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The object of the invention is to provide an anaerobic enterobacterium having a higher therapeutic effect on an anaerobic site such as a solid tumor tissue and an ischemic disease site. A bacterium of the genus Bifidobacterium, which is transformed with a plasmid co-expressing two types of heterologous polypeptides and comprising two types of secretory expression cassettes each sequentially comprising a promoter DNA functioning in the bacterium of the genus Bifidobacterium; a DNA encoding a secretory signal peptide; a DNA encoding a heterologous polypeptide; and a terminator DNA functioning in the bacterium of the genus Bifidobacterium, efficiently secretes the two types of heterologous peptides, i.e., two types of antibodies having anticancer effects, outside the bacterial cell.

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ABSTRACT

The object of the invention is to provide an anaerobic enterobacterium having a higher therapeutic effect on an anaerobic site such as a solid tumor tissue and an ischemic disease site. A bacterium of the genus *Bifidobacterium*, which is transformed with a plasmid co-expressing two types of heterologous polypeptides and comprising two types of secretory expression cassettes each sequentially comprising a promoter DNA functioning in the bacterium of the genus *Bifidobacterium*; a DNA encoding a secretory signal peptide; a DNA encoding a heterologous polypeptide; and a terminator DNA functioning in the bacterium of the genus *Bifidobacterium*, efficiently secretes the two types of heterologous peptides, i.e., two types of antibodies having anticancer effects, outside the bacterial cell.

DESCRIPTION

TITLE OF THE INVENTION

COEXPRESSION PLASMID

Technical Field

[0001]

The present invention relates to a co-expression plasmid characterized by comprising two types of secretory expression cassettes each sequentially comprising a promoter DNA functioning in a bacterium of the genus *Bifidobacterium*; a DNA encoding a secretory signal peptide; a DNA encoding a heterologous polypeptide; and a terminator DNA functioning in the bacterium of the genus *Bifidobacterium*. The present invention also relates to a bacterium of the genus *Bifidobacterium* transformed with the co-expression plasmid; a pharmaceutical composition comprising the bacterium; and the like.

Background Art

[0002]

A great many therapeutic agents have been proposed up to present for treating diseases such as cancer; however the therapeutic agents are all have a limit in effect as pointed out by researchers. Thus, it has been desired to develop therapeutic agents and therapies having higher effects. Recently, development of a therapy using a gene transporting carrier has advanced. For example, a transformed microorganism is proposed (see for example, Patent Document 1), which uses an anaerobic

enterobacterium having a nature of being accumulated in a hypoxic solid tumor when it is systemically administered and the transformant thereof expresses a gene encoding a protein having an antitumor activity or a protein having an activity to convert an antitumor substance precursor to an antitumor substance, in a target diseased site. Further development of this is expected as a new technology for delivering a drug which inevitably produces a side effect when it is systemically administered, to a tumor site effectively in a high concentration.

[0003]

In the meantime, a promoter, a DNA encoding a signal sequence, a DNA encoding a polypeptide or an expression cassette comprising cloning sites for inserting these DNAs (see, for example, Patent Document 2) is proposed.

Prior Art Documents

Patent Documents

[0004]

Patent Document 1: International Publication No. WO2009/128272

Patent Document 2: International Publication No. WO2010/126073

Summary of the Invention

Object to be Solved by the Invention

[0005]

The DNA encoding a signal sequence and proposed in the aforementioned documents is DNA of a signal sequence

found in a membrane protein and a secretory protein. Identification of a signal sequence having more excellent secretion efficiency is expected; at the same time, development of a plasmid expressing a secretory heterologous protein having a higher therapeutic effect and an anaerobic enterobacterium transformed with such a secretory expression plasmid has been desired.

[0006]

An object of the present invention is to provide an anaerobic enterobacterium having a higher therapeutic effect at an anaerobic site such as a solid tumor tissue and an ischemic disease site.

Means to Solve the Object

[0007]

The present inventors found that a bacterium of the genus *Bifidobacterium* transformed with a plasmid, which co-expresses two types of heterologous polypeptides, characterized by comprising two types of secretory expression cassettes each sequentially comprising a promoter DNA functioning in the bacterium of the genus *Bifidobacterium*; a DNA encoding a secretory signal peptide; a DNA encoding a heterologous polypeptide; and a terminator DNA functioning in the bacterium of the genus *Bifidobacterium*, has a significantly high cancer cell proliferation inhibitory activity, compared to single-expression strains of the heterologous polypeptides. Based on the finding, the present invention was accomplished.

[0008]

More specifically, the present invention is as follows.

[1] A co-expression plasmid comprising two types of secretory expression cassettes each sequentially comprising the following DNA (1) to (4) and expressed within a bacterium of the genus *Bifidobacterium*:

(1) a promoter DNA functioning in the bacterium of the genus *Bifidobacterium*;

(2) a DNA encoding a secretory signal peptide;

(3) a DNA encoding a heterologous polypeptide; and

(4) a terminator DNA functioning in the bacterium of the genus *Bifidobacterium*.

[2] The co-expression plasmid according to [1], wherein a DNA encoding a linker peptide is ligated downstream of the DNA encoding a secretory signal peptide.

[3] The co-expression plasmid according to [1] or [2], wherein the promoter functioning in the bacterium of the genus *Bifidobacterium* is one or two promoters selected from P30 promoter, P54 promoter and Hu promoter.

[4] The co-expression plasmid according to any one of [1] to [3], wherein the terminator functioning in the bacterium of the genus *Bifidobacterium* is one or two terminators selected from Hu terminator and T2 terminator.

[5] The co-expression plasmid according to any one of [1] to [4], wherein the secretory signal peptide has the following amino acid sequence a) or b):

a) an amino acid sequence represented by any one of SEQ ID Nos: 57, 59, 61, 63, 65, 67, 69, 71, 73, 75 and 77,

b) an amino acid sequence obtained by deleting, substituting or adding one or several amino acids in the

amino acid sequence represented by any one of SEQ ID Nos. 57, 59, 61, 63, 65, 67, 69, 71, 73, 75 and 77, and wherein a peptide consisting of the amino acid sequence serves as a secretory signal peptide in the bacterium of the genus *Bifidobacterium*.

[6] The co-expression plasmid according to any one of [1] to [5], wherein the heterologous polypeptide is a single-chain antibody.

[7] The co-expression plasmid according to [6], wherein the single-chain antibody is an anti-PD-1 antibody.

[8] The co-expression plasmid according to [6], wherein the single-chain antibody is an anti-CTLA-4 antibody.

[9] The co-expression plasmid according to [6], wherein the single-chain antibody is an anti-HER2 antibody.

[10] The co-expression plasmid according to any one of [1] to [5], wherein the heterologous polypeptide is a cytokine.

[11] The co-expression plasmid according to [10], wherein the cytokine is TNF-α.

[12] The co-expression plasmid according to [10], wherein the cytokine is IFN-γ.

[13] The co-expression plasmid according to any one of [1] to [5], wherein the heterologous polypeptide is a combination of the anti-PD-1 antibody and the anti-CTLA-4 antibody.

[14] The co-expression plasmid according to any one of [1] to [5], wherein the heterologous polypeptide is a combination of TNF-α and IFN-γ.

[15] The co-expression plasmid according to any one of [1] to [5], wherein the heterologous polypeptide is a combination of an anti-HER2 antibody and IFN-γ.

[16] A bacterium of the genus *Bifidobacterium* transformed with the co-expression plasmid according to any one of [1] to [15].

[17] The bacterium of the genus *Bifidobacterium* according to [16], wherein the bacterium is *Bifidobacterium longum*.

[18] A pharmaceutical composition comprising the bacterium of the genus *Bifidobacterium* according to [16] or [17].

Effect of the Invention

[0009]

According to the present invention, it is possible to provide a bacterium of the genus *Bifidobacterium* capable of efficiently secreting two types of heterologous polypeptides outside the bacterial cell by using two types of expression cassettes enabling excellent secretion of a heterologous polypeptide in combination in the same plasmid. If the two types of heterologous polypeptides are e.g., antibodies having an anticancer effect, such as an anti-PD-1 antibody and an anti-CTLA-4 antibody, a synergetic effect of the two types of heterologous polypeptides are obtained. Due to this, such a bacterium of the genus *Bifidobacterium* is extremely useful as an anticancer drug.

Brief Description of Drawings

[0010]

[Figure 1] The figure is a diagram showing the constitution of plasmid pSP3B-TNFα.

[Figure 2] The figure is a diagram showing the constitution of plasmid pHG-2.

[Figure 3] The figure is a diagram showing the constitution of plasmid pTNF11.

[Figure 4] The figure (a) is a diagram showing the constitution of plasmid phIFNg33; and the figure (b) is a diagram showing the constitution of plasmid phIFNg33TL.

[Figure 5] The figure is a graph showing cell proliferation inhibitory activity of a culture-supernatant crude purified product of an AG8TL strain to KPL-1 cell.

[Figure 6] The figure is a graph showing cell proliferation inhibitory activity of a culture-supernatant crude purified product of an AG8TL strain to MIA PaCa-2 cell.

[Figure 7] The figure is a graph showing an effect of the neutralization antibody added for neutralizing the cell proliferation inhibitory activity of the culture-supernatant crude purified product of an AG8TL strain.

[Figure 8] The figure (A) is a diagram showing the constitution of plasmid pHuSP7L20-hPD-1scFv03; (B) the constitution of pHuSP7L20-hCTLA-4scFv02; and (C) the constitution of pHuSP7L20-hCTLA-4scFv02FLAG.

[Figure 9] The figure (a) is a diagram showing the constitution of plasmid pPC1; and (b) the constitution of plasmid pCP1.

[Figure 10] The figure (A) is an electrophoretogram for checking the expression of anti-hPD-1scFv; and (B) is

an electrophoretogram for checking the expression of anti-hCTLA-4scFv.

[Figure 11] The figure is a graph showing the specific binding of anti-hPD-1scFv03 to a human PD-1 immobilized plate.

[Figure 12] The figure is a graph showing the specific binding of anti-hCTLA-4scFv02 to a human CTLA-4 immobilized plate.

[Figure 13] The figure is a graph showing competitive (binding) inhibition of the binding reaction between human PD-1 and human PD-L1 by anti-hPD-1scFv03.

[Figure 14] The figure is a graph showing competitive (binding) inhibition of the binding reaction between human CTLA-4 and human CD80 by anti-hCTLA-4scFv02.

[Figure 15] The figure is a graph showing competitive (binding) inhibition of the binding reaction between human CTLA-4 and human CD86 by anti-hCTLA-4scFv02.

[Figure 16] The figure is a graph showing binding of anti-hPD-1scFv03 purified from PC1 strain to a human PD-1 overexpressing cell.

[Figure 17] The figure is a graph showing binding of anti-hCTLA-4scFv02 purified from PC1 strain to a human CTLA-4 overexpressing cell.

[Figure 18] The figure is an electrophoretogram of western analysis for checking secretion of anti-HER2scFv and hIFN-γ by HG-2 strain.

[Figure 19] The figure is a graph showing proliferation inhibitory activity to HER2 positive cell by anti-HER2scFv in a concentrate of HG-2 strain culture supernatant.

[Figure 20] The figure is a graph showing cell proliferation inhibitory activity to HER2 positive cells by a concentrate of HG-2 strain culture supernatant.

[Figure 21] The figure (A) is a picture showing gram staining of HG-2 strain; and (B) is a picture showing gram staining of BEshuttle strain.

[Figure 22] The figure (A) is a picture showing immunohistochemical staining of HG-2 strain with an anti-hIFN-γ antibody; and (B) is a picture showing immunohistochemical staining of BEshuttle strain with anti-hIFN-γ antibody.

[Figure 23] The figure (A) is a picture showing immunohistochemical staining of HG-2 strain with an anti-histidine tag antibody; and (B) is a picture showing immunohistochemical staining of BEshuttle strain with an anti-histidine tag antibody.

[Figure 24] The figure is a schematic diagram showing the constitution of a co-expression plasmid (pPC2-pPC8).

[Figure 25] The figure is a diagram schematically showing a method for constructing a co-expression plasmid (pPC2-pPC8).

[Figure 26] The figure is a diagram schematically showing a method for constructing anti-human CTLA-4scFv02 expression plasmid.

[Figure 27] The figure is a diagram schematically showing a method for constructing pP30SPxLy-hCTLA-4scFv01-His.

[Figure 28] The figure is a diagram schematically showing a method for constructing pP30SPxLy-hCTLA-4scFv01-FLAG (pC1F-pC3F).

[Figure 29] The figure is a schematic diagram showing the constitution of a co-expression plasmid (pPC2TL-pPC8TL).

[Figure 30] The figure is a diagram schematically showing a method for constructing a co-expression plasmid (pPC2TL-pPC8TL).

[Figure 31] The figure is an electrophoretogram of western analysis for checking secretion of anti-hPD-1scFv03-His (a) and anti-hCTLA-4scFv01-FLAG (b) in individual strains.

[Figure 32] The figure is a diagram schematically showing a method for constructing an anti-hCTLA-4scFv01 single-expression strain (pC1TLB, pC2TLB, pC3TLB).

[Figure 33] The figure is a flow-cytometric analysis chart showing the binding of scFv derived from C1F strain, C2F strain and C3F strain to a human CTLA-4 expressing cell.

[Figure 34] The figure is a flow-cytometric analysis chart showing the binding of scFv derived from C1TLB strain, C2TLB strain and C3TLB strain to a human CTLA-4 expressing cell.

[Figure 35] The figure is a flow-cytometric analysis chart showing the binding of scFv derived from P1H strain, P2H strain and P3H strain to a human PD-1 expressing cell.

[Figure 36] The figure is a flow-cytometric analysis chart showing the binding of scFv derived from P1TL strain,

P2TL strain and P3TL strain to a human PD-1 expressing cell.

[Figure 37] The figure (a) is a flow-cytometric analysis chart showing the binding of an anti-human PD-1 antibody (EH12.2H7) to a human PD-1 expressing cell, (b) binding of an anti-human CTLA-4 antibody (L3D10) to a human CTLA-4 expressing cell; (c) binding of PC4-derived scFv to a human PD-1 expressing cell; and (d) binding of PC4-derived scFv to a human CTLA-4 expressing cell.

[Figure 38] The figure is a graph showing (binding) inhibitory activity of hPD-L1 to the binding between hPD-1scFv03-His purified from PC4 strain and hPD-1.

[Figure 39] The figure is a graph showing (binding) inhibitory activity of hCD80 to the binding between hCTLA-4scFv01-FLAG purified from PC4 strain and hCTLA-4.

[Figure 40] The figure is a graph showing (binding) inhibitory activity of hCD86 to the binding between hCTLA-4scFv01-FLAG purified from PC4 strain and hCTLA-4.

Mode of Carrying Out the Invention

[0011]

The co-expression plasmid of the present invention is not particularly limited as long as it is a plasmid comprising two types of secretory expression cassettes each sequentially comprising (1) a promoter DNA functioning in a bacterium of the genus *Bifidobacterium*; (2) a DNA encoding a secretory signal peptide; (3) a DNA encoding a heterologous polypeptide; and (4) a terminator DNA functioning in the bacterium of the genus *Bifidobacterium*, and capable of co-expressing two types of

heterologous polypeptides in the bacterium of the genus *Bifidobacterium* transformed with the plasmid. The 3' end of each of the above DNA fragments and the 5' end of the DNA fragment present immediately downstream may not be directly connected as long as the advantageous effect of the present invention can be obtained; however, the two ends are preferably directly connected.

[0012]

The promoter DNA of the present invention is not particularly limited as long as it is promoter DNA functioning in a bacterium of the genus *Bifidobacterium*. Examples thereof may include Hu promoter DNA, which is a promoter relating to expression of a gene encoding histone-like DNA binding protein derived from *Bifidobacterium longum*; P30 promoter DNA (J. Microbiology, 2012, 638-643); P54 promoter DNA, which is a promoter relating to expression of a gene encoding Elongation Factor Tu protein (J. Bacteriology, 2005, 5799-5808, J. Microbiology, 2012, 638-643); promoter DNA of Gap gene derived from *Bifidobacterium breve* (Biotechnol. Lett. 2008 30: 1983-1988); promoter DNA of AmyB gene derived from *Bifidobacterium longum* (Biotechnol. Lett. 2006 28: 163-168); 16SrRNA promoter DNA (Biotechnol. Lett. 2008 30: 165-172); promoter DNA of GAPDH (pr-BL1363) gene (Appl Environ Microbiol. 2006 72 (11): 7401-7405); P_RP_L promoter DNA (Cancer Gene Ther. 2007 14: 151-157); promoter DNA of p572 (β-glycosidase from B. animalis subsp lactis) gene (J. Microbiol Biotechnol. 2012 Dec; 22 (12): 1714-23); promoter (rplM promoter) DNA of p919 gene (J. Microbiol. 2012 Aug; 50 (4): 638-43); and promoter (rplR promoter)

DNA of p895 gene (J. Microbiology, 2012, 638-643). The promoters of individual expression cassettes excellent in secretion of the heterologous polypeptides are preferably different from each other. More specifically, a combination of Hu promoter DNA and P30 promoter DNA, a combination of Hu promoter DNA and P54 promoter DNA and a combination of P54 promoter DNA and P30 promoter DNA can be preferably mentioned.

[0013]

As the secretory signal peptide of the present invention, a) a peptide consisting of the amino acid sequence represented by any one of SEQ ID Nos. 57, 59, 61, 63, 65, 67, 69, 71, 73, 75 and 77 more specifically, any one of SP7, SP45, SP50, SP52, SP55, SP58, SP64, SP66, SP67, SP68 and SP69 can be mentioned. Of them, SP7 represented by SEQ ID No. 56 and SP69 represented by SEQ ID No. 66 can be preferably mentioned. Furthermore, b) a peptide consisting of an amino acid sequence obtained by deleting, substituting or adding one or several amino acids in the amino acid sequence represented by any one of SEQ ID Nos. 57, 59, 61, 63, 65, 67, 69, 71, 73, 75 and 77, and functioning as a secretory signal peptide in a bacterium of the genus *Bifidobacterium* (mutated secretory signal peptide) can be mentioned. The "amino acid sequence obtained by deleting, substituting or adding one or several amino acids" refers to an amino acid sequence obtained by deleting, substituting or adding, for example 1 to 5, preferably 1 to 3, more preferably 1 to 2 and further preferably one amino acid. The amino acid sequence of the mutated secretory signal peptide has a

sequence identity of preferably 90% or more, more preferably 95% or more and further preferably 98% or more, with the amino acid sequence represented by any one of SEQ ID Nos. 57, 59, 61, 63, 65, 67, 69, 71, 73, 75 and 77.

[0014]

The DNA encoding a secretory signal peptide consisting of the amino acid sequence as specified in a) is not particularly limited as long as it is DNA having a nucleotide sequence corresponding to the amino acid sequence specified in a). Accordingly, examples thereof, although different DNA due to degeneracy of codon may be included, may include DNA consisting of a nucleotide sequence (sequence listing, upper stage) represented by any one of SEQ ID Nos. 56, 58, 60, 62, 64, 66, 68, 70, 72, 74 and 76. These DNAs can be prepared by a method known to those skilled in the art, such as chemical synthesis and genetic engineering technique.

[0015]

The DNA encoding a secretory signal peptide consisting of the amino acid sequence as specified in b) is not particularly limited as long as it is a DNA having a nucleotide sequence corresponding to the amino acid sequence specified in b). Accordingly, although different DNAs due to degeneracy of codon are included, these DNAs can be prepared in accordance with a method known to those skilled in the art, such as chemical synthesis, genetic engineering technique and mutagenesis. Mutated DNA can be obtained, for example, by introducing a mutation into DNA in accordance with a method of bringing a drug serving as a mutagen into contact with DNA consisting of the

nucleotide sequence represented by any one of SEQ ID Nos. 56, 58, 60, 62, 64, 66, 68, 70, 72, 74 and 76; a method of irradiating the DNA with ultraviolet rays; or a method of applying a genetic engineering technique to the DNA. A genetic engineering technique, i.e., site-specific mutagenesis, is useful since it is a technique of introducing a predetermined mutation into a predetermined site, and is carried out in accordance with the method described in, e.g., Molecular Cloning: A laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY., 1989., Current Protocols in Molecular Biology, Supplement 1 to 38, John Wiley & Sons (1987-1997).

[0016]

The secretory signal peptide is preferably ligated to a linker peptide and used as a signal peptide-linker conjugate in view of expression/secretion efficiency of a heterologous polypeptide. Such a linker peptide is not particularly limited as long as it can be connected to the C terminal of the secretory signal peptide of the present invention and enhance expression/secretion efficiency of a heterologous polypeptide. A peptide consisting of an amino acid sequence having 0 to 30 amino acid residues is preferably mentioned as an example. More specifically, of the following 11 types of amino acid sequences, which follow the aforementioned individual secretory signal peptides identified from *Bifidobacterium longum* 105-A strain, amino acid sequences consisting of any number of amino acids within the range from 0 to 30th amino acid can be mentioned as the linker peptides.

[0017]

1) Linker peptide sequence represented by SEQ ID No. 79 (downstream of SP7);

2) Linker peptide sequence represented by SEQ ID No. 81 (downstream of SP45);

3) Linker peptide sequence represented by SEQ ID No. 83 (downstream of SP50);

4) Linker peptide sequence represented by SEQ ID No. 85 (downstream of SP52);

5) Linker peptide sequence represented by SEQ ID No. 87 (downstream of SP55);

6) Linker peptide sequence represented by SEQ ID No. 89 (downstream of SP58);

7) Linker peptide sequence represented by SEQ ID No. 91 (downstream of SP64);

8) Linker peptide sequence represented by SEQ ID No. 93 (downstream of SP66);

9) Linker peptide sequence represented by SEQ ID No. 95 (downstream of SP67);

10) Linker peptide sequence represented by SEQ ID No. 97 (downstream of SP68);

11) Linker peptide sequence represented by SEQ ID No. 99 (downstream of SP69).

The number of amino acid residues in the linker peptides mentioned above can be 0 to 30 residues, preferably, 0 to 25 residues, more preferably 0 to 20 residues, further preferably 0 to 15 residues, further more preferably 0 to 10 residues, and particularly preferably 1 to 10 residues.

[0018]

As the DNA sequences encoding linker peptides consisting of amino acid sequences consisting of any number of amino acids within the range from 0 to 30th amino acid of the above individual sequences, the following sequences can be mentioned:

1) DNA sequence represented by SEQ ID No. 78 (downstream of SP7);

2) DNA sequence represented by SEQ ID No. 80 (downstream of SP45);

3) DNA sequence represented by SEQ ID No. 82 (downstream of SP50);

4) DNA sequence represented by SEQ ID No. 84 (downstream of SP52);

5) DNA sequence represented by SEQ ID No. 86 (downstream of SP55);

6) DNA sequence represented by SEQ ID No. 88 (downstream of SP58);

7) DNA sequence represented by SEQ ID No. 90 (downstream of SP64);

8) DNA sequence represented by SEQ ID No. 92 (downstream of SP66);

9) DNA sequence represented by SEQ ID No. 94 (downstream of SP67);

10) DNA sequence represented by SEQ ID No. 96 (downstream of SP68);

11) DNA sequence represented by SEQ ID No. 98 (downstream of SP69).

[0019]

The DNA region encoding the secretory signal peptide-linker peptide conjugate of the present invention

refers to a DNA region from the 5' end of the DNA encoding each of the secretory signal peptides to the 3' end of the DNA encoding the linker peptide downstream of the secretory signal peptide (including both ends). Since the 3' end of the DNA encoding the secretory signal peptide and the 5' end of the DNA encoding the linker peptide are connected, the secretory signal peptide-linker peptide conjugate of the present invention is sometimes represented by SPxLy (where x is the number of a secretory signal peptide assigned in the specification and y is the number of amino acid residues of the linker peptide). Examples of the secretory signal peptide-linker peptide conjugate may include SP7L20, SP45L20, SP50L20, SP52L20, SP55L20, SP58L20, SP64L20, SP66L20, SP67L20, SP68L20 and SP69L20. For example, SP67L20 represents the secretory signal peptide-linker peptide conjugate obtained by connecting the first 20 amino acids of the sequence represented by SEQ ID No. 95 to the amino acid sequence represented by SEQ ID No. 73. Of the secretory signal peptide-linker peptide connected bodies, SP50L1 to L15, SP67L1 to L15, SP68L1 to L15 and SP69L1 to L15 are preferable; SP50L1 to L10, SP67L1 to L10, SP68L1 to L10 and SP69L1 to L10 are preferable; and SP50L5, SP67L1, SP67L10, SP68L1 and SP69L1 are particularly preferable. Note that, L1 to L15 represent L1, L2, L3, L4, L5, L6, L7, L8, L9, L10, L11, L12, L13, L14 and L15 and L1 to L10 represent L1, L2, L3, L4, L5, L6, L7, L8, L9 and L10.

[0020]

DNA encoding a heterologous polypeptide as mentioned above is not particularly limited as long as it is DNA

which encodes a polypeptide not derived from a bacterium of the genus *Bifidobacterium* and can be expressed by the secretory expression cassette of the present invention. Examples thereof may include DNA encoding a cytokine such as interferon (IFN)-α, β, γ, a granulocyte macrophage colony stimulating factor (GM-CSF), interleukin (IL)-1α, IL-1β, IL-2, IL-3, IL-4, IL-6, IL-7, IL-10, IL-12, IL-13, IL-15, IL-18, IL-21, IL-22, IL-27, a tumor necrosis factor (TNF)-α, lymphotoxin (LT)-β, a granulocyte colony stimulating factor (G-CSF), a macrophage colony stimulating factor (M-CSF) and a macrophage migration inhibitory factor (MIF); and DNA encoding an angiogenesis inhibitor such as endostatin, angiostatin and kringle (NH4). Besides these, DNA encoding cytosine deaminase, which is an enzyme of converting of 5-fluorocytosine (a prodrug of 5-fluorouracil) into 5-fluorouracil; and DNA encoding nitroreductase, which is an enzyme of reducing a nitro group of a prodrug of an anticancer agent having a nitroaromatic skeleton to activate it, can be preferably mentioned. In addition, DNA encoding an antibody polypeptide can be advantageously used. To such DNA encoding a heterologous polypeptide, DNA encoding an affinity tag such as histidine (His) tag and FLAG tag can be appropriately attached in order to facilitate an isolation treatment of the polypeptide.

[0021]

Examples of the antibody may include an antibody for use in a rheumatoid therapeutic agent targeting an interleukin-6 (IL-6) receptor; an antibody for use in a therapeutic agent for multiple sclerosis targeting α4-

integrin; an antibody having an anticancer effect targeting CD20, CD33, PD-1, CTLA-4, PD-L1, CD80, CD86, LAG3, TIM3 or KIR; an agonist antibody targeting e.g., OX40, CD137 or ICOS; and an antibody specifically binding to HER2, EGFR, CEA, HGF, EpCAM (CD326), cMET or CCR4.

[0022]

Of them, the anti-PD-1 antibody can be preferably mentioned as an example, because it binds to a PD-1 receptor expressed on an activated lymphocyte (T cell, B cell) to inhibit the binding of PD-L1 and PD-L2 expressed by a cancer cell to the PD-1 receptor, with the result that the immune reaction to the tumor cell is enhanced. Furthermore, the anti-CTLA-4 antibody suppresses the function of CTLA-4, which is known as an autoimmune function suppression molecule, thereby enhancing an antitumor immune response. More specifically, the anti-CTLA-4 antibody can be preferably mentioned as an example, because the anti-CTLA-4 antibody is considered to enhance the antitumor immune response by inhibiting the binding of CTLA-4 to CD80 and CD86 expressed in an antigen presenting cell to inhibit negative down-regulation of an immune response induced by the interaction between these molecules. Moreover, the anti-HER-2 (human EGFR-related2) antibody can be preferably mentioned as an example, because it is considered to specifically bind to HER2 protein, which is a gene product of oncogene HER2/neu (c-erbB-2), to suppress a proliferation signal, thereby exerting an anti-tumor effect.

[0023]

As the DNA encoding an antibody polypeptide as mentioned above, DNA encoding e.g., a chimeric antibody, a humanized antibody, Fab, Fab', F $(ab')_2$, and a single-chain antibody (scFv: single chain Fv) can be mentioned; however, DNA encoding a single-chain antibody, capable of recognizing and binding a target substance by itself, having an appropriate (not too big) molecular weight, and expressible when it is introduced into a bacterium of the genus *Bifidobacterium*, is preferable. DNAs encoding heterologous polypeptides including these antibodies can be also prepared by a method known in the art such as chemical synthesis and genetic engineering technique based on their sequence information appropriately obtained from published documents or database such as GenBank.

[0024]

As the aforementioned combination of heterologous polypeptides of the present invention, it is preferable to employ a combination of heterologous polypeptides which are expected to produce a synergistic combinational effect at an anaerobic disease site when locally expressed. This is because when a bacterium of the genus *Bifidobacterium* is systemically administered, it does not proliferate in a normal tissue but is selectively distributed and colonized at an anaerobic site such as a solid tumor tissue and an ischemic disease site. Specific examples of the combination may include a combination of TNF-α and IFN-γ; a combination of an anti-PD-1 antibody and an anti-CTLA-4 antibody; a combination of an anti-HER2 antibody and IFN-γ; a combination of an anti-PD-1 antibody and IFN-γ; a combination of an anti-PD-1 antibody and an anti-HER2

antibody; and a combination of an anti-PD-1 antibody and an anti-EpCAM antibody.

[0025]

The terminator DNA is not particularly limited as long as it is terminator DNA that functions in a bacterium of the genus *Bifidobacterium*. Specific examples thereof may include Hu terminator DNA derived from *Bifidobacterium longum*; d0013terminator DNA, which is a terminator of a lactate dehydrogenase gene derived from *Bifidobacterium longum*; T572terminator DNA derived from *Bifidobacterium animaris* (J. Microbiol Biotechnol. 2012 Dec; 22 (12): 1714-23); and BBa_B0015 (T2) terminator DNA, which is an artificially designed terminator. It is preferable to use different terminators for respective expression cassettes excellent in secretion of a heterologous polypeptide as mentioned above. Specific examples thereof may include Hu terminator DNA and d0013 terminator DNA in combination; Hu terminator DNA and T572 terminator DNA in combination; Hu terminator DNA and T2 terminator DNA in combination; d0013 terminator DNA and T572 terminator DNA in combination; d0013 terminator DNA and T2 terminator DNA in combination; and T572 terminator DNA and T2 terminator DNA in combination.

[0026]

As a method for producing a secretory expression cassette of the present invention, preferably a secretory expression cassette comprising a DNA encoding a linker peptide, it is possible to mention a method of sequentially connecting the following DNAs:

(1) a promoter DNA functioning in a bacterium of the genus *Bifidobacterium*;

(2) a DNA encoding a secretory signal peptide consisting of the amino acid sequence described in a) or b) below:

a) an amino acid sequence represented by any one of SEQ ID Nos. 57, 59, 61, 63, 65, 67, 69, 71, 73, 75 and 77;

b) an amino acid sequence obtained by deleting, substituting or adding one or several amino acids in the amino acid sequence represented by any one of SEQ ID Nos. 57, 59, 61, 63, 65, 67, 69, 71, 73, 75 and 77, wherein a peptide consisting of the amino acid sequence serves as a signal peptide in the bacterium of the genus *Bifidobacterium*;

(3) a DNA encoding a linker peptide;

(4) a DNA encoding a heterologous polypeptide; and

(5) a terminator DNA functioning in the bacterium of the genus *Bifidobacterium*,

in the order of (1) to (5) from upstream to downstream. The secretory expression cassette of the present invention can be prepared in accordance with a method described in a commercially available experimental technique, for example, Gene Manual (Kodansha Ltd.); Gene Manipulation Experimental Technique, edited by Yasutaka Takagi, Kodansha Ltd.; Molecular Cloning [Cold Spring Harbor Laboratory (1982)]; Molecular cloning second edition [Cold Spring Harbor Laboratory (1989)]; Methods in enzymology 194 (1991); and Gene experimental technique by yeast, Experimental Medicine, supplemental vol., YODOSHA CO., LTD. (1994).

[0027]

The plasmid vector that can be used for producing a co-expression plasmid comprising two types of secretory expression cassettes of the present invention is not particularly limited, as long as it is a plasmid vector into which two types of expression cassettes of the present invention can be inserted and as long as two types of secretory heterologous polypeptides are expressed when a bacterium of the genus *Bifidobacterium* is transformed with the plasmid vector. A shuttle plasmid vector further having an origin of replication such as a pUCori, which can function also in a bacterium other than a bacterium of the genus *Bifidobacterium*, for example, in *E. coli*, can be advantageously used.

[0028]

Examples of the plasmid vector having a plasmid replication unit functioning in a bacterium of the genus *Bifidobacterium* include pTB6 (Biosci Biotechnol Biochem. 2005 Feb; 69 (2): 422-5); pMB1 (Lett Appl Microbiol. 1990 Oct; 11 (4): 220-3); pTB4 (structural analysis and application of *Bifidobacterium longum*-derived plasmid pTB4, general subject of speech, poster presentation program, Molecular Biology Society of Japan, 1994); pFI2576 (J Microbiol Biotechnol. 2009 Apr; 19 (4): 403-8); pCIBAO (Appl Environ Microbiol. 2007 Dec; 73 (24): 7858-66); pBC1 (Plasmid. 2007 Mar; 57 (2): 165-74); pDOJH10S (Appl Environ Microbiol. 2006 Jan; 72 (1): 527-35); and PKJ50 (Microbiology 1999 Mar; 145 (Pt): 585-92). As the replication unit, pTB6rep unit consisting of a pTB6-derived OriV region and RepB gene can be preferably

mentioned. A shuttle plasmid vector further having an origin of replication such as a pUCori, which can function also in a bacterium other than a bacterium of the genus *Bifidobacterium*, for example in *E. coli*, can be used.

[0029]

The plasmid may contain a marker gene such as a drug resistance gene. Examples of the drug resistance marker gene include spectinomycin, chloramphenicol, erythromycin and ampicillin resistance genes.

[0030]

As a method for introducing the plasmid of the present invention into a bacterium of the genus *Bifidobacterium*, a gene introduction method known in the art, such as electroporation, may be mentioned.

[0031]

Examples of the bacterium of the genus *Bifidobacterium* in the present invention include *Bifidobacterium longum*, *Bifidobacterium breve* (*B. breve*), *Bifidobacterium adolescentis* (*B. adolescentis*), *Bifidobacterium bifidum* (*B. bifidum*), *Bifidobacterium pseudolongum* (*B. pseudolongum*), *Bifidobacterium thermophirum* (*B. thermophirum*), *Bifidobacterium infantis* (*B. infantis*), *Bifidobacterium animalis* (*B. animalis*), *Bifidobacterium angulatum* (*B. angulatum*), *Bifidobacterium asteroides* (*B. asteroides*), *Bifidobacterium boum* (*B. boum*), *Bifidobacterium catenulatum* (*B. catenulatum*), *Bifidobacterium choerinum* (*B. choerinum*), *Bifidobacterium coryneforme* (*B. coryneforme*), *Bifidobacterium cuniculi* (*B. cuniculi*), *Bifidobacterium denticolens* (*B. denticolens*), *Bifidobacterium dentium* (*B. dentium*), *Bifidobacterium*

gallicum (*B. gallicum*), *Bifidobacterium gallinarum* (*B. gallinarum*), *Bifidobacterium globosum* (*B. globosum*), *Bifidobacterium indicum* (*B. indicum*), *Bifidobacterium inopinatum* (*B. inopinatum*), *Bifidobacterium lactis* (*B. lactis*), *Bifidobacterium lactentis* (*B. lactentis*), *Bifidobacterium magnum* (*B. magnum*), *Bifidobacterium merycicum* (*B. merycicum*), *Bifidobacterium minimum* (*B. minimum*), *Bifidobacterium Mongolia Enns* (*B. Mongolia Enns*), *Bifidobacterium parvulorum* (*B. parvulorum*), *Bifidobacterium pseudocatenulatum* (*B. pseudocatenulatum*), *Bifidobacterium psychraerophilum* (*B. psychraerophilum*), *Bifidobacterium pullorum* (*B. pullorum*), *Bifidobacterium ruminale* (*B. ruminale*), *Bifidobacterium ruminantium* (*B. ruminantium*), *Bifidobacterium saeculare* (*B. saeculare*), *Bifidobacterium scardovii* (*B. scardovii*), *Bifidobacterium subtile* (*B. subtile*), *Bifidobacterium suis* (*B. suis*), and *Bifidobacterium thermacidophilum* (*B. thermacidophilum*). Of them, *Bifidobacterium longum*, *Bifidobacterium breve*, *Bifidobacterium adolescentis*, *Bifidobacterium bifidum* and *Bifidobacterium infantis*, which are known to be harbitually present within the human intestine regardless of age, are preferably used as a host cell, and *Bifidobacterium longum* is more preferably used. These bacteria all can be commercially available or can be easily obtained from e.g., a depository.

[0032]

The strain of each bacterium is not particularly limited. Examples of the strain of *Bifidobacterium longum* include *Bifidobacterium longum* 105-A strain, *Bifidobacterium longum* aE-194b strain, *Bifidobacterium*

longum bs-601 strain, *Bifidobacterium longum* M101-2 strain and *Bifidobacterium longum* ATCC-15707 strain. Of them, *Bifidobacterium longum* 105-A strain is preferable. As to *Bifidobacterium breve*, for example, *Bifidobacterium breve* standard strain (JCM1192), *Bifidobacterium breve* aS-1 strain and *Bifidobacterium breve* I-53-8W strain may be mentioned. Of them, *Bifidobacterium breve* standard strain and *Bifidobacterium breve* aS-1 strain are preferable. As to *Bifidobacterium infantis*, for example, *Bifidobacterium infantis* standard strain (JCM1222) and *Bifidobacterium infantis* I-10-5 strain may be mentioned. As to *Bifidobacterium lactentis*, for example, *Bifidobacterium lactentis* standard strain (JCM1210) may be mentioned. As to *Bifidobacterium bifidum* strain, for example, *Bifidobacterium bifidum* ATCC-11863 strain may be mentioned.

[0033]

The plasmid and transformed bacterium of the genus *Bifidobacterium* of the present invention can be prepared in accordance with a method described in a published experimental text, for example, Gene Manual (Kodansha Ltd.); Gene Manipulation Experimental Technique, edited by Yasutaka Takagi, Kodansha Ltd.; Molecular Cloning [Cold Spring Harbor Laboratory (1982)]; Molecular cloning second edition [Cold Spring Harbor Laboratory (1989)]; Methods in enzymology 194 (1991); and Gene experimental technique by yeast, Experimental medicine, supplemental vol., YODOSHA CO., LTD. (1994).

[0034]

The transformed bacterium of the genus *Bifidobacterium* does not proliferate in a normal tissue

but proliferates only in a tumor tissue under an anaerobic environment and can express two types of heterologous polypeptides useful for therapy within the tumor tissue.

[0035]

Accordingly, the transformed bacterium of the genus *Bifidobacterium* can be used in a pharmaceutical composition, in particular, an anticancer drug effective for treating a tumor under an anaerobic environment, preferably a solid tumor. Accordingly, the pharmaceutical composition of the present invention is not particularly limited as long as it contains the bacterium of the genus *Bifidobacterium* of the present invention capable of secreting heterologous polypeptides, preferably cytokines and antibodies having an anticancer effect, as an active ingredient, and may contain an optional ingredient such as a pharmacologically acceptable carrier, an excipient and a diluent as long as it does not prevent the action and effect of the polypeptide to be secreted.

[0036]

The administration target of the pharmaceutical composition of the present invention is a mammalian, preferably a human. Examples of the applicable target of the pharmaceutical composition of the present invention include colon carcinoma, head and neck cancer, breast cancer, lung cancer, esophageal carcinoma, gastric carcinoma, liver carcinoma, gallbladder carcinoma, cholangiocarcinoma, pancreatic cancer, pancreatic islet cell carcinoma, choriocarcinoma, colon carcinoma, renal cell carcinoma, adrenal cortical carcinoma, bladder carcinoma, testicular carcinoma, prostate cancer, testis

cancer, ovarian cancer, uterine cancer, choriocarcinoma, thyroid cancer, squamous cell carcinoma, skin cancer, brain tumor, malignant carcinoid tumor, osteosarcoma, soft tissue sarcoma, neuroblastoma, Wilms tumor, retinoblastoma and melanoma.

[0037]

As the dosage form of the pharmaceutical composition of the present invention, a liquid formulation or a solid formulation can be mentioned. The liquid formulation can be prepared by purifying the culture of the bacterium of the genus *Bifidobacterium* of the present invention, applicably adding, if necessary, saline, a replacement fluid or a pharmaceutical additive(s) and charging e.g., ampoules or vials with the resultant suspension. The solid formulation can be prepared by adding a protecting agent to the liquid formulation, charging ampoules or vials with the mixture, and freezing the mixture to obtain a frozen formulation or freeze-drying the mixture to obtain a lyophilized formulation. As a method of administering the pharmaceutical composition of the present invention, oral administration and parenteral administration both can be employed; however, parenteral administration is preferable; for example, intravenous administration and local administration can be mentioned.

[0038]

The dose of the pharmaceutical composition of the present invention is not particularly limited as long as a bacterium of the genus *Bifidobacterium* can grow in a diseased site and as long as it is a sufficient amount for the bacterium of the genus *Bifidobacterium* to express a

therapeutically effective amount of e.g., a cytokine or active antibody. The dose of the pharmaceutical composition can be appropriately selected depending upon the severity of disease and the weight, age and sex of the patient, and appropriately increased or decreased depending upon the degree of improvement. From an economic point of view and in order to avoid a side effect as much as possible, the dose is preferably as low as possible within the range where a requisite therapeutic effect is obtained.

[0039]

For example, in the case of intravenous administration, it is required to reduce a risk, in particular, e.g., embolization caused by bacterial mass. Thus, it is preferable that an injectable solution prepared to have a concentration as low as possible, is injected in a plurality of times by dividing the dose into the plural portions or continuously administered by diluting it with an appropriate replacement fluid. For example, in the case of an adult, the bifidobacterial cell of the present invention, a daily dose of 10^4 to 10^{12} cfu per body weight (1 kg), is divided in a plurality of times and administered for one to several days continuously or non-continuously at appropriate time intervals. More specifically, a formulation comprising the bifidobacterial cell of the present invention in a concentration of 10^4 to 10^{10} cfu/mL is directly administered or by diluting it with an appropriate replacement fluid, in a dose of 1 to 1000 mL per adult, once a day or by dividing the dose in a

plurality of times per day continuously for one to several days.

[0040]

For example, in the case of local administration, i.e., direct administration to a diseased tissue, a high-concentration injectable solution is desirably injected in a plurality of sites of the diseased tissue, since a bacterial cell is required to colonize and grow over the entire diseased tissue as much as possible. For example, in the case of an adult, the bifidobacterial cell of the present invention, more specifically, a daily dose of 10^4 to 10^{12} cfu per body weight (1 kg), is administered once or in a plurality of times per day, if necessary, for one to several days, continuously or non-continuously at appropriate time intervals. More specifically, a formulation comprising the bifidobacterial cell of the present invention in a concentration of 10^4 to 10^{10} cfu/mL is directly administered in a dose of 0.1 to 100 mL per adult, several times per day, if necessary, for one to several consecutive days.

[0041]

Now, the present invention will be more specifically described by way of Examples below; however, the technical scope of the present invention is not limited by these examples.

[0042]

[Example 1]

[Preparation of human TNF-α and human IFN-γ co-expression *Bifidobacterium*, AG8TL strain]

(Outline)

A co-expression plasmid, pAG8TL was prepared, which contains an expression cassette for secretory human TNF-α (human TNF-α secretory expression cassette) and an expression cassette for secretory human IFN-γ (human IFN-γ secretory expression cassette) and which serves as an *E. coli-Bifidobacterium* shuttle vector. *Bifidobacterium longum* 105-A strain was transformed with pAG8TL thus prepared by electroporation to obtain a human TNF-α and human IFN-γ co-expression *Bifidobacterium*, AG8TL strain. The primers used in Examples 1 to 3 are shown in the following Table 1.

[0043]

[Table 1]

Primer Name	DNA sequence (5' -> 3')	SEQ ID No.
GA1_primer	gagcagaaggTCACTGGGAGGCGCGACGGCCAC	SEQ ID No. 18
GA2_primer	AGTGAccttctgctcgtagcg	SEQ ID No. 19
GA5_primer_rev	ggtatgtaggcggtgctacag	SEQ ID No. 20
GA6_primer	caccgcctacatacctcgct	SEQ ID No. 21
GA100_primer	cggtgGTGCGCTCCTCCTCCCGTAC	SEQ ID No. 22
GA101_primer	TCACAGGGCGATGATGCC	SEQ ID No. 23
GA103_primer	AGGAGCGCACcaccgaactcgccttcgg	SEQ ID No. 24
GA104_primer	ATCATCGCCCTGTGAAACCGCTTCTCATTTCCATTTGCG	SEQ ID No. 25
GA113_primer	CGGTGCACACTAGTcctccaggacctc	SEQ ID No. 26
GA116_primer	gACTAGTGTGCACCGAATCGCGCTG	SEQ ID No. 27
IFNG1_primer	gaaggatgctttATGCAGGACCCGTACGTCAAGG	SEQ ID No. 28
IFNG2_primer	CATCATCACCACCACTGAccttctgctcgtagcg	SEQ ID No. 29
IFNG3_primer	GTGGTGGTGATGATGGTGCTGGGAGGCGCGACGGCC	SEQ ID No. 30
hIFNG4_primer	caggacccgtacgtcAAGG	SEQ ID No. 31
SP69-ins_F1_primer	caagaaggatgctttATGAATTATTTACGACAAAAAATTTCGG	SEQ ID No. 32
SP69-ins_R2_primer	gacgtacgggtcctgACCGCTATCAGTCGTGGTGTAAC	SEQ ID No. 33
pCDshuttle_R1_primer	cataaagcatccttcttgggtcag	SEQ ID No. 34
Hu-mCCL21-vecR1_primer	AAAGCATCCTTCTTGGGTCAGG	SEQ ID No. 35

[0044]

(Constitution of human TNF-α secretory expression cassette)

As the human TNF-α secretory expression cassette, a cassette sequentially comprising (1) a P30 promoter DNA, (2) a DNA encoding a signal peptide-linker conjugate, SP7L20, (3) a DNA encoding the amino acid sequence of a human TNF-α protein and (4) a d0013 terminator DNA, was prepared such that DNA (1) was placed upstream (the 5' end) and DNA (4) downstream (the 3' end).

[0045]

(Constitution of human IFN-γ secretory expression cassette)

As the human IFN-γ secretory expression cassette, a cassette sequentially comprising (1) a Hu promoter DNA, (2) a DNA encoding a signal peptide-linker conjugate, SP69L20, (3) a DNA encoding the amino acid sequence of human IFN-γ protein and (4) a Hu terminator DNA (derived from *Bifidobacterium longum*) was prepared such that DNA (1) was placed upstream (the 5' end) and DNA (4) downstream (the 3' end).

[0046]

(Preparation of plasmid pAG8TL)

In preparing plasmid pAG8TL, plasmid pAG8 was first prepared.

[0047]

(Preparation of plasmid pAG8)

(Preparation of hTNF-α insert fragment)

PCR amplification was carried out using plasmid pSP3B-TNFα (see, Figure 1) (1 ng) described in International Publication No. WO2011/093465, as a template

and a primer set of GA100_primer (forward) and GA101_primer (reverse) listed in Table 1. Each of the primer sequences was designed such that an insert fragment was overlapped with a vector fragment at the end of 15 bp. The PCR amplification was carried out by specifying the concentration of each primer as 0.2 μM and the reaction volume as 20 μL and using PrimeSTAR HS (Premix) kit (manufactured by Takara Bio Inc.) (hereinafter referred to as "STAR kit"). As the amplification program, a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 65°C for 5 seconds and a reaction at 75°C for 40 seconds was repeated 30 times and then an elongation reaction was carried out at 72°C for 30 seconds to prepare a h (human) TNF-α insert fragment amplified product of about 0.5 kbp.

[0048]

(Preparation of vector fragment comprising DNA encoding the amino acid sequence of human IFN-γ protein)

PCR amplification was carried out using a linearized fragment (SEQ ID No. 1) of plasmid pHG-2 (Figure 2) as a template and a primer set of GA104_primer (forward) and GA103_primer (reverse) listed in Table 1. Each of the primer sequences was designed such that an insert fragment was overlapped with a vector fragment at the end of 15 bp. The PCR amplification was carried out by specifying the concentration of each primer as 0.2 μM and the reaction volume as 20 μL, and using STAR kit. As the amplification program, a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 65°C for 5 seconds and a reaction at 75°C for 5 minutes was repeated 30 times and then an

elongation reaction at 72°C for 30 seconds was carried out to prepare a 5'-Hu promoter-SP69L20-human IFN-γ protein-His tag-Hu terminator-pTB rep unit-SPCM^r-pUCori-P30 promoter-SP7L20-3' vector fragment amplified product of about 5 kbp.

[0049]

The plasmid pHG-2 linearized fragment is a nucleotide sequence from 1st nucleotide to 4975th nucleotide of SEQ ID No. 1 and constituted as follows.

d0013 terminator: a nucleotide sequence from 16th nucleotide to 129th nucleotide of SEQ ID No. 1,

Hu promoter: a nucleotide sequence from 136th nucleotide to 496th nucleotide of SEQ ID No. 1,

SP69L20: a nucleotide sequence from 497th nucleotide to 646th nucleotide of SEQ ID No. 1,

human IFN-γ protein: a nucleotide sequence from 647th nucleotide to 1075th nucleotide of SEQ ID No. 1,

His tag: a nucleotide sequence from 1076th nucleotide to 1093rd nucleotide of SEQ ID No. 1,

Hu terminator: a nucleotide sequence from 1097th nucleotide to 1210th nucleotide of SEQ ID No. 1,

a bacterium of the genus *Bifidobacterium*, origin of replication pTB6 rep unit: a nucleotide sequence from 1217th nucleotide to 2812nd nucleotide of SEQ ID No. 1,

spectinomycin resistance gene SPCMr: a nucleotide sequence from 2819th nucleotide to 3897th nucleotide of SEQ ID No. 1,

E. coli origin of replication, pUCori: a nucleotide sequence from 3904th nucleotide to 4571st nucleotide of SEQ ID No. 1,

P30 promoter: a nucleotide sequence from 4572nd nucleotide to 4806th nucleotide of SEQ ID No. 1, and

SP7L20: a nucleotide sequence from 4807th nucleotide to 4965th nucleotide of SEQ ID No. 1.

[0050]

(In-fusion reaction 1)

The vector fragment amplified product and insert fragment amplified product prepared above were ligated by use of In-Fusion (registered trademark) HD Cloning kit (manufactured by Takara Bio Inc.) (hereinafter referred to as "HD kit "). More specifically, in a micro tube, the vector fragment amplified product (50 ng) and the insert fragment amplified product (13 ng) were added, and 5X In-Fusion HD Enzyme premix (2 μL) and Cloning Enhancer (1 μL) contained in the kit were further added. The volume of the reaction solution was adjusted with 0.1X TE buffer (1mM Tris-HCl, 0.1mM EDTA, pH7.5) to be 10 μL. The reaction solution was kept warm at 37°C for 15 minutes and further kept warm at 50°C for 15 minutes. The procedure other than this was carried out in accordance with the product manual of the kit to prepare in-fusion reaction solution 1.

[0051]

(Transformation of *E. coli* and determination of DNA sequence of plasmid pAG8)

Using in-fusion reaction solution 1 (1 μL), *E. coli* HST16CR competent cells (manufactured by Takara Bio Inc.)

were transformed in accordance with the product manual. After the transformation, the bacterial suspension was spread onto LB agar medium containing 75 μg/mL spectinomycin and cultured at 37°C overnight with shaking. *E. coli* colony formed on the agar medium was cultured in LB liquid medium containing 75 μg/mL spectinomycin at 37°C overnight. From this, a plasmid was extracted by use of QIAprepSpin Miniprep kit (manufactured by QIAGEN). In the plasmid extracted, the sequence of region 1 (5'-P30 promoter-SP7L20-human TNF-α protein-d0013 terminator-Hu promoter-SP69L20-human IFN-γ protein-His tag-Hu terminator-3') comprising the human TNF-α secretory expression cassette and the human IFN-γ secretory expression cassette, was determined by a sequencing reaction using Big Dye (registered trademark) Terminator v3.1 Cycle Sequencing kit (manufactured by Applied Biosystems). The plasmid extracted was designated as pAG8. The sequence of region 1 comprising the human TNF-α secretory expression cassette and the human IFN-γ secretory expression cassette of pAG8 is represented by SEQ ID No. 2.

[0052]

(Preparation of pAG8TL strain)

PCR amplification was carried out using pAG8 obtained above as a template and a primer set of GA2_primer (forward) and GA6_primer (reverse) listed in Table 1. The same procedure as in the above section (Preparation of plasmid pAG8) was repeated except that the amplification program, which is a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 65°C for 5

seconds and a reaction at 75°C for 3 minutes and 15 seconds, was repeated 30 times and then an elongation reaction was carried out at 72°C for 30 seconds, to prepare PCR amplification product A of about 3.3 kbp.

[0053]

PCR amplification was carried out using pAG8 mentioned above as a template and a primer set of GA5_primer_rev (forward) and GA1_primer (reverse) listed in Table 1. The same procedure as in the above section (Preparation of plasmid pAG8) was repeated except that the amplification program, which is a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 65°C for 5 seconds and a reaction at 75°C for 2 minutes and 10 seconds, was repeated 30 times, and then an elongation reaction was carried out at 72°C for 30 seconds to prepare PCR amplification product B of about 2.2 kbp.

[0054]

(In-fusion reaction 2)

The same procedure as in the above section (In-fusion reaction 1) was repeated except that PCR amplification product A (50 ng) and PCR amplification product B (33 ng) were used, to ligate PCR amplification product A and PCR amplification product B. In this manner, in-fusion reaction solution 2 was prepared.

[0055]

(Transformation of *E. coli* and determination of DNA sequence of plasmid pAG8TL)

Transformation of the *E. coli* HST16CR competent cells by using in-fusion reaction solution 2 obtained above and extraction of a plasmid from the recombinant *E.*

coli. were carried out in the same procedure as in the above section (Transformation of *E. coli* and determination of DNA sequence of plasmid pAG8). In the plasmid extracted, the sequence of region 2 (5'-P30 promoter-SP7L20-human TNF-α protein-d0013 terminator-Hu promoter-SP69L20-human IFN-γ protein-Hu terminator-3') comprising the human TNF-α secretory expression cassette and human IFN-γ secretory expression cassette was determined in the same procedure as above. The plasmid extracted was designated as pAG8TL. The sequence of region 2 comprising the human TNF-α secretory expression cassette and human IFN-γ secretory expression cassette of pAG8TL is represented by SEQ ID No. 3.

[0056]

(Transformation 1 of bacterium of the genus *Bifidobacterium*)

Bifidobacterium longum 105-A strain was transformed with plasmid pAG8TL (250 ng) extracted from the transformed *E. coli* by an electroporation system (Gene Pulser II, manufactured by Bio-Rad laboratories). Immediately after electric shock (2 kV, 25 μF, 200 Ω), a mixture of 800 μL of IMR liquid medium and 50 μL of vitamin C solution was added to a cuvette (2 mm gap) and collected in (transferred to) in a sterilized 2 mL-micro tube. The 2 mL (micro) tube with the cap loosen was placed together with a deoxygen/carbon dioxide generator (AnaeroPack (registered trademark) Kenki manufactured by Mitsubishi Gas Chemical Company, Inc.) in an airtight container and kept warm for 3 hours in an incubator set at 37°C.

[0057]

After kept warm, individual bacterial suspensions were each spread onto IMR agar medium containing 75 μg/mL spectinomycin. These plates were placed in an airtight container together with the deoxygen/carbon dioxide generator mentioned above and cultured in an incubator set at 37°C for 2 days.

[0058]

The colony formed on the IMR agar medium containing spectinomycin was determined as a transformant, *Bifidobacterium longum* 105-A/pAG8TL strain (hereinafter referred to as AG8TL strain).

[0059]

[Example 2]

[Preparation of Human TNF-α single-expression strain TNF11]

Human TNF-α single-expression strain TNF11 to be compared with co-expression *Bifidobacterium* AG8TL strain was prepared as follows.

[0060]

PCR amplification was carried out using pAG12 as a template and a primer set of GA113_primer (forward) and GA6_primer (reverse) listed in Table 1. The same procedure as in the above section (Preparation of plasmid pAG8) was repeated except that the amplification program, which is a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 65°C for 5 seconds and a reaction at 75°C for 3 minutes and 50 seconds was repeated 30 times and then an elongation reaction was carried out at 72°C

for 30 seconds to prepare PCR amplification product 60 of about 3.2 kbp.

[0061]

PCR amplification was carried out using pAG12 as a template and a primer set of GA5_primer_rev (forward) and GA116_primer (reverse) listed in Table 1. The same procedure as in the above section (Preparation of plasmid pAG8) was repeated except that the amplification program, which is a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 65°C for 5 seconds and a reaction at 72°C for one minute and 15 seconds, was repeated 30 times, and then an elongation reaction was carried out at 72°C for 30 seconds to prepare PCR amplification product 61 of about 1.2 kbp. Note that, the plasmid pAG12 used as a template was obtained by replacing SP/Linker of hIFN-γ of pAG8TL, i.e., SP69L20, by SP56L20.

[0062]

(In-fusion reaction 3)

The same procedure as in the above section (In-fusion reaction 1) was repeated except that PCR amplification product 60 (50 ng) and PCR amplification product 61 (20 ng) were used to ligate the PCR amplification product 60 and the PCR amplification product 61. In this manner, in-fusion reaction solution 3 was prepared.

[0063]

(Transformation of *E. coli* and determination of DNA sequence of plasmid pTNF11)

The same procedure as in the above section (Transformation of *E. coli* and determination of DNA

sequence of plasmid pAG8) was repeated by using in-fusion reaction solution 3 obtained above to carry out transformation of the *E. coli* HST16CR competent cells obtained above and extraction of a plasmid from the recombinant *E. coli*. In the plasmid extracted, the sequence of the human TNF-α secretory expression cassette (5'-P30 promoter-SP7L20-human TNF-α protein-d0013 terminator) was determined. The plasmid extracted was designated as pTNF11. The constitution of pTNF11 plasmid is shown in Figure 3.

[0064]

(Transformation 2 of bacterium of the genus *Bifidobacterium*)

Bifidobacterium longum 105-A strain was transformed by the electroporation system in the same procedure as in the above section (Transformation 1 of bacterium of the genus *Bifidobacterium*) except that plasmid pTNF11 (500 ng) extracted from the transformed *E. coli* mentioned above was used. The transformant obtained was designated as *Bifidobacterium longum* 105-A/pTNF11 strain (hereinafter referred to as TNF11 strain).

[0065]

[Example 3]

[Preparation of human IFN-γ single-expression strain hIFNg33TL]

Human IFN-γ single-expression strain hIFNg33TL to be compared with co-expression *Bifidobacterium* AG8TL strain was prepared as follows.

[0066]

Human IFN-γ single-expression strain hIFNg33TL to be compared with co-expression AG8TL strain was prepared. In preparation of plasmid phIFNg33TL, plasmid phIFNg33 was first prepared.

[0067]

(Artificial DNA synthesis of hIFN-γ gene)

Synthesis of hIFN-γ gene was ordered to GenScript Japan Inc. The codons of the hIFN-γ sequence were optimized for *Bifidobacterium longum* NCC2705 based on the amino acid sequence (Gln24-Gln166) of the mature protein of Accession#CAA31639 (plasmid delivered: pUC57-hIFNg). The DNA sequence of artificial synthesized hIFN-γ is represented by SEQ ID No. 4.

[0068]

PCR amplification was carried out using pBEshuttle (SEQ ID No. 5) as a template and a primer set of pCDshuttle_R1_primer (reverse) and IFNG2_primer (forward) listed in Table 1. The same procedure as in the above section (Preparation of plasmid pAG8) was repeated except that the amplification program, which is a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 55°C for 5 seconds and a reaction at 75°C for 3 minutes and 50 seconds, was repeated 30 times, and then an elongation reaction was carried out at 72°C for 30 seconds to prepare PCR amplification product, vector 1 of about 3.9 kbp.

[0069]

PCR amplification was carried out using pUC57-hIFNg (hIFNg in pUC57) mentioned above as a template and a primer set of IFNG1_primer (forward) and IFNG3_primer

(reverse) listed in Table 1. The same procedure as in the above section (Preparation of plasmid pAG8) was repeated except that the amplification program, which is a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 55°C for 5 seconds and a reaction at 75°C for 30 seconds, was repeated 30 times, and then an elongation reaction was carried out at 72°C for 30 seconds to prepare PCR amplification product, insert 1 of about 0.5 kbp.

[0070]

(In-fusion reaction 4)

The same procedure as in the above section (In-fusion reaction 1) was repeated except that PCR amplification product, vector 1 (50 ng) and PCR amplification product, insert 1 (12 ng) were used to ligate PCR amplification product, vector 1 and PCR amplification product, insert 1. In this manner, in-fusion reaction solution 4 was prepared.

[0071]

(Transformation of *E. coli* and determination of DNA sequence of non-secretory plasmid phIFNg)

E. coli HST16CR was transformed in the same manner as in the above section (Transformation of *E. coli* and determination of DNA sequence of plasmid pAG8) by using the in-fusion reaction solution 4 (2 µL). A plasmid was extracted from the recombinant *E. coli* and the sequence of full-length plasmid DNA was determined. The plasmid extracted was designated as phIFNg (non-secretory plasmid).

[0072]

(Preparation of secretory plasmid hIFNg33)

PCR amplification was carried out using phIFNg mentioned above as a template and a primer set of hIFNG4_primer (forward) and Hu-mCCL21-vecR1_primer (reverse) listed in Table 1. The same procedure as in the above section (Preparation of plasmid pAG8) was repeated except that the amplification program, which is a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 55°C for 5 seconds and a reaction at 75°C for 4 minutes and 20 seconds, was repeated 30 times, and then an elongation reaction was carried out at 72°C for 30 seconds to prepare PCR amplification product, vector 2 of about 4.3 kbp.

[0073]

PCR amplification was carried out using *Bifidobacterium longum* 105-A strain genomic DNA as a template and a primer set of SP69-ins_F1_primer (forward) and SP69-ins_R2_primer (reverse) listed in Table 1. The same procedure as in the above section (Preparation of plasmid pAG8) was repeated except that the amplification program, which is a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 55°C for 5 seconds and a reaction at 75°C for 15 seconds, was repeated 30 times, and then an elongation reaction was carried out at 72°C for 30 seconds to prepare PCR amplification product, insert 2 of about 0.2 kbp.

[0074]

(In-fusion reaction 5)

The same procedure as in the above section (In-fusion reaction 1) was repeated except that PCR amplification product, vector 2 (50 ng) and PCR

amplification product, insert 2 (5 ng) were used to ligate PCR amplification product, vector 2 and PCR amplification product, insert 2. In this manner, in-fusion reaction solution 5 was prepared.

[0075]

(Transformation of *E. coli* and determination of DNA sequence of plasmid phIFNg33)

The same procedure as in the above section (Transformation of *E. coli* and determination of DNA sequence of plasmid pAG8) was repeated by using in-fusion reaction solution 3 (2 μL) obtained above to carry out transformation of the *E. coli* HST16CR competent cells obtained above and extraction of a plasmid from the recombinant *E. coli*. In the plasmid extracted, the sequence of human IFN-γ secretory expression cassette (5'-Hu promoter-SP69L20-human IFN-γ protein-His tag-Hu terminator-3') was determined. The plasmid extracted was designated as phIFNg33. The constitution of plasmid phIFNg33 is shown in Figure 4 (a).

[0076]

(Transformation 3 of bacterium of the genus *Bifidobacterium*)

Bifidobacterium longum 105-A strain was transformed by the electroporation system in the same procedure as in the above section (Transformation 1 of bacterium of the genus *Bifidobacterium*) except that plasmid phIFNg33 (605 ng) extracted from the transformed *E. coli* mentioned above was used. The transformant obtained was designated as *Bifidobacterium longum* 105-A/ phIFNg33 strain (hereinafter referred to as hIFNg33 strain).

[0077]

PCR amplification was carried out using phIFNg33 mentioned above as a template and a primer set of GA5_primer_rev (forward) and GA1_primer (reverse) listed in Table 1. The same procedure as in the above section (Preparation of plasmid pAG8) was repeated except that the amplification program, which is a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 65°C for 5 seconds and a reaction at 75°C for 1 minute and 20 seconds, was repeated 30 times, and then an elongation reaction was carried out at 72°C for 30 seconds to prepare PCR amplification product C of about 1.2 kbp.

[0078]

PCR amplification was carried out using phIFNg33 mentioned above as a template and a primer set of GA2_primer (forward) and GA6_primer (reverse) listed in Table 1. The same procedure as in the above section (Preparation of plasmid pAG8) was repeated except that the amplification program, which is a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 65°C for 5 seconds and a reaction at 75°C for 3 minutes and 20 seconds, was repeated 30 times, and then an elongation reaction was carried out at 72°C for 30 seconds to prepare PCR amplification product D of about 3.3 kbp.

[0079]

(In-fusion reaction 6)

The same procedure as in the above section (In-fusion reaction 1) was repeated except that PCR amplification product C (18 ng) and PCR amplification product D (50 ng) were used to ligate PCR amplification

product C and PCR amplification product D. In this manner, in-fusion reaction solution 6 was prepared.

[0080]

(Transformation of *E. coli* and determination of DNA sequence of plasmid phIFNg33TL)

The same procedure as in the above section (Transformation of *E. coli* and determination of DNA sequence of plasmid pAG8) was repeated by using in-fusion reaction solution 6 obtained above to carry out transformation of the *E. coli* HST16CR competent cells obtained above and extraction of a plasmid from the recombinant *E. coli*. In the plasmid extracted, the sequence of human IFN-γ secretory expression cassette (5'-Hu promoter-SP69L20-human IFN-γ protein-Hu terminator-3') was determined. The plasmid extracted was designated as phIFNg33TL. The constitution of plasmid phIFNg33TL is shown in Figure 4 (b).

[0081]

(Transformation 4 of bacterium of the genus *Bifidobacterium*)

Bifidobacterium longum 105-A strain was transformed by the electroporation system in the same procedure as in the above section (Transformation 1 of bacterium of the genus *Bifidobacterium*) except that plasmid phIFNg33TL (435 ng) extracted from the transformed *E. coli* mentioned above was used. The transformant obtained was designated as *Bifidobacterium longum* 105-A/phIFNg33TL strain (hereinafter referred to as hIFNg33TL strain).

[0082]

[Example 4]

[Verification of hTNF-α and hIFN-γ secretion in AG8TL strain]

The presence or absence of secretion of human TNF-α protein and human IFN-γ protein in the culture supernatant of AG8TL strain was checked by ELISA as follows. A negative control strain, *Bifidobacterium longum* 105A/pBEshuttle strain (hereinafter referred to as BEshuttle strain) was subjected to the same analysis.

[0083]

(Culture of recombinant bacterium of the genus *Bifidobacterium*)

AG8TL strain, TNF11 strain, hIFNg33TL strain and BEshuttle strain each were inoculated in MRS (manufactured by Becton, Dickinson and Company) liquid medium (10 mL) supplemented with spectinomycin (final concentration 75 μg/mL) and 100 μL of a vitamin C solution (solution comprising ascorbic acid (35 g) and L-cysteine hydrochloride monohydrate (2 g) and sodium carbonate (11 g)/100 mL), and anaerobically cultured at 37°C for 24 hours. These were specified as activated culture solutions. Subsequently, to a medium (20 mL) obtained by adding DMEM (Cat No. 11885-084: manufactured by Life Technologies Corporation) and MRS in a ratio of 9: 1, vitamin C solution (100 μL) and spectinomycin (so as to obtain a 75 μg/mL), the above activated culture solution (100 μL) was inoculated. The resultant medium was anaerobically cultured at 37°C for 18 hours.

[0084]

(Collection of culture supernatant and ELISA)

After the anaerobic culture, the culture solutions of the individual strains were centrifuged. The culture supernatants were separately collected and subjected to ELISA. The same operation was applied to the BEshuttle strain and used as a negative control. As the ELISA kit for TNF-α measurement, Quantikine Human TNFα/TNFSF1A Immunoassay (manufactured by R&D systems) was used. As the ELISA kit for hIFN-γ measurement, Quantikine Human IFN-γ Immunoassay (manufactured by R&D systems) was used. The operation of ELISA was carried out in accordance with the product manual of each ELISA kit. The results are shown in Table 2.

[0085]

[Table 2]

(ng/mL culture supernatant)

Recombinant Bifidobacterium strain	Amount of hTNF-α secreted	Amount of hIFN-γ secreted
AG8TL	501	376
TNF11	515	N. D.
hIFNg33TL	N. D.	346
BEshuttle	N. D.	N. D.

[0086]

(Results)

As is apparent from Table 2, it was found that hTNF-α protein and hIFN-γ protein are both present in the culture supernatant of AG8TL strain. It was also found that hTNF-α protein is present in the culture supernatant of a positive control, i.e., single-expression strain TNF11; and that human IFN-γ protein is present in the culture supernatant of hIFNg33TL strain.

[0087]

[Example 5]

[Investigation of proliferative inhibitory activity of AG8TL strain on KPL-1 cell]

(Preparation of culture-supernatant crude purified product)

Each (7 mL) of the culture supernatants of AG8TL strain, TNF11 strain and hIFNg33TL strain mentioned above prepared in the same manner as in Example 4 (Culture of recombinant bacterium of the genus *Bifidobacterium*) and (Collection of culture supernatant and ELISA) was concentrated by use of *Amicon Ultra*-4, MWCO: 10k (manufactured by Millipore), washed three times with PBS buffer (pH7.4) and concentrated to prepare 0.5 mL of a culture-supernatant crude purified product. A crude purified product from the culture-supernatant of the negative control strain, i.e., *Bifidobacterium longum* 105A/pBEshuttle strain, was prepared in the same manner. The crude purified products from individual strains were subjected to measurement of cell proliferation inhibitory activity. The concentrations of hTNF-α and hIFN-γ were estimated by use of an ELISA kit for TNF-α measurement and an ELISA kit hIFN-γ measurement.

[0088]

(Preparation of KPL-1 cell and addition of test sample)

Cell proliferation inhibitory activity was measured by using a human breast cancer cell strain, i.e., KPL-1 cell (provided by Prof. Junichi Kurebayashi, Kawasaki Medical School). KPL-1 cells were cultured in 10% fetal

bovine serum (manufactured by EQUITECH-BIO)/Dulbecco's modified eagle medium (high glucose) (manufactured by Sigma Aldrich) in a 100-mm petri dish. After the (test) medium was removed, the cells were washed with PBS (-) (manufactured by Wako Pure Chemical Industries Ltd.). To the cells, 0.25% Trypsin-EDTA (manufactured by Life Technologies Corporation) was added. The cells were collected and centrifuged. The supernatant was removed and the cells were suspended in the test medium so as to obtain 6×10^3 cells/mL. In this manner, a cell suspension was prepared.

[0089]

The cell suspension was dispensed in individual wells of a 96-well plate in an amount of 0.1 mL per well and cultured in a CO_2 incubator set at 37°C. In the following day, exchange was made with the medium prepared by further adding a crude purified product from culture-supernatant of AG8TL strain (comprising 20 ng/mL hIFN-γ and 51.1 ng/mL hTNF-α) to the test medium mentioned above. In this way, stimulation was started. Medium were prepared by separately adding crude purified products from culture-supernatant of TNF11 strain and hIFNg33TL strain so as to obtain the same concentration in the case of AG8TL strain and subjected to the same treatment as positive controls. Four days after initiation of stimulation, the medium was removed and exchanged for a medium (1/10 volume) supplemented with Cell Counting kit-8 (manufactured by Dojindo Molecular Technologies, Inc.) and the reaction was further carried out for 3 hours. After the reaction, absorbance at 450 nm and 630 nm (reference

wavelength) was measured by a multi-mode plate reader (manufactured by DS Pharma Biomedical Co., Ltd.). The blank was calculated by subtracting the absorbance of a well comprising the medium and Cell Counting kit-8 alone and no cells. Note that, the value obtained from a test medium in which KPL-1 cells were continuously cultured without adding any one of the aforementioned culture-supernatant crude purified products was regarded as absorbance (100%) of an untreated district. The proliferation rate of KPL-1 cells in individual mediums was calculated. The results are shown in Figure 5.

[0090]

(Results)

As is apparent from Figure 5, a secretion from AG8TL strain, significantly suppressed proliferation of KPL-1 cell, compared to a secretion from TNF11 strain alone or hIFNg33TL strain alone. More specifically, TNF11 strain secreting hTNF-α exhibited a proliferation rate of 89.9%; hIFNg33TL strain secreting hIFN-γ exhibited a proliferation rate of 83%; whereas AG8TL strain secreting hTNF-α and hIFN-γ, exhibited a relative cell viability of 35.5%. It was verified that AG8TL strain has a strong proliferation inhibitory effect on a human breast cancer cell strain. Such a result is conceivably due to a combinational effect of the hTNF-α secretion and the hIFN-γ secretion.

[0091]

[Example 6]

[Investigation on proliferation inhibitory activity of AG8TL strain to MIA PaCa-2 cell]

Cell proliferation inhibitory activity to MIA PaCa-2 cell was investigated in the same procedure as in Example 5 except that a human pancreas cancer-derived cell, i.e., MIA PaCa-2 cell (obtained from the independent administrative institution, RIKEN Bio Resource Center) was used in place of KPL-1 cell. Note that, the concentration of MIA PaCa-2 cells suspension dispensed and seeded in 96-well plates was specified as 1×10^4 cells/mL. The results are shown in Figure 6.

[0092]

(Results)

As is apparent from Figure 6, a secretion from AG8TL strain, significantly suppressed proliferation of MIA PaCa-2 cell, compared to a secretion from TNF11 strain alone or hIFNg33TL strain alone. More specifically, TNF11 strain secreting hTNF-α exhibited a proliferation rate of 51.6%; hIFNg33TL strain secreting hIFN-γ exhibited a proliferation rate of 75.4%; whereas AG8TL strain secreting hTNF-α and hIFN-γ, exhibited a proliferation rate of 24.2%. It was verified that AG8TL strain has a strong proliferation inhibitory effect on the human pancreas cancer cell strain. Such a result is conceivably due to a combinational effect of the hTNF-α secretion and the hIFN-γ secretion.

[0093]

[Example 7]

[Investigation of the specificity in cell proliferation inhibitory activity of AG8TL strain by using neutralizing antibodies]

To verify that the cell proliferation inhibitory activity by crude purified product from the culture-supernatant of an AG8TL strain is due to secretion of hTNF-α and hIFN-γ, investigation using a neutralizing antibody, i.e., Human IFN-γ Antibody and Human TNF-α Antibody (both are manufactured by R&D systems) was made as follows.

[0094]

In the same procedure as in Example 6, MIA PaCa-2 cells were seeded onto a 96-well plate. On the following day, exchange was made with a medium comprising any one of the crude purified products from culture-supernatant of AG8TL strain, TNF11 strain, hIFNg33TL strain and BEshuttle strain (negative control) and the antibody shown in the following Table 3, in combination. The materials added to the medium are shown in the following Table 3. Cell proliferation rate was calculated in the same procedure as in Example 5. The results are shown in Figure 7.

[0095]

[Table 3]

Medium	culture-supernatant crude purified product	Secretion	Antibody
1)	AG8TL strain	hIFN-γ (20ng/mL) hTNF-α (51.1ng/mL)	
2)	AG8TL strain	hIFN-γ (20ng/mL) hTNF-α (51.1ng/mL)	Anti-hTNF-α antibody (6μg/mL) Anti-hIFN-γ antibody (1μg/mL)
3)	AG8TL strain	hIFN-γ (20ng/mL) hTNF-α (51.1ng/mL)	Anti-hTNF-α antibody (6μg/mL)
4)	AG8TL strain	hIFN-γ (20ng/mL) hTNF-α (51.1ng/mL)	Anti-hIFN-γ antibody (1μg/mL)
5)	TNF11 strain	hTNF-α (51.1ng/mL)	
6)	hIFNg33TL strain	hIFN-γ (20ng/mL)	
7)	BEshuttle strain		
8)	BEshuttle strain		Anti-hTNF-α antibody (6μg/mL) Anti-hIFN-γ antibody (1μg/mL)

[0096]

(Results)

As is apparent from Figure 7, MIA PaCa-2 cell proliferation rate was 15.3% at the time when the culture-supernatant crude purified product of an AG8TL strain was added; however, the cell proliferation rates at the time when the anti-hIFN-γ antibody alone and the anti-hTNF-α

antibody alone were added increased to 31.8% and 44.4%, respectively. The cell proliferation rate increased to 74.1% at the time when both anti-hIFN-γ antibody and anti-hTNF-α antibody were added. This is because the bioactivities of hTNF-α and hIFN-γ secreted from AG8TL strain wereneutralized by these antibodies. It is considered that proliferation inhibition of MIA PaCa-2 cell is due to hTNF-α and hIFN-γ secreted by AG8TL strain.
[0097]

[Example 8]

[Preparation of anti-hPD-1scFv03 and anti-hCTLA-4scFv02 co-expression *Bifidobacterium* PC1 strain and CP1 strain]

(Outline)

Co-expression plasmids pPC1 and pCP1 were prepared, which contain an expression cassette for secretory anti-hPD-1scFv03 (anti-human PD-1scFv03 secretory expression cassette) and an expression cassette for secretory anti-hCTLA-4scFv02FLAG (anti-human CTLA-4scFv02FLAG secretory expression cassette), respectively and which serve as *E. coli-Bifidobacterium* shuttle vector. *Bifidobacterium longum* 105-A strain was transformed with these plasmids pPC1 and pCP1 in accordance with electroporation to obtain anti-hPD-1scFv03 and anti-hCTLA-4scFv02 co-expression *Bifidobacterium* PC1 strain and CP1 strain. The primers used in Example 8 are shown in the following Table 4.
[0098]

[Table 4]

Primer Name	DNA sequence (5' -> 3')	SEQ ID No.
Ins-hPD-1 scFv03-F1	CAGGTCCAGCTGGTCGAATCGGGCGGCGGC	SEQ ID No. 36
Ins-hPD-1 scFv03-R1	ACGAGCAGAAGGTCAGTGGTGGTGATGATGGTGCTT	SEQ ID No. 37
TGA-Hu-Terminator-F	TGACCTTCTGCTCGTAGCGATTAC	SEQ ID No. 38
vec-SP7L20-R1	GACCAGCTGGACCTGCACCGAACTCGCCTTCGGGAA	SEQ ID No. 39
Ins-hCTLA-4 scFv02-F1	CAGGTCCAGCTGGTCGAATCGGGCGGCGGC	SEQ ID No. 40
Ins-hCTLA-4 scFv02-R1	ACGAGCAGAAGGTCAGTGATGATGATGATGATGCTT	SEQ ID No. 41
hCTLA-4 scFv02-FLAG-F1	GACTACAAGGACGACGACGACAAGTGACCTTCTGCTCGTAGCGAT	SEQ ID No. 42
hCTLA-4 scFv02-FLAG-R1	GTCGTCCTTGTAGTCCTTGATTTCCACCTTGGT	SEQ ID No. 43
InF_pTB6 rep_F1	ACTAGTCCTCCAGGACCTCGTCTACGAGGC	SEQ ID No. 44
InF_Hu Prom_F1	GTCTTCCTGCTGGCCTATGCATTGGGTTCC	SEQ ID No. 45
InF_Hu Term-Hu Prom_R1	GGCCAGCAGGAAGACCCGGAATAATACGGTTGGAC	SEQ ID No. 46
InF_Hu Term-pTB6_R1	TCCTGGAGGACTAGTCCGGAATAATACGGTTGGAC	SEQ ID No. 47

[0099]

(Preparation of anti-human PD-1scFv03 secretion plasmid pHuSP7L20-hPD-1scFv03)

In preparing pPC1 and pCP1, anti-human PD-1scFv03 secretion plasmid pHuSP7L20-hPD-1scFv03 was first prepared.

[0100]

(Constitution of anti-human PD-1scFv03 secretory expression cassette)

As an anti-human PD-1scFv03 secretory expression cassette, a cassette sequentially comprising (1) a Hu promoter DNA, (2) a DNA encoding a signal peptide-linker conjugate, SP7L20, (3) a DNA encoding the amino acid sequence of anti-hPD-1scFv03 (comprising a heavy chain sequence, a linker $(GGGGS)_3$ and a light chain sequence), (4) a DNA encoding a His tag sequence and (5) a Hu

terminator DNA was prepared such that DNA (1) was placed upstream (the 5' end) and DNA (5) downstream (the 3' end). As to the nucleotide sequence of DNA encoding the amino acid sequence of anti-hPD-1scFv03 (comprising a heavy chain sequence, a linker $(GGGGS)_3$ and a light chain sequence), the document shown in Table 5 (1) was referred to.

[0101]

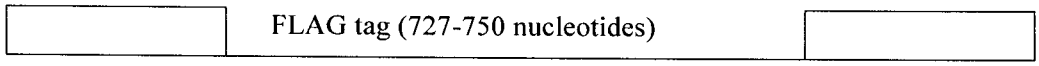
(Artificial DNA synthesis of anti-hPD-1scFv03)

Anti-hPD-1scFv03 represented by SEQ ID No. 11 was sub-cloned to *E. coli* plasmid pUC57 by GenScript Japan Inc. and used as artificially synthesized plasmid, pUC57-hPD-1scFv03.

[0102]

[Table 5]

Antibody		Reference document
(1) Anti-hPD-1 scFv03	SEQ ID No. 11 (729 nucleotide sequence) Heavy chain sequence (1-342 nucleotides) $(GGGGS)_3$ linker (343-387 nucleotides) Light chain sequence (388-708 nucleotides) Histidine tag (709-726 nucleotides)	Japanese Patent No. 5028700
(2) Anti-hCTLA-4 scFv02	SEQ ID No. 12 (747 nucleotide sequence) Heavy chain sequence (1-357 nucleotides) $(GGGGS)_3$ linker (358-402 nucleotides) Light chain sequence (403-726 nucleotides) Histidine tag (727-744 nucleotides)	Japanese Patent No. 4093757
(3) Anti-hCTLA-4 scFv02FLAG	SEQ ID No. 13 (753 nucleotide sequence) Heavy chain sequence (1-357 nucleotides) $(GGGGS)_3$ linker (358-402 nucleotides) Light chain sequence (403-726 nucleotides)	Japanese Patent No. 4093757



[0103]

(Preparation 1 of anti-hPD-1scFv03 insert fragment)

PCR amplification was carried out using plasmid, pUC57-hPD-1scFv03 (500 pg) as a template and a primer set of Ins-hPD-1scFv03-F1 primer (forward) and Ins-hPD-1scFv03-R1 primer (reverse) listed in Table 4. Each of the primer sequences was designed such that an insert fragment was overlapped with a vector fragment at the end of 15 bp. The PCR amplification was carried out by specifying the concentration of each primer as 0.2 μM and the reaction volume as 30 μL and using STAR kit. As the amplification program, a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 55°C for 5 seconds and a reaction at 72°C for 60 seconds was repeated 30 times. The insert PCR product amplified was electrophoresed on a 2% agarose gel and then purified by QIAquick Gel Extraction kit (hereinafter referred to as "QIAGel", manufactured by QIAGEN) to prepare an anti-hPD-1scFv03 insert fragment (1) of about 0.7 kbp.

[0104]

(Preparation of vector fragment (1) comprising DNA encoding the amino acid sequence of SP7L20)

PCR amplification was carried out using the vector linearized fragment (500 pg) represented by SEQ ID No. 14 as a template and a primer set of TGA-Hu-terminator-F primer (forward) and vec-SP7L20-R1 primer (reverse) listed in Table 4. The PCR amplification was carried out by specifying the concentration of each primer as 0.2 μM and

the reaction volume as 30 μL and using STAR kit. As the amplification program, a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 55°C for 5 seconds and a reaction at 72°C for 5 minutes, was repeated 30 times. The PCR product amplified was electrophoresed on a 0.8% agarose gel and then purified by QIAGel to prepare a 5'-Hu terminator-pTB6rep unit-SPCMr-pUCori-Hu promoter-SP7L20-3' vector fragment (1) of about 4.0 kbp.

[0105]

(In-fusion reaction 7)

The vector fragment (1) prepared above and the anti-hPD-1scFv03 insert fragment (1) were ligated by use of In-Fusion (registered trademark) HD Cloning kit. More specifically, the vector (fragment) and the insert (fragment) in the kit were added in a molar ratio of 1: 5 in a micro tube; then, 2 μL of 5X In-Fusion HD Enzyme premix was added; and the volume of the reaction solution was adjusted to be 10 μL. The reaction solution was kept warm at 50°C for 15 minutes. The procedure other than this was carried out in accordance with the product manual of the kit to prepare in-fusion reaction solution 7.

[0106]

(Transformation of *E. coli* and determination of DNA sequence of pHuSP7L20-hPD-1scFv03)

The same procedure as in the above section (Transformation of *E. coli* and determination of DNA sequence of plasmid pAG8) was repeated except that 5 μL of in-fusion reaction solution 7 was used to carry out transformation of the *E. coli* HST16CR competent cell obtained above and extraction of a plasmid from the

recombinant *E. coli*. Note that, after the transformation, *E. coli* colony formed on an agar medium was picked up and cultured in LB liquid medium containing 75 μg/mL spectinomycin while shaking at 30°C overnight. The sequence of anti-human PD-1scFv03 secretory expression cassette (5'-Hu promoter-SP7L20-anti-hPD-1scFv03-His tag-Hu terminator-3') in the plasmid extracted was determined in the same procedure as above. The plasmid extracted was designated as pHuSP7L20-hPD-1scFv03. The constitution of pHuSP7L20-hPD-1scFv03 is shown in Figure 8 (A) and the sequence thereof is represented by SEQ ID No. 6.

[0107]

(Transformation 5 of bacterium of the genus *Bifidobacterium*)

Bifidobacterium longum 105-A strain was transformed by the electroporation system in the same procedure as in the above section (Transformation 1 of bacterium of the genus *Bifidobacterium*) except that plasmid pHuSP7L20-hPD-1scFv03 (550 ng) extracted from the transformed *E. coli* mentioned above was used. The transformant obtained was designated as *Bifidobacterium longum* 105-A/pHuSP7L20-hPD-1scFv03 strain (hereinafter referred to as hPD-1scFv03 strain).

[0108]

(Preparation of anti-hCTLA-4scFv02 secretion plasmid pHuSP7L20-hCTLA-4scFv02)

In preparing pPC1 and pCP1, anti-hCTLA-4scFv02 secretion plasmid pHuSP7L20-hCTLA-4scFv02 without FLAG tag was first prepared.

[0109]

(Constitutions of anti-human CTLA-4scFv02 secretory expression cassette and anti-human CTLA-4scFv02FLAG secretory expression cassette)

As an anti-human CTLA-4scFv02 secretory expression cassette, a cassette sequentially comprising (1) a Hu promoter DNA, (2) a DNA encoding a signal peptide-linker conjugate, SP7L20, (3) a DNA encoding the amino acid sequence of anti-hCTLA-4scFv02 (comprising a heavy chain sequence, a linker $(GGGGS)_3$ and a light chain sequence), (4) a DNA encoding a His tag sequence and (5) a Hu terminator DNA, was prepared such that DNA (1) was placed upstream (the 5' end) and DNA (5) downstream (the 3' end). As to the nucleotide sequence of DNA encoding the amino acid sequence of anti-hCTLA-4scFv02 (comprising a heavy chain sequence, a linker $(GGGGS)_3$ and a light chain sequence), the document shown in Table 5 (2) was referred to.

[0110]

As an anti-human CTLA-4scFv02FLAG secretory expression cassette, a cassette sequentially comprising (1) a Hu promoter DNA, (2) a DNA encoding a signal peptide-linker conjugate, SP7L20, (3) a DNA encoding the amino acid sequence of anti-hCTLA-4scFv02 (comprising a heavy chain sequence, a linker $(GGGGS)_3$ and a light chain sequence), (4) a DNA encoding a FLAG tag sequence and (5) a Hu terminator DNA, was prepared such that DNA (1) was placed upstream (the 5' end) and DNA (5) downstream (the 3' end). As to the nucleotide sequence of DNA encoding the amino acid sequence of anti-hCTLA-4scFv02 (comprising a heavy chain sequence, a linker $(GGGGS)_3$ and a light

chain sequence), the document shown in Table 5 (3) was referred to.

[0111]

(Artificial DNA synthesis of hCTLA-4scFv02)

The fragment, hCTLA-4scFv02 represented by SEQ ID No. 12 was subcloned to *E. coli* plasmid pUC57 by GenScript Japan Inc. and used as artificially synthesized plasmid pUC57-hCTLA-4scFv02.

[0112]

(Preparation of anti-hCTLA-4scFv02 insert fragment)

The same procedure as in the above section (Preparation 1 of anti-hPD-1scFv03 insert fragment) was repeated except that the plasmid pUC57-hCTLA-4scFv02 as a template and a primer set of Ins-hCTLA-4scFv02-F1 (forward) and Ins-hCTLA-4scFv02-R1 (reverse) listed in Table 4 were used to prepare anti-hCTLA-4scFv02 insert fragment of about 0.8 kbp.

[0113]

(In-fusion reaction 8)

An in-fusion reaction solution was prepared in the same procedure as in the in-fusion reaction in the above section (In-fusion reaction 7) except that the vector fragment (1) and the anti-hCTLA-4scFv02 insert fragment were used.

[0114]

(Transformation of *E. coli* and determination of DNA sequence of pHuSP7L20-hCTLA-4scFv02)

The same procedure as in the above section (Transformation of *E. coli* and determination of DNA sequence of plasmid pHuSP7L20-hPD-1scFv03) was repeated

except that 5 μL of in-fusion reaction solution 8 was used to carry out transformation of *E. coli* HST08 competent cell. A sequencing reaction was carried out for determining the sequence of anti-human CTLA-4scFv02 secretory expression cassette (5'-Hu promoter-SP7L20-anti-hCTLA-4scFv02-His tag-Hu terminator-3') in the plasmid extracted. The plasmid extracted was designated as pHuSP7L20-hCTLA-4scFv02. The constitution of pHuSP7L20-hCTLA-4scFv02 is shown in Figure 8 (B) and the sequence thereof is represented by SEQ ID No. 7.

[0115]

[Preparation of anti-hCTLA-4scFv02FLAG single expression *Bifidobacterium* strain]

(Preparation of vector fragment comprising anti-hCTLA-4scFv02FLAG secretory expression cassette)

PCR amplification was carried out using plasmid pHuSP7L20-hCTLA-4scFv02 (500 pg) mentioned above as a template and a primer set of hCTLA-4scFv02-FLAG-F1 (forward) and hCTLA-4scFv02-FLAG-R1 (reverse) listed in Table 4. The primer sequences were designed such that both ends (15 bp.) of the PCR products were overlapped. The PCR amplification was carried out by specifying the concentration of each primer as 0.2 μM and the reaction volume as 30 μL and using the STAR kit. As the amplification program, a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 55°C for 5 seconds and a reaction at 72°C for 5 minutes, was repeated 30 times to prepare a vector fragment amplified product of about 4.8 kbp comprising an anti-hCTLA-4scFv02FLAG secretory expression cassette.

[0116]

(In-fusion reaction 9)

The vector fragment amplified product comprising the anti-human CTLA-4scFv02FLAG secretory expression cassette prepared above was subjected to an in-fusion reaction by use of In-Fusion (registered trademark) HD Cloning kit to close the ring by itself. More specifically, a reaction solution was prepared by mixing the vector fragment amplified product (5 μL) and Cloning Enhancer (2 μL), placed in a micro tube and kept warm at 37°C for 15 minutes, subsequently at 80°C for 15 minutes. Then, 0.5 μL of the reaction solution and 2 μL of 5X In-Fusion HD Enzyme premix were blended and the volume of the resultant reaction solution was adjusted to be 10 μL. The reaction solution was kept warm at 50°C for 15 minutes. The procedure other than this was carried out in accordance with the production manual of the kit to prepare in-fusion reaction solution 9.

[0117]

(Transformation of *E. coli* and determination of DNA sequence of pHuSP7L20-hCTLA-4scFv02FLAG)

The same procedure as in the above section (Transformation of *E. coli* and determination of DNA sequence of plasmid pHuSP7L20-hPD-1scFv03) was repeated except that 5 μL of in-fusion reaction solution 9 was used to carry out transformation of the *E. coli* HST08 competent cell obtained above and extraction of a plasmid from the recombinant *E. coli*. The sequence of anti-human CTLA-4scFv02FLAG secretory expression cassette (5'-Hu promoter-SP7L20-anti-human CTLA-4scFv02-FLAG tag-Hu terminator-3')

in the plasmid extracted was determined in the same procedure as above. The plasmid extracted was designated as pHuSP7L20-hCTLA-4scFv02FLAG. The constitution of pHuSP7L20-hCTLA-4scFv02FLAG is shown in Figure 8 (C) and the sequence thereof is represented by SEQ ID No. 8.

[0118]

(Transformation 6 of bacterium of the genus *Bifidobacterium*)

Bifidobacterium longum 105-A strain was transformed by the electroporation system in the same procedure as in the above section (Transformation 1 of bacterium of the genus *Bifidobacterium*) except that plasmid pHuSP7L20-hCTLA-4scFv02FLAG (256 ng) was used. The transformant obtained was designated as *Bifidobacterium longum* 105-A/pHuSP7L20-hCTLA-4scFv02FLAG strain (hereinafter referred to as hCTLA-4scFv02 strain).

[0119]

[Preparation of anti-hPD-1scFv03 and anti-hCTLA-4scFv02 co-expression strain]

PC1 strain and CP1 strain secreting both anti-hPD-1scFv03 and anti-hCTLA-4scFv02 were prepared. As is shown in Figure 9 (a), PC1 strain has a plasmid having a secretory expression cassette following the 3' end side of the origin of replication, pUCori, of *E. coli*. This secretory expression cassette is anti-human PD-1scFv03 secretory expression cassette, which is followed by anti-human CTLA-4scFv02 secretory expression cassette. As shown in Figure 9 (b), CP1 strain has a plasmid having a secretory expression cassette following the 3' end side of the origin of replication, pUCori, of *E. coli*. This

secretory expression cassette is anti-human CTLA-4scFv02 secretory expression cassette which is followed by anti-human PD-1scFv03 secretory expression cassette.

[0120]

[Preparation of PC1 strain]

(Preparation of anti-hCTLA-4scFv02FLAG insert fragment)

PCR amplification was carried out using plasmid pHuSP7L20-hCTLA-4scFv02FLAG (500 pg) (SEQ ID No. 8) as a template and a primer set of InF_Hu Prom_F1 (forward) and InF_Hu Term-pTB6_R1 (reverse) listed in Table 4. Each of the primer sequences was designed such that an insert fragment was overlapped with a vector fragment at the end of 15 bp. The PCR amplification was carried out by specifying the concentration of each primer as 0.2 μM and the reaction volume as 30 μL and using STAR kit. As the amplification program, a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 55°C for 5 seconds and a reaction at 72°C for 2 minutes, was repeated 30 times. The insert PCR product amplified was electrophoresed on a 0.8% agarose gel and then purified by use of QIAGel to prepare an anti-human CTLA-4scFv02FLAG insert fragment of about 1.4 kbp.

[0121]

(Preparation of vector fragment comprising anti-hPD-1scFv03 secretory expression cassette)

PCR amplification was carried out using plasmid pHuSP7L20-hPD-1scFv03 (500 pg) (SEQ ID No. 6) mentioned above as a template and a primer set of InF_pTB6rep_F1 (forward) and InF_HuTerm-HuProm_R1 (reverse) listed in

Table 4. Each of the primer sequences was designed such that an insert fragment was overlapped with a vector fragment at the end of 15 bp. The PCR amplification was carried out by specifying the concentration of each primer as 0.2 μM and the reaction volume as 30 μL and using PrimeSTAR HS (Premix) kit mentioned above. As the amplification program, a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 55°C for 5 seconds and a reaction at 72°C for 5 minutes, was repeated 30 times. The insert PCR product amplified was electrophoresed on a 0.8% agarose gel and then purified by QIAquick Gel Extraction kit to prepare a vector fragment (2) of about 4.7 kbp comprising anti-hPD-1scFv03 secretory expression cassette.

[0122]

(In-fusion reaction 10)

The vector fragment (2) prepared in the above and the anti-human CTLA-4scFv02FLAG insert fragment were ligated by use of In-Fusion (registered trademark) HD Cloning kit mentioned above. More specifically, the vector (fragment) and the insert (fragment) in the kit were added in a molar ratio of 1:3 in a micro tube; and then, 2 μL of 5X In-Fusion HD Enzyme premix was added; and the volume of the reaction solution was adjusted to be 10 μL. The reaction solution was kept warm at 50°C for 15 minutes. The procedure other than this was carried out in accordance with the product manual of the kit to prepare in-fusion reaction solution 10.

[0123]

(Transformation of *E. coli* and determination of DNA sequence of pPC1)

The same procedure as in the above section (Transformation of *E. coli* and determination of DNA sequence of plasmid pHuSP7L20-hPD-1scFv03) was repeated except that 2 μL of in-fusion reaction solution 10 was used to carry out transformation of the *E. coli* HST16CR competent cell obtained above and extraction of a plasmid from the recombinant *E. coli*. The sequences of anti-human PD-1scFv03 secretory expression cassette (5'-Hu promoter-SP7L20-anti-hPD-1scFv03-His tag-Hu terminator-3') and anti-human CTLA-4scFv02FLAG secretory expression cassette (5'-Hu promoter-SP7L20-anti-hCTLA-4scFv02-FLAG tag-Hu terminator-3') in the plasmid extracted were determined in the same procedure as above. The plasmid extracted was designated as pPC1. The constitution of pPC1 is shown in Figure 9 (a) and the DNA sequence thereof is represented by SEQ ID No. 9.

[0124]

(Transformation 7 of bacterium of the genus *Bifidobacterium*)

Bifidobacterium longum 105-A strain was transformed by the electroporation system in the same procedure as in the above section (Transformation 1 of bacterium of the genus *Bifidobacterium*) except that plasmid pPC1 (250 ng) mentioned above was used. The transformant obtained was designated as *Bifidobacterium longum* 105-A/pPC1 strain (hereinafter referred to as PC1 strain).

[0125]

[Preparation of CP1 strain]

(Preparation 2 of anti-hPD-1scFv03 insert fragment)

PCR amplification and purification by a gel extraction kit were carried out in the same procedure as in the above section (Preparation of anti-human CTLA-4scFv02FLAG insert fragment) except that plasmid pHuSP7L20-hPD-1scFv03 (500 pg) (SEQ ID No. 6) mentioned above was used as a template and a primer set of InF_HuProm_F1 (forward) and InF_HuTerm-pTB6_R1 (reverse) listed in Table 4 were used to prepare anti-hPD-1scFv03 insert fragment (2) of about 1.4 kbp.

[0126]

(Preparation of vector fragment comprising anti-human CTLA-4scFv02FLAG secretory expression cassette)

PCR amplification and purification by a gel extraction kit were carried out in the same procedure as in the above section (Preparation 2 of anti-hPD-1scFv03 insert fragment) except that plasmid pHuSP7L20-hCTLA-4scFv02FLAG (SEQ ID No. 8) mentioned above was used as a template and a primer set of InF_pTB6rep_F1 (forward) and InF_HuTerm-HuProm_R1 (reverse) listed in Table 4 were used to prepare a vector fragment (3) of about 4.8 kbp comprising anti-human CTLA-4scFv02FLAG secretory expression cassette.

[0127]

(In-fusion reaction 11)

In-fusion reaction solution 11 was prepared in the same procedure as in the above section (In-fusion reaction 10) except that the vector fragment (3) prepared above and the anti-hPD-1scFv03 insert fragment (2) were used.

[0128]

(Transformation of *E. coli* and determination of DNA sequence of pCP1)

Transformation of *E. coli* HST16CR competent cell and plasmid extraction from recombinant *E. coli* were carried out in the same procedure as in the above section (Transformation of *E. coli* and determination of DNA sequence of plasmid pHuSP7L20-hPD-1scFv03) except that 2 μL of in-fusion reaction solution 11 was used. The sequences of anti-human PD-1scFv03 secretory expression cassette (5'-Hu promoter-SP7L20-anti-hPD-1scFv03-His tag-Hu terminator-3') and anti-human CTLA-4scFv02FLAG secretory expression cassette (5'-Hu promoter-SP7L20-anti-hCTLA-4scFv02-FLAG tag-Hu terminator-3') in the plasmid extracted were determined in the same procedure as the above section (Transformation of *E. coli* and determination of DNA sequence of pPC1). The plasmid extracted was designated as pCP1. The constitution of pCP1 is shown in Figure 9 (b) and the DNA sequence of pCP1 is represented by SEQ ID No. 10.

[0129]

(Transformation 8 of bacterium of the genus *Bifidobacterium*)

Bifidobacterium longum 105-A strain was transformed by the electroporation system in the same procedure as in the above section (Transformation 1 of bacterium of the genus *Bifidobacterium*) except that plasmid pCP1 (250 ng) mentioned above was used. The transformant obtained was designated as *Bifidobacterium longum* 105-A/pCP1 strain (hereinafter referred to as CP1 strain).

[0130]

[Example 9]

[Verification of anti-hPD-1scFv and anti-hCTLA-4scFv secretion in PC1 strain and CP1 strain]

The presence or absence of anti-hPD-1scFv03 (labeled with a histidine tag) and anti-hCTLA-4scFv02 (labeled with FLAG tag) in the culture supernatants of PC1 strain and CP1 strain was checked by western blotting as follows. As a negative control strain, a BEshuttle strain was subjected to the same analysis.

[0131]

(Culture of recombinant bacterium of the genus *Bifidobacterium*)

Bifidobacterium strains, i.e., PC1 strain, CP1 strain, hPD-1scFv03 strain, hCTLA-4scFv02 strain and BEshuttle strain were cultured in the same procedure as in Example 4 (Culture of recombinant bacterium of the genus *Bifidobacterium*) and the culture supernatants were collected. The proteins in the culture supernatants were precipitated with trichloroacetic acid (TCA, manufactured by Wako Pure Chemical Industries Ltd.), washed with acetone, dissolved in a buffer for SDS-PAGE and treated with heat at 95°C for 3 minutes to obtain culture supernatant concentrates.

[0132]

(Western analysis)

Each of the culture supernatant concentrates (corresponding to about 0.1 mL of the culture solution) was electrophoresed by Mini-PROTEAN (registered trademark) TGX™ gel (4 to 20%) (manufactured by Bio-Rad). The resultant gel was transferred to PVDF membrane (iBlot

Transfer Stacks, manufactured by Life Technologies Corporation) by use of iBlot transfer device (manufactured by Life Technologies Corporation). After completion of blotting, the PVDF membrane was blocked with 2%ECL Prime Blocking agent (manufactured by GE Healthcare Japan) in TTBS. Electrophoresis and blotting to the membrane were carried out double. One of the PVDF membranes was used for detection of anti-hPD-1scFv03 using an anti-histidine antibody (THE His Tag Antibody, mAb, Mouse) (manufactured by GenScript) as a primary antibody and ECL peroxidase labelled anti-mouse antibody (manufactured by GE Healthcare) as a secondary antibody. The other PVDF membrane was used for detection of anti-hCTLA-4scFv02 using anti-FLAG antibody Monoclonal ANTI-FLAG M2 Antibody (produced in mouse) (manufactured by Sigma) as a primary antibody and an ECL peroxidase labelled anti-mouse antibody (manufactured by GE Healthcare) as a secondary antibody. After completion of the antibody reactions, the membranes were illuminated by Western Lightning Ultra (manufactured by PerkinElmer Co., Ltd.). These were analyzed by imaging equipment, MYECL Imager (manufactured by Thermo Scientific). The results are shown in Figure 10.

[0133]

(Results)

As is apparent from Figure 10, both anti-hPD-1scFv03 and anti-hCTLA-4scFv02 were detected in the culture supernatants of PC1 strain (lane 1 both in A and B) and CP1 strain (lane 2 both in A and B) comprising anti-human PD-1scFv03 secretory expression cassette having a His tag fused to the C terminal of the anti-hPD-1scFv03 and anti-

human CTLA-4scFv02FLAG secretory expression cassette having a FLAG tag fused to C terminal of the anti-hCTLA-4scFv02. The sizes of hPD-1scFv secretory proteins from PC1 strain (A: lane 1) and CP1 strain (A: lane 2) were the same as the size of hPD-1scFv secretory protein from hPD-1scFv03 single-expression strain (A: lane 3); and the sizes of hCTLA-4scFv secretory proteins from PC1 strain (B: lane 1) and CP1 strain (B: lane 2) were the same as the size of hCTLA-4scFv secretory protein from hCTLA-4scFv02 single-expression strain (B: lane 4). Accordingly, it was verified that co-expression *Bifidobacterium* strains, i.e., PC1 strain and CP1 strain, are recombinant *Bifidobacterium* strains capable of secreting both anti-hPD-1scFv and anti-hCTLA-4scFv in the culture supernatant.

[0134]

[Example 10]

[Verification of binding of antibody secreted from PC1 strain to hPD-1 and hCTLA-4]

The presence or absence of binding activity of anti-hPD-1scFv03, which was purified from the culture supernatant of co-expression strain PC1, to human PD-1 (hPD-1); and the presence or absence of binding activity of anti-hCTLA-4scFv02 to human CTLA-4 were checked by ELISA.

[0135]

To 96-well plates, hPD-1 (Recombinant Human hPD-1 Fc Chimera, manufactured by R&D systems); hCTLA-4 (Recombinant Human CTLA-4-Fc Chimera, manufactured by Biolegend); mPD-1 (Recombinant Human hPD-1 Fc Chimera, manufactured by R&D systems); and mCTLA-4 (Recombinant

mouse CTLA-4-Fc Chimera, manufactured by Biolegend), which were adjusted to be 1 μg/mL with 1X PBS, were dispensed in an amount of 100 μL for each and incubated at 4°C overnight for immobilization. After the liquid was removed, 1X PBS (350 μL) for each was dispensed and the liquid was removed. This operation was repeated three times for washing. To each of the plates, 1% BSA solution (350 μL) was dispensed at room temperature and incubated for 2 hours for blocking. After the liquid was removed, 1X PBS (350 μL) for each was dispensed and the liquid was removed. This operation was repeated three times for washing.

[0136]

Anti-hPD-1scFv03 and anti-hCTLA-4scFv02 were adjusted to be 1000 ng/mL, 100 ng/mL, 10 ng/mL and 1 ng/mL with a signal enhancing reagent (Signal Enhancer HIKARI, manufactured by Nacalai Tesque) and dispensed to the plates each in an amount of 100 μL after completion of the blocking. To blank wells, the signal enhancing reagent alone was dispensed in an amount of 100 μL. A seal was attached to the plates and the plates were incubated at room temperature for 2 hours to allow anti-hPD-1scFv03 to react with immobilized hPD-1, mPD-1 and hCTLA-4. Similarly, anti-hCTLA-4scFv02 was allowed to react with immobilized hCTLA-4, mCTLA-4 and hPD-1. After the liquid was removed, 1X PBS (350 μL for each) was dispensed and the liquid was removed. This operation was repeated three times for washing.

[0137]

The secondary antibody (Anti-Histag-Biotin, manufactured by MBL) was diluted with the signal enhancing reagent to 2000 fold. The dilution (100 µL) was dispensed to wells reacted with anti-hPD-1scFv03. Similarly, secondary antibody (THE (registered trademark) DYKDDDDK Tag Antibody [Biotin], mAb, mouse, manufactured by GenScript) was diluted with the signal enhancing reagent to 2500 fold. The dilution (100 µL) was dispensed to wells and allowed to react with anti-hCTLA-4scFv02. A seal was attached to the plates and the plates were incubated at room temperature for 2 hours. After the liquid was removed, 1X PBS (350 µL) was dispensed for each and the liquid was removed. This operation was repeated three times for washing.

[0138]

Three drops of each of solution A and solution B serving as the avidin-biotin marker enzyme complex (VECTASTAIN ABC kit, manufactured by Vector) were added to the signal enhancing reagent (7.5 mL). The mixture was dispensed in an amount of 100 µL for each plate. Thereafter, a seal was attached to the plates and the plates were incubated at room temperature for 30 minutes. After the liquid was removed, 1X PBS (350 µL for each) was dispensed and the liquid was removed. This operation was repeated three times for washing.

[0139]

A detection reagent was prepared by adding Color Solution A and Color Solution B (manufactured by R&D systems) in equal amounts, and dispensed in an amount of 200 µL for each. The plates were shielded from light and

incubated at room temperature for 20 minutes. Accurately 20 minutes later, Stop Solution (R&D Systems) was added in an amount of 50 μL for each to terminate the coloring reaction. Absorbance was measured at 450 nm and 570 nm (reference wavelength). The results of binding of anti-hPD-1scFv03 to a human PD-1 immobilized plate determined by ELISA are shown in the following Table 6 and Figure 11. Similarly, the results of binding of anti-hCTLA-4scFv02 to a human CTLA-4 immobilized plate are shown in the following Table 7 and Figure 12.

[0140]

[Table 6]

Binding of anti-hPD-1scFv03 to human PD-1 immobilized plate (OD value)

Concentration of anti-hPD-1scFv03	hPD-1 immobilized	hCTLA-4 immobilized	mPD-1 immobilized
1ng/mL	-0.030	-0.062	-0.059
10ng/mL	0.373	-0.055	-0.057
100ng/mL	3.774	-0.018	-0.049
1000ng/mL	3.788	0.341	0.107

[0141]

[Table 7]

Binding of anti-hCTLA-4scFv02 to human CTLA-4 immobilized plate (OD value)

Concentration of anti-hCTLA-4scFv02	hPD-1 immobilized	hCTLA-4 immobilized	mCTLA-4 immobilized
1ng/mL	-0.018	0.009	0.026
10ng/mL	-0.031	0.026	0.005
100ng/mL	-0.038	0.255	0.001
1000ng/mL	-0.001	1.419	0.051

[0142]

(Results)

As is apparent from Table 6 and Figure 11, anti-hPD-1scFv03 which was purified from the culture supernatant of co-expression strain PC1, bound to hPD-1. As is apparent from Table 7 and Figure 12, anti-hCTLA-4scFv02 bound to hCTLA-4. In contrast, anti-hPD-1scFv03 bound to neither hCTLA-4 nor mPD-1; and anti-hCTLA-4scFv02 bound to neither hPD-1 nor mCTLA-4. From these, it was verified that anti-hPD-1scFv03 and anti-hCTLA-4scFv02 secreted from co-expression strain PC1, specifically bind to the corresponding antigen proteins.

[0143]

[Example 11]

[Competitive inhibitory activity of anti-hPD-1scFv in binding reaction of human PD-L1 to human PD-1]

It is said that when ligand PD-L1 binds to PD-1, a negative signal is transmitted to T cell to suppress an immune response (immune tolerance). Then, Competitive inhibitory activity of anti-hPD-1scFv in binding reaction of human PD-L1 to human PD-1 was investigated.

[0144]

Whether anti-hPD-1scFv03 purified from the culture supernatant of co-expression strain PC1 has a competitive binding inhibition activity in binding of human PD-L1 (hPD-L1) to human PD-1 (hPD-1) was checked by ELISA. As a negative control, anti-hCTLA-4scFv02 was used.

[0145]

To 96-well plates, hPD-1 (Recombinant HumanPD-1 Fc Chimera, manufactured by R&D systems), the concentration

of which was adjusted to be 1 μg/mL with 1X PBS, was dispensed in an amount of 100 μL for each and incubated at 4°C overnight for immobilization. After the liquid was removed, 1X PBS (350 μL) was dispensed and the liquid was removed. This operation was repeated three times for washing.

[0146]

To the plates, 1% BSA solution (350 μL for each) was dispensed and incubated at room temperature for 2 hours for blocking. After the liquid was removed, 1X PBS (350 μL) for each was dispensed and the liquid was removed. This operation was repeated three times for washing. Ligand, hPD-L1 (R&D Systems, Recombinant HumanB7-H1/PD-L1 FcChimera) was adjusted to be 2000 ng/mL with the signal enhancing reagent.

[0147]

Anti-hPD-1scFv03 purified from a bacterium of the genus *Bifidobacterium* was adjsuted to be 20000 ng/mL, 2000 ng/mL, 200 ng/mL and 20 ng/mL with the signal enhancing reagent and mixed with hPD-L1 in equal amounts (120 μL). The resultant solutions was each (100 μL) dispensed to plates after completion of blocking. As a negative control, anti-hCTLA-4scFv02 purified from a bacterium of the genus *Bifidobacterium* was subjected to the same operation. To blank wells, the signal enhancing reagent alone (100 μL) was dispensed. A seal was attached to the plate and the plate was incubated at room temperature for 2 hours to allow hPD-L1 mixed with anti-hPD-1scFv03 to react with immobilized hPD-1. After the liquid was removed, 1X PBS (350 μL) was dispensed and the liquid was

removed. This operation was repeated three times for washing. A secondary antibody (Biotinanti-human CD274, manufactured by Biolegend) to hPD-L1 was adjusted to be 0.2 µg/mL with the signal enhancing reagent and dispensed in an amount of 100 µL for each. A seal was attached to the plate and the plate was incubated at room temperature for 30 minutes. After the liquid was removed, 1X PBS (350 µL) was dispensed and the liquid was removed. This operation was repeated three times for washing.

[0148]

One drop of solution A and solution B serving as the avidin-biotin marker enzyme complex were added to the signal enhancing reagent (2.5 mL). This was dispensed in an amount of 100 µL for each. Then, a seal was attached to the plate and the plate was incubated at room temperature for 30 minutes. After the liquid was removed, 1X PBS (350 µL) was dispensed and the liquid was removed. This operation was repeated three times for washing. A detection reagent was prepared by adding Color Solution A and Color Solution B (manufactured by R&D systems) in equal amounts and dispensed in an amount of 200 µL for each. The plate was shielded from light and incubated at room temperature for 20 minutes. Accurately 20 minutes later, 50 µL of Stop Solution (R&D Systems) for each was added to terminate the coloring reaction. Absorbance was measured at 450 nm and 570 nm (reference wavelength). The measurement results are shown in the following Table 8 and Figure 13.

[0149]

[Table 8]

Competitive inhibitory activity of anti-hPD-1scFv03 in binding reaction of human PD-L1 to human PD-1

Concentration of scFv	Binding (OD) to hPD-L1 (1000 ng/mL) and Inhibition rate (%)	
	Anti- hPD-1 scFv03	Anti- hCTLA-4 scFv02
10ng/mL	3.479 (-0.7%)	3.697 (-7.0%)
100ng/mL	3.503 (-1.4%)	3.473 (-0.5%)
1000ng/mL	3.309 (4.2%)	3.555 (-2.9%)
10000ng/mL	1.062 (69.3%)	3.596 (-4.1%)
Non-scFv	3.455 (0%)	

[0150]

(Results)

As is apparent from Table 8 and Figure 13, the inhibition rate of anti-hPD-1scFv03 (10000 ng/mL) to the binding of hPD-L1 (1000 ng/mL) to hPD-1 was 69.3%. From the result, it was demonstrated that anti-hPD-1scFv03 competitively inhibits the binding of hPD-L1 (1000 ng/mL) to hPD-1 at a concentration of 10000 ng/mL or more.

[0151]

[Example 11-1]

[Verification of competitive inhibitory activity of hCTLA-4scFv in binding reactions of human CD80 and human CD86 to human CTLA-4]

Using anti-hCTLA-4scFv02 purified from the culture supernatant of co-expression strain PC1 mentioned above, competitive inhibitory activity thereof in binding of each of human CD80 (hCD80) and human CD86 (hCD86) to human CTLA-4 (hCTLA-4) was checked by ELISA. As a negative control, anti-hPD-1scFv03 was used.

[0152]

To 96-well plates, hCTLA-4 (Recombinant Human CTLA-4-Fc Chimera, carrier-free, manufactured by BioLegend) adjusted to be 1 μg/mL with 1X PBS was dispensed in an amount of 100 μL for each and incubated at 4°C overnight for immobilization. After the liquid was removed, 1X PBS (350 μL for each) was dispensed and the liquid was removed. This operation was repeated three times for washing.

[0153]

To the plates, 1% BSA solution (350 μL for each) was dispensed, incubated at room temperature for 2 hours for blocking. After the liquid was removed, 1X PBS (350 μL for each) was dispensed and the liquid was removed. This operation was repeated three times for washing. Ligand hCD80 (R&D Systems, Recombinant Human B7-1/CD80 Fc Chimera) and hCD86 (R&D Systems, Recombinant Human B7-2/CD86 Fc Chimera) were separately adjusted to be 2000 ng/mL with the signal enhancing reagent.

[0154]

Anti-hCTLA-4scFv02 purified from a bacterium of the genus *Bifidobacterium* was adjusted to be 20000 ng/mL, 2000 ng/mL, 200 ng/mL and 20 ng/mL with the signal enhancing reagent, mixed with each of the hCD80 and hCD86 in equal amounts (120 μL). The resultant mixtures was each (100 μL) dispensed to the plates after completion of blocking. Anti-hPD-1scFv03 purified from a bacterium of the genus *Bifidobacterium* as a negative control was subjected to the same preparation. To blank wells, the signal enhancing reagent alone (100 μL) was dispensed. A seal was attached to the plates and the plates were incubated at room

temperature for 2 hours. Each of hCD80 and hCD86, which were separately mixed with anti-hCTLA-4scFv02, was allowed to react with the hCTLA-4 immobilized. After the liquid was removed, 1X PBS (350 µL for each) was dispensed and the liquid was removed. This operation was repeated three times for washing.

[0155]

A secondary antibody (R&D Systems, Human B7-1/CD80 Biotinylated Antibody) to hCD80 and a secondary antibody (R&D Systems, Biotinylated Anti-human B7-2 Antibody) to hCD86 were separately adjusted to be 2.5 µg/mL and 0.5 µg/mL with the signal enhancing reagent and each dispensed in an amount of 10 µL. A seal was attached to the plates and the plates were incubated at room temperature for 30 minutes. After the liquid was removed, 1X PBS (350 µL for each) was dispensed and the liquid was removed. This operation was repeated three times for washing.

[0156]

Three drops of solution A and solution B mentioned above serving as the avidin-biotin marker enzyme complex were added to the signal enhancing reagent (7.5 mL). This was dispensed in an amount of 100 µL for each. Then, a seal was attached to the plates and the plates were incubated at room temperature for 30 minutes. After the liquid was removed, 1X PBS (350 µL for each) was dispensed and the liquid was removed. This operation was repeated three times for washing. A detection reagent was prepared by adding Color Solution A and Color Solution B (R&D) in equal amounts and dispensed in an amount of 200 µL for each. The plate was shielded from light and incubated at

room temperature for 20 minutes. Accurately 20 minutes later, 50 μL of Stop Solution (R&D) was added to terminate the coloring reaction. Absorbance was measured at 450 nm and 570 nm (reference wavelength). The results are shown in the following Table 9 and Table 10 and Figure 14 and Figure 15.

[0157]

[Table 9]

Competitive inhibitory activity of anti-hCTLA-4scFv02 in binding reaction of human CD80 to human CTLA-4

Concentration of scFv	Binding (OD) to hCD80 (1000 ng/mL) and Inhibition rate (%)	
	Anti- hCTLA-4 scFv02	Anti- hPD-1 scFv03
10ng/mL	2.656 (15.7%)	2.638 (16.2%)
100ng/mL	2.403 (23.7%)	2.410 (23.5%)
1000ng/mL	1.774 (43.7%)	2.464 (21.8%)
10000ng/mL	0.577 (81.7%)	2.862 (9.1%)
Non-scFv	3.149 (0%)	

[0158]

[Table 10]

Competitive inhibitory activity of anti-hCTLA-4scFv02 in binding reaction of human CD86 to human CTLA-4

Concentration of scFv	Binding (OD) to hCD86 (1000 ng/mL) and Inhibition rate (%)	
	Anti- hCTLA-4 scFv02	Anti- hPD-1 scFv03
10ng/mL	2.085 (0.3%)	1.491 (28.7%)
100ng/mL	1.421 (32.0%)	1.396 (33.2%)
1000ng/mL	0.627 (70.0%)	1.265 (39.5%)
10000ng/mL	0 (100.0%)	1.526 (27.0%)
Non-scFv	2.091 (0%)	

[0159]

(Results)

As is apparent from Table 9 and Table 10 and Figure 14 and Figure 15, the inhibition rates of anti-hCTLA-4scFv02 (1000 ng/mL) to the binding of hCD80 and hCD86 (1000 ng/mL) to hCTLA-4 are as follows. In the case of anti-hCTLA-4scFv02 (1000 ng/mL), the inhibition rates thereof were 43.7% and 70.0%, respectively. In the case of anti-hCTLA-4scFv02 (10000 ng/mL), the inhibition rates thereof were 81.7% and 100.0%, respectively. It was demonstrated that anti-hCTLA-4scFv02 competitively inhibits the binding of each of hCD80 and hCD86 (1000 ng/mL) to hCTLA-4 at a concentration of 1000 ng/mL or more.

[0160]

[Example 12]

[Investigation on binding of each of anti-hPD-1scFv and anti-hCTLA-4scFv purified from a co-expression

Bifidobacterium to cells by using antigen overexpressing cell strain]

The presence or absence of binding of anti-hPD-1scFv purified from the culture supernatant of co-expression strain PC1 mentioned above to a human PD-1 (hPD-1) expressing cells, and the presence or absence of binding of anti-hCTLA-4scFv to a human CTLA-4 (hCTLA-4) expressing cells were checked by using the following antigen-protein overexpressing cells in accordance with flow cytometry.

[0161]

HEK293T cells overexpressing hPD-1 (hPD-1 and rat CD2 are bicistronically expressed) and HEK293T cells overexpressing hCTLA-4 (hCTLA-4 and rat CD2 are bicistronically expressed) were used. HEK293T cells overexpressing hPD-1 and HEK293T cells overexpressing hCTLA-4 were each cultured in DMEM medium containing inactivated 10% fetal bovine serum and seeded in a 100-mm dish (manufactured by Greiner Japan). After the culture supernatants of hPD-1 overexpressing HEK293T cells and hCTLA-4 overexpressing HEK293T cells were removed, these cells were separately washed twice with PBS (Ca^{2+}, Mg^{2+}-free phosphate buffer). A trypsin/EDTA solution (1 mL)(manufactured by Wako Pure Chemical Industries Ltd.) diluted 10 fold with PBS was added. The cells were incubated at room temperature for one minute. Thereafter, 10 mL of DMEM medium containing inactivated 10% fetal bovine serum was added. The resultant cells were transferred to 15 mL-centrifuge tubes (manufactured by BD FALCON) and spun by a low-speed centrifuge (manufactured by TOMY SEIKO CO., LTD.) at 1000 rpm for 5 minutes. After

centrifugation, the supernatant was removed. DMEM medium (1 mL) containing inactivated 10% fetal bovine serum was added and the number of cells was counted. Further, the DMEM medium was added to prepare a cell suspension of 1×10^5 cells/mL and dispensed in 1.5-mL tubes (manufactured by Ina-optika Corporation) so as to contain 1×10^5 cells/mL/tube. The hPD-1 overexpressing HEK293T cells and hCTLA-4 overexpressing HEK293T cells dispensed in 1.5-mL tubes were each spun by a micro refrigerated centrifuge (manufactured by TOMY SEIKO CO., LTD.) at 5000 rpm and 4°C for one minute and then the supernatants were removed. The remaining cell pellets in the tube were washed twice with 0.5 mL of PBS. Thereafter, anti-hPD-1scFv03 and anti-hCTLA-4scFv02 (100 µL for each), which were purified from a recombinant bacterium of the genus *Bifidobacterium* and prepared to be 10 µg/mL, were added to respective tubes and incubated on ice for 30 minutes. Thirty minutes later, FACS buffer (PBS containing 1% BSA and 0.1%NaN_3) was added in the tubes (500 µL). After centrifugation was carried out by a micro refrigerated centrifuge at 5000 rpm and 4°C for one minute, the supernatants were removed and washing operation was carried out. The same operation was repeated once more and the washing operation was carried out twice. Anti-His-tag Alexa Fluor 488 antibody (manufactured by MBL) and anti-DDDDK-tag Alexa Fluor 488 antibody (manufactured by MBL) diluted with FACS buffer to be 0.5 µg/mL were added in an amount of 100 µL to the 1.5-mL tubes, sufficiently mixed by pipetting and incubated on ice for 30 minutes. Thirty minutes later, the FACS buffer was added to the tubes (500 µL), centrifuged by a micro

refrigerated centrifuge at 5000 rpm and 4°C for one minute. After the supernatants were removed, a washing operation was carried out. The same operation was repeated once more and the washing operation was carried out twice. After the FACS buffer (500 µL) was added, the cells suspended with the FACS buffer were transferred to 5 mL polystyrene round-bottom tubes (manufactured by Becton, Dickinson and Company). A propidium iodide solution (5 µL) diluted with the FACS buffer to be 5 µg/mL was added and analysis was carried out by use of BD FACS canto II flow cytometer (manufactured by Becton, Dickinson and Company) and flow cytometry analysis software Kaluza ver 1.2 (manufactured by Beckman Coulter, Inc.). The binding results of anti-PD-1scFv03 purified from PC1 strain to human PD-1 overexpressing cells are shown in Figure 16. Similarly, the binding results of anti-CTLA-4scFv02 purified from co-expression strain PC1 to human CTLA-4 overexpressing cells are shown in Figure 17.

[0162]

(Results)

As is apparent form Figure 16, it was verified that anti-PD-1scFv03 purified from PC1 strain specifically binds to human PD-1 high expressing cells (rat CD2 high expressing cells). Similarly, as is apparent form Figure 17, it was verified that anti-CTLA-4scFv02 purified from PC1 strain specifically binds to a human CTLA-4 high expressing cells (rat CD2 high expressing cell). From these results, it was verified that the scFv purified from co-expression strain PC1, specifically binds to human PD-1 and human CTLA-4 overexpressing cells.

[0163]

[Example 13]

[Preparation of anti-HER2scFv and hIFN-γ co-expression *Bifidobacterium* strain, HG-2]

(Outline)

A co-expression plasmid, pHG-2, which contains an expression cassette for secretory anti-HER2scFv (anti-HER2scFv secretory expression cassette) and an expression cassette for secretory hIFN-γ (human IFN-γ secretory expression cassette), and which serves as an *E. coli-Bifidobacterium* shuttle vector, was prepared (Figure 2). *Bifidobacterium longum* 105-A strain was transformed with pHG-2 thus prepared by electroporation to obtain an anti-HER2scFv and hIFN-γ co-expression *Bifidobacterium*, HG-2 strain. The primers used in Example 13 are shown in the following Table 11.

[0164]

[Table 11]

Primer Name	DNA sequence (5' -> 3')	SEQ ID No.
pTB6_Vec_F1	ACTAGTCCTCCAGGACCTCGTCTAC	SEQ ID No. 48
Hu-term_Vec_R1	CCGGAATAATACGGTTGGACAAC	SEQ ID No. 49
Hu-HuT_ins_F1	accgtattattccggGGATCCGTCTTCCTGCTGG	SEQ ID No. 50
HuT-pTB6_ins_R1	tcctggaggactagtCCGGAATAATACGGTTGGACAAC	SEQ ID No. 51
Hu_Vec_F1	GGATCCGTCTTCCTGCTGG	SEQ ID No. 52
bHER2-His_Vec_R1	TCAGTGATGATGATGATGATGCTTG	SEQ ID No. 53
d0013+T_ins_F1	catcatcatcactgaAACCGCTTCTCATTTCCATTTG	SEQ ID No. 54
d0013+T_ins_R1	caggaagacggatccGTGCACCGAATCGCGCT	SEQ ID No. 55

[0165]

As the anti-HER2scFv secretory expression cassette, a cassette sequentially comprising (1) a P30 promoter DNA, (2) a DNA encoding a signal peptide-linker conjugate, SP7L20, (3) a DNA encoding the amino acid sequence of anti-HER2scFv, (4) a DNA encoding a His tag sequence and (5) a d0013 terminator DNA was prepared such that DNA (1) was placed upstream (the 5' end) and DNA (5) downstream (the 3' end).

[0166]

(Preparation of plasmid pHG-2)

In preparing plasmid pHG-2, plasmid pHG-1 was first prepared.

[0167]

(Preparation of plasmid pHG-1)

(Preparation of hIFNg insert fragment)

PCR amplification was carried out using a plasmid phIFNg33 (SEQ ID No. 16) as a template and a primer set of Hu-HuT_ins_F1 (forward) and HuT-pTB6_ins_R1 (reverse) listed in Table 11. Each of the primer sequences was designed such that an insert fragment was overlapped with a vector fragment at the end of 15 bp. The PCR amplification was carried out by specifying the concentration of each primer as 0.2 μM and the reaction volume as 20 μL and using STAR kit. As the amplification program, a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 65°C for 5 seconds and a reaction at 72°C for 72 seconds, was repeated 30 times and then an elongation reaction was carried out at 72°C for 30 seconds to prepare hIFNg insert fragment amplified product of about 1.1 kbp.

[0168]

(Preparation of vector fragment comprising DNA encoding the amino acid sequence of anti-HER2scFv protein)

PCR amplification was carried out using pP30SP7L20-bHER2 (SEQ ID No. 15) as a template and a primer set of pTB6_Vec_F1 (forward) and Hu-term_Vec_R1 (reverse) listed in Table 11. Each of the primer sequences was designed such that an insert fragment was overlapped with a vector fragment at the end of 15 bp. The PCR amplification was carried out by specifying the concentration of each primer as 0.2 μM and the reaction volume as 20 μL and using STAR kit. As the amplification program, a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 65°C for 5 seconds and a reaction at 72°C for 4 minutes 45 seconds, was repeated 30 times and then an elongation reaction was carried out at 72°C for 30 seconds to prepare a vector fragment amplified product of about 4.6 kbp.

[0169]

(In-fusion reaction 12)

The vector fragment amplified product of 4.6 kbp and the insert fragment amplified product of about 1.1 kbp prepared above were ligated by use of In-Fusion (registered trademark) HD Cloning kit (manufactured by Takara Bio Inc.). First, in the website of Clontech: In-Fusion (registered trademark) Molar Ratio Calculator (http://bioinfo.clontech.com/infusion/molarRatio.do), the requisite insert amount and vector amount were calculated and the molar ratio of the vector to the insert was specified as 1:2. Two μL of 5X In-Fusion HD Enzymes premix, Cloning Enhancer (1 μL) and the requisite amounts

of insert and vector were mixed and the total amount of the reaction system was adjusted with 0.1X TE buffer (1 mM Tris-HCl, 0.1 mM EDTA, pH7.5) to be 10 μL. The reaction was carried out at 37°C for 15 minutes and then 50°C for 15 minutes. The reaction solution was incubated at 4°C. The procedure other than this was carried out in accordance with the product manual of the kit to prepare in-fusion reaction solution 12.

[0170]

(Transformation of *E. coli* and determination of DNA sequence of plasmid pHG-1)

The same procedure as in the above section (Transformation of *E. coli* and determination of DNA sequence of plasmid pAG8) was repeated by using in-fusion reaction solution 12 obtained above to carry out transformation of the *E. coli* HST16CR competent cell obtained above and extraction of a plasmid from the recombinant *E. coli*. In the plasmid extracted, the sequences of anti-HER2scFv secretory expression cassette and human IFN-γ secretory expression cassette were determined in the same procedure as above. The plasmid extracted was designated as pHG-1. The DNA sequence of plasmid pHG-1 is represented by SEQ ID No. 17.

[0171]

(Preparation of pHG-2 strain)

The terminator of anti-HER2scFv expression secretion cassette of plasmid pHG-1 was changed from Hu terminator to d0013 terminator. PCR amplification was carried out using pHG-1 mentioned above as a template and a primer set of Hu_Vec_F1 (forward) and bHER2-His_Vec_R1 (reverse)

listed in Table 11. As the amplification program, a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 65°C for 5 seconds and a reaction at 72°C for 5 minutes 45 seconds, was repeated 30 times, and then an elongation reaction was carried out at 72°C for 30 seconds. Other than this, the same procedure as in the above section (Preparation of plasmid pAG8) was repeated to obtain a vector fragment amplified product of about 5.6 kbp.

[0172]

PCR amplification was carried out using genomic DNA of *Bifidobacterium longum* 105-A as a template and a primer set of d0013+T_ins_F1 (forward) and d0013+T_ins_R1 (reverse) listed in Table 11. As the amplification program, a cycle consisting of a reaction at 98°C for 10 seconds, a reaction at 65°C for 5 seconds and a reaction at 72°C for 35 seconds, was repeated 30 times, and then an elongation reaction was carried out at 72°C for 30 seconds. Other than this, the same procedure as in the above section (Preparation of plasmid pAG8) was repeated to obtain an insert fragment amplified product of about 144 bp.

[0173]

(In-fusion reaction 13)

The same procedure as in the above (In-fusion reaction 10) was repeated except that the vector fragment and the insert fragment prepared above were used to prepare in-fusion reaction solution 13.

[0174]

(Transformation of *E. coli* and determination of DNA sequence of plasmid pHG-2)

The same procedure as in the above section (Transformation of *E. coli* and determination of DNA sequence of plasmid pAG8) was repeated by using in-fusion reaction solution 13 to carry out transformation of the *E. coli* HST16CR competent cell obtained above and extraction of a plasmid from the recombinant *E. coli*. In the plasmid extracted, the DNA sequences of anti-HER2scFv secretory expression cassette and human IFN-γ secretory expression cassette were determined in the same procedure. The plasmid extracted was designated as pHG-2. The DNA sequence of plasmid pHG-2 is represented by SEQ ID No. 100.

[0175]

(Transformation 9 of bacterium of the genus *Bifidobacterium*)

Bifidobacterium longum 105-A strain was transformed by the electroporation system in the same procedure as in the above section (Transformation 1 of bacterium of the genus *Bifidobacterium*) except that plasmid pHG-2 (500 ng) extracted from the transformed *E. coli* was used. The transformant obtained was designated as *Bifidobacterium longum* 105-A/pHG-2 (hereinafter referred to as HG-2 strain).

[0176]

[Example 14]

[Verification of anti-HER2scFv and hIFN-γ secretion in HG-2 strain]

The presence or absence of anti-HER2scFv and hIFN-γ in the culture supernatant of HG-2 strain was verified by western blotting as follows.

[0177]

(Culture of recombinant bacterium of the genus *Bifidobacterium*)

Bacteria of the genus *Bifidobacterium*, i.e., HG-2 strain; a single-expression strain P30SP7L20-bHER2 (plasmid pP30SP7L20-bHER2 introduced strain) having one of the human IFN-γ secretory expression cassettes; and hIFNg33 strain; were cultured in the same procedure as in Example 4, (Culture of recombinant bacterium of the genus *Bifidobacterium*) and the culture supernatants were collected. The proteins in the culture supernatants were precipitated by trichloroacetic acid (TCA, manufactured by Wako Pure Chemical Industries Ltd.), washed with acetone, dissolved in a buffer for SDS-PAGE and treated with heat at 95°C for 3 minutes to obtain culture supernatant concentrates.

[0178]

(Western analysis)

Each of the culture supernatant concentrates (corresponding to 0.1 mL of the culture solution) was treated in the same manner as in Example 9, (Western analysis). After blotting, the membrane was treated with blocking reagent. Thereafter, using an anti-histidine antibody, namely, THE His Tag Antibody, mAb, Mouse as a primary antibody and ECL peroxidase labelled anti-mouse antibody (mentioned above) as a secondary antibody, an antibody reaction was carried out. After the reaction,

the membrane was illuminated by Western Lightning Ultra. This was analyzed by imaging equipment, Fluor-S Max (manufactured by BIO-RAD). The results are shown in Figure 18.

[0179]

(Results)

As is apparent from Figure 18, both anti-HER2scFv and hIFN-γ in HG-2 strain were detected. The sizes of these proteins were the same as those of the proteins separately secreted from the corresponding single-expression strains. Note that, lane M shows a marker; lane 1 shows a protein secreted from anti-HER2scFv single-expression strain (P30SP7L20-bHER2 strain); lane 2 shows a protein secreted from hIFN-γ single-expression strain (hIFNg33 strain); and lane 3 shows proteins secreted from anti-HER2scFv and hIFN-γ co-expression strain (HG-2 strain).

[0180]

[Example 15]

[Investigation on physiological activity of secretion from HG-2 strain]

(Preparation of culture supernatant concentrate and determination of hINF-γ protein expression-level)

Each of HG-2 strain, P30SP7L20-bHER2 strain, hIFNg33 strain, and BEshuttle strain was inoculated in MRS liquid medium (75 μg/mL spectinomycin and 1% vitamin C-added liquid)in a ratio of 1% and anaerobically cultured at 37°C for 24 hours (activation culture). The activated culture solution (0.5%) was inoculated in DMEM/MRS (75 μg/mL spectinomycin, 0.5% vitamin C-added liquid) medium, which

was prepared by adding MRS liquid medium (2 mL) to 18 mL of DMEM medium (low glucose, manufactured by Life technologies). This medium was anaerobically cultured (main culture) at 37°C for 18 hours. The culture solution (20 mL) was centrifuged. The culture supernatant (13 mL) was collected and filtered by a filter having a pore size of 0.2 μm to remove bacterial cells. The culture supernatant (10 mL) was concentrated by Amicon Ultra-4 (manufactured by Millipore) and replaced with PBS (-) to obtain a concentrate of 500 μL (*Bifidobacterium* culture supernatant concentrate (PBS substitution)). Each of the bacterium culture supernatant concentrates was subjected to hIFN-γ measurement by an ELISA kit in accordance with ELISA to measure the amount of IFN-γ in the culture supernatant. The measurement results are shown in the following Table 12.

[0181]

[Table 12]

Measurement of human IFN-γ concentration in bifidobacterium culture supernatant concentrate

Sample name	hIFN-γ conc. (ng/mL)
BEshuttle strain	0
P30SP7L20-bHER2 strain (anti-HER2scFv single expression strain)	0
hIFNg33 strain (hIFN-γ single expression strain)	3697
HG-2 strain (anti-HER2scFv/hIFN-γ co-expression strain)	6851

[0182]

(Cancer cell proliferation inhibitory activity of anti-HER2scFv in secretion form HG-2 strain)

As the physiological activity of anti-HER2scFv, cell proliferation inhibitory activity thereof was measured by adding anti-HER2scFv (PBS substitution), which was obtained by His-tag purification from the culture supernatant of P30SP7L20-bHER2 strain, to HER2 positive cell (NCI-N87 (stomach cancer) cell). More specifically, NCI-N87 cells were cultured in RPMI 1640 medium (10% (v/v) FBS) at 37°C in the condition of 5% CO_2, and then seeded in a 96-well plate in a ratio of 2×10^4 cells for each and cultured at 37°C in the condition of 5% CO_2 for 24 hours. Thereafter, the old medium was removed by suction and fresh RPMI 1640 medium (10% (v/v) FBS) was added in an amount of 98 μL for each. Subsequently, measurement samples, PBS (-) and anti-HER2scFv, which were adjusted to have a concentration in the range of 244 ng/mL to 1 mg/mL, were added separately in an amount of 2 μL for each. This plate was cultured at 37°C in a condition of 5% CO_2 for 5 days.

[0183]

After 5-day culture, the medium was removed by suction, a mixture, which was obtained by adding Cell Counting kit-8 (1 mL) to 9 mL of fresh RPMI 1640 medium (10% (v/v) FBS), was added in an amount of 100 μL for each. The plate was kept warm for further three hours at 37°C in the condition of 5% CO_2. The absorbance was measured at a wavelength of 450 nm and 630 nm (reference wavelength) to determine cell proliferation inhibitory activity to the HER2 positive cells. The measurement results are shown in Figure 19.

[0184]

(Results)

As is apparent from Figure 19, anti-HER2scFv purified from the *Bifidobacterium longum* Re-105A/pP30SP7L20-bHER2 exhibited cell proliferation inhibitory activity to NCI-N87 stomach cancer cell in a dose dependent manner. Thus, it was verified that anti-HER2scFv secreted from recombinant bacterium of the genus *Bifidobacterium* has a physiological activity.

[0185]

[Example 16]

(Cancer cell proliferation inhibitory activity 1 of anti-HER2scFv and hIFN-γ secreted from HG-2 strain)

The physiological activities of anti-HER2scFv and hINF-γ, which were secreted from a co-expression strain, were checked by determining cell proliferation inhibitory activity thereof by adding the concentrate (PBS substitution) of the culture supernatant of a bacterium of the genus *Bifidobacterium* (HG-2 strain) obtained in Example 15 to HER2 positive cell (NCI-N87 cell). More specifically, NCI-N87 cells were cultured in RPMI 1640 medium (10% (v/v) FBS) at 37°C, in the condition of 5% CO_2, seeded in a 96-well plate in an amount of 2×10^4 cells for each and cultured at 37°C in the condition of 5% CO_2 for 24 hours. From the cell culture solution, the old medium was removed by suction and fresh RPMI 1640 medium (10% (v/v) FBS) was added in an amount of 90 μL for each. Subsequently, as measurement samples, PBS (-), a BEshuttle strain culture supernatant concentrate (negative control), a P30SP7L20-bHER2 strain culture supernatant concentrate, a hINFg33 strain culture supernatant concentrate (prepared

by diluting with PBS so as to have a hIFN-γ concentration of 2000 ng/mL), a HG-2 strain culture supernatant concentrate and recombinant hIFN-γ (positive control, the hIFN-γ concentration was adjusted so as to be 2000 ng/mL) were added in an amount of 10 μL for each. This plate was subjected to culture at 37°C in the condition of 5% CO_2 for 5 days.

[0186]

The cell proliferation inhibitory activity was measured in the same manner as described in the above section (Cell proliferation inhibitory activity of anti-HER2scFv). The results are shown in Figure 20.

[0187]

As is apparent from Figure 20, the culture supernatant concentrate derived from anti-HER2scFv/hIFN-γ co-expression strain, i.e., HG-2 strain, exhibited extremely strong cell proliferation inhibitory activity to NCI-N87 cell. In contrast, the concentrate derived from the culture supernatant of hIFN-γ single-expression strain, i.e., hINFg33 strain, exhibited the same cell proliferation inhibitory activity as recombinant hIFN-γ (manufactured by PeploTech) used as a standard at a hIFN-γ concentration of 200 ng/mL. The concentrate derived from P30SP7L20-bHER2 strain was determined to have the same cell proliferation inhibitory activity as in Example 15. The cell proliferation inhibitory activity of anti-HER2scFv/hIFN-γ co-expression strain, i.e., HG-2 strain, is higher than the activity of either one of strains: hIFN-γ single-expression strain and P30SP7L20-bHER2 strain, simultaneously checked. This activity is conceivably due

to synergistic combinational effect of both hIFN-γ and anti-HER2scFv proteins secreted together.

[0188]

[Example 17]

[Verification of secretion from anti-HER2scFv and hIFN-γ co-expression strain within tumor]

Using a human stomach cancer cell strain NCI-N87 bearing nude mouse, localization of a bacterium of the genus *Bifidobacterium* within a tumor was checked by gram staining. The localization of anti-HER2scFv and hIFN-γ within the tumor was checked by immunohistochemical staining with an anti-histidine tag antibody and anti-hIFN-γ antibody.

[0189]

Human stomach cancer cell strain NCI-N87 (ATCC) was cultured in RPMI 1640 medium (manufactured by Wako Pure Chemical Industries Ltd.) comprising 10%FBS (manufactured by EQUITECH-BIO, INC.) and transplanted into nude mice (produced by Japan SLC, Inc.) to prepare tumor bearing mice. To mice having a tumor size of about 470 mm^3, a simple frozen preparation of an anti-HER2scFv and hIFN-γ co-expression strain (HG-2 strain); and a simple frozen preparation of the BEshuttle strain expressing neither one of them and serving as a control, were administered in a dose of 6×10^8 cfu from the tail vein. Note that, a 10% maltose solution was administered in a dose of 1 mL at a frequency of two times a day for 5 days. On Day 7 after the administration, the tumor was excised out, embedded in O. C. T. compound (manufactured by Sakura Finetek) and frozen. Thin specimens were prepared by Cryostat

microtome Leica CM1900 (manufactured by Leica), separately placed on slides and subjected to tissue staining.

[0190]

(Gram staining)

The thin specimens on slides were dried in air and soaked in 4% PFA (manufactured by Wako Pure Chemical Industries Ltd.) for 10 minutes to fix. After fixation, the thin specimens were pre-stained with Bartholomew & Mittwer M crystal violet solution (manufactured by MUTO PURE CHEMICALS Co., Ltd.) for 2 minutes, and allowed to react with Bartholomew & Mittwer M iodine/sodium hydroxide solution (manufactured by MUTO PURE CHEMICALS Co., Ltd.) for one minute. The thin specimens were decolored with Bartholomew & Mittwer M acetone/ethyl alcohol mixture (manufactured by MUTO PURE CHEMICALS Co., Ltd.) and stained with Bartholomew & Mittwer M 0.1% fuchsin solution (manufactured by MUTO PURE CHEMICALS Co., Ltd.) for one minute. After staining, the thin specimens were washed with purified water, dewatered with 99.5% ethanol (manufactured by Wako Pure Chemical Industries Ltd.), cleared in Lemosol (manufactured by Wako Pure Chemical Industries Ltd.) and enclosed with Entellan new (manufactured by MERCK KGaA). The results are shown in Figure 21.

[0191]

(Immunohistochemical staining with anti-histidine tag antibody and anti-hIFN-γ antibody)

The thin specimens on slides were dried in air and soaked in 4% PFA (manufactured by Wako Pure Chemical Industries Ltd.) for about 4 hours to fix. After fixation,

the thin specimens were washed with purified water for one minute and three times with 1X PBS (-) for 5 minutes. Moisture around the tissue was wiped out. A line was drawn around the tissue by Dako pen (manufactured by Dako). To the tissue, 3% BSA-PBS was added dropwise and allowed to react for 60 minutes to inhibit non-specific binding. Anti-His-tag mAb-Alexa Fluor (registered trademark) 594 (manufactured by MBL) and FITC Anti-human IFN-γ antibody (manufactured by BioLegend) were mixed and diluted with Can Get Signal (registered trademark) immunostain (manufactured by TOYOBO CO., LTD.) to prepare an antibody reaction solution. The antibody reaction solution was added dropwise onto the tissue and allowed to react at 4°C overnight. After the antibody reaction, washing with 1X PBS (-) for 5 minutes was carried out three times. The specimen(s) was enclosed with VECTASHIELD (registered trademark) Mounting Medium with DAPI. The stained slice was observed by a microscope DM5000B (manufactured by Leica) and the image thereof was photographed. The results are shown in Figures 22 and 23.

[0192]

(Results)

As is apparent from Figure 21, in gram staining, bacterial cells, i.e., HG-2 strain (A) and BEshuttle strain (B) (Figure 21 (A) and (B), pointed by the arrow) each were found to be present in the tumor tissue. Also, in immunohistochemical staining with an anti-hIFN-γ antibody, hIFN-γ positive images were found in the tumor tissue of the mouse having a cancer and administered with a co-expression strain, i.e., HG-2 strain (Figure 22,

green color portions pointed by arrows). Furthermore, in immunohistochemical staining with a histidine tag, histidine tag positive images (anti-HER2scFv and hIFN-γ) were found (Figure 23, red color portions pointed by arrows). From these results, it was verified that when HG-2 strain is intravenously administered to a mouse having a human stomach cancer NCI-N87, the HG-2 strain is colonized in the tumor and hIFN-γ and anti-HER2scFv proteins are secreted from the HG-2 strain and simultaneously present in the tumor.

[0193]

[Example 18]

[Preparation of co-expression strain for anti-human PD-1scFv03-His and anti-human CTLA-4scFv01-FLAG]

E. coli-Bifidobacterium shuttle vector secreting anti-human PD-1scFv03-His and anti-human CTLA-4scFv01-FLAG, i.e., co-expression plasmids pPC2, pPC3, pPC4, pPC5, pPC6, pPC7 and pPC8 were prepared. *Bifidobacterium longum* 105-A strain was separately transformed with these 7 types of co-expression plasmids.

[0194]

The constitutions of anti-human PD-1scFv03-His expression cassettes and anti-human CTLA-4scFv01-FLAG expression cassettes in the 7 types of co-expression plasmids pPC2 to pPC8 are shown in Table 13-1 and Table 13-2. To avoid homologous sequences within a plasmid molecule, the promoter DNA, a terminator DNA and DNA sequences encoding a tag and signal peptide-linker peptide conjugate in each expression cassette were designed not to have the same constitution.

[0195]

[Table 13-1]

	anti-hPD-1 scFv03-His expression cassette				
plasmid	promoter	SP/Linker	scFv	tag	terminator
pPC2	Hu	SP50L5	anti-hPD-1 scFv03	His	Hu
pPC3	Hu	SP50L5	anti-hPD-1 scFv03	His	Hu
pPC4	Hu	SP67L10	anti-hPD-1 scFv03	His	Hu
pPC5	Hu	SP67L10	anti-hPD-1 scFv03	His	Hu
pPC6	Hu	SP69L1	anti-hPD-1 scFv03	His	Hu
pPC7	Hu	SP69L1	anti-hPD-1 scFv03	His	Hu
pPC8	Hu	SP69L1	anti-hPD-1 scFv03	His	Hu

[0196]

[Table 13-2]

	anti-hCTLA-4 scFv01-FLAG expression cassette				
plasmid	promoter	SP/Linker	scFv	tag	terminator
pPC2	P30	SP67L1	anti-hCTLA-4 scFv01	FLAG	T2
pPC3	P30	SP68L1	anti-hCTLA-4 scFv01	FLAG	T2
pPC4	P30	SP50L5	anti-hCTLA-4 scFv01	FLAG	T2
pPC5	P30	SP68L1	anti-hCTLA-4 scFv01	FLAG	T2
pPC6	P30	SP50L5	anti-hCTLA-4 scFv01	FLAG	T2
pPC7	P30	SP67L1	anti-hCTLA-4 scFv01	FLAG	T2
pPC8	P30	SP68L1	anti-hCTLA-4 scFv01	FLAG	T2

[0197]

(Constitution of anti-hPD-1scFv03-His secretory expression cassette)

As the anti-hPD-1scFv03-His secretory expression cassette, a cassette sequentially comprising (1) a Hu promoter DNA, (2) a DNA encoding a signal peptide-linker peptide conjugate, (3) a DNA encoding an anti-hPD-1scFv03

amino acid sequence, (4) a DNA encoding a His tag sequence and (5) a Hu terminator DNA was prepared such that DNA (1) was placed upstream (the 5' end) and DNA (5) downstream (the 3' end). As the signal peptide-linker peptide conjugate, SP50L5 (secretory signal peptide-linker peptide conjugate obtained by binding a sequence consisting of the initial five amino acids (Ala Thr Leu Thr Pro) of the amino acid sequence represented by SEQ ID No. 83 to the amino acid sequence represented by SEQ ID No. 61); SP67L10 (secretory signal peptide-linker peptide conjugate obtained by binding a sequence consisting of initial ten amino acids (Ala Gly Val Asp Tyr Leu Pro Thr Ile Gly) of the amino acid sequence represented by SEQ ID No. 95 to the amino acid sequence represented by SEQ ID No. 73); or SP69L1 (secretory signal peptide-linker peptide conjugate obtained by binding the initial amino acid (Asp) of the amino acid sequence represented by SEQ ID No. 99 to the amino acid sequence represented by SEQ ID No. 77) was used.

[0198]

(Constitution of anti-hCTLA-4scFv01-FLAG secretory expression cassette)

As the anti-hCTLA-4scFv01-FLAG secretory expression cassette, a cassette sequentially comprising (1) a P30 promoter DNA, (2) a DNA encoding signal peptide-linker peptide conjugate, (3) a DNA encoding an anti-hCTLA-4scFv01 amino acid sequence, (4) a DNA encoding a FLAG tag sequence and (5) a T2 terminator DNA was prepared such that DNA (1) was placed upstream (the 5' end) and DNA (5) downstream (the 3' end). As the signal peptide-linker peptide conjugate, SP50L5 (secretory signal peptide-linker

peptide conjugate obtained by binding a sequence consisting of the initial five amino acids (Ala Thr Leu Thr Pro) of the amino acid sequence represented by SEQ ID No. 83 to the amino acid sequence represented by SEQ ID No. 61); SP67L1 (secretory signal peptide-linker peptide conjugate obtained by binding the initial amino acid (Ala) of the amino acid sequence represented by SEQ ID No. 95 to the amino acid sequence represented by SEQ ID No. 73); or SP68L1 (secretory signal peptide-linker peptide conjugate obtained by binding the initial amino acid (Asp) of the amino acid sequence represented by SEQ ID No. 97 to the amino acid sequence represented by SEQ ID No. 75) was used.
[0199]

The basic constitutions of co-expression plasmids pPC2 to pPC8 are shown in Figure 24.

The production method thereof is schematically shown in Figure 25.

(1) In the first step, template plasmids (pHuSPxLy-hPD-1scFv03-His (SPxLy = SP50L5, SP67L10, SP69L1)) to be used in preparation of co-expression plasmids pPC2 to pPC8 was prepared.

(2) In the second step, plasmid pHuSPxLy-hPD-1scFv03-His-T2 (SPxLy = SP50L5, SP67L10, SP69L1) was prepared by inserting T2 terminator fragment downstream of an anti-hPD-1scFv03-His expression cassette of an anti-hPD-1scFv03-His expression plasmid, pHuSPxLy-hPD-1scFv03-His (SPxLy = SP50L5, SP67L10, SP69L1).

(3) In the third step, an anti-hCTLA-4scFv01-FLAG expression cassette (except a terminator) was inserted between Hu terminator and T2 terminator of the plasmid

prepared in the second step to prepare co-expression plasmids pPC2 to pPC8.

[0200]

(First step)

Step A: PCR products of a linear vector (vector) prepared by using plasmid pHuSP7L20-hPD-1scFv03 (histidine tag is added to the 3' end of scFv sequence) prepared in Example 8 as template DNA and SP50 insert (insert), SP67 insert and SP69 insert prepared from 105-A genomic DNA, were prepared. A primer was designed such that terminal 15 bp of a PCR product has the same sequence as that of the terminal 15 bp to the adjacent PCR product to be ligated in the in-fusion reaction of the next step. The elongation reaction time was set assuming that one minute is required for elongation of 1 kbp. The PCR amplification step herein is referred to as "PCR amplification step 1".

[0201]

The linear vector and each of the inserts were ligated by an in-fusion reaction using HD kit. In the same manner as in (Example 1: Transformation of *E. coli* and determination of DNA sequence of plasmid pAG8), the *E. coli* HST16CR competent cells were transformed by using the in-fusion reaction solution; a plasmid was extracted from recombinant *E. coli*; and the sequence of "anti-hPD-1scFv03-His expression cassette region" (including Hu promoter to Hu terminator) in the plasmid extracted, was determined (hereinafter, the step from the in-fusion reaction to the sequence determination will be carried out in the same procedure as this). The plasmids extracted

were designated as pHuSP50L20-hPD-1scFv03-His, pHuSP67L20-hPD-1scFv03-His and pHuSP69L20-hPD-1scFv03-His.

[0202]

Step B: PCR products of SP50L5 insert, SP67L10 insert and SP69L1 insert, which were prepared respectively from the pHuSP50L20-hPD-1scFv03-His, pHuSP67L20-hPD-1scFv03-His and pHuSP69L20-hPD-1scFv03-His, were prepared.

[0203]

The linear vector prepared in Step A and each of the SP50L5 insert, SP67L10 insert and SP69L1 insert prepared above were ligated by the HD kit in the same manner as above and the sequence of the "anti-hPD-1scFv03-His expression cassette region" was determined. The plasmids extracted were designated as pHuSP50L5-hPD-1scFv03-His, pHuSP67L10-hPD-1scFv03-His and pHuSP69L1-hPD-1scFv03-His.

[0204]

(Second step)

(Preparation of T2 terminator)

Synthesis of the sequence (SEQ ID No. 101) was ordered to GenScript Japan Inc. and the sequence was designated as T2 terminator, which was integrated in plasmid vector pUC57 (Bba-B0015 in pUC57) and delivered.

[0205]

Using the pHuSP50L5-hPD-1scFv03-His, pHuSP67L10-hPD-1scFv03-His and pHuSP69L1-hPD-1scFv03-His separately as template DNA and PC1_primer (SEQ ID No. 102) as a forward primer and PC2_primer (SEQ ID No. 103) as a reverse primer, PCR products were prepared in the same manner as in the above step "PCR amplification 1" and designated as A1, A2

and A3 linear vectors. The sizes of the PCR products were 4751 bp, 4697 bp and 4661 bp, respectively.

[0206]

Using the Bba-B0015 in pUC57 as template DNA, and PC3_primer (SEQ ID No. 104) as a forward primer and PC4_primer (SEQ ID No. 105) as a reverse primer, PCR product B (159 bp) was prepared.

[0207]

[Table 14]

Plasmid to be constructed	Combination in in-fusion reaction
pHuSP50L5-hPD-1 scFv03-His-T2	A1 and B
pHuSP67L10-hPD-1 scFv03-His-T2	A2 and B
pHuSP69L1-hPD-1 scFv03-His-T2	A3 and B

[0208]

PCR products were ligated in accordance with the combinations shown in Table 14 by use of the HD kit and the sequence of each of "anti-hPD-1scFv03-His expression cassette-T2 terminator regions"(5'-Hu promoter-SPxLy-human PD-1scFv03-His-T2 terminator-3') was determined. The plasmids extracted were designated as pHuSP50L5-hPD-1scFv03-His-T2, pHuSP67L10-hPD-1scFv03-His-T2 and pHuSP69L1-hPD-1scFv03-His-T2.

[0209]

[Preparation of plasmid phCTLAz]

The Hu promoter of the plasmid pHuSP7L20-hCTLA-4scFv02FLAG prepared in Example 8 was replaced by P30 promoter, as schematically shown in Figure 26.

[0210]

A PCR product (4410 bp) of a linear vector was prepared by using pHuSP7L20-hCTLA-4scFv02FLAG as template DNA, and vec_F5_primer (SEQ ID No. 106) as a forward primer and vec_R2_primer (SEQ ID No. 107) as a reverse primer. A PCR product (245 bp) of an insert was prepared by using 105-A genomic DNA as a template, and P30_F1_primer (SEQ ID No. 108) as a forward primer and P30_R1_primer (SEQ ID No. 109) as a reverse primer. The linear vector and insert thus prepared were ligated as mentioned above by use of the HD kit and the sequence of the full-length plasmid extracted was determined. The plasmid extracted was designated as phCTLA1.

[0211]

(Preparation of plasmid phCTLAz (z = 4, 10, 11))

The signal peptide sequence of the plasmid phCTLA1 prepared above was changed from SP7 to SP50, SP67 and SP68.

[0212]

PCR amplification was carried out in the same manner as in the above step "PCR amplification 1" using template DNA molecules and primers listed in Table 15 to prepare a vector and inserts.

[0213]

[Table 15]

Template and primer used in PCR

PCR product	Template DNA	Fow. Primer (Forward primer)	Rev. primer (Reverse primer)	Size of PCR product
Vector	phCTLA1	hCTLA3_primer (SEQ ID No. 110)	P30_R1_primer (SEQ ID No. 109)	4466 bp
Insert-SP50	*B. longum* 105A genome	SP50-ins_F2_primer (SEQ ID No. 111)	SP50-ins_R6_primer (SEQ ID No. 112)	258 bp
Insert-SP67	*B. longum* 105A genome	SP67-ins_F2_primer (SEQ ID No. 113)	SP67-ins_R6_primer (SEQ ID No. 114)	189 bp
Insert-SP68	*B. longum* 105A genome	SP68-ins_F2_primer (SEQ ID No. 115)	SP68-ins_R6_primer (SEQ ID No. 116)	219 bp

[0214]

The vector and each of the inserts listed in Table 15 were ligated in the same manner as above by using HD kit, and the sequences of "anti-hCTLA-4scFv02-FLAG cassette regions" were determined. The plasmids extracted were designated as phCTLA4 (in the case of SP50), phCTLA10 (in the case of SP67) and phCTLA11 (in the case of SP68).

[0215]

(Preparation of pP30SPx'Ly'-hCTLA-4scFv01-His)

Preparation is schematically shown in Figure 27.

[0216]

(Preparation of anti-CTLA-4 single-chain antibody expression cassette)

Referring to [Preparation of pHuSP7L20-scFv-CTLA-4-1] in WO2015/166640, pHuSP7L20-scFv-CTLA-4-1 was prepared and designated as pHuSP7L20-hCTLA-4scFv01-His in this Example.

[0217]

PCR products were prepared in the same manner as in the above step "PCR amplification 1" by using template DNA molecules and primers listed in Table 16.

[0218]

[Table 16]

PCR product	Template DNA	Fow. primer	Rev. primer	Size of PCR product
A	pHuSP7L20-hCTLA-4 scFv01-His	hCTLA3_primer	GA6_primer (SEQ ID No. 117)	4020 bp
B-SP50L5	phCTLA4	GA5_primer_rev (SEQ ID No. 118)	SP50L5-ins_R1_hPD1_03 (SEQ ID No. 128)	671 bp
B-SP67L1	phCTLA10	GA5_primer_rev	SP67L1-ins_R1_hPD1_03 (SEQ ID No. 129)	590 bp
B-SP68L1	phCTLA11	GA5_primer_rev	SP68L1-ins_R1_hPD1_03 (SEQ ID No. 130)	620 bp

[0219]

The PCR product A and each of PCR products B listed in Table 16 were ligated in the same manner as above by using HD kit, and the sequences of "anti-hCTLA-4scFv01-His cassette regions" were determined. The plasmids extracted were designated as pP30SP50L5-hCTLA-4scFv01-His, pP30SP67L1-hCTLA-4scFv01-His and pP30SP68L1-hCTLA-4scFv01-His.

[0220]

[Preparation of plasmid pP30SPx'Ly'-hCTLA-4scFv01-FLAG]

The histidine tag of plasmid pP30SPx'Ly'-hCTLA-4scFv01-His (SPx'Ly' = SP50L5, SP67L1, SP68L1) prepared above was changed to FLAG tag as schematically shown in Figure 28. Hereinafter, in the case of anti-hCTLA-4scFv, expression "SPx'Ly'" may be sometimes employed.

[0221]

PCR products were prepared by using the template DNA molecules and primers listed in Table 17 in the same manner as in the above step "PCR amplification 1".

[0222]

[Table 17]

PCR product	Template DNA	Fow. primer	Rev. primer	Size of PCR product
A	pP30SP50L5-hCTLA-4 scFv01-His	hCTLA15_primer (SEQ ID No. 120)	GA6_primer	3280 bp
B-SP50L5	pP30SP50L5-hCTLA-4 scFv01-His	GA5_primer_rev	hCTLA16_primer (SEQ ID No. 119)	1417 bp
B-SP67L1	pP30SP67L1-hCTLA-4 scFv01-His	GA5_primer_rev	hCTLA16_primer	1336 bp
B-SP68L1	pP30SP68L1-hCTLA-4 scFv01-His	GA5_primer_rev	hCTLA16_primer	1366 bp

[0223]

PCR product A and each of PCR products B listed in Table 17 were ligated by using HD kit in the same manner as mentioned above and the sequences of "anti-hCTLA-4 scFv01-FLAG cassette regions" were determined. The plasmids extracted were designated as pP30SP50L5-hCTLA-4scFv01-FLAG (hereinafter referred to as pC1F), pP30SP67L1-hCTLA-4scFv01-FLAG (hereinafter referred to as pC2F) and pP30SP68L1-hCTLA-4scFv01-FLAG (hereinafter referred to as pC3F).

[0224]

(Third step)

PCR products were prepared by using the template DNA and primers listed in Table 18 in the same manner as in the above step "PCR amplification 1".

[0225]

[Table 18]

PCR product	Template DNA	Fow. primer	Rev. primer	Size of PCR product
A7	pHuSP50L5-hPD-1 scFv03-His-T2	PC5_primer (SEQ ID No. 121)	PC2_primer	4886 bp
A8	pHuSP67L10-hPD-1 scFv03-His-T2	PC5_primer	PC2_primer	4832bp
A9	pHuSP69L1-hPD-1 scFv03-His-T2	PC5_primer	PC2_primer	4796 bp
B1	pC1F	PC6_primer (SEQ ID No. 122)	PC7_primer (SEQ ID No. 123)	1210 bp
B2	pC2F	PC6_primer	PC7_primer	1129 bp
B3	pC3F	PC6_primer	PC7_primer	1159 bp

[0226]

[Table 19]

Plasmid to be constructed	Combination in in-fusion reaction
pPC2	A7 and B2
pPC3	A7 and B3
pPC4	A8 and B1
pPC5	A8 and B3
pPC6	A9 and B1
pPC7	A9 and B2
pPC8	A9 and B3

[0227]

The PCR products listed in Table 18 were ligated in accordance with the combinations listed in Table 19 by using HD kit in the same manner as mentioned above and DNA sequences of the full-length plasmids extracted were determined. The plasmids extracted were designated as pPC2, pPC3, pPC4, pPC5, pPC6, pPC7 and pPC8. The individual plasmids will be outlined below.

[0228]

(pPC2 scFv expression cassette sequences (2618 bp) (SEQ ID No. 131))

1..1384 Anti-human PD-1scFv03-His expression cassette

1..361 Hu promoter

362..544 SP50L5

545..1249 anti-human PD-1scFv03

1250..1267 His tag

1268..1270 stop codon

1271..1384 Hu terminator

1385..2618 Anti-human CTLA-4scFv01-FLAG expression cassette

1385..1619 P30 promoter

1620..1721 SP67L1

1722..2462 anti-human CTLA-4scFv01

2463..2486 FLAG tag

2487..2489 stop codon

2490..2618 T2 terminator

[0229]

(pPC3 scFv expression cassette sequences (2648 bp) (SEQ ID No. 132))

1..1384 Anti-human PD-1scFv03-His expression cassette

1..361 Hu promoter

362..544 SP50L5

545..1249 anti-human PD-1scFv03

1250..1267 His tag

1268..1270 stop codon

1271..1384 Hu terminator

1385..2648 Anti-human CTLA-4scFv01-FLAG expression cassette

1385..1619 P30 promoter

1620..1751 SP68L1

1752..2492 anti-human CTLA-4scFv01

2493..2516 FLAG tag

2517..2519 stop codon

2520..2648 T2 terminator

[0230]

(pPC4 scFv expression cassette sequences (2645 bp) (SEQ ID No. 133))

1..1330 Anti-human PD-1scFv03-His expression cassette

1..361 Hu promoter

362..490 SP67L10

491.. 1195 anti-human PD-1scFv03

1196..1213 His tag

1214..1216 stop codon

1217..1330 Hu terminator

1331..2645 Anti-human CTLA-4scFv01-FLAG expression cassette

1331..1565 P30 promoter

1566..1748 SP50L5

1749..2489 anti-human CTLA-4scFv01

2490..2513 FLAG tag

2514..2516 stop codon

2517..2645 T2 terminator

[0231]

(pPC5 scFv expression cassette sequences (2594 bp) (SEQ ID No. 134))

1..1330 Anti-human PD-1scFv03-His expression cassette

1..361 Hu promoter

362..490 SP67L10

491..1195 anti-human PD-1scFv03

1196..1213 His tag

1214..1216 stop codon

1217..1330 Hu terminator

1331..2594 Anti-human CTLA-4scFv01-FLAG expression cassette

1331..1565 P30 promoter

1566..1697 SP68L1

1698..2438 anti-human CTLA-4scFv01

2439..2462 FLAG tag

2463..2465 stop codon

2466..2594 T2 terminator

[0232]

(pPC6 scFv expression cassette sequences (2609 bp) (SEQ ID No. 135))

1..1294 Anti-human PD-1scFv03-His expression cassette

1..361 Hu promoter

362..454 SP69L1

455..1159 anti-human PD-1scFv03

1160..1177 His tag

1178..1180 stop codon

1181..1294 Hu terminator

1295..2609 Anti-human CTLA-4scFv01-FLAG expression cassette

1295..1529 P30 promoter

1530..1712 SP50L5

1713..2453 anti-human CTLA-4scFv01

2454..2477 FLAG tag

2478..2480 stop codon

2481..2609 T2 terminator

[0233]

(pPC7 scFv expression cassette sequences (2528 bp) (SEQ ID No. 136))

1..1294 Anti-human PD-1scFv03-His expression cassette

1..361 Hu promoter

362..454 SP69L1

455..1159 anti-human PD-1scFv03

1160..1177 His tag

1178..1180 stop codon

1181..1294 Hu terminator

1295..2528 Anti-human CTLA-4scFv01-FLAG expression cassette

1295..1529 P30 promoter

1530..1631 SP67L1

1632..2372 anti-human CTLA-4scFv01

2373..2396 FLAG tag

2397..2399 stop codon

2400..2528 T2 terminator

[0234]

(pPC8 scFv expression cassette sequences (2558 bp) (SEQ ID No. 137))

1..1294 Anti-human PD-1scFv03-His expression cassette

1..361 Hu promoter

362..454 SP69L1

455..1159 anti-human PD-1scFv03

1160..1177 His tag

1178..1180 stop codon

1181..1294 Hu terminator

1295..2558 Anti-human CTLA-4scFv01-FLAG expression cassette

1295..1529 P30 promoter

1530..1661 SP68L1

1662..2402 anti-human CTLA-4scFv01

2403..2426 FLAG tag

2427..2429 stop codon

2430..2558 T2 terminator

[0235]

(Transformation 10 of bacterium of the genus *Bifidobacterium*)

Bifidobacterium longum 105-A strain was transformed by the electroporation system in the same procedure as in the above section (Transformation 1 of bacterium of the genus *Bifidobacterium*) except that the plasmids pPC2, pPC3, pPC4, pPC5, pPC6, pPC7 and pPC8, extracted from the transformed *E. coli* mentioned above were used each in an amount of 5 μL. The transformants obtained were

designated as *Bifidobacterium longum* 105-A/pPC2 (hereinafter referred to as PC2 strain); *Bifidobacterium longum* 105-A/pPC3 (hereinafter referred to as PC3 strain); *Bifidobacterium longum* 105-A/pPC4 (hereinafter referred to as PC4 strain); *Bifidobacterium longum* 105-A/pPC5 (hereinafter referred to as PC5 strain); *Bifidobacterium longum* 105-A/pPC6 (hereinafter referred to as PC6 strain); *Bifidobacterium longum* 105-A/pPC7 (hereinafter referred to as PC7 strain) and *Bifidobacterium longum* 105-A/pPC8 (hereinafter referred to as PC8 strain).

[0236]

[Example 19]

[Preparation of PC2TL strain to PC8TL strain]

In view of clinical development, from the plasmids prepared in Example 18, scFv marker tag (histidine tag/FLAG tag) and a plasmid origin of replication pUCori, working in *E. coli* were removed to prepare anti-human PD-1scFv03 and anti-human CTLA-4scFv01 co-expression plasmids, pPC2TL to pPC8TL. *Bifidobacterium longum* 105-A was transformed with each of these plasmids to obtain co-expression strains, PC2TL strain to PC8TL strain.

[0237]

Basic constitution of the plasmid in co-expression strains, PC2TL strain to PC8TL strain, is shown in Figure 29. In 7 types of non-tagged co-expression plasmids pPC2TL to pPC8TL, the constitutions of anti-human PD-1scFv03 expression cassette and anti-human CTLA-4scFv01 expression cassette are shown in the following Table 20 and Table 21.

[0238]

[Table 20]

	Anti-hPD-1 scFv03 expression cassette (not tagged)				
Plasmid	Promoter	SP/Linker	scFv	Tag	Terminator
pPC2TL	Hu	SP50L5	anti-hPD-1 scFv03	-	Hu
pPC3TL	Hu	SP50L5	anti-hPD-1 scFv03	-	Hu
pPC4TL	Hu	SP67L10	anti-hPD-1 scFv03	-	Hu
pPC5TL	Hu	SP67L10	anti-hPD-1 scFv03	-	Hu
pPC6TL	Hu	SP69L1	anti-hPD-1 scFv03	-	Hu
pPC7TL	Hu	SP69L1	anti-hPD-1 scFv03	-	Hu
pPC8TL	Hu	SP69L1	anti-hPD-1 scFv03	-	Hu

[0239]

[Table 21]

	Anti-hCTLA-4 scFv01 expression cassette (not tagged)				
Plasmid	Promoter	SP/Linker	scFv	Tag	Terminator
pPC2TL	P30	SP67L1	anti-hCTLA-4 scFv01	-	T2
pPC3TL	P30	SP68L1	anti-hCTLA-4 scFv01	-	T2
pPC4TL	P30	SP50L5	anti-hCTLA-4 scFv01	-	T2
pPC5TL	P30	SP68L1	anti-hCTLA-4 scFv01	-	T2
pPC6TL	P30	SP50L5	anti-hCTLA-4 scFv01	-	T2
pPC7TL	P30	SP67L1	anti-hCTLA-4 scFv01	-	T2
pPC8TL	P30	SP68L1	anti-hCTLA-4 scFv01	-	T2

[0240]

(Constitution of anti-hPD-1scFv03 secretory expression cassette)

As anti-hPD-1scFv03 secretory expression cassette, a cassette sequentially comprising (1) a Hu promoter DNA, (2) a DNA encoding a signal peptide-linker peptide conjugate, (3) a DNA encoding an anti-hPD-1scFv03 amino

acid sequence and (4) a Hu terminator DNA was prepared such that DNA (1) was placed upstream (the 5' end) and DNA (4) downstream (the 3' end). As the signal peptide-linker peptide conjugate, SP50L5, SP67L10 or SP69L1 was used.

[0241]

(Constitution of anti-hCTLA-4scFv01 secretory expression cassette)

As anti-hCTLA-4scFv01 secretory expression cassette, a cassette sequentially comprising (1) a P30 promoter DNA, (2) a DNA encoding a signal peptide-linker peptide conjugate, (3) a DNA encoding an anti-hCTLA-4scFv01 amino acid sequence and (4) a T2 terminator DNA was prepared such that DNA (1) was placed upstream (the 5' end) and DNA (4) downstream (the 3' end). As the signal peptide-linker peptide conjugate, SP50L5, SP67L1 or SP68L1 was used.

[0242]

(Method for producing pPC2TL to pPC8TL)

The method for producing a plasmid is constituted of the following 4 steps as schematically shown in Figure 30.

(1) In the first step, a template plasmid, pHuSPxLy-hPD-1scFv03TL to be used in preparation of co-expression plasmids, pPC2TL-pPC8TL was prepared.

(2) In the second step, a T2 terminator fragment was inserted downstream of anti-PD-1scFv03 expression cassette of anti-hPD-1scFv03 (not tagged) expression plasmid to prepare plasmid pHuSPxLy-hPD-1scFv03TL-T2 (SPxLy = SP50L5, SP67L10, SP69L1).

(3) In the third step, anti-hCTLA-4scFv01 (not tagged) expression cassette (note that a terminator is excluded) was inserted between Hu terminator and T2

terminator of the plasmid prepared in the second step to prepare plasmids pPC2TLS to pPC8TLS.

(4) In the fourth step, the fragment of pUCori, which serves as a plasmid origin of replication in *E. coli*, of the plasmid prepared in the third step, was cleaved out with a restriction enzyme(s). Thereafter, the plasmid was allowed to self-close to prepare a co-expression plasmids (not tagged, non-shuttle) pPC2TL to pPC8TL.

[0243]

(First step)

PCR product A-1 (3261 bp) was prepared in the same manner as in the above step "PCR amplification 1" using pHuSP50L5-hPD-1scFv03-His as mentioned above as template DNA, pCDshuttle_F1_ primer (SEQ ID No. 126) as a forward primer and GA6_ primer as a reverse primer. PCR products, B-SP50L5 (1502 bp), B-SP67L10 (1448 bp) and B-SP69L1 (1412 bp) were prepared in the same manner as in the above step "PCR amplification 1" using pHuSP50L5-hPD-1scFv03-His, pHuSP67L10-hPD-1scFv03-His and pHuSP69L1-hPD-1scFv03-His, respectively as template DNA, and GA5_primer_rev primer as a forward primer and hPD1scFv03-1_ primer (SEQ ID No. 127) as a reverse primer.

[0244]

PCR product A-1 and each of B-SP50L5, B-SP67L10 and B-SP69L1 were ligated by using HD kit in the same manner as mentioned above and the sequences of "anti-hPD-1scFv03 expression cassette regions" (including from Hu promoter to Hu terminator) were determined. The plasmids extracted were designated as pHuSP50L5-hPD-1scFv03TL, pHuSP67L10-hPD-1scFv03TL and pHuSP69L1-hPD-1scFv03TL.

[0245]

(Second step)

PCR products were prepared by using the template DNA and primers listed in Table 22, in the same manner as in the above step "PCR amplification 1".

[0246]

[Table 22]

PCR product	Template DNA	Fow. primer	Rev. primer	Size of PCR product
A4	pHuSP50L5-hPD-1 scFv03TL	PC1_primer	PC2_primer	4733 bp
A5	pHuSP67L10-hPD-1 scFv03TL	PC1_primer	PC2_primer	4679 bp
A6	pHuSP69L1-hPD-1 scFv03TL	PC1_primer	PC2_primer	4643 bp
B	Bba-B0015 in pUC57	PC3_primer	PC4_primer	159 bp

[0247]

[Table 23]

Plasmid to be constructed	Combination in in-fusion reaction
pHuSP50L5-hPD-1 scFv03TL-T2	A4 and B
pHuSP67L10-hPD-1 scFv03TL-T2	A5 and B
pHuSP69L1-hPD-1 scFv03TL-T2	A6 and B

[0248]

The PCR products listed in Table 22 were ligated in accordance with the combinations listed in Table 23 by using HD kit in the same manner as mentioned above and the sequence of "anti-hPD-1scFv03 (not tagged) expression cassette-T2 terminator region" (5'-Hu promoter-SPxLy-human PD-1scFv03-Hu terminator-T2 terminator-3') was determined. The plasmids extracted were designated as pHuSP50L5-hPD-

1scFv03TL-T2, pHuSP67L10-hPD-1scFv03TL-T2 and pHuSP69L1-hPD-1scFv03TL-T2.

[0249]

(Third step)

PCR products were prepared by using the template DNA and primers listed in Table 24 in the same manner as in the above step "PCR amplification 1".

[0250]

[Table 24]

PCR product	Template DNA	Fow. primer	Rev. primer	Size of PCR product
A10	pHuSP50L5-hPD-1 scFv03TL-T2	PC8_primer (SEQ ID No. 124)	PC2_primer	4868 bp
A11	pHuSP67L10-hPD-1 scFv03TL-T2	PC8_primer	PC2_primer	4814 bp
A12	pHuSP69L1-hPD-1 scFv03TL-T2	PC8_primer	PC2_primer	4778 bp
B4	pP30SP50L5-hCTLA-4 scFv01-FLAG	PC6_primer (SEQ ID No. 122)	PC9_primer (SEQ ID No. 125)	1186 bp
B5	pP30SP67L1-hCTLA-4 scFv01-FLAG	PC6_primer	PC9_primer	1105 bp
B6	pP30SP68L1-hCTLA-4 scFv01-FLAG	PC6_primer	PC9_primer	1135 bp

[0251]

[Table 25]

Plasmid to be constructed	Combination in in-fusion reaction
pPC2TLS	A10 and B5
pPC3TLS	A10 and B6
pPC4TLS	A11 and B4
pPC5TLS	A11 and B6
pPC6TLS	A12 and B4
pPC7TLS	A12 and B5
pPC8TLS	A12 and B6

[0252]

The PCR products listed in Table 24 were ligated in accordance with the combinations listed in Table 25 by using HD kit in the same manner as mentioned above and DNA sequences of the full-length plasmids extracted were determined in the same manner as above.

The plasmids extracted were designated as pPC2TLS, pPC3TLS, pPC4TLS, pPC5TLS, pPC6TLS, pPC7TLS and pPC8TLS.

[0253]

(Fourth step)

To plasmids pPC2TLS to pPC8TLS (2 μg for each), which were prepared in the third step of the plasmid preparation, restriction enzymes, BamHI and BglII (manufactured by Thermo Scientific Inc.) were added each in an amount of 10 units. The mixture was kept warm at 37°C for 3 hours to cleave the plasmids. BamHI and BglII recognition sites are present one for each in the adjacent

portions to pUCori, which is the plasmid origin of replication in *E. coli*, as shown in Figure 30. After the treatment with the restriction enzymes, an aliquot was taken from the DNA solution and subjected to 0.8% agarose gel electrophoresis. In this manner, it was verified that the plasmid DNA was cleaved into fragments having predetermined sizes (about 5.3 kbp and about 0.7 kbp).

[0254]

(Separation and purification by agarose gel electrophoresis)

After the treatment with the restriction enzymes, the DNA fragments were separated by agarose gel electrophoresis. DNA band of about 5.3 kbp was cut out and DNA was extracted by use of QIAGel. The concentration of DNA was estimated by agarose gel electrophoresis.

[0255]

(Self-closing of DNA)

The purified DNA mentioned above (about 5.3 kbp) was subjected to a self-ligation reaction using Rapid DNA Ligation kit (manufactured by Thermo Scientific Inc.). After completion of the reaction, the solution was purified by QIAquick PCR Purification Kit (manufactured by QIAGEN) to prepare pPC2TL, pPC3TL, pPC4TL, pPC5TL, pPC6TL, pPC7TL and pPC8TL.

[0256]

(Transformation 11 of bacterium of the genus *Bifidobacterium*)

Bifidobacterium longum 105-A strain was transformed by the electroporation system in the same procedure as in the above section (Transformation 1 of bacterium of the

genus *Bifidobacterium*) except that plasmid pPC2TL, pPC3TL, pPC4TL, pPC5TL, pPC6TL, pPC7TL and pPC8TL prepared in the fourth step of the plasmid preparation were used each in an amount of 5 μL. The transformants obtained were designated as *Bifidobacterium longum* 105-A/pPC2TL (hereinafter referred to as PC2TL strain); *Bifidobacterium longum* 105-A/pPC3TL (hereinafter referred to as PC3TL strain); *Bifidobacterium longum* 105-A/pPC4TL (hereinafter referred to as PC4TL strain); *Bifidobacterium longum* 105-A/pPC5TL (hereinafter referred to as PC5TL strain); *Bifidobacterium longum* 105-A/pPC6TL (hereinafter referred to as PC6TL strain); *Bifidobacterium longum* 105-A/pPC7TL (hereinafter referred to as PC7TL strain); and *Bifidobacterium longum* 105-A/pPC8TL (hereinafter referred to as PC8TL strain).

[0257]

(Determination of plasmid DNA sequence)

To each of the bacterium of the genus *Bifidobacterium* transformant cells obtained in the above section (Transformation 11 of bacterium of the genus *Bifidobacterium*), 1.5 mL of a lysozyme-Proteinase K mixture, which was prepared by adding lysozyme (manufactured by Wako Pure Chemical Industries Ltd.) and Proteinase K (manufactured by QIAGEN) in a concentration of 40 mg/mL and 0.4 mg/mL, respectively with the addition of 1 mM Tris-HCl-0.1mM EDTA buffer (pH8.0), was added and suspended. Each of the mixtures was kept warm at 37°C for about 1.5 hours. After centrifugal separation was carried out and the supernatant was removed, the resultant bacterial cells were subjected to extraction of plasmid

DNA by QIAprep Spin Miniprep Kit (manufactured by QIAGEN). In the same manner as in the above section (Example 1: Transformation of *E. coli* and determination of DNA sequence of plasmid pAG8), the DNA sequence of the full-length plasmid extracted was determined. The plasmids extracted were designated as plasmid pPC2TL, pPC3TL, pPC4TL, pPC5TL, pPC6TL, pPC7TL and pPC8TL as outlined below. The sequences of expression cassettes of plasmids pPC2TL, pPC3TL, pPC4TL, pPC5TL, pPC6TL, pPC7TL and pPC8TL are the same as in pPC2, pPC3, pPC4, pPC5, pPC6, pPC7 and pPC8 sequences except that neither of the sequences of expression cassettes of anti-hPD-1scFv03 and anti-hCTLA-4scFv01 contain a tag sequence.

[0258]

(pPC2TL: scFv expression cassette sequence (2576 bp) (SEQ ID No. 138))

1..1366 Anti-human PD-1scFv03 expression cassette

1..361	Hu promoter
362..544	SP50L5
545..1249	anti-human PD-1scFv03
1250..1252	stop codon
1253..1366	Hu terminator

1367..2576 Anti-human CTLA-4scFv01 expression cassette

1367..1601	P30 promoter
1602..1703	SP67L1
1704..2444	anti-human CTLA-4scFv01
2445..2447	stop codon
2448..2576	T2 terminator

[0259]

(pPC3TL scFv expression cassette sequence (2606 bp) (SEQ ID No. 139))

1..1366 Anti-human PD-1scFv03 expression cassette

1..361 Hu promoter

362..544 SP50L5

545..1249 anti-human PD-1scFv03

1250..1252 stop codon

1253..1366 Hu terminator

1367..2606 Anti-human CTLA-4scFv01 expression cassette

1367..1601 P30 promoter

1602..1733 SP68L1

1734..2474 anti-human CTLA-4scFv01

2475..2477 stop codon

2478..2606 T2 terminator

[0260]

(pPC4TL scFv expression cassette sequence (2603 bp) (SEQ ID No. 140))

1..1312 Anti-human PD-1scFv03 expression cassette

1..361 Hu promoter

362..490 SP67L10

491.. 1195 anti-human PD-1scFv03

1196..1198 stop codon

1199..1312 Hu terminator

1313..2603 Anti-human CTLA-4scFv01 expression cassette

1313..1547 P30 promoter

1548..1730 SP50L5

1731..2471 anti-human CTLA-4scFv01

2472..2474 stop codon

2475..2603 T2 terminator

[0261]

(pPC5TL scFv expression cassette sequence (2552 bp) (SEQ ID No. 141))

1..1312 Anti-human PD-1scFv03 expression cassette

1..361 Hu promoter

362..490 SP67L10

491..1195 anti-human PD-1scFv03

1196..1198 stop codon

1199..1312 Hu terminator

1313..2552 Anti-human CTLA-4scFv01 expression cassette

1313..1547 P30 promoter

1548..1679 SP68L1

1680..2420 anti-human CTLA-4scFv01

2421..2423 stop codon

2424..2552 T2 terminator

[0262]

(pPC6TL scFv expression cassette sequence (2567 bp) (SEQ ID No. 142))

1..1276 Anti-human PD-1scFv03 expression cassette

1..361 Hu promoter

362..454 SP69L1

455..1159 anti-human PD-1scFv03

1160..1162 stop codon

1163..1276 Hu terminator

1277..2567 Anti-human CTLA-4scFv01 expression cassette

1277..1511 P30 promoter

1512..1694 SP50L5

1695..2435 anti-human CTLA-4scFv01

2436..2438 stop codon

2439..2567 T2 terminator

[0263]

(pPC7TL scFv expression cassette sequence (2486 bp) (SEQ ID No. 143))

1..1276 anti-human PD-1scFv03 expression cassette

1..361 Hu promoter

362..454 SP69L1

455..1159 anti-human PD-1scFv03

1160..1162 stop codon

1163..1276 Hu terminator

1277..2486 anti-human CTLA-4scFv01 expression cassette

1277..1511 P30 promoter

1512..1613 SP67L1

1614..2354 anti-human CTLA-4scFv01

2355..2357 stop codon

2358..2486 T2 terminator

[0264]

(pPC8TL scFv expression cassette sequence (2516 bp) (SEQ ID No. 144))

1..1276 Anti-human PD-1scFv03 expression cassette

1..361 Hu promoter

362..454 SP69L1

455..1159 anti-human PD-1scFv03

1160..1162 stop codon

1163..1276 Hu terminator

1277..2516 Anti-human CTLA-4scFv01 expression cassette

1277..1511 P30 promoter

1512..1643 SP68L1

1644..2384 anti-human CTLA-4scFv01

2385..2387 stop codon

2388..2516 T2 terminator

[0265]

[Example 20]

[Verification of scFv secretion in co-expression *Bifidobacterium* strain]

The presence or absence of secretions of anti-hPD-1scFv03-His and anti-hCTLA-4scFv01-FLAG in culture supernatants of co-expression strains, PC2 to PC8, was checked by western blotting as follows. The BEshuttle strain mentioned above was used as a negative control strain and subjected to the same analysis.

[0266]

(Culture of recombinant bacterium of the genus *Bifidobacterium*)

PC2 strain to PC8 strain, PC1 strain prepared in Example 8 and BEshuttle strain were cultured in the same procedure as in Example 4 (Culture of recombinant bacterium of the genus *Bifidobacterium*) and the culture supernatants were collected. The culture supernatants were concentrated by TCA in the same procedure as in

Example 9 (Culture of recombinant bacterium of the genus *Bifidobacterium*) to obtain individual culture supernatant concentrates.

[0267]

(Western analysis)

Western analysis using each of the culture supernatant concentrates (corresponding to about 0.01 mL of the culture solution) was carried out in the same manner as in Example 9 (Western analysis). Note that transferring from the gel to membrane was carried out by using Trans-Blot Turbo (manufactured by Bio-Rad) and the 2%ECL Advance Blocking Agent mentioned above was used as a blocking solution. The results for anti-hPD-1scFv03-His are shown in Figure 31 (A) and the results for anti-hCTLA-4scFv01-FLAG are shown in Figure 31 (B).

[0268]

(Results)

As is apparent from Figures 31A and B, both secretions of anti-hPD-1scFv03-His and anti-hCTLA-4scFv01-FLAG were observed in the culture supernatants of PC2 strain (lane 1 both in A and B), PC3 strain (lane 2 both in A and B), PC4 strain (lane 3 both in A and B), PC5 strain (lane 4 both in A and B), PC6 strain (lane 5 both in A and B), PC7 strain (lane 6 both in A and B), PC8 strain (lane 7 both in A and B) and PC1 strain (lane 9 both in A and B) (note that in PC1 strain, anti-hCTLA-4scFv02-FLAG was secreted). The amounts of anti-hPD-1scFv03-His and anti-hCTLA-4scFv01-FLAG secreted from PC2 strain to PC8 strain were each larger than the amount of single-chain antibody secreted from PC1 strain.

[0269]

From the above results, it was verified that co-expression *Bifidobacterium* strains, PC2 strain to PC8 strain, are recombinant *Bifidobacterium* strains that can secrete both anti-hPD-1scFv and anti-hCTLA-4scFv in the culture supernatants and secrete a single-chain antibody in a larger amount than PC1 strain.

[0270]

[Example 21]

[Verification of scFv secretion in co-expression strains, PC2TL strain to PC8TL strain]

The antibodies secreted from the co-expression strains, PC2TL strain to PC8TL strain, were subjected to ELISA for checking the presence or absence of binding activity to human PD-1 (hPD-1) and binding activity to human CTLA-4.

[0271]

(Culture of recombinant bacterium of the genus *Bifidobacterium*)

Co-expression *bifidobacteria*, PC2TL strain to PC8TL strain prepared in Example 19 above and BEshuttle strain were cultured in the same procedure as in Example 4 (Culture of recombinant bacterium of the genus *Bifidobacterium*) and the culture supernatants were collected. The culture supernatants of PC2TL strain to PC8TL strain were appropriately diluted with the culture supernatant of BEshuttle strain and used as samples for ELISA.

[0272]

(Evaluation on secretion of anti-hPD-1scFv03)

To 96-well plates (MaxiSorp, Type C, manufactured by Nunc), 1 μg/mL hPD-1 solution (using Recombinant Human PD-1 Fc Chimera (R&D Systems) as an antigen and prepared with 1X PBS) was dispensed in an amount of 100 μL for each. To the upper portion of the plates, a seal was attached and the plates were incubated at 4°C overnight to immobilize the antigen. After the solution was removed from the plates, 1X PBS was dispensed in an amount of 400 μL for each and the liquid was removed. This operation was repeated three times for washing (hereinafter referred to as "PBS washing").

[0273]

To the plates washed, 1% BSA solution (350 μL for each) was dispensed. The plates were incubated at room temperature for 2 hours for blocking and PBS washing was carried out.

[0274]

To the plates, the samples for ELISA were dispensed. To the blank wells, the culture supernatant (100 μL) of BEshuttle strain was dispensed. As a positive control, a solution (100 μL), which was prepared by adding anti-hPD-1scFv03 purified product to the culture supernatant of BEshuttle strain so as to obtain a concentration of 100 ng/mL, was dispensed. A seal was attached to the upper portions of the plates and the plates were incubated at room temperature for 2 hours to carry out the scFv binding reaction to the antigen immobilized and PBS washing was carried out.

[0275]

After completion of the scFv binding reaction to the antigen immobilized, 100 μL of 0.2 μg/mL biotinylated Protein L solution (prepared by diluting Biotinylated Protein L: manufactured by Thermo Scientific with Solution B of Signal Enhancer HIKARI: manufactured by Nacalai Tesque Inc., up to a concentration of 0.2 μg/mL) was added to the plates. A seal was attached to the upper portions of the plates and the plates were incubated at room temperature for one hour (binding reaction of biotinylated Protein L to scFv light chain, κ chain) and PBS washing was carried out.

[0276]

After the binding reaction of the biotinylated Protein L, 100 μL of an avidin-biotin marker enzyme complex preparation solution (prepared by adding Solution A and B (30 μL for each) of avidin-biotin marker enzyme complex VECTASTAIN ABC Kit: manufactured by VECTOR Laboratories, to Solution B (7.5 mL) of Signal Enhancer HIKARI, followed by stirring) was added to the plates. A seal was attached to the upper portions of the plates and the plates were incubated at room temperature for 30 minutes and PBS washing was carried out.

[0277]

To the plates to which the enzyme was bound, 200 μL of a detection reagent (comprising Color Reagent A and Color Reagent B: both are manufactured by R&D systems and blended in equal amounts) was added. The plates were shielded from light and incubated at room temperature. Accurately 20 minutes later, the stop solution (50 μL) mentioned above was added to terminate a color reaction.

Absorbance was measured at 450 nm and 570 nm (reference wavelength) by a plate reader.

[0278]

(Evaluation on secretion of anti-hCTLA-4scFv01)

Evaluation was carried out in the same manner as in the above section (Evaluation on secretion of anti-hPD-1scFv03) except the points: 1) the antigen was immobilized to a plate by using a 1 μg/mL hCTLA-4 solution (Recombinant Human CTLA-4-Fc Chimera (manufactured by BioLegend) prepared in the addition of 1X PBS), 2) as the positive control, anti-hCTLA-4scFv01 purified product added in the culture supernatant of BEshuttle strain was used and 3) the concentration of biotinylated Protein L solution was specified as 0.05 μg/mL. The results are shown in Table 26.

[0279]

[Table 26]

Binding activity of scFv to immobilized antigen

Sample	Binding (OD450-570) to immobilized antigen	
	hPD-1 immobilized	hCTLA-4 immobilized
PC2TL	0.812	1.312
PC3TL	0.792	1.286
PC4TL	0.363	1.788
PC5TL	0.305	1.39
PC6TL	0.528	1.116
PC7TL	0.549	1.457
PC8TL	0.733	1.320
P.C. 1 (anti-hPD-1 scFv03)	0.667	n.t.
P.C. 2 (anti-hCTLA-4 scFv01)	n.t.	1.074

n.t.: not treated, P.C.: Positive control

[0280]

(Results)

As is apparent from Table 26, culture supernatants of ELISA co-expression strains, PC2TL strain to PC8TL strain, had binding activities to both of human PD-1 and human CTLA-4 immobilized. Note that it was already verified that the binding activities of such scFv molecules are specific to the corresponding antigens (data are not shown).

[0281]

[Example 22]

Anti-hPD-1scFv03-His single-expression strain and anti-hCTLA-4scFv01-FLAG single-expression strain to be

compared with co-expression *bifidobacteria*, PC2 strain to PC8 strain, are shown in Table 27.

[0282]

[Table 27]

Co-expression strain	Comparable anti-hPD-1scFv03-His single expression strain	Comparable anti-hCTLA-4scFv01-FLAG single expression strain
PC2	P1H	C2F
PC3	P1H	C3F
PC4	P2H	C1F
PC5	P2H	C3F
PC6	P3H	C1F
PC7	P3H	C2F
PC8	P3H	C3F

[0283]

(Transformation 12 of bacterium of the genus *Bifidobacterium*)

Bifidobacterium longum 105-A strain was transformed by the electroporation system in the same procedure as in the above section (Transformation 1 of bacterium of the genus *Bifidobacterium*) by using plasmid pHuSP50L5-hPD-1scFv03-His, pHuSP67L10-hPD-1scFv03-His and pHuSP69L1-hPD-1scFv03-His prepared in Example 18. The transformants obtained were designated as *Bifidobacterium longum* 105-A/pHuSP50L5-hPD-1scFv03-His (hereinafter referred to as P1H strain); *Bifidobacterium longum* 105-A/pHuSP67L10-hPD-1scFv03-His (hereinafter referred to as P2H strain); and *Bifidobacterium longum* 105-A/pHuSP69L1-hPD-1scFv03-His (hereinafter referred to as P3H strain).

[0284]

Similarly, *Bifidobacterium longum* 105-A strain was transformed by using plasmid pC1F, pC2F and pC3F prepared in Example 18 to obtain *Bifidobacterium longum* 105-A/pC1F (hereinafter referred to as C1F strain); *Bifidobacterium longum* 105-A/pC2F (hereinafter referred to as C2F strain); and *Bifidobacterium longum* 105-A/pC3F (hereinafter referred to as C3F strain).

[0285]

[Example 23]

Anti-hPD-1scFv03 single-expression strain and anti-hCTLA-4scFv01 single-expression strain to be compared with co-expression *bifidobacteria* PC2TL-PC8TL strain are shown in Table 28.

[0286]

[Table 28]

Co-expression strain	Comparable anti-hPD-1scFv03 single expression strain	Comparable anti-hCTLA-4scFv01 single expression strain
PC2TL	P1TL	C2TLB
PC3TL	P1TL	C3TLB
PC4TL	P2TL	C1TLB
PC5TL	P2TL	C3TLB
PC6TL	P3TL	C1TLB
PC7TL	P3TL	C2TLB
PC8TL	P3TL	C3TLB

[0287]

(Transformation 13 of bacterium of the genus *Bifidobacterium*)

Bifidobacterium longum 105-A strain was transformed by the electroporation system in the same procedure as in the above section (Transformation 1 of bacterium of the genus *Bifidobacterium*) by using plasmid pHuSP50L5-hPD-1scFv03TL, pHuSP67L10-hPD-1scFv03TL and pHuSP69L1-hPD-1scFv03TL prepared in Example 19. The transformants obtained were designated as *Bifidobacterium longum* 105-A/pHuSP50L5-hPD-1scFv03TL (hereinafter referred to as P1TL strain); *Bifidobacterium longum* 105-A/pHuSP67L10-hPD-1scFv03TL (hereinafter referred to as P2TL strain); and *Bifidobacterium longum* 105-A/pHuSP69L1-hPD-1scFv03TL (hereinafter referred to as P3TL strain).

[0288]

(Preparation of anti-hCTLA-4scFv01 single-expression strain)

A method for producing anti-hCTLA-4scFv01 expression plasmids pC1TLB, pC2TLB and pC3TLB is schematically shown in Figure 32. The method consists of two steps: T2 substitution step of removing FLAG tag from plasmids pC1F, pC2F and pC3F and replacing with T2 terminator; and pUCori removal step of removing pUCori from plasmids pC1TL, pC2TL and pC3TL.

[0289]

(T2 substitution step)

Using the template DNA and primers listed in Table 29, PCR products were prepared in the same manner as in the above step "PCR amplification 1".

[0290]

[Table 29]

PCR product	Template DNA	Fow. primer	Rev. primer	Size of PCR product
A1	pC1F	PC1_primer	PC9_primer	4538 bp
A2	pC2F	PC1_primer	PC9_primer	4457 bp
A3	pC3F	PC1_primer	PC9_primer	4487 bp
B	BBa-B0015 in pUC57	PC8_primer	PC4_primer	150 bp

[0291]

PCR products were used in combination in accordance with the following three types: A1 and B (for preparation of pC1TL), A2 and B (for preparation of pC2TL) and A3 and B (for preparation of pC3TLpreparation) and ligated by using HD kit in the same manner as mentioned above. The DNA sequences of the full-length plasmids extracted were determined in the same procedure as above. The plasmids extracted were designated as pC1TL, pC2TL and pC3TL.

[0292]

(pUCori removal step)

pUCori was removed from pC1TL, pC2TL and pC3TL in the same manner as in the third step of the method for producing co-expression plasmids, pPC2TL to pPC8TL in Example 19 to obtain pC1TLB, pC2TLB and pC3TLB.

[0293]

(Transformation 14 of bacterium of the genus *Bifidobacterium*)

Bifidobacterium longum 105-A strain was transformed by the electroporation system in the same procedure as in the above section (Transformation 1 of bacterium of the genus *Bifidobacterium*) by using plasmid pC1TLB, pC2TLB and

pC3TLB. The transformants obtained were designated as *Bifidobacterium longum* 105-A/pC1TLB (hereinafter referred to as C1TLB strain); *Bifidobacterium longum* 105-A/pC2TLB (hereinafter referred to as C2TLB strain); and *Bifidobacterium longum* 105-A/pC3TLB (hereinafter referred to as C3TLB strain).

[0294]

[Example 24]

(Binding to cell)

Binding of anti-hCTLA-4scFv01-FLAG purified from the anti-hCTLA-4scFv01-FLAG single-expression strain to be compared with co-expression strains, PC2 to PC8 was investigated.

[0295]

Anti-hCTLA-4scFv01-FLAG was purified from each of the culture supernatants of anti-hCTLA-4scFv01-FLAG single expression *Bifidobacterium* C1F strain, C2F strain and C3F strain by Protein L column. The purified scFv (100 µL) prepared to have a concentration of 1 µg/mL was added to HEK293T cells, in which intracellular domain deficient hCTLA-4 was overexpressed by use of a retrovirus vector, and incubated on ice for 30 minutes. After the cells were washed, biotinylated Protein L (100 µL) was added and incubated for 30 minutes to allow protein L to bind to scFv bound to the cell. After the cells were washed, Brilliant Violet 421 Streptavidin (100 µL) prepared to have a concentration of 0.5 µg/mL was added and incubated for 30 minutes. The cells were washed and then analysis was carried out by flow cytometry. The results are shown in Figure 33.

[0296]

(Results)

As is apparent from Figure 33, scFv molecules derived from C1F strain, C2F strain and C3F strain all bound to intracellular domain deficient hCTLA-4 expression HEK293T cells. From this, it was presumed that anti-hCTLA-4scFv01-FLAG secreted from each of co-expression strains, PC4 and PC6, which secrete the same scFv as in C1F strain; co-expression strains, PC2 strain and PC7 strain, which secrete the same scFv as in C2F strain; and co-expression strains, PC3 strain, PC5 strain and PC8 strain, which secrete the same scFv as in C3F strain can also bind to hCTLA-4 expressing cells. From Figure 33 (a), binding to a Mock cells not expressing hCTLA-4 was slightly observed; however, binding of anti-hCTLA-4scFv01-FLAG purified from co-expression PC4 strain to the Mock cells were not observed from the results of Figure 37 (d). From this, binding to scFv molecules derived from C1F strain, C2F strain and C3F strain to the Mock cells is considered as non-specific binding.

[0297]

[Example 25]

(Binding of anti-hCTLA-4scFv01 purified from anti-hCTLA-4scFv01 single-expression strain to cells)

Anti-hCTLA-4scFv01 was purified from each of the culture supernatants of anti-hCTLA-4scFv01 single-expression strains, C1TLB, C2TLB and C3TLB to be compared with co-expression strains, PC2TL to PC8TL, by Protein L column. To HEK293T cells in which intracellular domain deficient hCTLA-4 was overexpressed by use of a retrovirus

vector, purified scFv (100 µL) prepared to have a concentration of 1 µg/mL was added and incubated on ice for 30 minutes. After the cells were washed, biotinylated Protein L (100 µL) was added and incubated for 30 minutes to allow protein L to bind to scFv bound to the cells. After the cells were washed, Brilliant Violet 421 Streptavidin (100 µL) prepared to have a concentration of 0.5 µg/mL was added and incubated for 30 minutes. The cells were washed and then analysis was carried out by flow cytometry. The results are shown in Figure 34.

[0298]

(Results)

As is apparent form Figure 34, scFv molecules derived from C1TLB strain, C2TLB strain and C3TLB strain all bound to hCTLA-4 expressing cells. From this, it was presumed that anti-hCTLA-4scFv01 secreted from each of co-expression strains, PC4TL strain and PC6TL strain, which secrete the same scFv as in C1TLB strain; co-expression strains, PC2TL strain and PC7TL strain, which secrete the same scFv as in C2TLB strain; and co-expression strains, PC3TL strain, PC5TL strain and PC8TL strain, which secrete the same scFv as in C3TLB strain can also bind to hCTLA-4 expressing cells.

[0299]

[Example 26]

(Binding of anti-PD-1scFv03-His purified from anti-hPD-1scFv03-His single-expression strain to cells)

Anti-PD-1scFv03-His was purified from each of the culture supernatants of anti-PD-1scFv03-His single-expression strains, P1H, P2H and P3H to be compared with

co-expression strains, PC2 to PC8, by Protein L. To HEK293T cells in which hPD-1 was overexpressed by use of a retrovirus vector, purified scFv (100 µL) prepared to have a concentration of 1 µg/mL was added and incubated on ice for 30 minutes. After the cells were washed, biotinylated Protein L (100 µL) was added, incubated for 30 minutes to allow protein L to bind to scFv bound to the cells. After the cells were washed, Brilliant Violet 421 Streptavidin (100 µL) prepared to have a concentration of 0.5 µg/mL was added and incubated for 30 minutes. The cells were washed and then analysis was carried out by flow cytometry. The results are shown in Figure 35.

[0300]

(Results)

As is apparent form Figure 35, scFv molecules derived from P1H strain, P2H strain and P3H strain all bound to hPD-1 expressing cells. From this, it was presumed that anti-PD-1scFv03-His secreted from each of co-expression strains, PC2 and PC3, which secrete the same scFv as in P1H strain; co-expression strains, PC4 and PC5, which secrete the same scFv as in P2H strain; and co-expression strains, PC6, PC7 and PC8, which secrete the same scFv as in P3H strain can also bind to hPD-1 expressing cells.

[0301]

[Example 27]

(Binding of anti-hPD-1scFv03 purified from anti-hPD-1scFv03 single-expression strain to cells)

Anti-hPD-1scFv03 was purified from each of the culture supernatants of PD-1scFv03 single expression

bifidobacteria, P1TL strain, P2TL strain and P3TL strain, to be compared with co-expression strains, PC2TL strain to PC8TL strain, by Protein L column. To HEK293T cells in which hPD-1 was overexpressed by use of a retrovirus vector, purified scFv (100 µL) prepared to have a concentration of 1 µg/mL was added and incubated on ice for 30 minutes. After the cells were washed, biotinylated Protein L (100 µL) was added and incubated for 30 minutes to allow protein L to bind to scFv bound to the cells. After the cells were washed, Brilliant Violet 421 Streptavidin (100 µL) prepared to have a concentration of 0.5 µg/mL was added and incubated for 30 minutes. The cells were washed and then analysis was carried out by flow cytometry. The results are shown in Figure 36.

[0302]

(Results)

As is apparent from Figure 36, scFv molecules derived from P1TL strain, P2TL strain and P3TL strain all bound to hPD-1 expressing cells. From this, it was presumed that anti-PD-1scFv03 secreted from each of co-expression strains, PC2TL and PC3TL, which secrete the same scFv as in P1TL strain; co-expression strains, PC4TL and PC5TL, which secrete the same scFv as in P2TL strain; and co-expression strains, PC6TL, PC7TL and PC8TL, which secrete the same scFv as in P3TL strain can bind to hPD-1 expressing cells.

[0303]

[Example 28]

(Binding of scFv purified from co-expression strain PC4 to cells)

From the culture supernatant of co-expression strain PC4, anti-hPD-1scFv03-His was purified by use of a purification column for His-tag; and anti-hCTLA-4scFv01-FLAG was purified by use of a purification column for FLAG tag. Binding of anti-hPD-1scFv03 to hPD-1 expressing cells were checked in the same manner as in [Example 26]. Binding of anti-hCTLA-4scFv01 to hCTLA-4 expressing cells were checked in the same manner as in [Example 24]. As positive controls, PE-labeled anti-hPD-1 antibody (clone: EH12.2H7) and PE-labeled anti-hCTLA-4 antibody (clone: L3D10) were used. The results are shown in Figure 37.

[0304]

From Figure 37 (a), it was found that a positive control, i.e., PE-labeled anti-hPD-1 antibody (clone: EH12.2H7) binds to hPD-1 expressing cells and binds to neither Mock cells nor hCTLA-4 expressing cells. From (b), it was found that PE-labeled anti-hCTLA-4 antibody (clone: L3D10) binds to hCTLA-4 expressing cells and binds to neither Mock cells nor hPD-1 expressing cells. As is apparent from Figures 37 (c) and (d), it was found that anti-hPD-1scFv03-His and anti-hCTLA-4scFv01-FLAG purified from co-expression strain PC4 bind to the corresponding antigen expressing cells.

[0305]

[Example 29]

(Affinity of anti-hCTLA-4scFv01-FLAG purified from anti-hCTLA-4scFv01-FLAG single-expression strain for antigen (Biacore))

Anti-hCTLA-4scFv01-FLAG was purified by Protein L column from each of the culture supernatants of anti-

hCTLA-4scFv01-FLAG single expression *bifidobacteria* C1F, C2F and C3F to be compared with co-expression strains, PC2 to PC8. The binding kinetic parameter of each hCTLA-4scFv01-FLAG purified to hCTLA-4 (fusion protein with human IgG1Fc) was measured by surface plasmon resonance using Biacore system (manufactured by GE Healthcare). To a substrate having a carboxyl methyl dextran-coated gold membrane, a mouse anti-human IgG (Fc) monoclonal antibody (manufactured by GE Healthcare) was bound in accordance with amine coupling, and Fc-fused recombinant hCTLA-4 (Biolegend) was captured as a ligand. As an analyte, scFv purified was used. Samples of scFv having a concentration of 0.7404, 2.2222, 6.6667, 20 and 60 nM were prepared and sequentially subjected to binding in order of increasing concentration in accordance with single cycle kinetics. Data obtained were analyzed by BIA evaluation software (manufactured by GE Healthcare). The results are shown in Table 30.

[0306]

[Table 30]

sample	ka (1/Ms)	kd (1/s)	KD (M)
C1F	9.18E+05	9.96E-04	1.08E-09
C2F	4.52E+05	1.03E-03	2.28E-09
C3F	3.47E+05	1.03E-03	2.97E-09
C1TLB	6.61E+05	9.80E-04	1.48E-09
C2TLB	9.81E+05	9.61E-04	9.80E-10
C3TLB	7.73E+05	9.96E-04	1.29E-09

[0307]

(Results)

As is apparent from Table 30, scFvs secreted from each of the single-expression strains C1F, C2F and C3F exhibits a high KD value, meaning that scFvs have affinity for hCTLA-4. It is presumed that anti-hCTLA-4scFv secreted from each of co-expression strains, PC4 and PC6, also has affinity for hCTLA-4, similarly to scFv derived from C1F strain. It is also presumed that scFv secreted from each of co-expression strains, PC2 and PC7, presumably has affinity for hCTLA-4 similarly to scFv derived from C2F strain; and that scFv secreted from each of co-expression strains, PC3, PC5 and PC8, has affinity for hCTLA-4 similarly to scFv derived from C3F strain.

[0308]

[Example 30]

(Affinity of anti-hCTLA-4scFv01 purified from anti-hCTLA-4scFv01 single-expression strain for antigen (Biacore))

Anti-hCTLA-4scFv01 was purified by Protein L column from each of the culture supernatants of anti-hCTLA-4scFv01 single expression *bifidobacteria*, C1TLB strain, C2TLB strain and C3TLB strain to be compared with co-expression strains, PC2TL to PC8TL. The binding kinetic parameter to hCTLA-4 (fusion protein with human IgG1Fc) was measured by surface plasmon resonance using Biacore system. Thereafter, analysis was carried out in the same manner as in Example 29. The results are shown in Table 30.

[0309]

(Results)

As is apparent from Table 30, scFvs secreted from each of single-expression strains, C1TLB, C2TLB and C3TLB exhibits a high KD value, meaning that scFvs have affinity for hCTLA-4. It is presumed that scFv secreted from each of co-expression strains, PC4TL and PC6TL, also has affinity for hCTLA-4, similarly to scFv derived from C1TLB strain. It is also presumed that scFv secreted from each of co-expression strains, PC2TL and PC7TL, has affinity for hCTLA-4 similarly to scFv derived from C2TLB strain; and that scFv secreted from each of co-expression strains, PC3TL, PC5TL and PC8TL, has affinity for hCTLA-4 similarly to scFv derived from C3TLB strain.

[0310]

[Example 31]

(Affinity of anti-hPD-1scFv03-His purified from anti-hPD-1scFv03-His single-expression strain for antigen (Biacore))

Anti-hPD-1scFv03-His was purified by Protein L column from each of the culture supernatants of anti-hPD-1scFv03-His single expression *bifidobacteria*, P1H strain, P2H strain and P3H strain, to be compared with co-expression strains, PC2 to PC8. The binding kinetic parameter with hPD-1 (fusion protein with human IgG1Fc) was measured by surface plasmon resonance using Biacore system. Analysis was carried out in the same manner as in Example 29 except that Fc fused recombinant hPD-1 (R&D) was captured as a ligand. The results are shown in Table 31.

[0311]

[Table 31]

sample	ka (1/Ms)	kd (1/s)	KD (M)
P1H	4.79E+05	1.29E-03	2.69E-09
P2H	4.60E+05	1.86E-03	4.04E-09
P3H	4.33E+05	1.99E-03	4.60E-09
P1TL	3.44E+05	1.34E-03	3.89E-09
P2TL	3.35E+05	1.96E-03	5.85E-09
P3TL	3.03E+05	2.07E-03	6.83E-09

[0312]

(Results)

As is apparent from Table 31, scFvs secreted from each of single-expression strains, P1H, P2H and P3H exhibited a high KD value, meaning that scFvs have affinity for hPD-1. It is presumed that scFv secreted from each of co-expression strains, PC2 and PC3, also has affinity for hPD-1, similarly to scFv derived from P1H strain. It is also presumed that scFv secreted from each of co-expression strains, PC4 and PC5 has affinity for hPD-1, similarly to scFv derived from P2H strain; and that scFv secreted from each of co-expression strains, PC6, PC7 and PC8 has affinity for hPD-1, similarly to scFv derived from P3H strain.

[0313]

[Example 32]

(Affinity of anti-hPD-1scFv03 purified from anti-hPD-1scFv03 single-expression strain for antigen (Biacore))

Anti-hPD-1scFv03 was purified by Protein L column from each of the culture supernatants of anti-hPD-1scFv03 single expression *bifidobacteria*, P1TL strain, P2TL strain and P3TL strain to be compared with co-expression strains, PC2TL to PC8TL. The binding kinetic parameter with hPD-1 (fusion protein with human IgG1Fc) was measured by surface plasmon resonance using Biacore system. Analysis was carried out in the same manner as in Example 29 except that Fc fused recombinant hPD-1 (R&D) was captured as a ligand. The results are shown in Table 31.

[0314]

(Results)

As is apparent from Table 31, scFvs secreted from each of single-expression strains P1TL, P2TL and P3TL exhibited a high KD value, meaning that scFvs have affinity for hPD-1. It is presumed that scFv secreted from each of co-expression strains, PC2TL and PC3TL, also has affinity for hPD-1, similarly to scFv derived from P1TL strain. It is also presumed that scFv secreted from each of co-expression strains, PC4TL and PC5TL has affinity for hPD-1, similarly to scFv derived from P2TL strain; and that scFv secreted from each of co-expression strains, PC6TL, PC7TL and PC8TL has affinity for hPD-1, similarly to scFv derived from P3TL strain.

[0315]

[Example 33]

(Inhibitory activity of scFv derived from co-expression strain PC4 to antigen-ligand binding)

From the culture supernatant of co-expression strain PC4, anti-hPD-1scFv03-His was purified by the purification

column for His-tag and anti-hCTLA-4scFv01-FLAG was purified by the purification column for FLAG-tag. Competitive activities (ELISA) of these with a ligand in binding to the corresponding antigens were evaluated.

[0316]

(Competitive activity of human PD-L1 to binding between anti-hPD-1scFv03 to antigen)

The same operation as in Example 21 (Evaluation on secretion of anti-hPD-1scFv03) was repeated except the sample addition operation. As a sample, a mixture of 2 nM (immobilization concentration) of anti-hPD-1scFv03-His obtained by His-tag purification of the culture supernatant of co-expression strain PC4 and a human PD-L1 solution (Recombinant Human B7-H1/PD-L1Fc Chimera, R&D systems, hereinafter referred to as hPD-L1) different in concentration (640 nM, 320 nM, 160 nM, 80 nM, 40 nM, 20 nM, 5nM, 0.5 nM, 0 nM) was used. In control wells, a sample of 2 nM anti-hCTLA-4scFv01-FLAG (derived from C1F strain) and a sample of 640 nM hPD-L1 were added. The binding inhibition rate of hPD-L1 to binding of hPD-1/anti-hPD-1scFv03-His was calculated in accordance with Expression 1. The resultant (binding) inhibitory activities of hPD-L1 to the binding between hPD-1scFv03 purified from PC4 strain and hPD-1 are shown in Table 32.

[0317]

[Expression 1]

$$\text{Binding inhibition rate (\%)} = 100 - \frac{\text{(Absorbance in the presence of hPD-L1)}}{\text{(Absorbance in the absence of hPD-L1)}} \times 100 \qquad \text{(Formula 1)}$$

[0318]

[Table 32]

Sample		Binding	Inhibition rate
hPD-1 scFv03-His	hPD-L1	A450-570	(%)
2 nM	0 nM	1.942	0
2 nM	0.5 nM	1.426	26.6
2 nM	5 nM	1.359	30
2 nM	20 nM	1.481	23.7
2 nM	40 nM	1.021	47.4
2 nM	80 nM	0.851	56.2
2 nM	160 nM	0.649	66.6
2 nM	320 nM	0.396	79.6
2 nM	640 nM	0.272	86
no	640 nM	0	-
hCTLA-4 scFv	no	-0.001	-

[0319]

As is apparent from Table 32, as the hPD-L1 concentration increased, absorbance decreased. Binding of anti-hPD-1scFv03-His to an antigen decreased in a hPD-L1-concentration dependent manner. In contrast, in the case where 2nM anti-hCTLA-4scFv01-FLAG was added, binding to the plate was not observed. From this, binding of anti-hPD-1scFv03-His to human PD-1 immobilized can be said to be specific binding. In the case where hPD-L1 alone was added, binding to the plate was not observed. From this, what was bound to the plate when anti-hPD-1scFv03-His/hPD-L1 mixture was added can be said to be anti-hPD-1scFv03-His. The binding inhibition rate of hPD-L1 against the binding between hPD-1 and anti-hPD-1scFv03-His is shown in Figure 38. The binding between hPD-1 and anti-hPD-1scFv03-His is inhibited by hPD-L1 in a concentration-dependent manner.

[0320]

(Competitive activity of ligand to binding between anti-hCTLA-4scFv01 and antigen)

The same operation as in Example 21 (Evaluation on secretion of anti-hCTLA-4scFv01) was repeated except the sample addition operation. As samples, a mixture of 1 nM anti-hCTLA-4scFv01-FLAG obtained by FLAG-tag purification from co-expression strain PC4 and human CD80 (Recombinant Human B7-1/CD80 Fc Chimera, R&D Systems, hereinafter referred to as hCD80) different in concentration (30 nM, 20 nM, 10 nM, 5 nM, 3 nM and 0 nM); and a mixture of 1 nM anti-hCTLA-4 scFv01-FLAG and human CD86 (Recombinant Human B7-1/CD86 Fc Chimera, R&D Systems, hereinafter referred to as hCD86) different in concentration (100 nM, 80 nM, 60 nM, 40 nM, 20 nM and 0 nM) were used. In control wells, 1 nM anti-hPD-1scFv03-His (derived from P2H strain), 30 nM hCD80 alone, or 100 nM hCD86 alone were added. The binding inhibition rate of hCD80 or hCD86 to binding of hCTLA-4/anti-hCTLA-4scFv01-FLAG was calculated in accordance with Expression 2.

[0321]

[Expression 2]

$$\text{Binding inhibition rate (\%)} = 100 - \frac{\text{(Absorbance in the presence of ligand*)}}{\text{(Absorbance in the absence of ligand*)}} \times 100 \qquad \text{(Formula 2)}$$

*Ligand: hCD80 or hCD86

[0322]

(Results)

The results of competitive ELISA are shown in Table 33 (hCD80 was used as a ligand) and Table 34 (hCD86 was used as a ligand).

[0323]

[Table 33]

Binding inhibitory activity of hCD80 to binding between hCTLA-4scFv01-FLAG purified from PC4 strain and hCTLA-4

Sample		Binding	Inhibition
hCTLA-4scFv01-FLAG	hCD80	A450-570	(%)
1 nM	0 nM	1.027	0
1 nM	3 nM	1.352	-31.6
1nM	5 nM	0.489	52.4
1nM	10 nM	0.407	60.4
1nM	20 nM	0.248	75.9
1nM	30 nM	0.185	82
no	30 nM	0.004	-
hPD-1 scFv	no	-0.020	-

[0324]

[Table 34]

Binding inhibitory activity of hCD86 to binding between hCTLA-4scFv01-FLAG purified from PC4 strain and hCTLA-4

Sample		Binding	Inhibition
hCTLA-4scFv01-FLAG	hCD86	A450-570	(%)
1 nM	0 nM	1.027	0
1 nM	20 nM	1.084	-5.6
1nM	40 nM	0.799	22.2
1nM	60 nM	0.614	40.2
1nM	80 nM	0.521	49.3
1nM	100 nM	0.374	63.6
no	100 nM	0.009	-
hPD-1 scFv	no	-0.020	-

[0325]

As is apparent from Table 33 and Table 34, as the concentration of hCD80 or hCD86 increased, absorbance decreased. Binding of anti-hCTLA-4scFv01-FLAG to an

antigen decreased in a hCD80- or hCD86-concentration dependent manner. In contrast, in the case where 1 nM anti-hPD-1scFv03-His was added, binding to the plate was not observed. From this, the binding of hCTLA-4scFv01-FLAG to human CTLA-4 immobilized can be said to be specific binding. In the case where hCD80 alone or hCD86 alone was added, binding to the plate was not observed. From this, what was bound to the plate when the mixture of anti-hCTLA-4scFv01-FLAG and hCD80 or the mixture of anti-hCTLA-4scFv01-FLAG and hCD86 was added, can be said to be anti-hCTLA-4scFv01-FLAG.

[0326]

The binding inhibition rates of hCD80 and hCD86 to binding between hCTLA-4 and anti-hCTLA-4scFv01-FLAG are shown in Figure 39 and Figure 40, respectively. The binding between hCTLA-4 and anti-hCTLA-4scFv01-FLAG is inhibited by hCD80 or hCD86 in a concentration-dependent manner.

[0327]

From the results mentioned above, it was successfully verified that anti-hPD-1scFv03-His and anti-hCTLA-4scFv01-FLAG secreted from co-expression strain PC4 have activities to competitively bind to the corresponding antigens with the respective ligands.

CLAIMS

1. A co-expression plasmid suitable for expressing polypeptides within a bacterium of the genus Bifidobacterium, the co-expression plasmid comprising two types of secretory expression cassettes each sequentially comprising the following DNAs (1) to (4):

(1) a promoter DNA functioning in the bacterium of the genus Bifidobacterium;

(2) a DNA encoding a secretory signal peptide having an amino acid sequence represented by any one of SEQ ID Nos: 57, 59, 61, 63, 65, 67, 69, 71, 73, 75 and 77;

(3) a DNA encoding a heterologous polypeptide; and

(4) a terminator DNA functioning in the bacterium of the genus Bifidobacterium.

2. The co-expression plasmid according to Claim 1, wherein a DNA encoding a linker peptide is ligated downstream of the DNA encoding a secretory signal peptide.

3. The co-expression plasmid according to Claim 1 or 2, wherein the promoter functioning in the bacterium of the genus Bifidobacterium is one or two promoters selected from P30 promoter, P54 promoter and Hu promoter.

4. The co-expression plasmid according to any one of Claims 1 to 3, wherein the terminator functioning in the bacterium of the genus Bifidobacterium is one or two terminators selected from Hu terminator and T2 terminator.

5. The co-expression plasmid according to any one of Claims 1 to 4, wherein at least one heterologous polypeptide is a single-chain antibody.

6. The co-expression plasmid according to Claim 5, wherein the single-chain antibody which is the at least one heterologous polypeptide is an anti-PD-1 antibody.

7. The co-expression plasmid according to Claim 5, wherein the single-chain antibody which is the at least one heterologous polypeptide is an anti-CTLA-4 antibody.

8. The co-expression plasmid according to Claim 5, wherein the single-chain

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antibody which is the at least one heterologous polypeptide is an anti-HER2 antibody.

9. The co-expression plasmid according to any one of Claims 1 to 4, wherein at least one heterologous polypeptide is a cytokine.

10. The co-expression plasmid according to Claim 9, wherein the cytokine which is the at least one heterologous polypeptide is TNF-α.

11. The co-expression plasmid according to Claim 9, wherein the cytokine which is the at least one heterologous polypeptide is IFN-γ.

12. The co-expression plasmid according to any one of Claims 1 to 4, wherein a first heterologous polypeptide expressed by one cassette of the two types of secretory expression cassettes is an anti-PD-1 antibody and a second heterologous polypeptide expressed by the other cassette of the two types of secretory expression cassettes is an anti-CTLA-4 antibody.

13. The co-expression plasmid according to any one of Claims 1 to 4, wherein a first heterologous polypeptide expressed by one cassette of the two types of secretory expression cassettes is TNF-α and a second heterologous polypeptide expressed by the other cassette of the two types of secretory expression cassettes is IFN-γ.

14. The co-expression plasmid according to any one of Claims 1 to 4, wherein a first heterologous polypeptide expressed by one cassette of the two types of secretory expression cassettes is an anti-HER2 antibody and a second heterologous polypeptide expressed by the other cassette of the two types of secretory expression cassettes is IFN-γ.

15. A bacterium of the genus Bifidobacterium transformed with the co-expression plasmid according to any one of Claims 1 to 14.

16. The bacterium of the genus Bifidobacterium according to Claim 15, wherein the bacterium is Bifidobacterium longum.

17. A pharmaceutical composition comprising the bacterium of the genus Bifidobacterium according to Claim 15 or 16, and at least one ingredient selected from a pharmacologically acceptable carrier, excipient and diluent.

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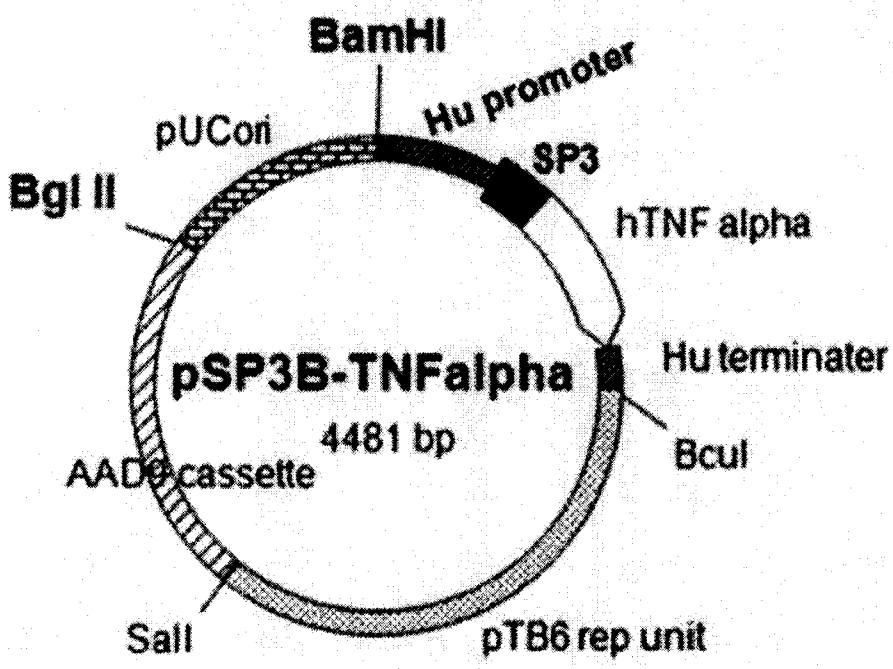
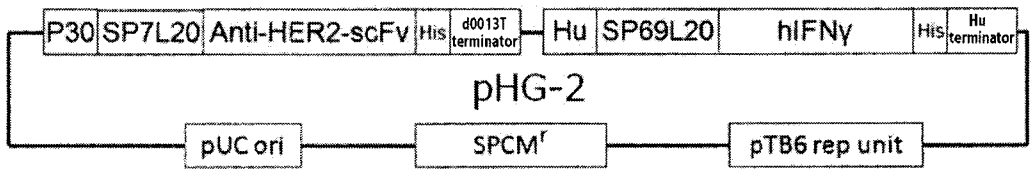
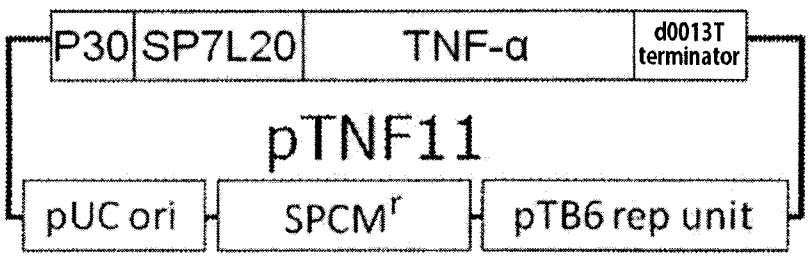
[Figure 1]



[Figure 2]

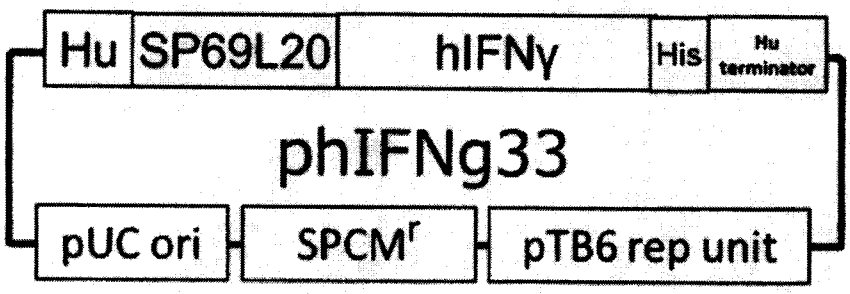


[Figure 3]

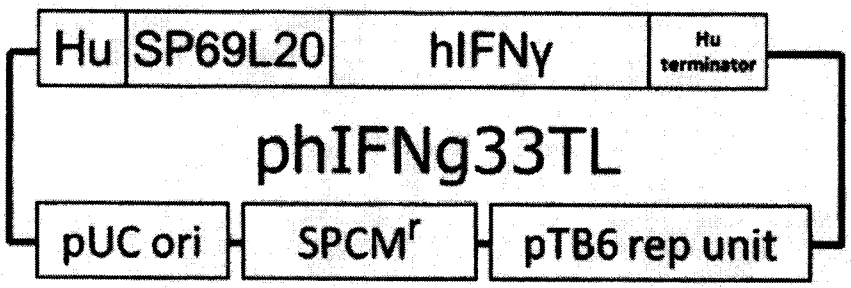


[Figure 4]

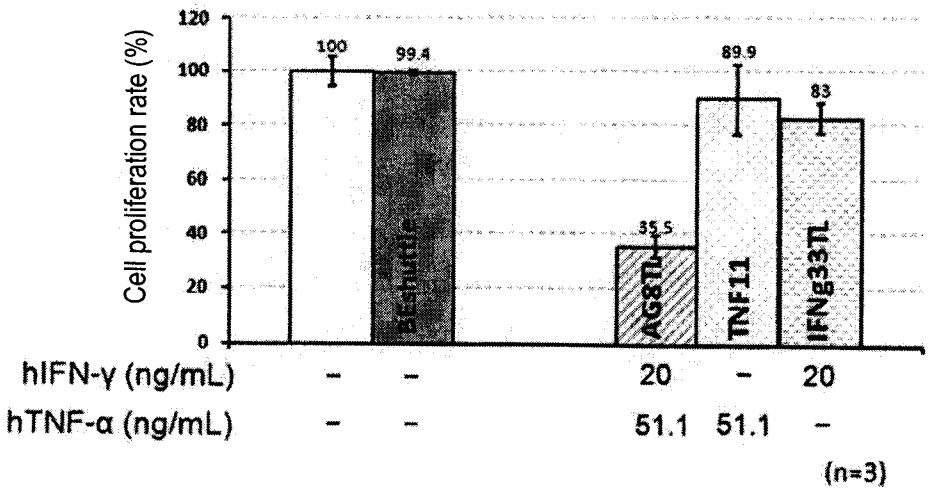
(a)



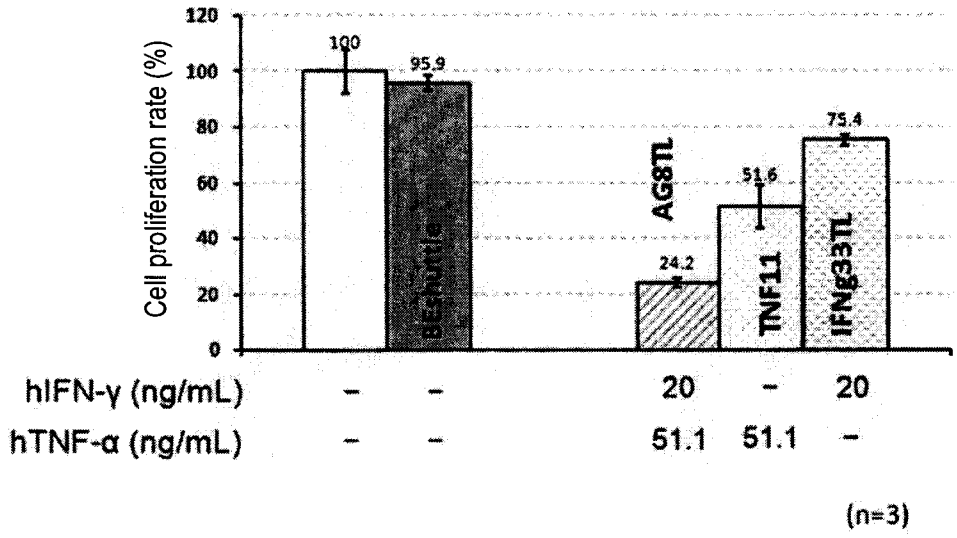
(b)



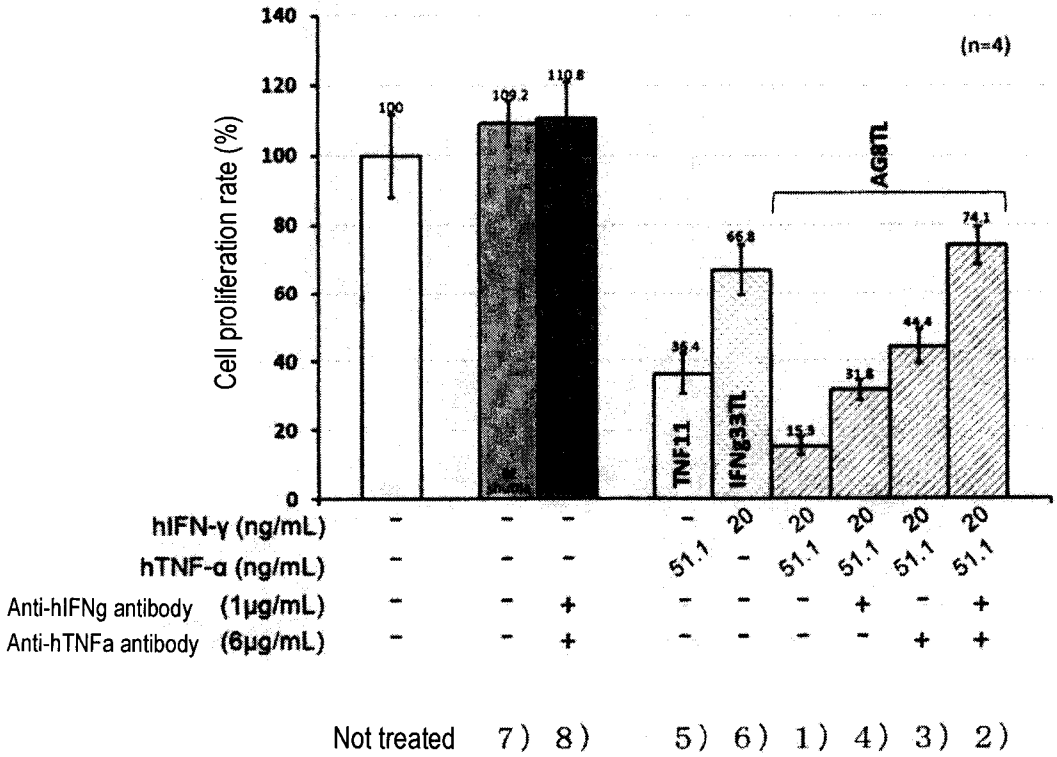
[Figure 5]



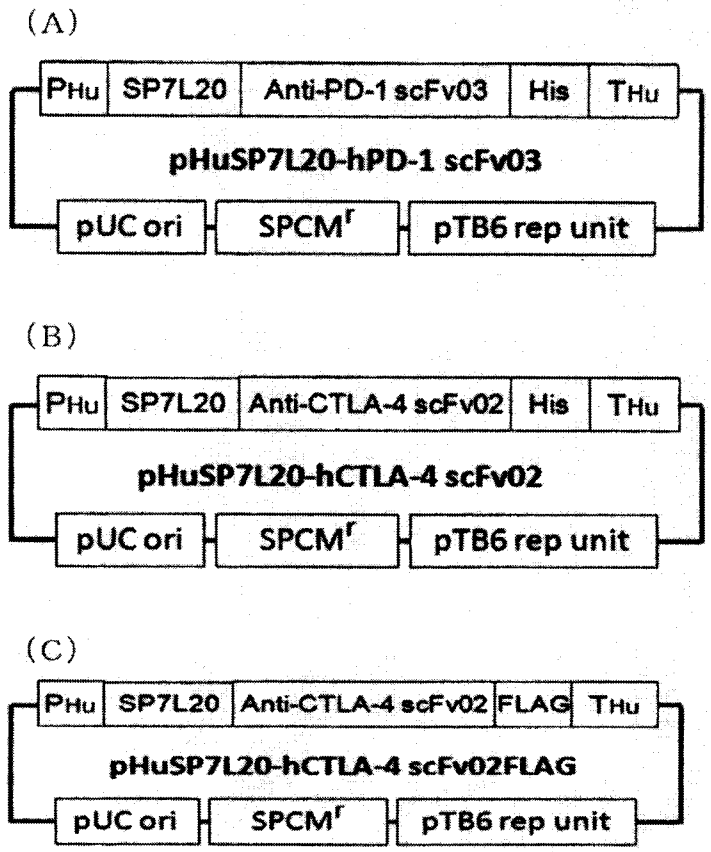
[Figure 6]



[Figure 7]

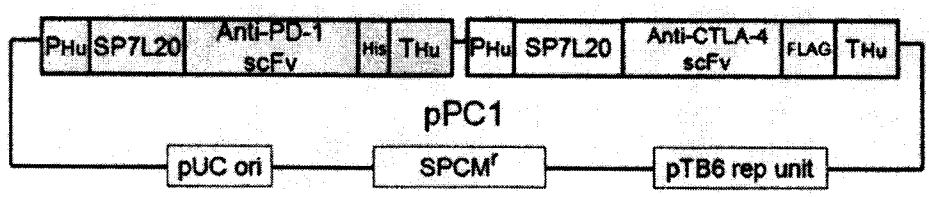


[Figure 8]

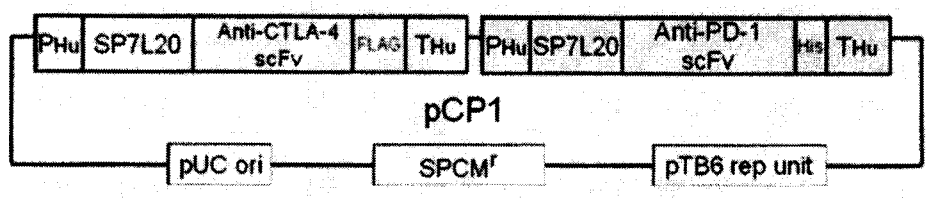

(A)
PHu
SP7L20
Anti-PD-1 scFv03
His
THu
pHuSP7L20-hPD-1 scFv03
pUC ori
SPCMr
pTB6 rep unit
(B)
PHu
SP7L20
Anti-CTLA-4 scFv02
His
THu
pHuSP7L20-hCTLA-4 scFv02
pUC ori
SPCMr
pTB6 rep unit
(C)
PHu
SP7L20
Anti-CTLA-4 scFv02
FLAG
THu
pHuSP7L20-hCTLA-4 scFv02FLAG
pUC ori
SPCMr
pTB6 rep unit

[Figure 9]

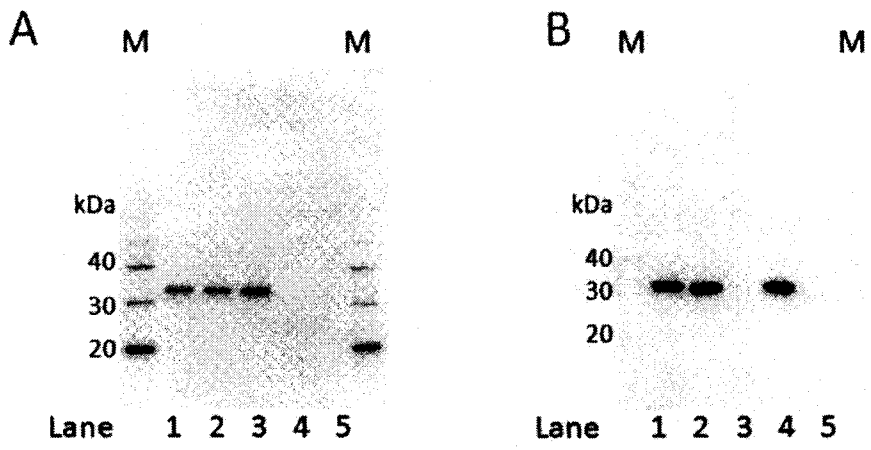
(a)


PHu
SP7L20
Anti-PD-1 scFv
His
THu
PHu
SP7L20
Anti-CTLA-4 scFv
FLAG
THu
pPC1
pUC ori
SPCM^r
pTB6 rep unit

(b)


PHu
SP7L20
Anti-CTLA-4 scFv
FLAG
THu
PHu
SP7L20
Anti-PD-1 scFv
His
THu
pCP1
pUC ori
SPCM^r
pTB6 rep unit

[Figure 10]



A : Detection of anti-hPD-1scFv03 by anti-His tag antibody

B : Detection of anti-hCTLA-4scFv02 FLAG by anti-FLAG tag antibody

Lane 1: Co-expression strain PC1 (anti-hPD-1scFv03 fused with His-tag at the C terminus, anti-hCTLA-4scFv02 fused with FLAG-tag at the C terminus)

Lane 2: Co-expression strain CP1 (anti-hPD-1scFv03 fused with His-tag at the C terminus, anti-hCTLA-4scFv02 fused with FLAG-tag at the C terminus)

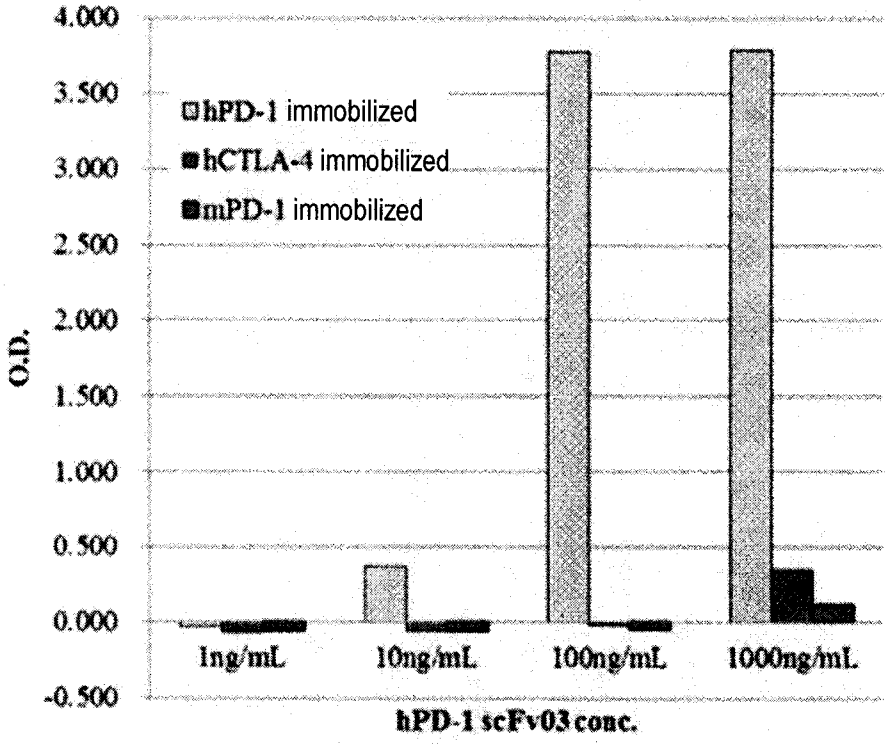
Lane 3: Anti-hPD-1scFv03 single expression strain (anti-hPD-1scFv03 fused with His-tag at the C terminus)

Lane 4: Anti-hCTLA-4scFv02 single expression strain (anti-hCTLA-4scFv02 fused with FLAG-tag at the C terminus)

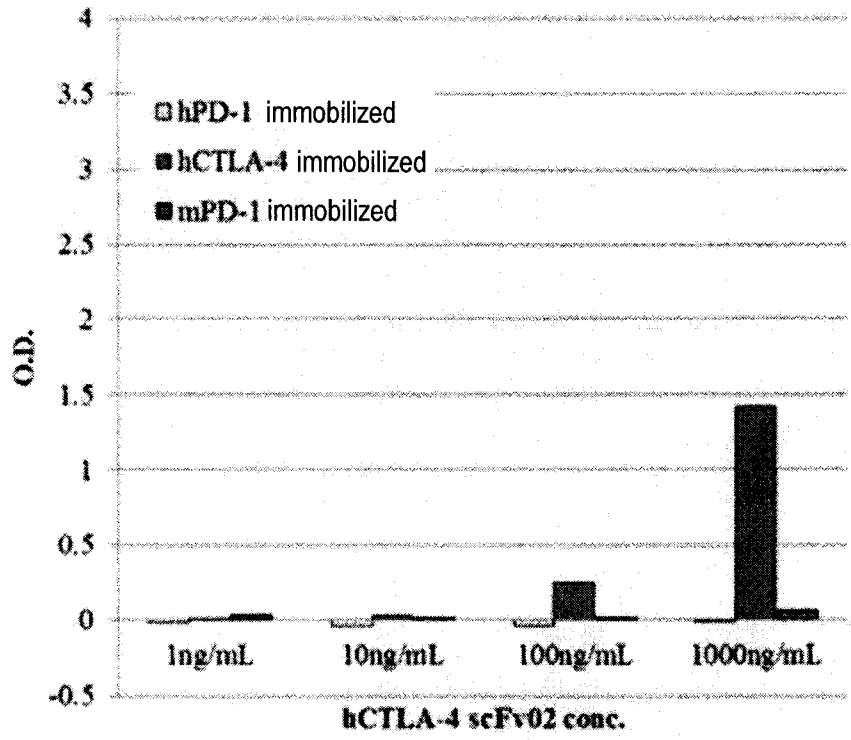
Lane 5: Negative control strain, BEshuttle

M: Molecular-weight marker

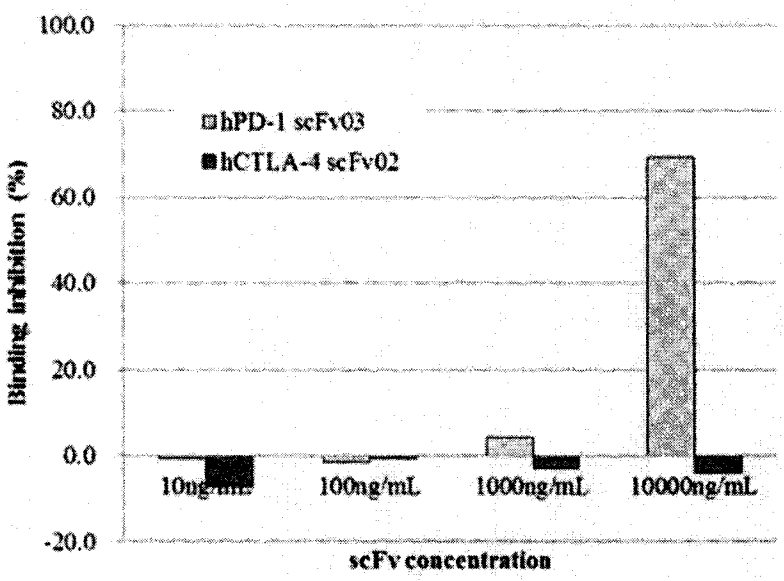
[Figure 11]


4.000
3.500
3.000
2.500
2.000
1.500
1.000
0.500
0.000
-0.500
O.D.
hPD-1 immobilized
hCTLA-4 immobilized
mPD-1 immobilized
1ng/mL
10ng/mL
100ng/mL
1000ng/mL
hPD-1 scFv03 conc.

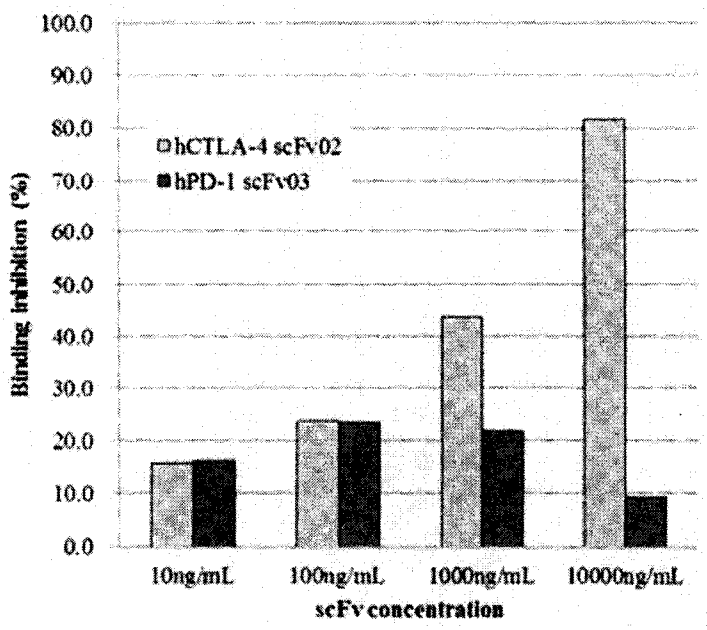
[Figure 12]


4
3.5
3
2.5
2
1.5
1
0.5
0
-0.5
O.D.
hPD-1 immobilized
hCTLA-4 immobilized
mPD-1 immobilized
1ng/mL
10ng/mL
100ng/mL
1000ng/mL
hCTLA-4 scFv02 conc.

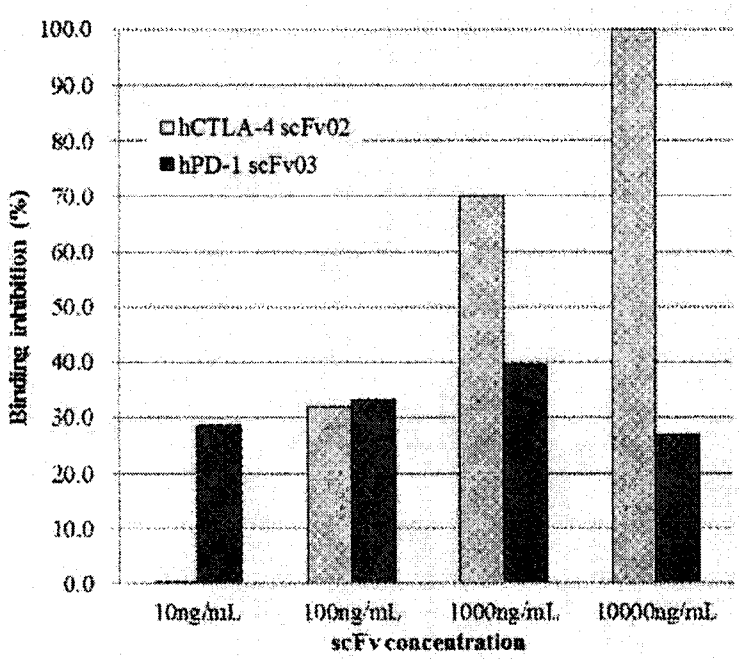
[Figure 13]



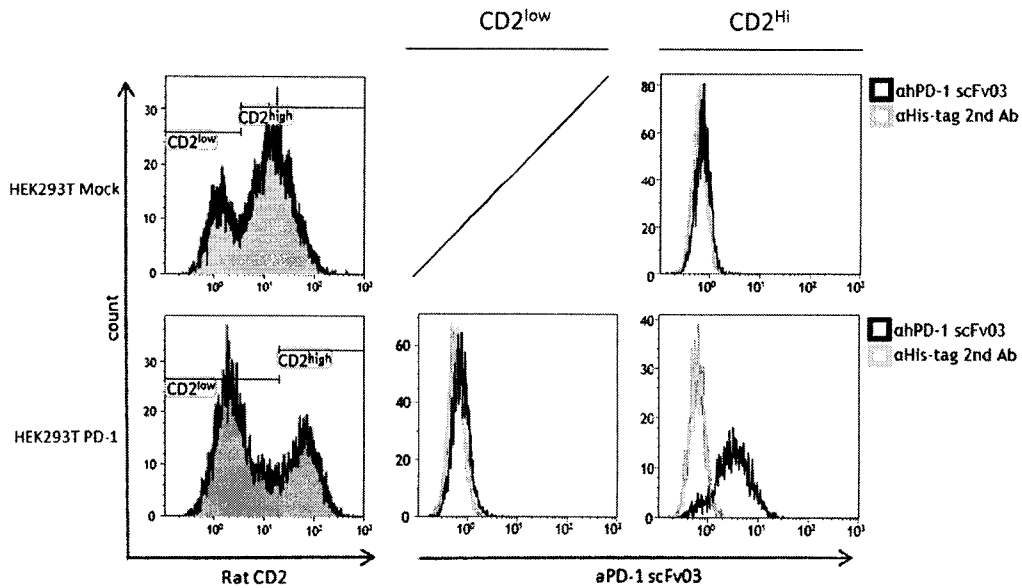
[Figure 14]



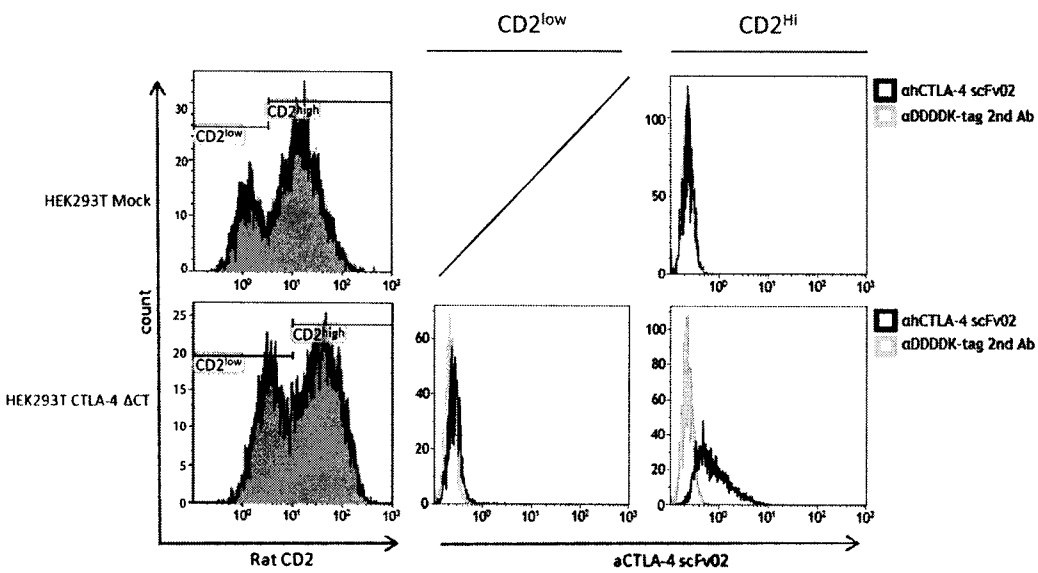
[Figure 15]



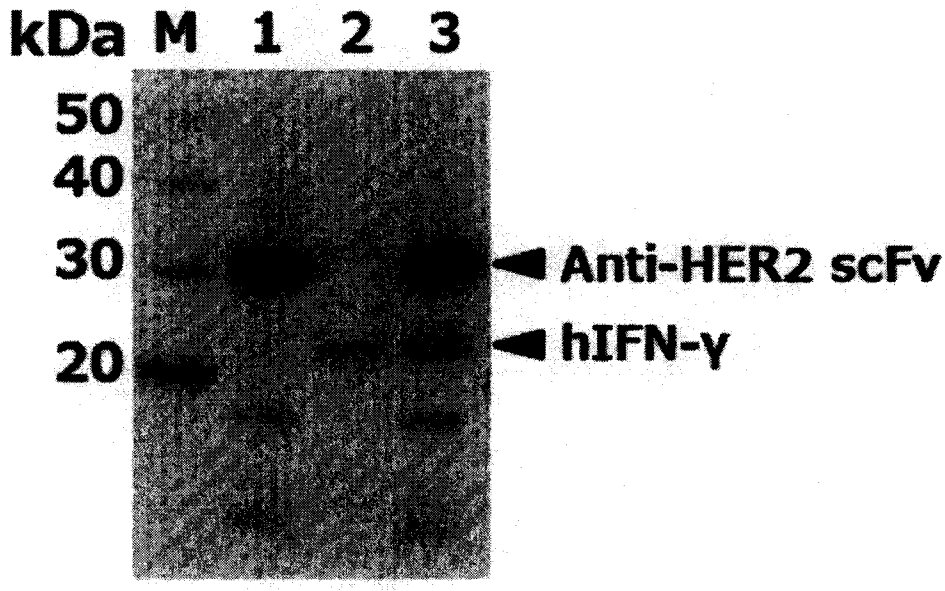
[Figure 16]



[Figure 17]



[Figure 18]

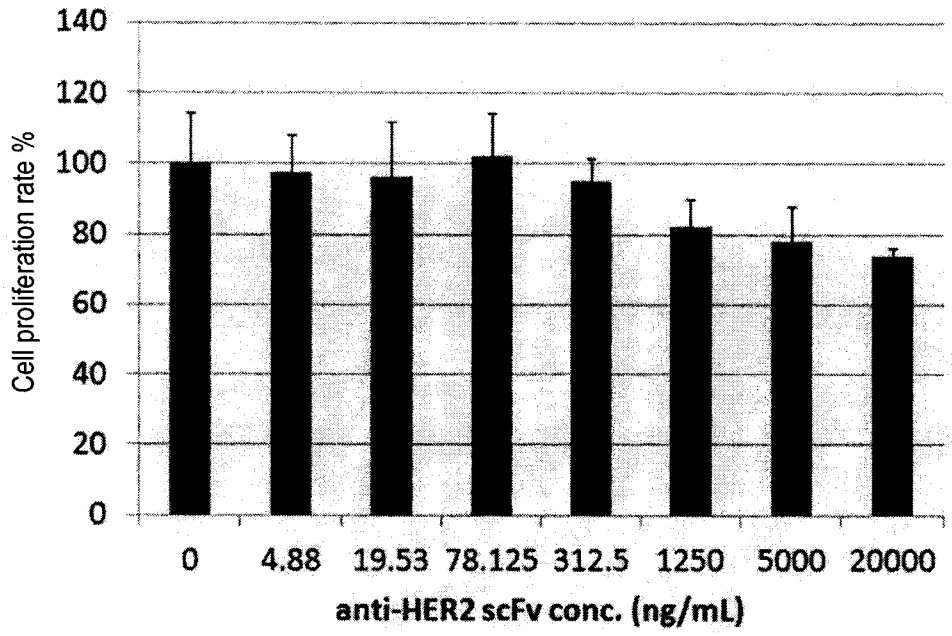


Lane 1: P30SP7L20-bHER2 strain (anti-HER2 scFv single expression)

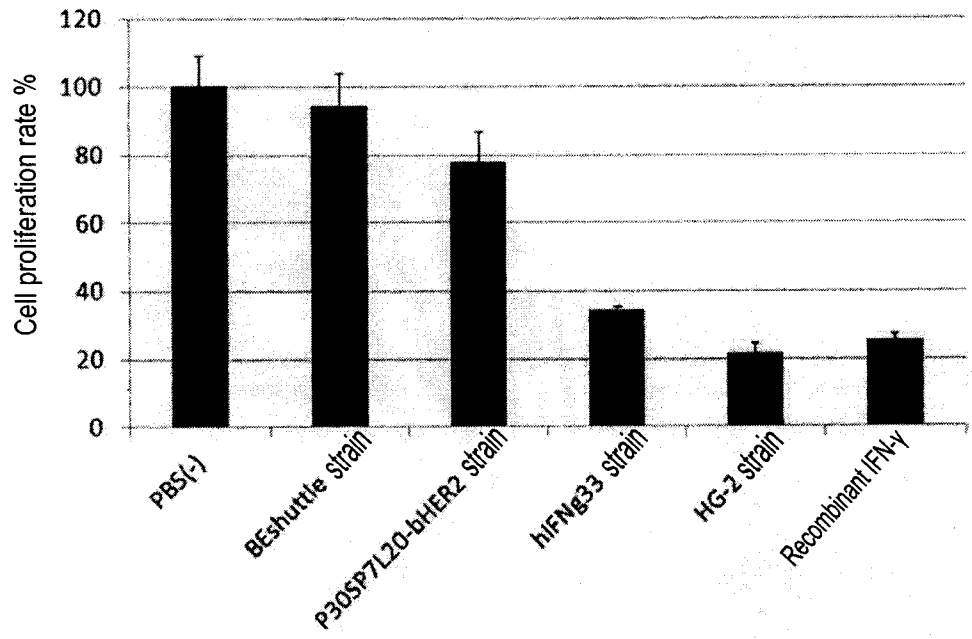
Lane 2: hIFNg33 strain (IFN-γ single expression)

Lane 3: HG-2 strain (co-expression)

[Figure 19]

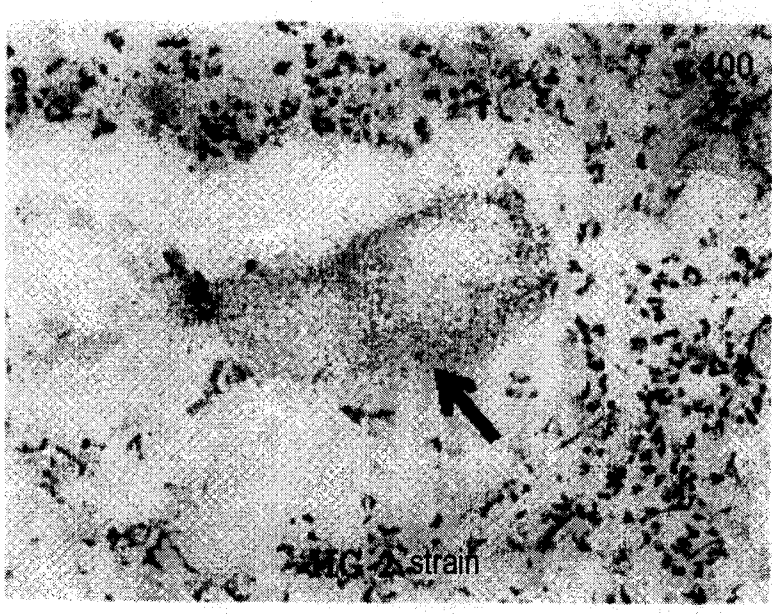

140
120
100
80
60
40
20
0
Cell proliferation rate %
0
4.88
19.53
78.125
312.5
1250
5000
20000
anti-HER2 scFv conc. (ng/mL)

[Figure 20]

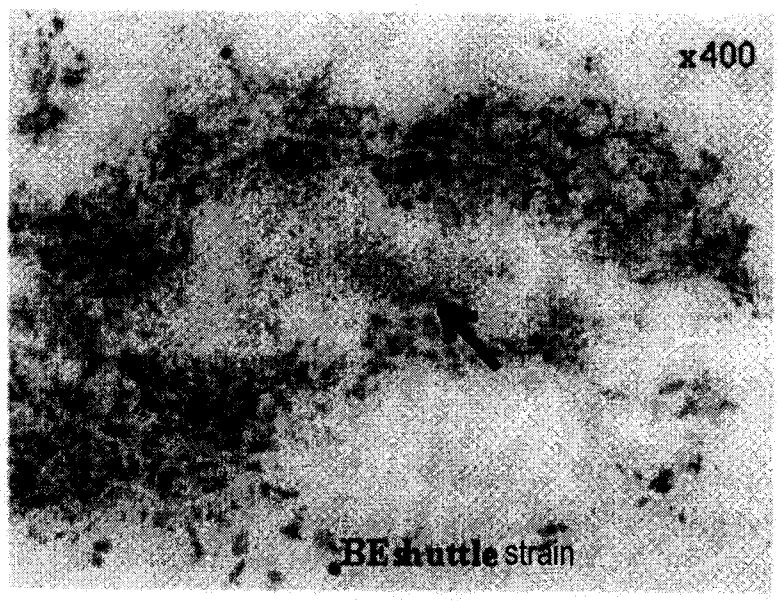

120
100
80
60
40
20
0
Cell proliferation rate %
PBS(-)
BEshuttle strain
P30SP7L20-bHER2 strain
hIFNg33 strain
HG-2 strain
Recombinant IFN-γ

[Figure 21]

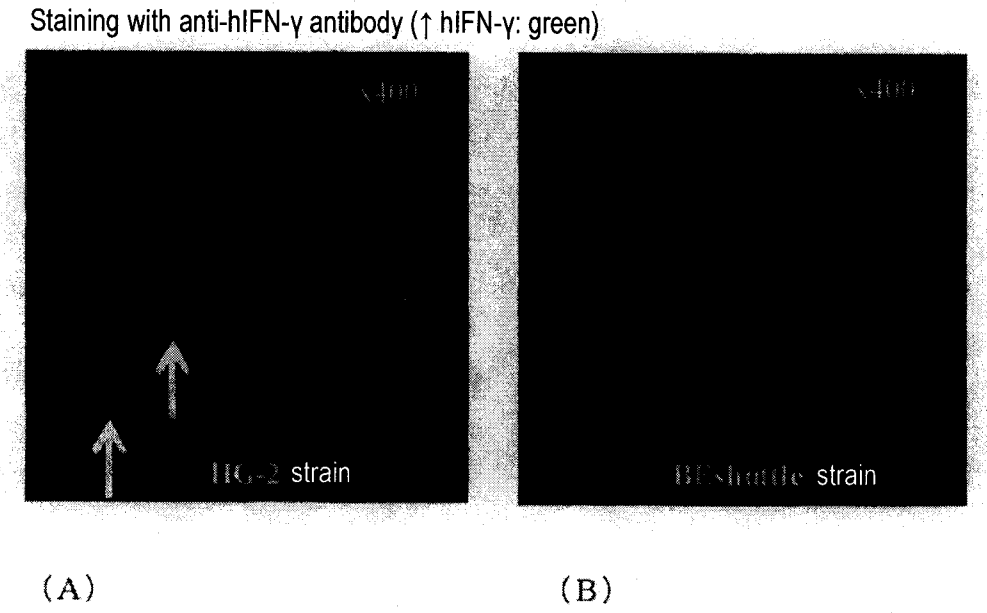
(A)



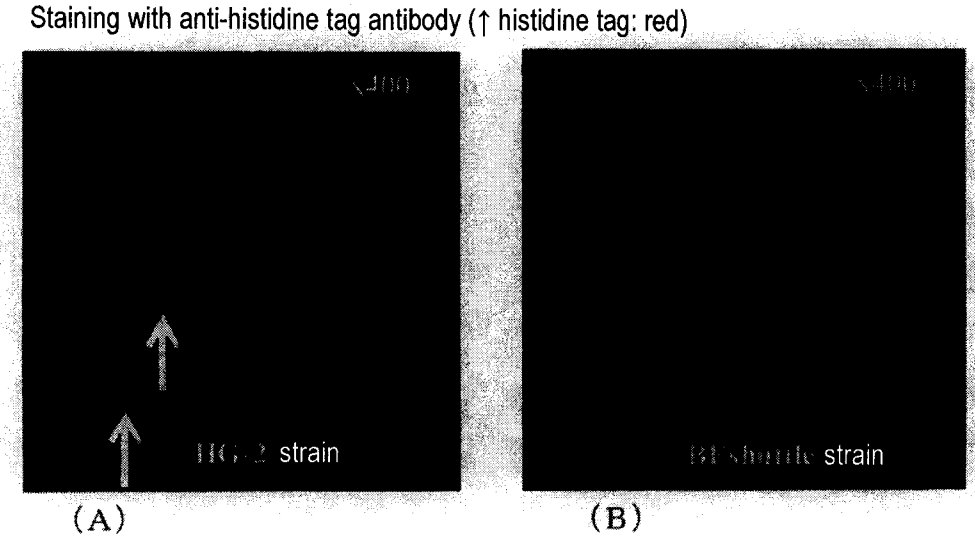
(B)



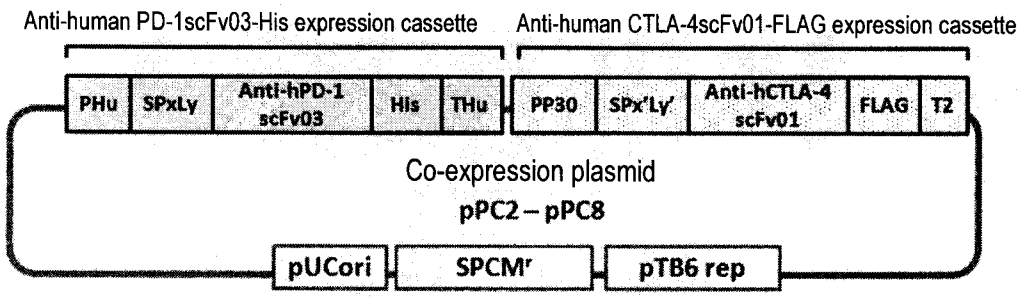
[Figure 22]



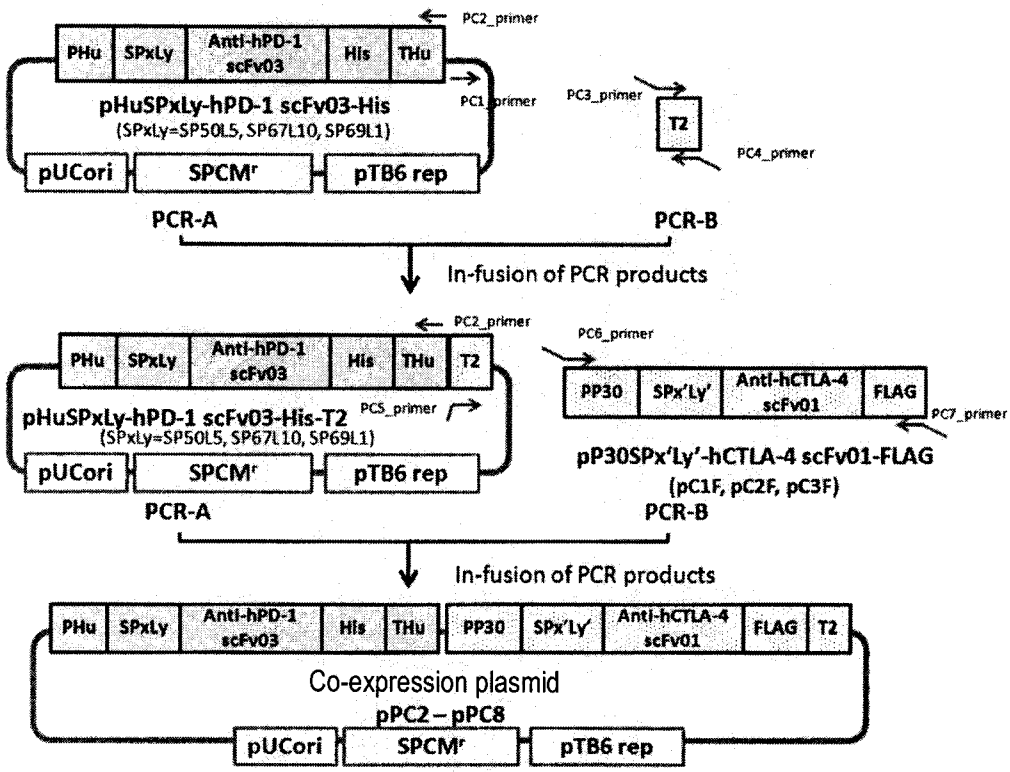
[Figure 23]



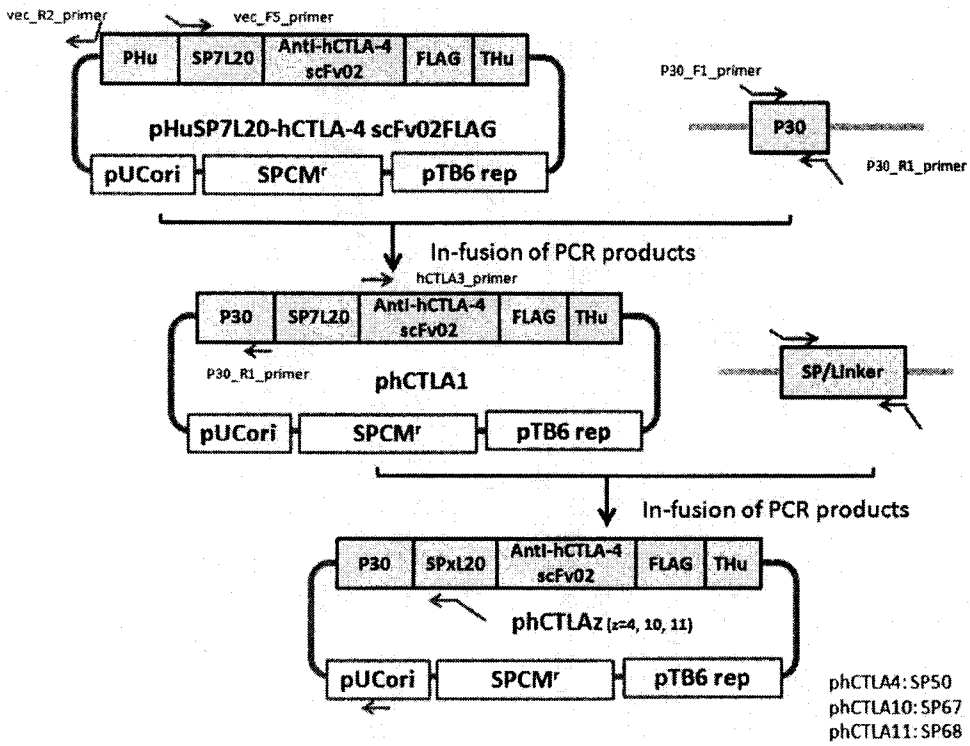
[Figure 24]



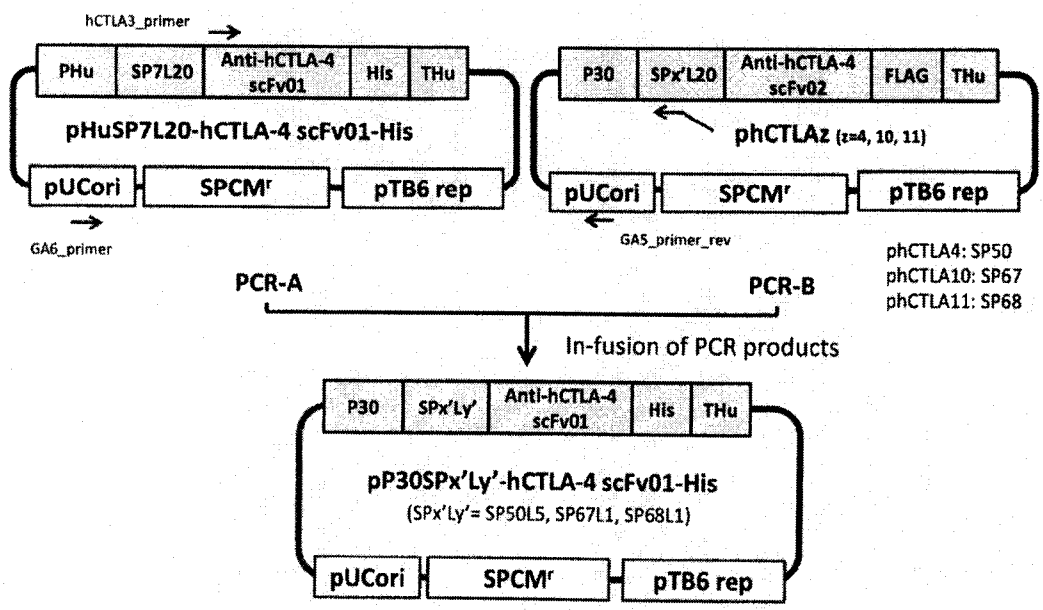
[Figure 25]



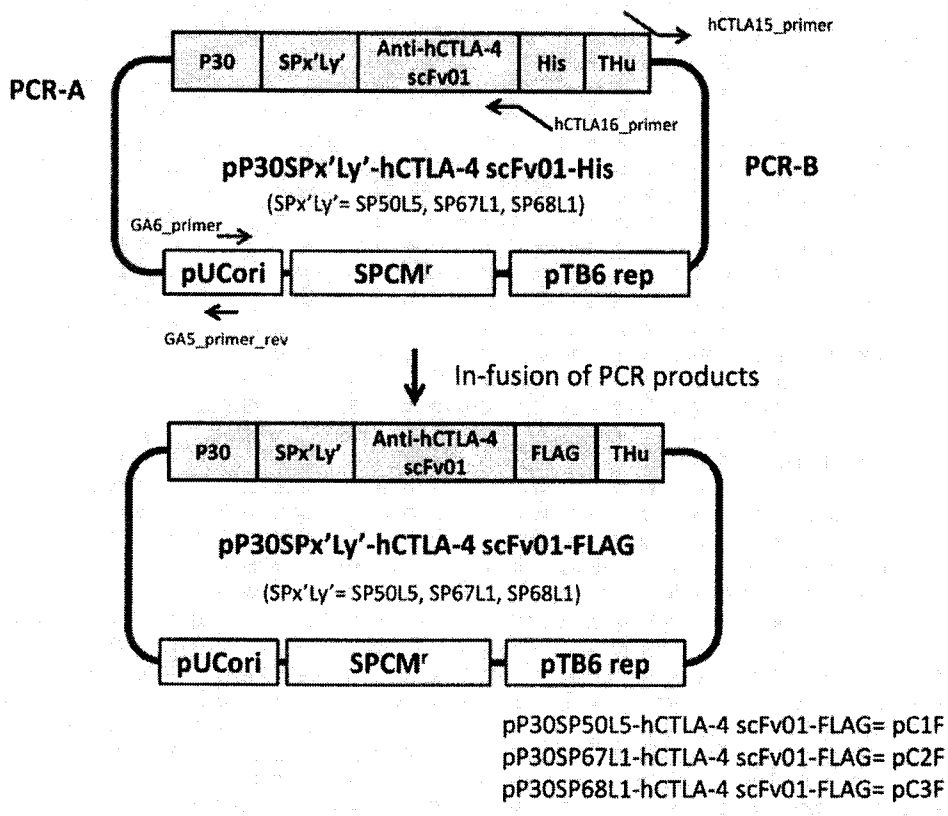
[Figure 26]



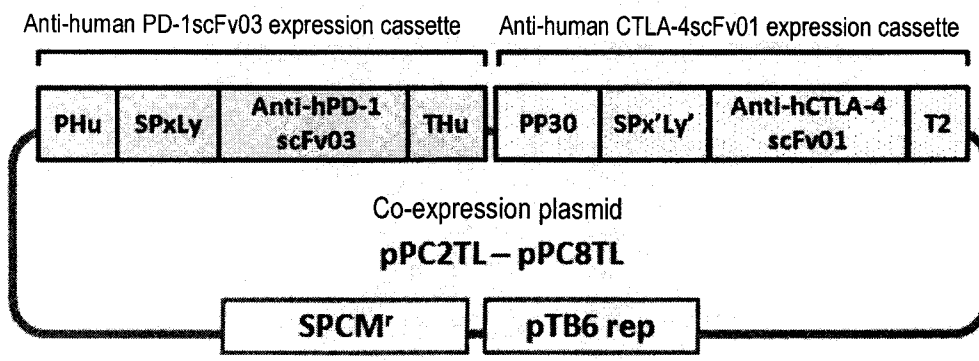
[Figure 27]



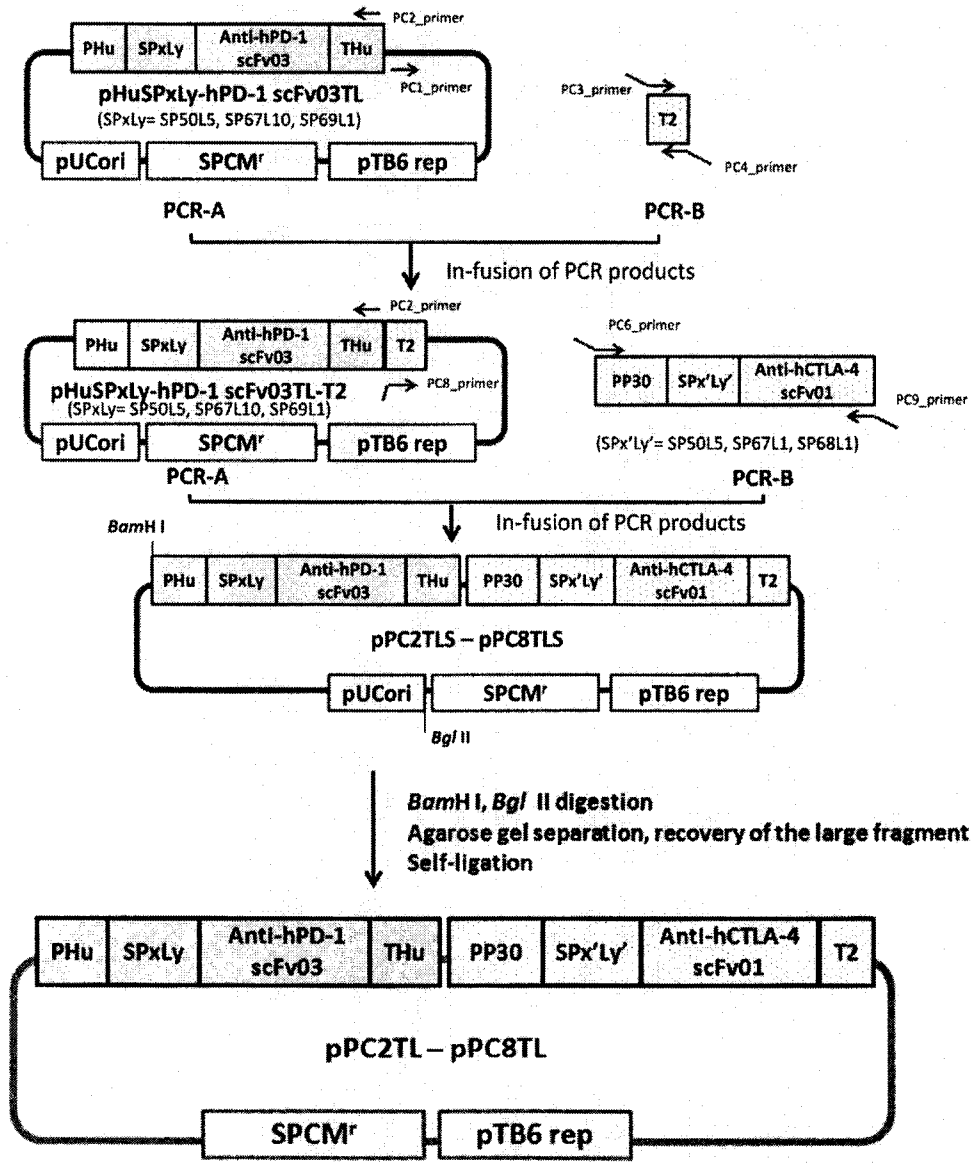
[Figure 28]



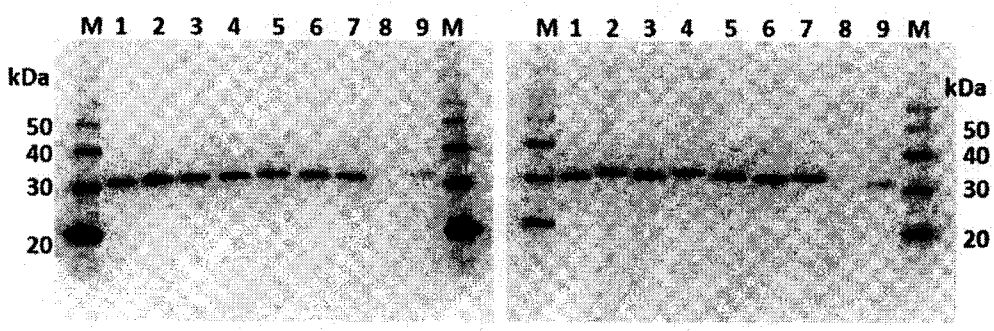
[Figure 29]



[Figure 30]


PC2_primer
PHu
SPxLy
Anti-hPD-1 scFv03
THu
PC1_primer
pHuSPxLy-hPD-1 scFv03TL
(SPxLy= SP50L5, SP67L10, SP69L1)
pUCori
SPCMr
pTB6 rep
PC3_primer
T2
PC4_primer
PCR-A
PCR-B
In-fusion of PCR products
PC2_primer
PHu
SPxLy
Anti-hPD-1 scFv03
THu
T2
PC8_primer
pHuSPxLy-hPD-1 scFv03TL-T2
(SPxLy= SP50L5, SP67L10, SP69L1)
pUCori
SPCMr
pTB6 rep
PCR-A
PC6_primer
PP30
SPx'Ly'
Anti-hCTLA-4 scFv01
PC9_primer
(SPx'Ly'= SP50L5, SP67L1, SP68L1)
PCR-B
In-fusion of PCR products
BamH I
PHu
SPxLy
Anti-hPD-1 scFv03
THu
PP30
SPx'Ly'
Anti-hCTLA-4 scFv01
T2
pPC2TLS – pPC8TLS
pUCori
SPCMr
pTB6 rep
Bgl II
BamH I, Bgl II digestion
Agarose gel separation, recovery of the large fragment
Self-ligation
PHu
SPxLy
Anti-hPD-1 scFv03
THu
PP30
SPx'Ly'
Anti-hCTLA-4 scFv01
T2
pPC2TL – pPC8TL
SPCMr
pTB6 rep

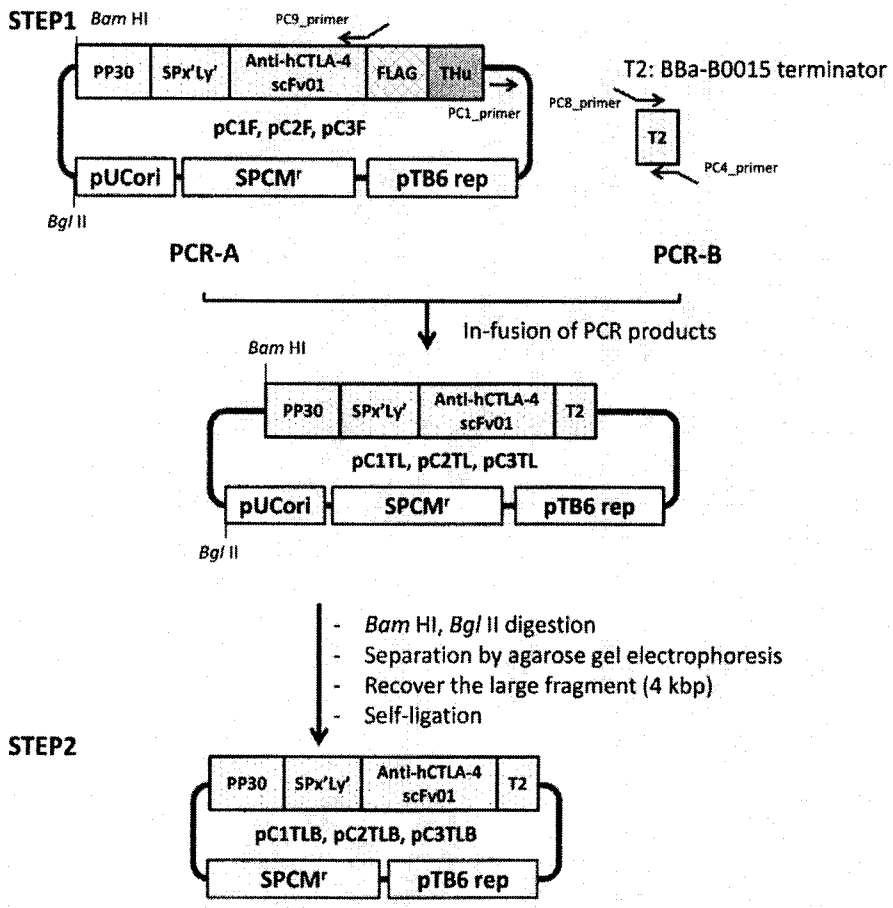
[Figure 31]



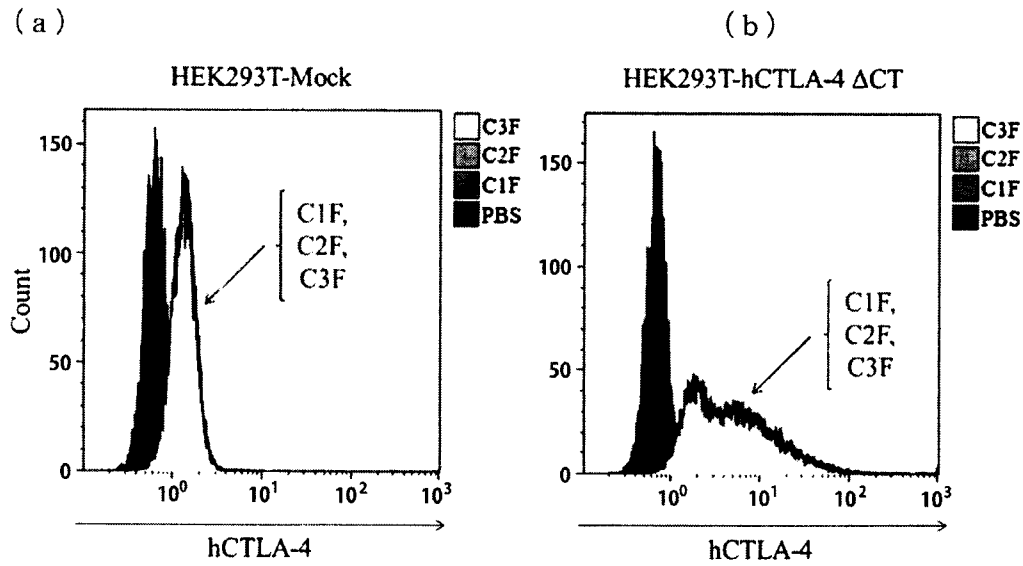
(A) Detection of anti-hPD-1 scFv03-His (B) Detection of anti-hCTLA-4 scFv01-FLAG

1. PC2 (SP50L5-hPD-1 scFv03-His, SP67L1-hCTLA4-scFv01-FLAG)
2. PC3 (SP50L5-hPD-1 scFv03-His, SP68L1-hCTLA4-scFv01-FLAG)
3. PC4 (SP67L10-hPD-1 scFv03-His, SPSP50L5-hCTLA4-scFv01-FLAG)
4. PC5 (SP67L10-hPD-1 scFv03-His, SP68L1-hCTLA4-scFv01-FLAG)
5. PC6 (SP69L1-hPD-1 scFv03-His, SP50L5-hCTLA4-scFv01-FLAG)
6. PC7 (SP69L1-hPD-1 scFv03-His, SP67L1-hCTLA4-scFv01-FLAG)
7. PC8 (SP69L1-hPD-1 scFv03-His, SP68L1-hCTLA4-scFv01-FLAG)
8. BEshuttle (N.C.)
9. PC1

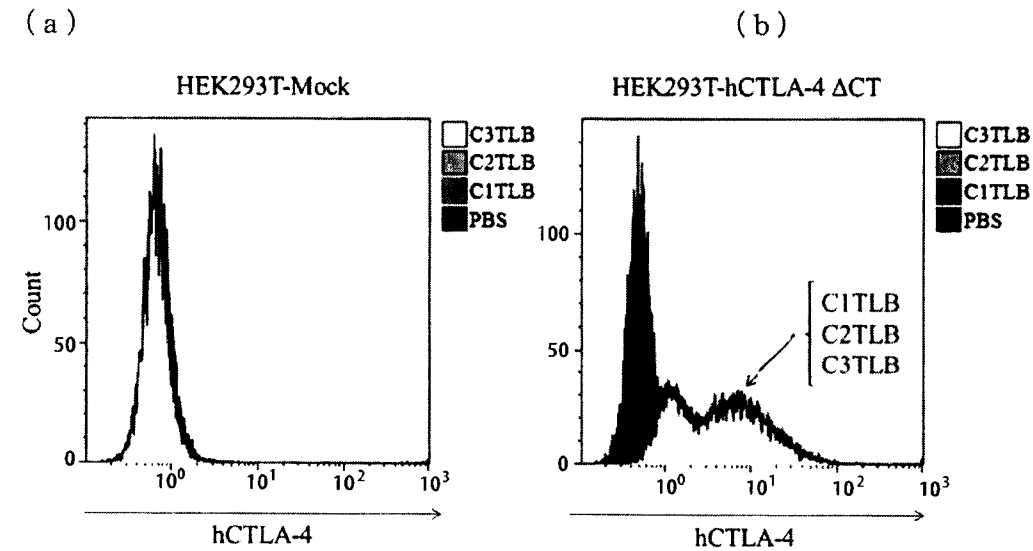
[Figure 32]


STEP1
Bam HI
PC9_primer
PP30
SPx'Ly'
Anti-hCTLA-4 scFv01
FLAG
THu
PC1_primer
pC1F, pC2F, pC3F
pUCori
SPCMr
pTB6 rep
Bgl II
T2: BBa-B0015 terminator
PC8_primer
T2
PC4_primer
PCR-A
PCR-B
In-fusion of PCR products
Bam HI
PP30
SPx'Ly'
Anti-hCTLA-4 scFv01
T2
pC1TL, pC2TL, pC3TL
pUCori
SPCMr
pTB6 rep
Bgl II
- Bam HI, Bgl II digestion
- Separation by agarose gel electrophoresis
- Recover the large fragment (4 kbp)
- Self-ligation
STEP2
PP30
SPx'Ly'
Anti-hCTLA-4 scFv01
T2
pC1TLB, pC2TLB, pC3TLB
SPCMr
pTB6 rep

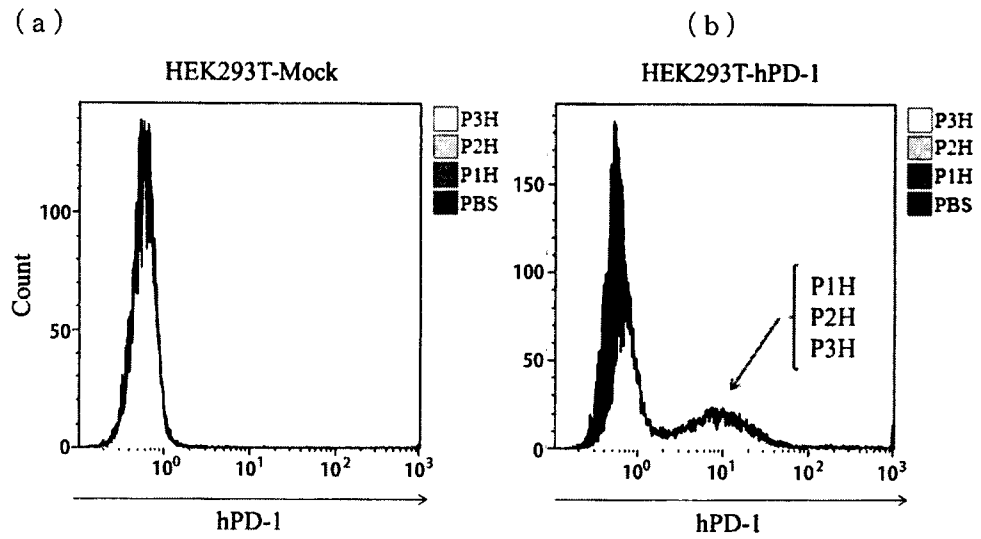
[Figure 33]



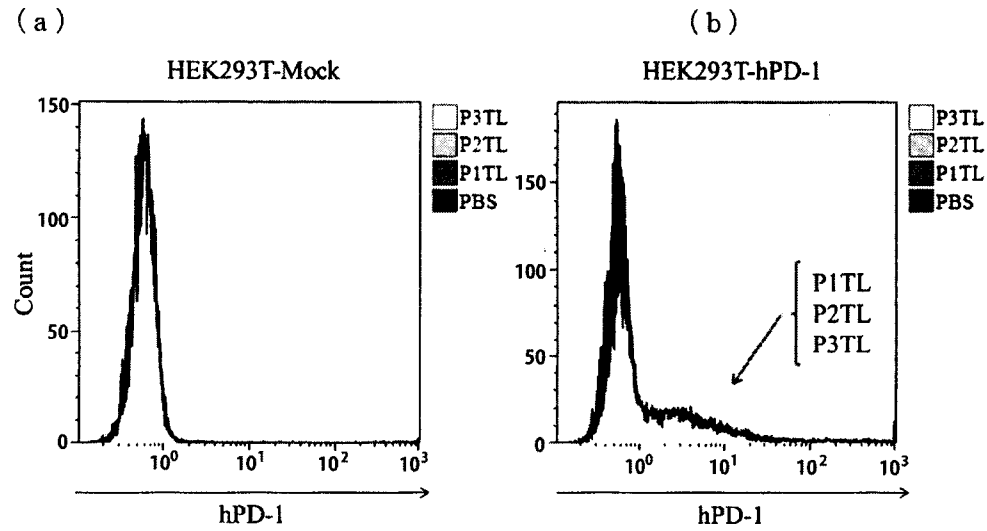
[Figure 34]



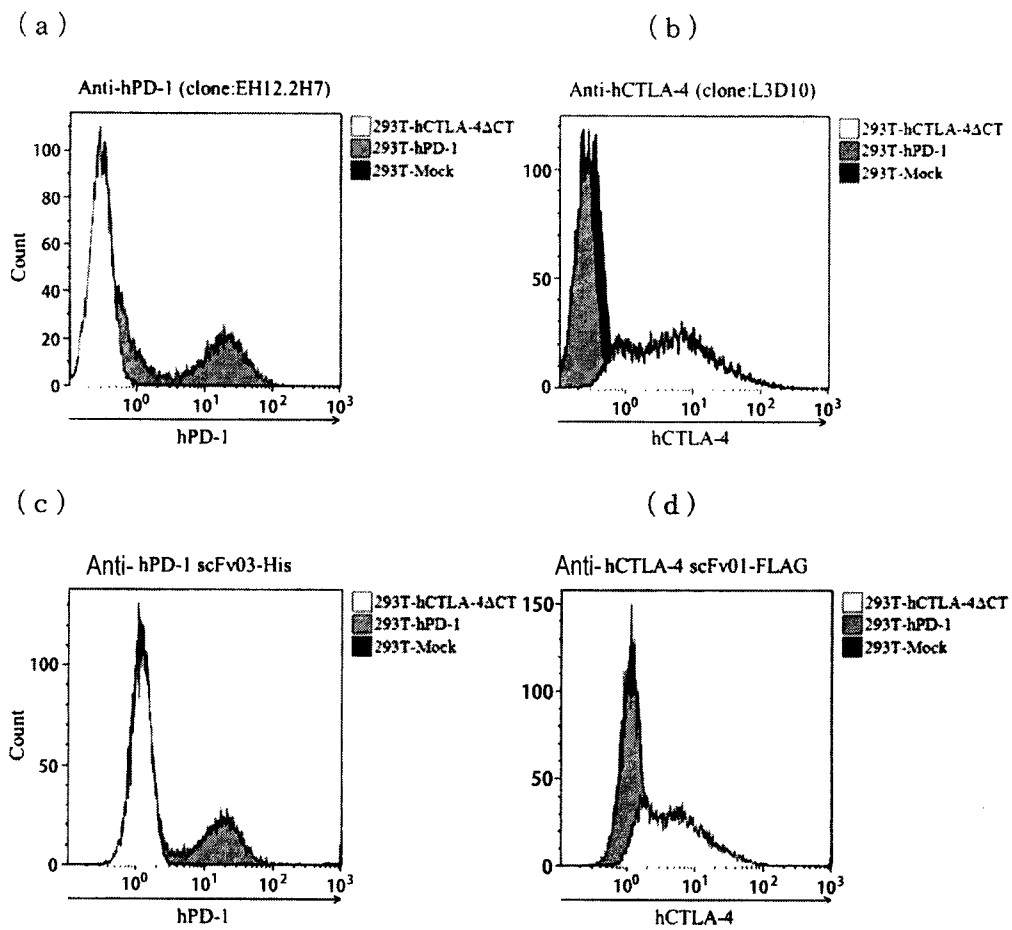
[Figure 35]



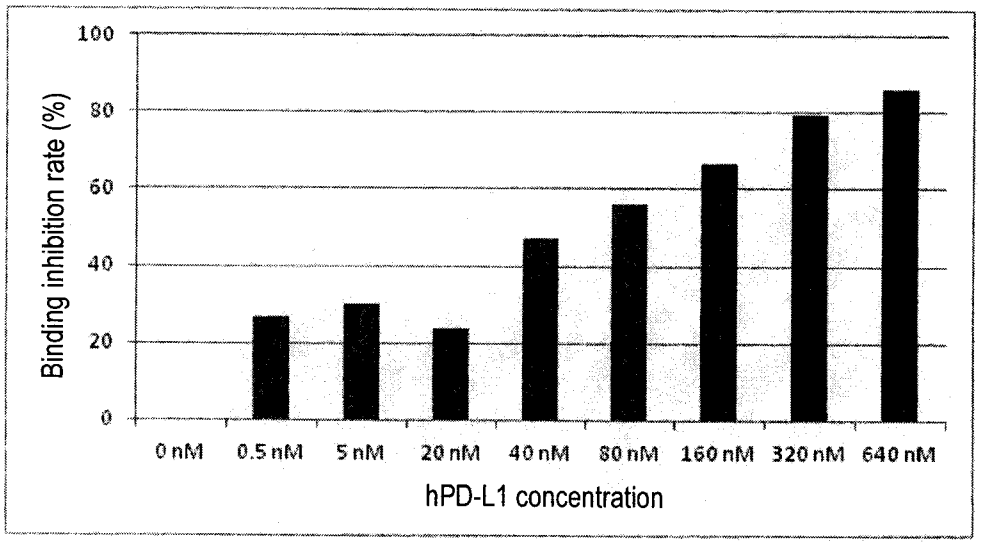
[Figure 36]



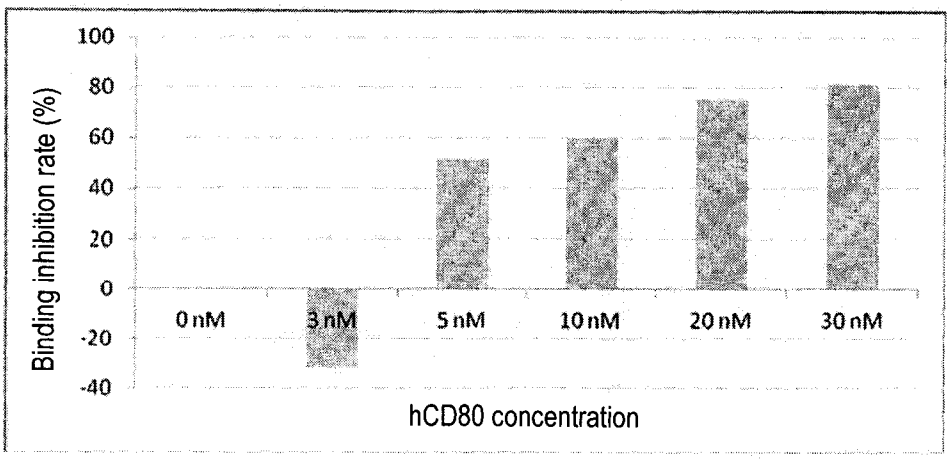
[Figure 37]


(a)
Anti-hPD-1 (clone:EH12.2H7)
293T-hCTLA-4ΔCT
293T-hPD-1
293T-Mock
Count
hPD-1
(b)
Anti-hCTLA-4 (clone:L3D10)
293T-hCTLA-4ΔCT
293T-hPD-1
293T-Mock
hCTLA-4
(c)
Anti- hPD-1 scFv03-His
293T-hCTLA-4ΔCT
293T-hPD-1
293T-Mock
Count
hPD-1
(d)
Anti- hCTLA-4 scFv01-FLAG
293T-hCTLA-4ΔCT
293T-hPD-1
293T-Mock
hCTLA-4

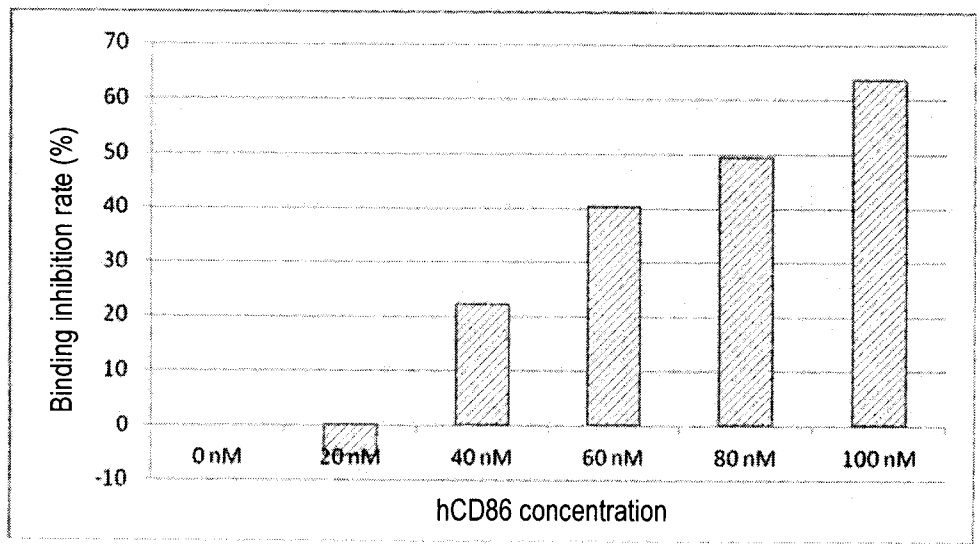
[Figure 38]



[Figure 39]



[Figure 40]


70
60
50
40
30
20
10
0
-10
Binding inhibition rate (%)
0 nM
20 nM
40 nM
60 nM
80 nM
100 nM
hCD86 concentration