRIKCGSLYMLCTGNSSHSSWD NQCQCTSSATRNTTKQVTPQPREEQKERK TTEMQSPMQPVDQASLPGHCREPPPWEN EATERIYHFVVGQMVYYQCVQGYRALH RGPAESVCKMTHGKTRWTQPQLICTG	56	127
IL-2 Complex 8	IL-2 (C125A, T41C) Pair 8	APTSSSTKKTQLQLEHLLLDLQMILNGIN NYKNPKLTRMLCFKFYMPKKATELKHLQ CLEEELKPLEEVLNLAQSKNFHLRPRDLIS NINVIVLELKGSETTFMCEYADETATIVEF LNRWITFAQSIISTLT	33	104
	CD25-EC D (I118C) Pair 8	ELCDDDPPEIPHATFKAMAYKEGTMLNC ECKRGFRRIKSGSLYMLCTGNSSHSSWD NQCQCTSSATRNTTKQVTPQPREEQKERK TTEMQSPMQPVDQASLPGHCREPPPWEN EATERCYHFVVGQMVYYQCVQGYRALH RGPAESVCKMTHGKTRWTQPQLICTG	57	128

IL-2 Complex 9	IL-2 (C125A, K35C) Pair 9	APTSSSTKKTQLQLEHLLLDLQMILNGIN NYKNPCLTRMLTFKFYMPKKATELKHLQ CLEEELKPLEEVLNLAQSKNFHLRPRDLIS NINVIVLELKGSETTFMCEYADETATIVEF LNRWITFAQSIISTLT	34	105
	CD25-EC D (D4C) Pair 9	ELCCDDPPEIPHATFKAMAYKEGTMLNC ECKRGFRRIKSGSLYMLCTGNSSHSSWD NQCQCTSSATRNTTKQVTPQPPEEQKERK TTEMQSPMQPVDQASLPGHCREPPPWEN EATERIYHFVVGQMVYYYQCVQGYRALH RGPAESVCKMTHGKTRWTQPQLICTG	58	129
IL-2 Complex 10	IL-2 (C125A, T37C) Pair 10	APTSSSTKKTQLQLEHLLLDLQMILNGIN NYKNPKLCRMLTFKFYMPKKATELKHLQ CLEEELKPLEEVLNLAQSKNFHLRPRDLIS NINVIVLELKGSETTFMCEYADETATIVEF LNRWITFAQSIISTLT	35	106
	CD25-EC D (D4C) Pair 10	ELCCDDPPEIPHATFKAMAYKEGTMLNC ECKRGFRRIKSGSLYMLCTGNSSHSSWD NQCQCTSSATRNTTKQVTPQPPEEQKERK TTEMQSPMQPVDQASLPGHCREPPPWEN EATERIYHFVVGQMVYYYQCVQGYRALH RGPAESVCKMTHGKTRWTQPQLICTG	59	130
IL-2 Complex 11	IL-2 (C125A, R38C) Pair 11	APTSSSTKKTQLQLEHLLLDLQMILNGIN NYKNPKLTCMLTFKFYMPKKATELKHLQ CLEEELKPLEEVLNLAQSKNFHLRPRDLIS NINVIVLELKGSETTFMCEYADETATIVEF LNRWITFAQSIISTLT	36	107
	CD25-EC D (D4C) Pair 11	ELCCDDPPEIPHATFKAMAYKEGTMLNC ECKRGFRRIKSGSLYMLCTGNSSHSSWD NQCQCTSSATRNTTKQVTPQPPEEQKERK TTEMQSPMQPVDQASLPGHCREPPPWEN EATERIYHFVVGQMVYYYQCVQGYRALH RGPAESVCKMTHGKTRWTQPQLICTG	60	131
IL-2 Complex 12	IL-2 (C125A, R38C) Pair 12	APTSSSTKKTQLQLEHLLLDLQMILNGIN NYKNPKLTCMLTFKFYMPKKATELKHLQ CLEEELKPLEEVLNLAQSKNFHLRPRDLIS NINVIVLELKGSETTFMCEYADETATIVEF LNRWITFAQSIISTLT	37	108
	CD25-EC D (D5C) Pair 12	ELCDCDPPEIPHATFKAMAYKEGTMLNC ECKRGFRRIKSGSLYMLCTGNSSHSSWD NQCQCTSSATRNTTKQVTPQPPEEQKERK TTEMQSPMQPVDQASLPGHCREPPPWEN EATERIYHFVVGQMVYYYQCVQGYRALH RGPAESVCKMTHGKTRWTQPQLICTG	61	132
IL-2 Complex 13	IL-2 (C125A, T41C) Pair 13	APTSSSTKKTQLQLEHLLLDLQMILNGIN NYKNPKLTRMLCFKFYMPKKATELKHLQ CLEEELKPLEEVLNLAQSKNFHLRPRDLIS NINVIVLELKGSETTFMCEYADETATIVEF LNRWITFAQSIISTLT	38	109

	CD25-EC D (L42C) Pair 13	ELCDDDPPEIPHATFKAMAYKEGTMLNC ECKRGFRRIKSGSCYMLCTGNSSHSSWD NQCQCTSSATRNTTKQVTPQPPEEQKERK TTEMQSPMQPVDQASLPGHCREPPPWEN EATERIYHFVVGQMVYYQCVQGYRALH RGPAESVCKMTHGKTRWTQPQLICTG	62	133
IL-2 Complex 14	IL-2 (C125A, T41C) Pair 14	APTSSSTKKTQLQLEHLLLDLQMILNGIN NYKNPKLTRMLCFKFYMPKKATELKHLQ CLEEELKPLEEVLNLAQSKNFHLRPRDLIS NINVIVLELKGSETTFMCEYADETATIVEF LNRWITFAQSIISTLT	39	110
	CD25-EC D (Y119C) Pair 14	ELCDDDPPEIPHATFKAMAYKEGTMLNC ECKRGFRRIKSGSLYMLCTGNSSHSSWD NQCQCTSSATRNTTKQVTPQPPEEQKERK TTEMQSPMQPVDQASLPGHCREPPPWEN EATERICHFVVGQMVYYQCVQGYRALH RGPAESVCKMTHGKTRWTQPQLICTG	63	134
IL-2 Complex 15	IL-2 (C125A, K43C) Pair 15	APTSSSTKKTQLQLEHLLLDLQMILNGIN NYKNPKLTRMLTFCFYMPKKATELKHLQ CLEEELKPLEEVLNLAQSKNFHLRPRDLIS NINVIVLELKGSETTFMCEYADETATIVEF LNRWITFAQSIISTLT	40	111
	CD25-EC D (R35C) Pair 15	ELCDDDPPEIPHATFKAMAYKEGTMLNC ECKRGFCRIKSGSLYMLCTGNSSHSSWD NQCQCTSSATRNTTKQVTPQPPEEQKERK TTEMQSPMQPVDQASLPGHCREPPPWEN EATERIYHFVVGQMVYYQCVQGYRALH RGPAESVCKMTHGKTRWTQPQLICTG	64	135
IL-2 Complex 16	IL-2 (C125A, K43C) Pair 16	APTSSSTKKTQLQLEHLLLDLQMILNGIN NYKNPKLTRMLTFCFYMPKKATELKHLQ CLEEELKPLEEVLNLAQSKNFHLRPRDLIS NINVIVLELKGSETTFMCEYADETATIVEF LNRWITFAQSIISTLT	41	112
	CD25-EC D (R36C) Pair 16	ELCDDDPPEIPHATFKAMAYKEGTMLNC ECKRGFRCIKSGSLYMLCTGNSSHSSWD NQCQCTSSATRNTTKQVTPQPPEEQKERK TTEMQSPMQPVDQASLPGHCREPPPWEN EATERIYHFVVGQMVYYQCVQGYRALH RGPAESVCKMTHGKTRWTQPQLICTG	65	136
IL-2 Complex 17	IL-2 (C125A, F44C) Pair 17	APTSSSTKKTQLQLEHLLLDLQMILNGIN NYKNPKLTRMLTFKCYMPKKATELKHLQ CLEEELKPLEEVLNLAQSKNFHLRPRDLIS NINVIVLELKGSETTFMCEYADETATIVEF LNRWITFAQSIISTLT	42	113
	CD25-EC D (L42C) Pair 17	ELCDDDPPEIPHATFKAMAYKEGTMLNC ECKRGFRRIKSGSCYMLCTGNSSHSSWD NQCQCTSSATRNTTKQVTPQPPEEQKERK TTEMQSPMQPVDQASLPGHCREPPPWEN EATERIYHFVVGQMVYYQCVQGYRALH RGPAESVCKMTHGKTRWTQPQLICTG	66	137

IL-2 Complex 18	IL-2 (C125A, K43C) Pair 18	APTSSSTKKTQLQLEHLLLDLQMILNGIN NYKNPKLTRMLTFCFYMPKKATELKHLQ CLEEELKPLEEVLNLAQSKNFHLRPRDLIS NINVIVLELKGSETTFMCEYADETATIVEF LNRWITFAQSIISTLT	43	114
	CD25-EC D (L42C) Pair 18	ELCDDDPPEIPHATFKAMAYKEGTMLNC ECKRGFRRIKSGSCYMLCTGNSSHSSWD NQCQCTSSATRNTTKQVTPQPPEEQKERK TTEMQSPMQPVDQASLPGHCREPPPWEN EATERIYHFVVGQMVYYQCVQGYRALH RGP AESVCKMTHGKTRWTQPQLICTG	67	138
IL-2 Complex 19	IL-2 (C125A, E61C) Pair 19	APTSSSTKKTQLQLEHLLLDLQMILNGIN NYKNPKLTRMLTFKFYMPKKATELKHLQ CLECELKPLEEVLNLAQSKNFHLRPRDLIS NINVIVLELKGSETTFMCEYADETATIVEF LNRWITFAQSIISTLT	44	115
	CD25-EC D (K38C) Pair 19	ELCDDDPPEIPHATFKAMAYKEGTMLNC ECKRGFRRICSGSLYMLCTGNSSHSSWDN QCQCTSSATRNTTKQVTPQPPEEQKERKTT EMQSPMQPVDQASLPGHCREPPPWENEA TERIYHFVVGQMVYYQCVQGYRALHRG PAESVCKMTHGKTRWTQPQLICTG	68	139
IL-2 Complex 20	IL-2 (C125A, E62C) Pair 20	APTSSSTKKTQLQLEHLLLDLQMILNGIN NYKNPKLTRMLTFKFYMPKKATELKHLQ CLEECLKPLEEVLNLAQSKNFHLRPRDLIS NINVIVLELKGSETTFMCEYADETATIVEF LNRWITFAQSIISTLT	45	116
	CD25-EC D (K38C) Pair 20	ELCDDDPPEIPHATFKAMAYKEGTMLNC ECKRGFRRICSGSLYMLCTGNSSHSSWDN QCQCTSSATRNTTKQVTPQPPEEQKERKTT EMQSPMQPVDQASLPGHCREPPPWENEA TERIYHFVVGQMVYYQCVQGYRALHRG PAESVCKMTHGKTRWTQPQLICTG	69	140
IL-2 Complex 21	IL-2 (C125A, K64C) Pair 21	APTSSSTKKTQLQLEHLLLDLQMILNGIN NYKNPKLTRMLTFKFYMPKKATELKHLQ CLEEELCPLEEVLNLAQSKNFHLRPRDLIS NINVIVLELKGSETTFMCEYADETATIVEF LNRWITFAQSIISTLT	46	117
	CD25-EC D (S39C) Pair 21	ELCDDDPPEIPHATFKAMAYKEGTMLNC ECKRGFRRIKCGSLYMLCTGNSSHSSWD NQCQCTSSATRNTTKQVTPQPPEEQKERK TTEMQSPMQPVDQASLPGHCREPPPWEN EATERIYHFVVGQMVYYQCVQGYRALH RGP AESVCKMTHGKTRWTQPQLICTG	70	141
IL-2 Complex 22	IL-2 (C125A, K64C) Pair 22	APTSSSTKKTQLQLEHLLLDLQMILNGIN NYKNPKLTRMLTFKFYMPKKATELKHLQ CLEEELCPLEEVLNLAQSKNFHLRPRDLIS NINVIVLELKGSETTFMCEYADETATIVEF LNRWITFAQSIISTLT	47	118

	CD25-EC D (G40C) Pair 22	ELCDDDDPPEIPHATFKAMAYKEGTMLNC ECKRGFRRIKSCSLYMLCTGNSSHSSWDN QCQCTSSATRNTTKQVTPQPEEQKERKTT EMQSPMQPVDQASLPGHCREPPPWENEA TERIYHFVVGQMVYYQCVQGYRALHRG PAESVCKMTHGKTRWTQPQLICTG	71	142
IL-2 Complex 23	IL-2 (C125A, K64C) Pair 23	APTSSSTKKTQLQLEHLLLDLQMILNGIN NYKNPKLTRMLTFKFYMPKKATELKHLQ CLEEELCPLEEVNLNAQSKNFHLRPRDLIS NINVIVLELKGSETTFMCEYADETATIVEF LNRWITFAQSIISTLT	48	119
	CD25-EC D (S41C) Pair 23	ELCDDDDPPEIPHATFKAMAYKEGTMLNC ECKRGFRRIKSGCLYMLCTGNSSHSSWD NQCQCTSSATRNTTKQVTPQPEEQKERK TTEMQSPMQPVDQASLPGHCREPPPWEN EATERIYHFVVGQMVYYQCVQGYRALH RGP AESVCKMTHGKTRWTQPQLICTG	72	143
IL-2 Complex 24	IL-2 (C125A, P65C) Pair 24	APTSSSTKKTQLQLEHLLLDLQMILNGIN NYKNPKLTRMLTFKFYMPKKATELKHLQ CLEEELKCLEEVNLNAQSKNFHLRPRDLI SNINVIVLELKGSETTFMCEYADETATIVE FLNRWITFAQSIISTLT	49	120
	CD25-EC D (K38C) Pair 24	ELCDDDDPPEIPHATFKAMAYKEGTMLNC ECKRGFRRICSGSLYMLCTGNSSHSSWDN QCQCTSSATRNTTKQVTPQPEEQKERKTT EMQSPMQPVDQASLPGHCREPPPWENEA TERIYHFVVGQMVYYQCVQGYRALHRG PAESVCKMTHGKTRWTQPQLICTG	73	144

The expression hosts were mammalian cells (HEK293 or CHO).

Example 1: Preparation of IL-2 (C125A), IL-2 mutants and IL-2 complexes

In this example, IL-2 wt (C125A), IL-2 mutants 1-4 and IL-2 complexes 1-4 were selected for expression, respectively. The HPC4 tag attached to the C-terminus of the molecule was used for purification and preparation.

1.1 Construction of expression plasmid

The genes of IL-2 wt C125A (SEQ ID NO: 74), IL-2 mutants 1-4, and IL-2 complexes 1-4 (IL-2 Pair 1-4 and CD25-ECD Pair 1-4 for IL-2 complexes) were synthesized by Suzhou Jinweizhi Biotechnology Co., Ltd. Then the overlapping PCR was performed in accordance with the procedures as described in “Molecular Cloning” to obtain the target fragments.

Subsequently, the fragments and pTT5 universal vectors were recombined, ligated, transformed (DH10B), sequenced and preserved to obtain the desired plasmids of the IL-2 wt (C125A), IL-2 mutants 1-4 and IL-2 complexes 1-4 (plasmids of IL-2 Pair 1-4 and CD25-ECD Pair 1-4 for the IL-2 complexes). Or PCR, DpnI digestion, DH10B transformation, sequencing, and bacteria preservation were performed in accordance with the procedures as described in “Agilent Quik Change Lightning Site - Directed Mutagenesis Kit” to obtain the desired plasmids of IL-2 wt (C125A), IL-2 mutants 1-4 and IL-2 complexes 1-4.

1.2 Extraction of plasmids and preparation of HEK293 cells

1.2.1 Extraction of plasmids

The preparation of the plasmids of IL-2 wt (C125A), IL-2 mutants 1-4 and IL-2 complexes 1-4 was performed in accordance with the procedures as described in “Qiagen Mini-prep Kit” and “Qiagen Endofree Maxi-prep Kit”.

1.2.2 Preparation of HEK293 cells

Freshly passaged HEK293 cells (National Research Council, Canada) with a density of 1 to 1.2×10^6 cells/mL were used for transient expression.

1.3 Transient expression of HEK293

1.3.1 Preparation of reagents

A) G418 solution: 250 mg of Geneticin™ was weighed, dissolved in 4.5 mL ultrapure water, added with ultrapure water to give a volume of 5 mL, filtered with a 0.22 µm filter membrane, and stored at -20 °C;

B) PEI solution: 50 mg of PEI was weighed, dissolved in 4.5 mL ultrapure water, adjusted with 1 M NaOH to pH 7.0, added with ultrapure water to give a volume of 50 mL, filtered with a 0.22 µm filter membrane, and store at -20 °C;

C) Medium: 10 mL of Pluronicd™ F-68 and 500 µL of G418 were added to 1 L of FreeStyle™ 293 Expression Medium;

D) The plasmid was prepared in advance in a 2 mL de-endotoxin centrifuge tube; and

E) A freshly passaged cell suspension of 1 to 1.2×10^6 cells/mL was prepared according to the volume required for transfection.

1.3.2 Preparation of transfection reagent - plasmid complex

Liquid A: Plasmid 1 $\mu\text{g/mL}$ + Opti-MEM™ 33.3 $\mu\text{L/mL}$

Liquid B: PEI 2 $\mu\text{g/mL}$ + Opti-MEM™ 33.3 $\mu\text{L/mL}$

Liquid B was introduced into Liquid A and incubated for 10 minutes. Then the cell suspension was added.

During transfection, plasmids of IL-2 wt (C125A) and IL-2 mutants 1-4 were transfected separately; and the plasmids of IL-2 complexes 1-4 were transfected after mixing the two plasmids.

1.3.3 Replacement of liquids

After incubating at 115rpm, 36.8 °C and 5% CO₂ for 4 h, the culture was centrifuged at 800g for 5 minutes and replaced with FreeStyle™ 293 Expression Medium free of F68 and G418.

1.3.4 Expression of the culture and harvest

After incubating at 115rpm, 36.8 °C and 5% CO₂ for 5 days, the culture was centrifuged at 8500 rpm for 15 minutes and the cell supernatant was collected.

1.4 Preparation of purified product

All the derivatives related to IL-2 had an HPC4 tag at the C-terminus. Therefore, they could be affinity purified with fillers coupled with HPC4 antibody, and then further purified by gel filtration chromatography (Superdex 200) to obtain proteins with higher purity. SDS-PAGE analysis was performed according to the method as described in *Molecular Cloning*.

The results were shown in Fig. 1, indicating that after the plasmids were transfected into the cells, the corresponding proteins and complexes of IL-2 wt (C125A), IL-2 mutants 1-4 and IL-2 complexes 1-4 were produced. The construction of plasmids, and the expression and purification of proteins were successful.

Example 2: Determination of the affinity of IL-2 wt (C125A), IL-2 mutants 1-4 and IL-2 complexes 1-4 to IL2R $\beta\gamma$ and IL2R α by biolayer interferometry (BLI)

1. Experimental Materials

The proteins used in the experiment were produced by Beijing Zhidao Biotechnology Co., Ltd.

IL2R α -his (available from Beijing Zhidao Biotechnology Co., Ltd.), IL2R $\beta\gamma$ -Fc (available from Beijing Zhidao Biotechnology Co., Ltd.) and IL-2 mutants were obtained by transient expression with HEK293 and affinity purification. The buffer formulation was as follows: 10 mM HEPES, 150 mM sodium chloride, 3 mM EDTA, 0.1% BSA and 0.05% Tween 20. The ProA sensor was purchased from Pall Fortebio (Cat. No. #18-5010). The HISIK sensor was purchased from Pall Fortebio (Cat. No. #18-5120). The BLI equipment was Octet RED96 from Pall Fortebio. Data acquisition and analysis were carried out using software Data acquisition 11.0 and Data Analysis 11.0, respectively.

2. Experimental methods

1) Preparation of IL2R $\beta\gamma$ -Fc

IL2R $\beta\gamma$ -Fc was diluted with the buffer solution to a concentration of 10 μ g/mL, and then added to column 2 of the 96-well assay plate. The program was set to Loading, 600s.

2) Preparation of IL2R α -his

IL2R α -his was diluted with the buffer solution to a concentration of 10 μ g/mL, and then added to column 3 of the 96-well assay plate. The program was set to Loading, 600s.

3) Preparation of samples

The IL-2 derivative was diluted with the buffer solution to 100 nM, and then serially diluted downward by 6 gradients (7 gradients in total) at a ratio of 1:1 to a concentration of 1.625 nM and a concentration of 0 nM. They were added to columns 5-9 of the 96-well assay plate, respectively. The program was set to Association, 200s. The buffers were added to columns 1, 4, 10 and 11 of the 96-well assay plate, and the glycine with pH 1.7 was added to column 12. The loading volume of the above samples or solutions was 200 μ L.

4) Detection of affinity with IL2R $\beta\gamma$ -Fc

8 ProA sensors were respectively placed in A-H of column 1 of the sensor holder. In the software Data acquisition 11.0, the detection conditions were set as follows. 1 Pre-wetting: Baseline, 60s, Position: Column 1. 2 Cycle detection: column 2: loading, 600s; column 4: Baseline 1, 60s; columns 5-9: Association, 200s; column 10: Dissociation, 600s; column 11: neutralization; and column 12: regeneration.

5) Detection of binding to IL2R α

8 HISIK sensors were respectively placed in A-H of column 2 of the sensor holder. In the software Data acquisition 11.0, the detection conditions were set as follows. 1 pre-wet: Baseline, 60s, position: column 1. 2 Cycle detection: column 3: loading, 600s; column 4: Baseline 1, 60s; columns 5-9: Association, 200s; column 10: Dissociation, 600s; column 11: neutralization; and column 12: regeneration.

6) Data analysis

The software Data Analysis 11.0 was used to analyze the data. KD value was calculated by Fitting curve, and concentration 0 was used as the control to subtract background.

3. Results

In Fig. 2, as can be seen from the binding curves of IL-2 derivatives and IL2R α receptors, all the IL-2 derivatives hardly bond to IL2R α receptors, except that the binding of IL-2 mutant 1 to IL2R α receptor was greatly weakened. In Fig. 3, as can be seen from the binding curves of IL-2 derivatives and IL2R $\beta\gamma$ receptors, there was no significant change compared with IL-2 wt C125A (Fig. 3).

Example 3: Proliferation test of T cells

The proliferation test of CTLL-2 (T cells) was a commonly used cell-level assay to determine the activity of interleukins to stimulate immune cells. Therefore, the proliferation test of CTLL-2 cells was used to examine the biological activity of the IL-2 derivatives.

1) Preparation of CTLL-2 cells: The cells were resuspended in culture medium containing FBS and Rat-T-Stim.

2) Loading of samples: The cells were seeded in a 96-well culture plate with 0.1 mL per well. At the same time, the samples of proteins of IL-2 derivatives 11, 18, 21 and 28 (i.e., proteins prepared in Example 1) to be tested were diluted in multiples. 0.1 mL was added to each well, and 3 replicate wells were set for each dilution concentration. In addition, the culture media control well (100 μ L cells + 100 μ L culture media) was also set. They were incubated at 37 °C and 5% CO₂ for 72 hours.

3) MTS addition: 20 μL of CellTiter96[®] AQueous One Solution Reagent was added to each well, and incubated at 37 °C and 5% CO₂ for 2 to 4 hours.

4) Detection: The absorbance (A) was detected with a microplate reader at a wavelength of 490 nm and the EC₅₀ value was calculated.

5) Results: Representative IL-2 complex 2 and IL-2 mutant 4 were selected, all of which had the proliferation activity of CTLL-2 (T cell). It indicated that the signal transduction function of the complexes of β and γ receptor subunits was not seriously affected (Fig. 4).

We claim:

1. An IL-2 derivative, characterized in that the IL-2 derivative is introduced with at least one cysteine residue based on wild-type IL-2, and the binding plane of the IL-2 derivative and α receptor subunit is partially or completely blocked, while the affinity of the IL-2 derivative to the complex of β and γ receptor subunits is basically retained.
2. The IL-2 derivative according to claim 1, characterized in that the at least one cysteine residue introduced based on wild-type IL-2 is capable of:
 - a) forming an intramolecular disulfide bond of the IL-2 derivative; or
 - b) enabling the IL-2 derivative to bind to a blocking module through an intermolecular disulfide bond.
3. The IL-2 derivative according to claim 2, characterized in that the at least one cysteine residue is introduced by means of point mutation.
4. The IL-2 derivative according to claim 3, characterized in that a first cysteine residue and a second cysteine residue are introduced by means of point mutation based on wild-type IL-2; and one or both of the first cysteine residue and the second cysteine residue are amino acids related to the binding plane of the wild-type IL-2 and α receptor subunit, or amino acids in the vicinity thereof.
5. The IL-2 derivative according to claim 4, characterized in that the first cysteine residue is an amino acid at position 37, position 38, position 41, position 42, position 43, position 44, position 45, position 61 or position 62 of the wild-type IL-2, or an amino acid in the vicinity thereof; and the second cysteine residue is an amino acid at position 61, position 62, position 65, position 68 or position 72 of the wild-type IL-2, or an amino acid in the vicinity thereof.
6. The IL-2 derivative according to claim 4, characterized in that the first cysteine residue is an amino acid point mutant selected from the group consisting of: K35C, L36C, R38C, M39C, L40C, T41C, F42C, K43C, F44C and E61C.
7. The IL-2 derivative according to claim 4, characterized in that the second cysteine residue is an amino acid point mutant selected from the group consisting of: V69C, E62C, P65C, T111C,

Y107C, A112C, T113C, I114C, L72C and A73C.

8. The IL-2 derivative according to claim 4, characterized in that a combination of the first cysteine residue and the second cysteine residue that form the intramolecular disulfide bond is a combination of amino acid point mutants selected from the group consisting of: M39C and V69C; F44C and E62C; F44C and P65C; F42C and V69C; E61C and Y107C; F42C and P65C; F42C and T111C; F42C and A112C; F42C and T113C; T41C and A112C; L40C and A112C; T113C and T113C; L40C and I114C; M39C and L72C; M39C and A73C; R38C and V69C; R38C and L72C; L36C and V69C; L36C and L72C; L36C and A73C; K35C and V69C; and K43C and A112C.

9. The IL-2 derivative according to claim 4, characterized in that a center-of-mass vector distance between the first cysteine residue and the second cysteine residue is less than 6Å.

10. The IL-2 derivative according to claim 3, characterized in that a third cysteine residue is introduced by means of point mutation based on wild-type IL-2; and the third cysteine residue is an amino acid related to the binding plane of wild-type IL-2 and α receptor subunit, or an amino acid in the vicinity thereof.

11. The IL-2 derivative according to claim 10, characterized in that the third cysteine residue is an amino acid at position 37, position 38, position 41, position 42, position 43, position 44, position 45, position 61, position 62, position 65, position 68 or position 72 of the wild-type IL-2, or an amino acid in the vicinity thereof.

12. The IL-2 derivative according to claim 10, characterized in that the third cysteine residue is an amino acid point mutant selected from the group consisting of: P34C, K35C, T37C, R38C, T41C, K43C, F44C, Y45C, E61C, E62C, K64C, P65C, E68C and L72C.

13. The IL-2 derivative according to claim 10, characterized in that the blocking module has or is introduced with a fourth cysteine residue; and the third cysteine residue on the IL-2 derivative and the fourth cysteine residue on the blocking module are capable of forming an intermolecular disulfide bond.

14. The IL-2 derivative according to claim 13, characterized in that a center-of-mass vector

distance between the third cysteine residue and the fourth cysteine residue is less than 6Å.

15. The IL-2 derivative according to claim 13, characterized in that the blocking module is an extracellular segment of the α receptor subunit.

16. The IL-2 derivative according to claim 3, characterized in that the cysteine residue at position 125 of the wild-type IL-2 is converted into another amino acid residue by means of point mutation.

17. The IL-2 derivative according to claim 16, characterized in that a point mutant at position 125 of the wild-type IL-2 is C125A.

18. The IL-2 derivative according to claim 3, characterized in that the amino acid sequence of the IL-2 derivative is shown in SEQ ID NO: 3 to SEQ ID NO: 24, or in SEQ ID NO: 26 to SEQ ID NO: 40.

19. A complex, comprising:

1) an IL-2 derivative, which is introduced with a third cysteine residue based on wild-type IL-2; and

2) a blocking module, which has or is introduced with a fourth cysteine residue; wherein the third cysteine residue on the IL-2 derivative and the fourth cysteine residue on the blocking module are capable of forming an intermolecular disulfide bond, thereby forming a complex of the IL-2 derivative and the blocking module; and wherein the binding plane of the IL-2 derivative and α receptor subunit is partially or completely blocked, while the affinity of the IL-2 derivative to the complex of β and γ receptor subunits is basically retained.

20. The complex according to claim 19, characterized in that the third cysteine residue is an amino acid related to the binding plane of the wild-type IL-2 and the α receptor subunit, or an amino acid in the vicinity thereof.

21. The complex according to claim 20, characterized in that a center-of-mass vector distance between the third cysteine residue and the fourth cysteine residue is less than 6Å.

22. The complex according to claim 19, characterized in that the third cysteine residue is an

amino acid point mutant selected from the group consisting of: P34C, K35C, T37C, R38C, T41C, K43C, F44C, Y45C, E61C, E62C, K64C, P65C, E68C and L72C.

23. The complex according to claim 19, characterized in that the blocking module is an extracellular segment of the α receptor subunit, the amino acid sequence of which is shown in SEQ ID NO: 25.

24. The complex according to claim 23, characterized in that the fourth cysteine residue on the extracellular segment of the α receptor subunit is an amino acid point mutant selected from the group consisting of: D4C, D5C, M25C, N27C, R35C, R36C, K38C, S39C, G40C, S41C, L42C, I118C, Y119C and H120C.

25. The complex according to claim 23, characterized in that a combination of the third cysteine residue and the fourth cysteine residue that form the intramolecular disulfide bond is a combination of amino acid point mutants selected from the group consisting of: T41C and N27C; P34C and D4C; E68C and L42C; Y45C and R35C; R38C and H120C; L72C and M25C; E61C and S39C; T41C and I118C; K35C and D4C; T37C and D4C; R38C and D4C; R38C and D5C; T41C and L42C; T41C and Y119C; K43C and R35C; K43C and R36C; F44C and L42C; K43C and L42C; E61C and K38C; E62C and K38C; K64C and S39C; K64C and G40C; K64C and S41C; and P65C and K38C.

26. The complex according to claim 19, characterized in that a point mutant at position 125 of the wild-type IL-2 is C125A.

27. The complex according to claim 23, characterized in that a combination of the amino acid sequence of the IL-2 derivative and the sequence of the extracellular segment of the α receptor subunit is a combination selected from the group consisting of: SEQ ID NO: 26 and SEQ ID NO: 50; SEQ ID NO: 27 and SEQ ID NO: 51; SEQ ID NO: 28 and SEQ ID NO: 52; SEQ ID NO: 29 and SEQ ID NO: 53; SEQ ID NO: 30 and SEQ ID NO: 54; SEQ ID NO: 31 and SEQ ID NO: 55; SEQ ID NO: 32 and SEQ ID NO: 56; SEQ ID NO: 33 and SEQ ID NO: 57; SEQ ID NO: 34 and SEQ ID NO: 58; SEQ ID NO: 35 and SEQ ID NO: 59; SEQ ID NO: 36 and SEQ ID NO: 60; SEQ ID NO: 37 and SEQ ID NO: 61; SEQ ID NO: 38 and SEQ ID NO: 62; SEQ ID NO: 39 and SEQ ID NO: 63; SEQ ID NO: 40 and SEQ ID NO: 64; SEQ ID NO: 41 and SEQ ID NO: 65; SEQ ID NO: 42 and SEQ ID NO: 66; SEQ ID NO: 43 and SEQ ID NO:

67; SEQ ID NO: 44 and SEQ ID NO: 68; SEQ ID NO: 45 and SEQ ID NO: 69; SEQ ID NO: 46 and SEQ ID NO: 70; SEQ ID NO: 47 and SEQ ID NO: 71; SEQ ID NO: 48 and SEQ ID NO: 72; and SEQ ID NO: 49 and SEQ ID NO: 73.

28. An isolated polynucleotide, which encodes the IL-2 derivative according to any one of claims 1-18 or the complex according to any one of claims 19-27.

29. An expression vector, comprising the isolated polynucleotide according to claim 28.

30. A host cell, comprising the isolated polynucleotide according to claim 28.

31. A composition, comprising the IL-2 derivative according to any one of claims 1 to 18 or the complex according to any one of claims 19 to 27, and a pharmaceutically acceptable carrier.

32. Use of the IL-2 derivative according to any one of claims 1 to 18 or the complex according to any one of claims 19 to 27 for preparing drugs or preparations for treating diseases.

33. Use of the IL-2 derivative according to any one of claims 1 to 18 or the complex according to any one of claims 19 to 27 for preparing a composition for stimulating the immune system of an individual.

34. A method for producing an IL-2 derivative, which includes culturing the host cell according to claim 30 under conditions suitable for expressing the IL-2 derivative.

35. A method for producing a complex, which includes culturing the host cell according to claim 30 under conditions suitable for expressing the complex.