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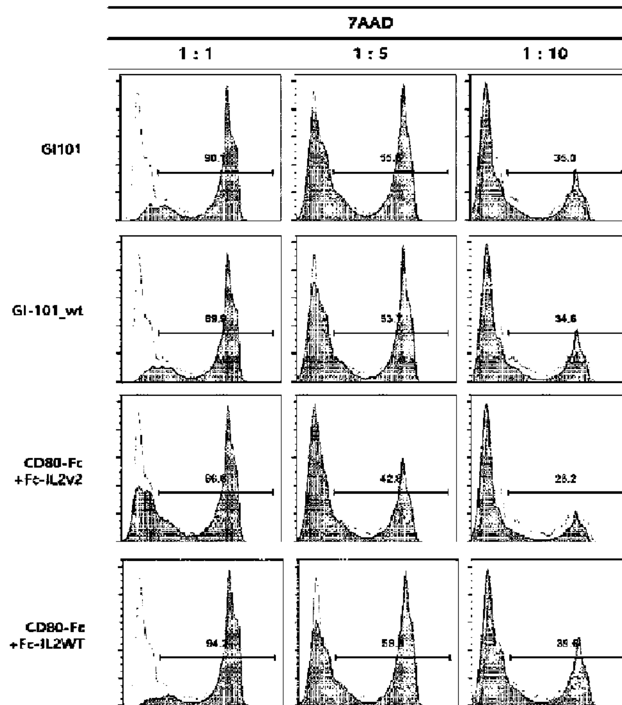
(54) **Titre : COMPOSITION POUR LA CULTURE DE CELLULES TUEUSES NATURELLES ET PROCEDE DE PREPARATION DE CELLULES TUEUSES NATURELLES L'UTILISANT**

(54) **Title: COMPOSITION FOR CULTURING NATURAL KILLER CELLS AND METHOD FOR PREPARING NATURAL KILLER CELLS BY USING SAME**



(57) **Abrégé/Abstract:**

The invention relates to: a composition for culturing natural killer cells, comprising, as an active ingredient, a fusion protein comprising an IL-2 protein and a CD80 protein; and a method for preparing natural killer cells by using same. Particularly, a composition for culturing natural killer cells, comprising, as an active ingredient, a fusion protein comprising IL-2 or a variant thereof and CD80 or a fragment thereof, of the present invention, promotes the proliferation of natural killer cells, induces the expression of CD16 and NKp46, and increases the expression and secretion of granzyme B and perforin, thereby being effectively usable in the preparation of natural killer cells having an excellent anticancer immune function.

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Abstract:

The invention relates to: a composition for culturing natural killer cells, comprising, as an active ingredient, a fusion protein comprising an IL-2 protein and a CD80 protein; and a method for preparing natural killer cells by using same. Particularly, a composition for culturing natural killer cells, comprising, as an active ingredient, a fusion protein comprising IL-2 or a variant thereof and CD80 or a fragment thereof, of the present invention, promotes the proliferation of natural killer cells, induces the expression of CD16 and NKp46, and increases the expression and secretion of granzyme B and perforin, thereby being effectively usable in the preparation of natural killer cells having an excellent anticancer immune function.

COMPOSITION FOR CULTURING NATURAL KILLER CELLS AND METHOD FOR PREPARING NATURAL KILLER CELLS BY USING SAME

Technical Field

5 The present invention relates to a composition for culturing natural killer cells and a method for producing natural killer cells using the same.

Background Art

Various treatments such as surgical operation, radiation therapy, and 10 chemotherapy have been developed to treat cancer, but as several side effects have been reported, immunotherapy using immune function of a patient has been recently developed. In particular, immunotherapy using natural killer cells which can undergo large-scale production and freezing has been studied.

Specifically, natural killer cells are a type of lymphocyte that are distributed 15 in bone marrow, spleen, peripheral lymph nodes and peripheral blood of the body. They make up about 10% of peripheral blood lymphocytes, and play an important role in innate immune response (Ann Rev Immunol., 24: 257-286, 2006)). In addition, natural killer cells are positive for CD56 and CD16, but negative for CD3. Natural killer cells kill cells by release of cytoplasmic granules containing perforin 20 and granzyme. Natural killer cells secrete various cytokines such as IFN-γ, TNF-α, GM-CSF and IL-10.

In addition, natural killer cells express several receptors on the cell surface, and these receptors are involved in cell adhesion, activation of capacity to kill cells, or inhibition of capacity to kill cells. However, most natural killer cells in the body 25 of a normal subject exist in an inactive state. Therefore, there is a need for activated

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natural killer cells to eliminate cancer. In addition, for natural killer cells present in the body of a cancer patient, natural killer cells have functional defects due to immune evasion mechanism of cancer cells. Therefore, it is very important to activate natural killer cells to use natural killer cells as a therapeutic agent. Further, it is essential to develop a technique to massively proliferate and freeze natural killer cells in blood from a normal subject or a patient because the number of natural killer cells present in the body is limited.

Meanwhile, IL-2, also called as T-cell growth factor (TCGF), is a globular glycoprotein that plays a central role in production, survival, and homeostasis of lymphocyte. IL-2 has a size of 15.5 kDa to 16 kDa protein and consists of 133 amino acids. IL-2 mediates various immune responses by binding to the IL-2 receptor which has three distinct subunits.

In addition, CD80 is known as B7-1 and one of B7 family members among membrane-bound proteins involved in immune regulation by binding to the ligand and thus transmitting costimulatory responses and coinhibitory responses. CD80 is a transmembrane protein expressed on the surface of T cells, B cells, dendritic cells, and monocytes. CD80 is known to bind to CD28, CTLA4 (CD152), and PD-L1.

As such, it is widely known that natural killer cells are important for anti-cancer treatment, but specific methods that can amplify natural killer cells to use them effectively are still insufficient.

Detailed Description of the Invention

Technical Problem

Accordingly, the present inventors prepared natural killer cells by using a fusion protein dimer comprising IL-2 protein and CD80 protein, as a result of

researching to prepare activated natural killer cells in a large amount. In addition, the present inventors have identified that natural killer cells thus prepared has increased activity and exhibits excellent anticancer effects, and thus have completed the present invention.

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Solution to Problem

To achieve the above object, in accordance with an exemplary embodiment, provided is a composition for culturing natural killer cells including, as an active ingredient, a fusion protein dimer comprising IL-2 or a variant thereof and CD80 or a

10 fragment thereof.

In accordance with another exemplary embodiment, provided is a method for culturing natural killer cells including: i) isolating cells that do not express CD3 from peripheral blood mononuclear cells (PBMC); ii) isolating cells that express CD56 from the cells that do not express CD3, isolated in the above step; and iii) culturing

15 the isolated cells in the presence of a fusion protein dimer comprising IL-2 or a variant thereof and CD80 or a fragment thereof.

In accordance with yet another exemplary embodiment, provided is natural killer cells prepared by the method for culturing natural killer cells.

In accordance with still another exemplary embodiment, provided is a

20 pharmaceutical composition for preventing or treating cancer including the natural killer cells as an active ingredient.

In accordance with yet still another exemplary embodiment, provided is a method for culturing natural killer cells including: i) isolating cells that do not express CD3 from PBMCs; and ii) culturing the isolated cells in the presence of a

25 fusion protein dimer comprising IL-2 or a variant thereof and CD80 or a fragment

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thereof.

In accordance with another exemplary embodiment, provided is natural killer cells prepared by the method for culturing natural killer cells.

In accordance with yet another exemplary embodiment, provided is a pharmaceutical composition for preventing or treating cancer including the natural killer cells as an active ingredient.

In accordance with still another exemplary embodiment, provided is a method for culturing natural killer cells including: i) isolating cells that express CD56 from PBMCs; and ii) culturing the isolated cells in the presence of a fusion protein dimer comprising IL-2 or a variant thereof and CD80 or a fragment thereof.

In accordance with yet still another exemplary embodiment, provided is natural killer cells prepared by the method for culturing natural killer cells.

In accordance with another exemplary embodiment, provided is a pharmaceutical composition for preventing or treating cancer including the natural killer cells as an active ingredient.

In accordance with yet another exemplary embodiment, provided is a method for promoting the activity of natural killer cells in PBMCs including culturing PBMCs in the presence of a fusion protein dimer comprising IL-2 or a variant thereof and CD80 or a fragment thereof.

In accordance with still another exemplary embodiment, provided are PBMCs prepared by the method for promoting the activity of natural killer cells in the PBMCs.

In accordance with yet still another exemplary embodiment, provided is a pharmaceutical composition for preventing or treating cancer including the PBMCs as an active ingredient.

Effect of the Invention

The composition of the present invention for culturing natural killer cells including, as an active ingredient, a fusion protein comprising IL-2 or a variant thereof and CD80 or a fragment thereof promotes proliferation of natural killer cells, induces expression of CD16 and NKp46, and increases expression and secretion of granzyme B and perforin, and thus may be usefully used in the production of natural killer cells having excellent anticancer immune function.

Brief Description of Drawings

Exemplary embodiments can be understood in more detail from the following description taken in conjunction with the accompanying drawings, in which:

FIG. 1A is a schematic diagram of a fusion protein dimer used in the present invention;

FIG. 1B shows an image of SDS-PAGE confirming the obtained fusion protein dimer (GI-101);

FIG. 1C shows a content of the fusion protein dimer (GI-101) according to the absorbance;

FIG. 1D shows a result of size exclusion chromatography (SEC) analysis of the obtained fusion protein dimer (GI-101);

FIG. 2A shows images of SDS-PAGE confirming the obtained hCD80-Fc fusion protein dimer;

FIG. 2B shows results of size exclusion chromatography (SEC) analysis of the obtained hCD80-Fc fusion protein dimer;

FIG. 3A shows an image of SDS-PAGE confirming the obtained Fc-IL2v2

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fusion protein dimer;

FIG. 3B shows results of size exclusion chromatography (SEC) analysis of the obtained Fc-IL2v2 fusion protein dimer;

FIG. 3C shows images of SDS-PAGE confirming the obtained Fc-IL2wt fusion protein dimer;

FIG. 3D shows results of size exclusion chromatography (SEC) analysis of the obtained Fc-IL2wt fusion protein dimer;

FIG. 4A shows an image of SDS-PAGE confirming the obtained hCD80-Fc-IL2wt fusion protein dimer;

FIG. 4B shows results of size exclusion chromatography (SEC) analysis of the obtained hCD80-Fc-IL2wt fusion protein dimer;

FIGS. 5A and 5B show the number of NK cells when cultured in an AIM-V (5% SR) condition;

FIGS. 6A and 6B show the viability of NK cells when cultured in an AIM-V (5% SR) condition;

FIGS. 7A and 7B show the number of NK cells when cultured in an AIM-V (5% hABS) condition;

FIGS. 8A and 8B show the viability of NK cells when cultured in an AIM-V (5% hABS) condition;

FIGS. 9A and 9B show the number of NK cells when cultured in an X-VIVO (5% hABS) condition;

FIGS. 10A and 10B show the viability of NK cells when cultured in an X-VIVO (5% hABS) condition;

FIGS. 11A and 11B show the number of NK cells when cultured in an NK MACS (5% hABS) condition;

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FIGS. 12A and 12B show the viability of NK cells when cultured in an NK MACS (5% hABS) condition;

FIG. 13 shows the result of FACS analysis confirming the purity of natural killer cells cultured in a composition containing an AIM-V (5% SR) medium;

5 FIG. 14 shows the result of FACS analysis confirming the purity of natural killer cells cultured in a composition containing an AIM-V (5% hABS) medium;

FIG. 15 shows the result of FACS analysis confirming the purity of natural killer cells cultured in a composition containing an X-VIVO15 (5% hABS) medium;

FIG. 16 shows the result of FACS analysis confirming the purity of natural 10 killer cells cultured in a composition containing an NK MACS (5% hABS) medium;

FIG. 17 shows the analysis result for activation markers of natural killer cells cultured in a composition containing an AIM-V (5% SR) medium;

FIG. 18 shows the analysis result for inhibition markers of natural killer cells cultured in a composition containing an AIM-V (5% SR) medium;

15 FIG. 19 shows the analysis result for activation markers of natural killer cells cultured in a composition containing an AIM-V (5% hABS) medium;

FIG. 20 shows the analysis result for inhibition markers of natural killer cells cultured in a composition containing an AIM-V (5% hABS) medium;

FIG. 21 shows the analysis result for activation markers of natural killer cells 20 cultured in a composition containing an X-VIVO (5% hABS) medium;

FIG. 22 shows the analysis result for inhibition markers of natural killer cells cultured in a composition containing an X-VIVO (5% hABS) medium;

FIG. 23 shows the analysis result for activation markers of natural killer cells cultured in a composition containing an NK MACS (5% hABS) medium;

25 FIG. 24 shows the analysis result for inhibition markers of natural killer cells

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cultured in a composition containing an NK MACS (5% hABS) medium;

FIG. 25 shows the analysis result for cytotoxicity markers of natural killer cells cultured in a composition containing an AIM-V (5% SR) medium;

FIG. 26 shows the analysis result for cytotoxicity markers of natural killer cells cultured in a composition containing an AIM-V (5% hABS) medium;

FIG. 27 shows the analysis result for cytotoxicity markers of natural killer cells cultured in a composition containing an X-VIVO15 (5% hABS) medium;

FIG. 28 shows the analysis result for cytotoxicity markers of natural killer cells cultured in a composition containing an NK MACS (5% hABS) medium;

FIG. 29 shows the results of analyzing the degranulation ability of natural killer cells cultured for 21 days in a composition containing GI-101 (50 nM), GI-101_wt (50 nM), CD80-Fc (50 nM)+Fc-IL2v2 (50 nM), or CD80-Fc (50 nM)+Fc-IL2wt (50 nM) in an AIM-V (5% hABS) medium;

FIG. 30 shows the results of analyzing the cancer cell-killing effect of natural killer cells cultured for 21 days against in a composition containing GI-101 (50 nM), GI-101_wt (50 nM), CD80-Fc (50 nM)+Fc-IL2v2 (50 nM), or CD80-Fc (50 nM)+Fc-IL2wt (50 nM) in an AIM-V (5% hABS) medium;

FIG. 31 shows the results of analyzing the degranulation ability of natural killer cells cultured for 21 days in a composition containing GI-101 (50 nM), GI-101_wt (50 nM), CD80-Fc (50 nM)+Fc-IL2v2 (50 nM), or CD80-Fc (50 nM)+Fc-IL2wt (50 nM) in an X-VIVO15 (5% hABS) medium;

FIG. 32 shows the results of analyzing the cancer cell-killing effect of natural killer cells cultured for 21 days in a composition containing GI-101 (50 nM), GI-101_wt (50 nM), CD80-Fc (50 nM)+Fc-IL2v2 (50 nM), or CD80-Fc (50 nM)+Fc-IL2wt (50 nM) in an X-VIVO15 (5% hABS) medium;

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FIG. 33 shows the results of analyzing the degranulation ability of natural killer cells cultured for 21 days in a composition containing GI-101 (50 nM), GI-101_wt (50 nM), CD80-Fc (50 nM)+Fc-IL2v2 (50 nM), or CD80-Fc (50 nM)+Fc-IL2wt (50 nM) in an NK MACS (5% hABS) medium; and

5 FIG. 34 shows the results of analyzing the cancer cell-killing effect of natural killer cells cultured for 21 days in a composition containing GI-101 (50 nM), GI-101_wt (50 nM), CD80-Fc (50 nM)+Fc-IL2v2 (50 nM), or CD80-Fc (50 nM)+Fc-IL2wt (50 nM) in an NK MACS (5% hABS) medium.

10 **Best Mode for Carrying out the Invention**

Composition and medium for NK cell proliferation

An aspect of the present invention provides a composition for culturing a natural killer (NK) cell including, as an active ingredient, a fusion protein dimer comprising IL-2 or a variant thereof and CD80 or a fragment thereof. In addition, a 15 natural killer cell culture medium including the fusion protein dimer as an active ingredient is provided.

The composition for culturing the natural killer cell may further include any one selected from the group consisting of a medium, a serum, a supplement, and a combination thereof.

20 The NK cell culture medium may be a medium in which the fusion protein dimer comprising IL-2 protein and CD80 protein is added to a cell culture medium. In this case, the cell culture medium may include any one selected from the group consisting of amino acids, sugars, inorganic salts, and vitamins. Preferably, the cell culture medium may include all of amino acids, sugars, inorganic salts, and vitamins. 25 As a specific embodiment, the NK cell culture medium may include at least one of

components in Table 1 to Table 4 below.

As used herein, the term "cell culture medium" means a medium used for culturing cells, specifically NK cells, and more specifically CD3-CD56+ cells. This includes components required by cells for cell growth and survival *in vitro*, or includes components that help cell growth and survival. Specifically, the components may be vitamins, essential or non-essential amino acids, and trace elements. The medium may be a medium used for culturing cells, preferably eukaryotic cells, and more preferably NK cells.

The cell culture medium according to the present invention may include an amino acid component, a vitamin component, an inorganic salt component, other component, and purified water, wherein:

a) the amino acid component is at least one amino acid selected from the group consisting of glycine, L-alanine, L-valine, L-leucine, L-isoleucine, L-threonine, L-serine, L-cysteine, L-methionine, L-aspartic acid, L-asparagine, L-glutamic acid, L-glutamine, L-lysine, L-arginine, L-histidine, L-phenylalanine, L-tyrosine, L-tryptophan, L-proline, β-alanine, γ-aminobutyric acid, ornithine, citrulline, homoserine, triiodothyronine, thyroxine and dioxy phenylalanine, or a combination thereof, and preferably at least one amino acid selected from the group consisting of glycine, L-alanine, L-arginine, L-cysteine, L-glutamine, L-histidine, L-lysine, L-methionine, L-proline, L-serine, L-threonine and L-valine, or a combination thereof;

b) the vitamin component is at least one vitamin selected from the group consisting of biotin, calcium D-pantothenate, folic acid, niacinamide, pyridoxine hydrochloride, riboflavin, thiamine hydrochloride, vitamin B12, choline chloride, i-inositol and ascorbic acid, or a combination thereof, and preferably at least one vitamin selected from the group consisting of i-inositol, thiamine hydrochloride,

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niacinamide and pyridoxine hydrochloride, or a combination thereof;

c) the inorganic salt component is at least one inorganic salt selected from the group consisting of calcium chloride ($CaCl_2$)(anhydrous), copper sulfate pentahydrate ($CuSO_4$-$5H_2O$), iron (III) sulfate heptahydrate ($FeSO_4$-$7H_2O$), magnesium chloride (anhydrous), magnesium sulfate ($MgSO_4$)(anhydrous), potassium chloride (KCl), sodium chloride (NaCl), disodium hydrogen phosphate (Na_2HPO_4), sodium dihydrogen phosphate monohydrate (NaH_2PO_4-H_2O), zinc sulfate heptahydrate ($ZnSO_4$-$7H_2O$), iron (III) nitrate nonahydrate ($Fe(NO_3)_3\cdot 9H_2O$) and sodium hydrogen carbonate ($NaHCO_3$), or a combination thereof, and preferably at least one inorganic salt selected from the group consisting of sodium chloride (NaCl), sodium hydrogen carbonate ($NaHCO_3$), potassium chloride (KCl), calcium chloride ($CaCl_2$)(anhydrous) and sodium dihydrogen phosphate monohydrate (NaH_2PO_4-H_2O), or a combination thereof;

d) the other component is at least one other component selected from the group consisting of D-glucose (dextrose), sodium pyruvate, hypoxanthine Na, thymidine, linoleic acid, lipoic acid, adenosine, cytidine, guanosine, uridine, 2'-deoxyadenosine, 2'-deoxycytidine HCl and 2'-deoxyguanosine, or a combination thereof, and it may preferably be sodium pyruvate; and

e) the purified water is used to dissolve the amino acid, vitamin, inorganic salt, and other component, and may be obtained by one or more processes of distillation, or purified through a filter.

In addition, the cell culture medium according to the present invention may further include a growth factor or a cytokine. The growth factor may be IGF, bFGF, TGF, HGF, EGF, VEGF, PDGF, or the like alone or at least two thereof, but is not limited thereto. The cytokine may be IL-1, IL-4, IL-6, IFN-γ, IL-10, IL-15, IL-17,

IL-21, or the like alone or at least two thereof, but is not limited thereto.

In addition, the cell culture medium according to the present invention may further include an antibody for activating natural killer cells. The antibody for activating natural killer cells may be an anti-CD3 antibody, an anti-CD2 antibody, an anti-CD335 antibody, or the like alone or at least two thereof, but is not limited thereto. In addition, a bead to which the antibody for activating natural killer cells is bound may be included. Also, a fusion protein including two or more types of antibodies or variable region fragments thereof for activating natural killer cells may be used.

In particular, the NK culture medium may further include any one selected from the group consisting of IL-15, IL-21, and a combination thereof.

The IL-15 and IL-21 may be a type of interleukin (IL), and mean proteinaceous bioactive substances produced by immunocompetent cells such as lymphocytes or monocytes and macrophages. The IL-15 and IL-21 may be used when culturing natural killer cells using mononuclear cells as source cells by promoting proliferation of natural killer cells, but there is a problem of low proliferation rate and purity when only these are used alone or in combination (Biossel L. et al., Biology of Blood and Marrow Transplantation, 14, 1031-1038, 2008).

Specifically, the medium may be a conventional medium for culturing animal cells, such as DMEM (Dulbecco's Modified Eagle's Medium), EDM (Endothelial differentiation medium), MEM (Minimal Essential Medium), BME (Basal Medium Eagle), RPMI 1640, F-10, F-12, a-MEM (a-Minimal Essential Medium), G-MEM (Glasgow's Minimal Essential Medium), Iscove's Modified Dulbecco's Medium, AIM-V Medium, X-VIVO™ 15 Medium, NK MACS Medium. In an embodiment

of the present invention, AIM-V Medium, X-VIVO™ 15 Medium and NK MACS Medium were used as a medium.

The term "serum" as used in the present invention means clear supernatant separated from blood after the blood has been completely clotted. In addition, it is required to add serum to a synthetic medium for culturing animal cells, and it is common to use bovine, horse, or human serum. For bovine-derived serum, fetal bovine serum (FBS), newborn bovine serum, calf serum, bovine serum, or the like may be used depending on the timing of blood collection. For human-derived serum, human serum from a donor whose blood type is AB is used, and human AB serum which is free of antibodies to A and B blood type antigens so that can minimize immune reactivity may be used. In addition, CTS Immune Cell SR, or the like may be used as an alternative to the "serum." In an embodiment of the present invention, human AB serum or CTS Immune Cell SR was used.

GLUTAMAX (GIBCO®), a L-Glutamine alternative, may be used as the supplement to improve stability and cell activity during cell culture. In addition, the supplement may be NK MACS supplement (Miltenyi Biotec, 130-113-102).

Fusion protein dimer comprising IL-2 protein and CD80 protein

As used herein, the term "IL-2" or "interleukin-2", unless otherwise stated, refers to any wild-type IL-2 obtained from any vertebrate source, including mammals, for example, primates (such as humans) and rodents (such as mice and rats). IL-2 may be obtained from animal cells, and also includes one obtained from recombinant cells capable of producing IL-2. In addition, IL-2 may be wild-type IL-2 or a variant thereof.

In the present specification, IL-2 or a variant thereof may be collectively expressed by the term "IL-2 protein" or "IL-2 polypeptide". IL-2, an IL-2 protein, an

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IL-2 polypeptide, and an IL-2 variant specifically bind to, for example, an IL-2 receptor. This specific binding may be identified by methods known to those skilled in the art.

An embodiment of IL-2 may have the amino acid sequence of SEQ ID NO: 35 or SEQ ID NO: 36. Here, IL-2 may also be in a mature form. Specifically, the mature IL-2 may not comprise a signal sequence, and may have the amino acid sequence of SEQ ID NO: 10. Here, IL-2 may be used under a concept encompassing a fragment of wild-type IL-2 in which a portion of N-terminus or C-terminus of the wild-type IL-2 is truncated.

In addition, the fragment of IL-2 may be in a form in which 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 contiguous amino acids are truncated from N-terminus of a protein having the amino acid sequence of SEQ ID NO: 35 or SEQ ID NO: 36. In addition, the fragment of IL-2 may be in a form in which 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, or 25 contiguous amino acids are truncated from C-terminus of a protein having the amino acid sequence of SEQ ID NO: 35 or SEQ ID NO: 36.

As used herein, the term “IL-2 variant” refers to a form in which a portion of amino acids in the full-length IL-2 or the above-described fragment of IL-2 is substituted. That is, an IL-2 variant may have an amino acid sequence different from wild-type IL-2 or a fragment thereof. However, an IL-2 variant may have activity equivalent or similar to the wild-type IL-2. Here, “IL-2 activity” may, for example, refer to specific binding to an IL-2 receptor, which specific binding can be measured by methods known to those skilled in the art.

Specifically, an IL-2 variant may be obtained by substitution of a portion of amino acids in the wild-type IL-2. An embodiment of the IL-2 variant obtained by

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amino acid substitution may be obtained by substitution of at least one of the 38th, 42nd, 45th, 61st, and 72nd amino acids in the amino acid sequence of SEQ ID NO: 10.

Specifically, the IL-2 variant may be obtained by substitution of at least one of the 38th, 42nd, 45th, 61st, or 72nd amino acid in the amino acid sequence of SEQ ID NO: 10 with another amino acid. In addition, when IL-2 is in a form in which a portion of N-terminus in the amino acid sequence of SEQ ID NO: 35 is truncated, the amino acid at a position complementarily corresponding to that in the amino acid sequence of SEQ ID NO: 10 may be substituted with another amino acid. For example, when IL-2 has the amino acid sequence of SEQ ID NO: 35, its IL-2 variant may be obtained by substitution of at least one of 58th, 62nd, 65th, 81st, or 92nd amino acid in the amino acid sequence of SEQ ID NO: 35 with another amino acid. These amino acid residues correspond to the respective 38th, 42nd, 45th, 61st, and 72nd amino acid residues in the amino acid sequence of SEQ ID NO: 10. According to an embodiment, one, two, three, four, five, six, seven, eight, nine, or ten amino acids may be substituted as long as such IL-2 variant maintains IL-2 activity. According to another embodiment, one to five amino acids may be substituted.

In an embodiment, an IL-2 variant may be in a form in which two amino acids are substituted. Specifically, the IL-2 variant may be obtained by substitution of the 38th and 42nd amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 38th and 45th amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 38th and 61st amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 38th and 72nd amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an

embodiment, the IL-2 variant may be obtained by substitution of the 42nd and 45th amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 42nd and 61st amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 42nd and 72nd amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 45th and 61st amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 45th and 72nd amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 61st and 72nd amino acids in the amino acid sequence of SEQ ID NO: 10.

Furthermore, an IL-2 variant may be in a form in which three amino acids are substituted. Specifically, the IL-2 variant may be obtained by substitution of the 38th, 42nd, and 45th amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 38th, 42nd, and 61st amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 38th, 42nd, and 72nd amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 38th, 45th, and 61st amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 38th, 45th, and 72nd amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 38th, 61st, and 72nd amino acids in the amino acid sequence of SEQ ID NO: 10. In

addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 42nd, 45th, and 61st amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 42nd, 45th, and 72nd amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 45th, 61st, and 72nd amino acids in the amino acid sequence of SEQ ID NO: 10.

In addition, an IL-2 variant may be in a form in which four amino acids are substituted. Specifically, the IL-2 variant may be obtained by substitution of the 38th, 42nd, 45th, and 61st amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 38th, 42nd, 45th, and 72nd amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 38th, 45th, 61st, and 72nd amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of the 38th, 42nd, 61st, and 72nd amino acids in the amino acid sequence of SEQ ID NO: 10. In addition, in an embodiment, the IL-2 variant may be obtained by substitution of 42nd, 45th, 61st, and 72nd amino acids in the amino acid sequence of SEQ ID NO: 10.

Furthermore, an IL-2 variant may be in a form in which five amino acids are substituted. Specifically, the IL-2 variant may be obtained by substitution of each of the 38th, 42nd, 45th, 61st, and 72nd amino acids in the amino acid sequence of SEQ ID NO: 10 with another amino acid.

Here, the "another amino acid" introduced by the substitution may be any one selected from the group consisting of alanine, arginine, asparagine, aspartic acid, cysteine, glutamic acid, glutamine, histidine, isoleucine, leucine, lysine, methionine,

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phenylalanine, proline, serine, threonine, tryptophan, tyrosine, and valine. However, regarding amino acid substitution for the IL-2 variant, in the amino acid sequence of SEQ ID NO: 10, the 38th amino acid cannot be substituted with arginine, the 42nd amino acid cannot be substituted with phenylalanine, the 45th amino acid cannot be substituted with tyrosine, the 61st amino acid cannot be substituted with glutamic acid, and the 72nd amino acid cannot be substituted with leucine.

Regarding amino acid substitution for an IL-2 variant, in the amino acid sequence of SEQ ID NO: 10, the 38th amino acid, arginine, may be substituted with an amino acid other than arginine. Preferably, regarding amino acid substitution for an IL-2 variant, in the amino acid sequence of SEQ ID NO: 10, the 38th amino acid, arginine, may be substituted with alanine (R38A).

Regarding amino acid substitution for an IL-2 variant, in the amino acid sequence of SEQ ID NO: 10, the 42nd amino acid, phenylalanine, may be substituted with an amino acid other than phenylalanine. Preferably, regarding amino acid substitution for an IL-2 variant, in the amino acid sequence of SEQ ID NO: 10, the 42nd amino acid, phenylalanine, may be substituted with alanine (F42A).

Regarding amino acid substitution for an IL-2 variant, in the amino acid sequence of SEQ ID NO: 10, the 45th amino acid, tyrosine, may be substituted with an amino acid other than tyrosine. Preferably, regarding amino acid substitution for an IL-2 variant, in the amino acid sequence of SEQ ID NO: 10, the 45th amino acid, tyrosine, may be substituted with alanine (Y45A).

Regarding amino acid substitution for an IL-2 variant, in the amino acid sequence of SEQ ID NO: 10, the 61st amino acid, glutamic acid, may be substituted with an amino acid other than glutamic acid. Preferably, regarding amino acid substitution for an IL-2 variant, in the amino acid sequence of SEQ ID NO: 10, the

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61st amino acid, glutamic acid, may be substituted with arginine (E61R).

Regarding amino acid substitution for an IL-2 variant, in the amino acid sequence of SEQ ID NO: 10, the 72nd amino acid, leucine, may be substituted with an amino acid other than leucine. Preferably, regarding amino acid substitution for an IL-2 variant, in the amino acid sequence of SEQ ID NO: 10, the 72nd amino acid, leucine, may be substituted with glycine (L72G).

Specifically, an IL-2 variant may be obtained by at least one substitution selected from the group consisting of R38A, F42A, Y45A, E61R, and L72G, in the amino acid sequence of SEQ ID NO: 10.

Specifically, an IL-2 variant may be obtained by amino acid substitutions at two, three, four, or five positions among the positions selected from the group consisting of R38A, F42A, Y45A, E61R, and L72G.

In addition, an IL-2 variant may be in a form in which two amino acids are substituted. Specifically, an IL-2 variant may be obtained by the substitutions, R38A and F42A. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, R38A and Y45A. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, R38A and E61R. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, R38A and L72G. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, F42A and Y45A. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, F42A and E61R. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, F42A and L72G. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, E61R and L72G.

Furthermore, an IL-2 variant may be in a form in which three amino acids are substituted. Specifically, an IL-2 variant may be obtained by the substitutions, R38A,

F42A, and Y45A. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, R38A, F42A, and E61R. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, R38A, F42A, and L72G. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, R38A, Y45A, and E61R. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, R38A, Y45A, and L72G. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, F42A, Y45A, and E61R. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, F42A, Y45A, and L72G. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, F42A, E61R, and L72G. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, Y45A, E61R, and L72G.

In addition, an IL-2 variant may be in a form in which four amino acids are substituted. Specifically, an IL-2 variant may be obtained by the substitutions, R38A, F42A, Y45A, and E61R. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, R38A, F42A, Y45A, and L72G. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, R38A, F42A, E61R, and L72G. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, R38A, Y45A, E61R, and L72G. In addition, in an embodiment, an IL-2 variant may be obtained by the substitutions, F42A, Y45A, E61R, and L72G.

Furthermore, an IL-2 variant may be obtained by the substitutions, R38A, F42A, Y45A, E61R, and L72G.

Preferably, an embodiment of the IL-2 variant may comprise which are any one selected from the following substitution combinations (a) to (d) in the amino acid sequence of SEQ ID NO: 10:

(a) R38A/F42A

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(b) R38A/F42A/Y45A

(c) R38A/F42A/E61R

(d) R38A/F42A/L72G

Here, when IL-2 has the amino acid sequence of SEQ ID NO: 35, an amino acid substitution may be present at a position complementarily corresponding to that in the amino acid sequence of SEQ ID NO: 10. In addition, even when IL-2 is a fragment of the amino acid sequence of SEQ ID NO: 35, an amino acid substitution may be present at a position complementarily corresponding to that in the amino acid sequence of SEQ ID NO: 10.

Specifically, an IL-2 variant may have the amino acid sequence of SEQ ID NO: 6, 22, 23, or 24.

In addition, an IL-2 variant may be characterized by having low *in vivo* toxicity. Here, the low *in vivo* toxicity may be a side effect caused by binding of IL-2 to the IL-2 receptor alpha chain (IL-2Rα). Various IL-2 variants have been developed to ameliorate the side effect caused by binding of IL-2 to IL-2Rα, and such IL-2 variants may be those disclosed in US Patent No. 5,229,109 and Korean Patent No. 1667096. In particular, IL-2 variants described in the present application have low binding ability for the IL-2 receptor alpha chain (IL-2Rα) and thus have lower *in vivo* toxicity than the wild-type IL-2.

As used herein, the term “CD80”, also called “B7-1”, is a membrane protein present in dendritic cells, activated B cells, and monocytes. CD80 provides co-stimulatory signals essential for activation and survival of T cells. CD80 is known as a ligand for the two different proteins, CD28 and CTLA-4, present on the surface of T cells. CD80 may consist of 288 amino acids, and may specifically have the amino acid sequence of SEQ ID NO: 11. In addition, as used herein, the term “CD80

protein" refers to the full-length CD80 or a CD80 fragment.

As used herein, the term "CD80 fragment" refers to a truncated form of CD80. In addition, the CD80 fragment may be an extracellular domain of CD80. An embodiment of the CD80 fragment may be obtained by elimination of the 1st to 34th amino acids from N-terminus which are a signal sequence of CD80. Specifically, an embodiment of the CD80 fragment may be a protein consisting of the 35th to 288th amino acids in SEQ ID NO: 11. In addition, an embodiment of the CD80 fragment may be a protein consisting of the 35th to 242nd amino acids in SEQ ID NO: 11. In addition, an embodiment of the CD80 fragment may be a protein consisting of the 35th to 232nd amino acids in SEQ ID NO: 11. In addition, an embodiment of the CD80 fragment may be a protein consisting of the 35th to 139th amino acids in SEQ ID NO: 11. In addition, an embodiment of the CD80 fragment may be a protein consisting of the 142nd to 242nd amino acids in SEQ ID NO: 11. In an embodiment, a CD80 fragment may have the amino acid sequence of SEQ ID NO: 2.

In addition, the IL-2 protein and the CD80 protein may be attached to each other via a linker or a carrier. Specifically, the IL-2 or a variant thereof and the CD80 (B7-1) or a fragment thereof may be attached to each other via a linker or a carrier. In the present description, the linker and the carrier may be used interchangeably.

The linker links two proteins. An embodiment of the linker may include 1 to 50 amino acids, albumin or a fragment thereof, an Fc domain of an immunoglobulin, or the like. Here, the Fc domain of immunoglobulin refers to a protein that comprises heavy chain constant region 2 (CH2) and heavy chain constant region 3 (CH3) of an immunoglobulin, and does not comprise heavy and light chain variable regions and light chain constant region 1 (CH1) of an immunoglobulin. The

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immunoglobulin may be IgG, IgA, IgE, IgD, or IgM, and may preferably be IgG4. Here, Fc domain of wild-type immunoglobulin G4 may have the amino acid sequence of SEQ ID NO: 4.

In addition, the Fc domain of an immunoglobulin may be an Fc domain variant as well as wild-type Fc domain. In addition, as used herein, the term "Fc domain variant" may refer to a form which is different from the wild-type Fc domain in terms of glycosylation pattern, has a high glycosylation as compared with the wild-type Fc domain, or has a low glycosylation as compared with the wild-type Fc domain, or a deglycosylated form. In addition, an aglycosylated Fc domain is included therein. The Fc domain or a variant thereof may be adapted to have an adjusted number of sialic acids, fucosylations, or glycosylations, through culture conditions or genetic manipulation of a host.

In addition, glycosylation of the Fc domain of an immunoglobulin may be modified by conventional methods such as chemical methods, enzymatic methods, and genetic engineering methods using microorganisms. In addition, the Fc domain variant may be in a mixed form of respective Fc regions of immunoglobulins, IgG, IgA, IgE, IgD, and IgM. In addition, the Fc domain variant may be in a form in which some amino acids of the Fc domain are substituted with other amino acids. An embodiment of the Fc domain variant may have the amino acid sequence of SEQ ID NO: 12.

The fusion protein may have a structure in which, using an Fc domain as a linker (or carrier), a CD80 protein and an IL-2 protein, or an IL-2 protein and a CD80 protein are linked to N-terminus and C-terminus of the linker or carrier, respectively (Fig. 1A). Linkage between N-terminus or C-terminus of the Fc domain, and CD80 or IL-2 may optionally be achieved by a linker peptide.

Specifically, a fusion protein may consist of the following structural formula (I) or (II):

N'-X-[linker (1)]$_n$-Fc domain-[linker (2)]$_m$-Y-C' (I)

N'-Y-[linker (1)]$_n$-Fc domain-[linker (2)]$_m$-X-C' (II)

5 Here, in the structural formulas (I) and (II),

N′ is the N-terminus of the fusion protein,

C′ is the C-terminus of the fusion protein,

X is a CD80 protein,

Y is an IL-2 protein,

10 the linkers (1) and (2) are peptide linkers, and

n and m are each independently 0 or 1.

Preferably, the fusion protein may consist of the structural formula (I). The IL-2 protein is as described above. In addition, the CD80 protein is as described above. According to an embodiment, the IL-2 protein may be an IL-2 variant with 15 one to five amino acid substitutions as compared with the wild-type IL-2. The CD80 protein may be a fragment obtained by truncation of up to about 34 contiguous amino acid residues from the N-terminus or C-terminus of the wild-type CD80. Alternatively, the CD80 protein may be an extracellular immunoglobulin-like domain having the activity of binding to the T cell surface receptors CTLA-4 and 20 CD28.

Specifically, the fusion protein may have the amino acid sequence of SEQ ID NO: 9, 26, 28, or 30. According to another embodiment, the fusion protein includes a polypeptide having a sequence identity of 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99%, or 100% to the amino acid sequence of 25 SEQ ID NO: 9, 26, 28, or 30. Here, the identity is, for example, percent homology,

and may be determined through homology comparison software such as BlastN software of the National Center of Biotechnology Information (NCBI).

The peptide linker (1) may be included between the CD80 protein and the Fc domain. The peptide linker (1) may consist of 5 to 80 contiguous amino acids, 20 to 60 contiguous amino acids, 25 to 50 contiguous amino acids, or 30 to 40 contiguous amino acids. In an embodiment, the peptide linker (1) may consist of 30 amino acids. In addition, the peptide linker (1) may comprise at least one cysteine. Specifically, the peptide linker (1) may comprise one, two, or three cysteines. In addition, the peptide linker (1) may be derived from the hinge of an immunoglobulin. In an embodiment, the peptide linker (1) may be a peptide linker consisting of the amino acid sequence of SEQ ID NO: 3.

The peptide linker (2) may consist of 1 to 50 contiguous amino acids, 3 to 30 contiguous amino acids, or 5 to 15 contiguous amino acids. In an embodiment, the peptide linker (2) may be $(G4S)_n$ (where n is an integer of 1 to 10). Here, in $(G4S)_n$, n may be 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10. In an embodiment, the peptide linker (2) may be a peptide linker consisting of the amino acid sequence of SEQ ID NO: 5.

In another aspect of the present invention, provided is a dimer obtained by binding of two fusion proteins, each of which comprises an IL-2 protein and a CD80 protein. The fusion protein comprising IL-2 or a variant thereof and CD80 or a fragment thereof is as described above.

Here, the binding between the fusion proteins constituting the dimer may be achieved by, but is not limited to, a disulfide bond formed by cysteines present in the linker. The fusion proteins constituting the dimer may be the same or different fusion proteins from each other. Preferably, the dimer may be a homodimer. An embodiment of the fusion protein constituting the dimer may be a protein having the

amino acid sequence of SEQ ID NO: 9.

NK cell culture method 1

Another aspect of the present invention provides a method for culturing a natural killer cell, including: i) isolating a cell that do not express CD3 from
5 peripheral blood mononuclear cells (PBMC); ii) isolating a cell that express CD56 from the cell that do not express CD3 isolated in the above step; and iii) culturing the isolated cells in the presence of a fusion protein dimer comprising IL-2 or a variant thereof and CD80 or a fragment thereof.

The term "PBMC" as used in the present invention means a peripheral blood
10 mononuclear cell. The PBMC is composed of lymphocytes (T cells, B cells, natural killer cells) and monocytes, and can be isolated from whole blood by Ficoll and centrifugation. The PBMC may be isolated from whole blood obtained from an individual.

The fusion protein dimer is as described in detail for a composition for
15 culturing natural killer cells. The fusion protein dimer may be treated at a concentration of 1 nM to 500 nM. Specifically, the fusion protein dimer may be treated at a concentration of 1 nM to 500 nM, 5 nM to 300 nM, or 10 nM to 150 nM. In an embodiment of the present invention, the fusion protein dimer was treated at a concentration of 1.6 nM or 50 nM.

20 A method for culturing the isolated cells may be performed using a method widely known in the art. Specifically, the culture temperature in the step of culturing the isolated cells may be 27°C to 40°C, or 30°C to 37°C. In an embodiment of the present invention, culture was performed at a temperature of 37°C. In addition, in the step of culturing the isolated cells, CO_2 concentration condition during culture
25 may be 1% to 10%, and preferably, it may be cultured in a 5% CO_2 condition.

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In the step of culturing the isolated cells, culture period may be 5 days to 25 days, 6 days to 23 days, or 7 days to 21 days. In an embodiment of the present invention, culture period was 20 days, and a significant difference in proliferation appeared from the 5th day.

5 **Obtained NK cells and use thereof**

Another aspect of the present invention provides natural killer cells prepared by the method for culturing natural killer cells.

The natural killer cells may have increased expression of CD16 and NKp46. The natural killer cells may have increased expression of granzyme B and perforin.
10 The natural killer cells cultured according to the method for culturing natural killer cells may be frozen and the function of cells is not impaired even when thawed again.

Due to high expression of activating receptors such as CD16 and NKp46, the natural killer cells exhibit increased killing capacity against a cancer cell line and increased secretion of granzyme B and perforin, and thus an excellent anticancer
15 effect may be expected. Therefore, a therapeutic agent effective for treating cancer may be prepared, using a large amount of activated natural killer cells which are clinically applicable. In addition, the natural killer cells may have high expression of NKp30 or DNAM1.

Still another aspect of the present invention provides a pharmaceutical
20 composition for preventing or treating cancer including the natural killer cells as an active ingredient.

In addition, natural killer cells prepared by the method for culturing natural killer cells may be included in amount of 10 to 95% by weight based on the total weight of the pharmaceutical composition. Further, the pharmaceutical composition
25 may further include, in addition to the active ingredient, at least one active ingredient

that exhibits the same or similar functions.

A dosage of the pharmaceutical composition may be adjusted according to various factors including type of disease, severity of disease, kinds and content of active ingredients and other ingredients included in the composition, kinds of formulation, and age, weight, general health condition, gender, and diet of a patient, time of administration, route of administration, and secretion rate of a composition, duration of treatment, and simultaneously used drugs.

However, for a desirable effect, a dosage of the pharmaceutical composition may be $1X10^2$ cells/kg to $1.0X10^{13}$ cells/kg, and $1X10^7$ cells/kg to $1.5X10^{11}$ cells/kg based on the natural killer cells, which is an active ingredient. In this case, the dose may be administered once a day, or may be divided in several times.

In addition, the pharmaceutical composition may be administered to an individual by various methods known in the art. The route of administration may be appropriately selected by a person skilled in the art in consideration of the method of administration, volume of body fluid, viscosity, or the like.

The cancer may be any one selected from the group consisting of gastric cancer, liver cancer, lung cancer, colorectal cancer, breast cancer, prostate cancer, ovarian cancer, pancreatic cancer, cervical cancer, thyroid cancer, larynx cancer, acute lymphoblastic leukemia, brain tumor, neuroblastoma, retinoblastoma, head and neck cancer, salivary cancer, and lymphoma.

Treatment method using the obtained NK cells

Another aspect of the present invention provides a method for treating cancer including administering the NK cell to an individual having cancer. In this case, the NK cells and cancer are as described above. Still another aspect of the present invention provides use of the NK cell to treat cancer.

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NK cell culture method 2

Another aspect of the present invention provides a method for culturing a natural killer cell, including: i) isolating a cell that do not express CD3 from PBMCs; and ii) culturing the isolated cell in the presence of a fusion protein dimer comprising IL-2 or a variant thereof and CD80 or a fragment thereof.

Another aspect of the present invention provides natural killer cells prepared by the method for culturing natural killer cell. Yet another aspect of the present invention provides a pharmaceutical composition for preventing or treating cancer, including the natural killer cell as an active ingredient. Still another aspect of the present invention provides a method for treating cancer including administering the NK cell to an individual having cancer. In this case, the NK cell and cancer are as described above. Yet still another aspect of the present invention provides use of the NK cell to treat cancer.

Due to high expression of activating receptors such as CD16 and NKp46, the natural killer cells exhibit increased killing capacity against a cancer cell line and increased secretion of granzyme B and perforin, and thus an excellent anticancer effect may be expected. Therefore, a therapeutic agent effective for treating cancer may be prepared, using a large amount of activated natural killer cells which are clinically applicable. In addition, the natural killer cells may have high expression of NKp30 or DNAM1.

The cancer may be any one selected from the group consisting of gastric cancer, liver cancer, lung cancer, colorectal cancer, breast cancer, prostate cancer, ovarian cancer, pancreatic cancer, cervical cancer, thyroid cancer, larynx cancer, acute lymphoblastic leukemia, brain tumor, neuroblastoma, retinoblastoma, head and neck cancer, salivary cancer, and lymphoma.

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NK cell culture method 3

Another aspect of the present invention provides a method for culturing natural killer cells, including: i) isolating a cell that express CD56 from PBMCs; and ii) culturing the isolated cell in the presence of a fusion protein dimer comprising IL-2 or a variant thereof and CD80 or a fragment thereof.

Yet another aspect of the present invention provides a natural killer cell prepared by the method for culturing natural killer cell. Due to high expression of activating receptors such as CD16 and NKp46, the natural killer cells exhibit increased killing capacity against a cancer cell line and increased secretion of granzyme B and perforin, and thus an excellent anticancer effect may be expected. Therefore, a therapeutic agent effective for treating cancer may be prepared, using a large amount of activated natural killer cells which are clinically applicable. In addition, the natural killer cells may have high expression of NKp30 or DNAM1.

Yet another aspect of the present invention provides a pharmaceutical composition for preventing or treating cancer, including the natural killer cell as an active ingredient. Still another aspect of the present invention provides a method for treating cancer including administering the NK cell to an individual having cancer. In this case, NK cells and cancer are as described above. Yet still another aspect of the present invention provides use of the NK cells to treat cancer.

The cancer may be any one selected from the group consisting of gastric cancer, liver cancer, lung cancer, colorectal cancer, breast cancer, prostate cancer, ovarian cancer, pancreatic cancer, cervical cancer, thyroid cancer, larynx cancer, acute lymphoblastic leukemia, brain tumor, neuroblastoma, retinoblastoma, head and neck cancer, salivary cancer, and lymphoma.

NK cell culture method 4

Another aspect of the present invention provides a method for promoting the activity of natural killer cells in PBMCs, including culturing PBMCs in the presence of a fusion protein dimer comprising IL-2 or a variant thereof and CD80 or a fragment thereof.

5 Yet another aspect of the present invention provides PBMCs prepared by the method for promoting the activity of natural killer cells in the PBMCs. The natural killer cells in PBMC have high expression of an activating receptor such as CD16 and NKp46 so that increase the cell killing capacity against cancer cell lines and secretion of granzyme B and perforin, and thus excellent anticancer effects can be 10 expected. Therefore, a therapeutic agent effective for treating cancer may be prepared using PBMCs including a large amount of activated natural killer cells which are clinically applicable. In addition, the natural killer cells in PBMCs may have high expression of NKp30 or DNAM1.

Still another aspect of the present invention provides a pharmaceutical 15 composition for preventing or treating cancer including the PBMCs as an active ingredient. Yet still another aspect of the present invention provides a method for treating cancer including administering the NK cells to an individual having cancer. In this case, NK cells and cancer are as described above. Yet still another aspect of the present invention provides use of the NK cells to treat cancer.

20 The cancer may be any one selected from the group consisting of gastric cancer, liver cancer, lung cancer, colorectal cancer, breast cancer, prostate cancer, ovarian cancer, pancreatic cancer, cervical cancer, thyroid cancer, larynx cancer, acute lymphoblastic leukemia, brain tumor, neuroblastoma, retinoblastoma, head and neck cancer, salivary cancer, and lymphoma.

25 **Mode for Carrying out the Invention**

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Hereinafter, the present invention will be described in more detail by way of the following examples. However, the following examples are only for illustrating the present invention, and the scope of the present invention is not limited thereto.

5 **I. Preparation of GI-101, natural killer cells, and natural killer cell culture composition**

Preparatory Example 1. Preparation of a hCD80-Fc-IL-2 variant (2M): GI-101

In order to produce a fusion protein including a human CD80 fragment, a Fc 10 domain, and an IL-2 variant, a polynucleotide including a nucleotide sequence (SEQ ID NO: 8) encoding a fusion protein comprising a signal peptide (SEQ ID NO: 1), a CD80 fragment (SEQ ID NO: 2), a linker-conjugated Ig hinge (SEQ ID NO: 3), a Fc domain (SEQ ID NO: 4), a linker (SEQ ID NO: 5), and an IL-2 variant (2M) in which two amino acids are substituted (R38A, F42A) (SEQ ID NO: 6) in this order 15 from N-terminus was synthesized through Invitrogen GeneArt Gene Synthesis service of ThermoFisher Scientific Inc., and cloned into a pcDNA3_4 vector. In addition, the vector was introduced into CHO cells (EXPI-CHO^{TM}) to express a fusion protein of SEQ ID NO: 9. After introducing the vector, cells were cultured in an environment of 37°C, 125 RPM, and 8% CO_2 for 7 days, and then collected to 20 purify a fusion protein. The purified fusion protein dimer was named as "GI-101."

Purification was performed using chromatography including MabSelect SuRe protein A resin. The fusion protein was bound under the condition of 25 mM Tris, 25 mM NaCl, and pH 7.4. Then, it was eluted with 100 mM NaCl and 100 mM acetic acid at pH 3. After putting 20% of 1M Tris-HCl at pH 9 into a collection tube, 25 the fusion protein was collected. The collected fusion protein was dialyzed into PBS

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buffer for 16 hours to change.

Then, absorbance at a wavelength of 280 nm over time was measured by using size exclusion chromatography with TSKgel G3000SWXL column (TOSOH Bioscience) to obtain a high concentration of fusion protein. At this time, the isolated and purified fusion protein was subjected to SDS-PAGE under the reducing (R) or non-reducing (NR) conditions, and stained with coomassie blue to confirm its purity (FIG. 1B). It was confirmed that the fusion protein was included at a concentration of 2.78 mg/ml as detected using NanoDrop (FIG. 1C). Also, the result analyzed using size exclusion chromatography is as shown in FIG. 1D.

Preparatory Example 2. Preparation of a Fc-IL-2 variant (2M) dimer: Fc-IL-2v2

In order to produce a fusion protein comprising a Fc domain and an IL-2 variant, a polynucleotide including a nucleotide sequence (SEQ ID NO: 45) encoding a fusion protein comprising a signal peptide (SEQ ID NO: 1), an Ig hinge (SEQ ID NO: 38), a Fc domain (SEQ ID NO: 4), a linker (SEQ ID NO: 5), and an IL-2 variant (2M) in which two amino acids are substituted (R38A, F42A) (SEQ ID NO: 6) in this order from N-terminus was synthesized through Invitrogen GeneArt Gene Synthesis service of ThermoFisher Scientific Inc., and cloned into a pcDNA3_4 vector. In addition, the vector was introduced into CHO cells (EXPI-CHO^{TM}) to express a fusion protein of SEQ ID NO: 44. After introducing the vector, the cells were cultured in an environment of 37°C, 125 RPM, and 8% CO_2 for 7 days, and then collected to purify a fusion protein dimer. The purified fusion protein dimer was named as “Fc-IL2v2.”

The purification and collection of the fusion protein were performed in the same manner as in the Preparatory Example 1. The isolated and purified fusion

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protein was subjected to SDS-PAGE under the reducing (R) or non-reducing (NR) conditions, and stained with coomassie blue to confirm its purity (FIG. 3A). As a result, it was confirmed that the fusion protein forms a dimer. Also, the result analyzed using size exclusion chromatography is as shown in FIG. 3B.

5 **Preparatory Example 3. Preparation of a Fc-IL-2 dimer: Fc-IL-2wt**

In order to produce a fusion protein comprising a Fc domain and a wild-type IL-2, a polynucleotide including a nucleotide sequence (SEQ ID NO: 43) encoding a fusion protein comprising a signal peptide (SEQ ID NO: 1), an Ig hinge (SEQ ID NO: 38), a Fc domain (SEQ ID NO: 4), a linker (SEQ ID NO: 5), and a wild-type IL-
10 2 (SEQ ID NO: 10) in this order from N-terminus was synthesized through Invitrogen GeneArt Gene Synthesis service of ThermoFisher Scientific Inc., and cloned into a pcDNA3_4 vector. In addition, the vector was introduced into CHO cells (EXPI-CHO™) to express a fusion protein of SEQ ID NO: 42. After introducing the vector, the cells were cultured in an environment of 37°C, 125 RPM,
15 and 8% CO_2 for 7 days, and then collected to purify a fusion protein dimer. The purified fusion protein dimer was named as "Fc-IL2wt."

The purification and collection of the fusion protein were performed in the same manner as in the Preparatory Example 1. The isolated and purified fusion protein was subjected to SDS-PAGE under the reducing (R) or non-reducing (NR)
20 conditions, and stained with coomassie blue to confirm its purity (FIG. 3C). As a result, it was confirmed that the fusion protein forms a dimer. Also, the result analyzed using size exclusion chromatography is as shown in FIG. 3D.

Preparatory Example 4. Preparation of a hCD80-Fc-IL-2 wild-type dimer: hCD80-Fc-IL-2wt

25 In order to produce a fusion protein comprising a human CD80 fragment, a

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Fc domain, and an IL-2 wile-type protein, a polynucleotide including a nucleotide sequence (SEQ ID NO: 41) encoding a fusion protein comprising a signal peptide (SEQ ID NO: 1), a CD80 fragment (SEQ ID NO: 2), a linker-conjugated Ig hinge (SEQ ID NO: 3), a Fc domain (SEQ ID NO: 4), a linker (SEQ ID NO: 5), and IL-2
5 wild-type (SEQ ID NO: 10) in this order from N-terminus was synthesized through Invitrogen GeneArt Gene Synthesis service of ThermoFisher Scientific Inc., and cloned into a pcDNA3_4 vector. In addition, the vector was introduced into CHO cells (EXPI-CHO™) to express a fusion protein of SEQ ID NO: 46. After introducing the vector, the cells were cultured in an environment of 37°C, 125 RPM,
10 and 8% CO_2 concentration for 7 days, and then collected to purify a fusion protein dimer. The purified fusion protein dimer was named as "hCD80-Fc-IL2wt.".

Purification was performed using chromatography including MabSelect SuRe protein A resin. The fusion protein was bound under the condition of 25 mM Tris, 25 mM NaCl, and pH 7.4. Then, it was eluted with 100 mM NaCl and 100 mM
15 acetic acid at pH 3. After putting 20% of 1M Tris-HCl at pH 9 into a collection tube, the fusion protein was collected. The collected fusion protein was dialyzed into PBS buffer for 16 hours to change.

Then, absorbance at a wavelength of 280 nm over time was measured by using size exclusion chromatography with TSKgel G3000SWXL column (TOSOH
20 Bioscience) to obtain a high concentration of fusion protein. At this time, the isolated and purified fusion protein was subjected to SDS-PAGE under the reducing (R) or non-reducing (NR) conditions, and stained with coomassie blue to confirm its purity (FIG. 4A). As a result, it was confirmed that the fusion protein forms a dimer. Also, the result analyzed using size exclusion chromatography is as shown in FIG.
25 4B.

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Preparatory Example 5. Preparation of a hCD80-Fc dimer: hCD80-Fc

In order to produce a fusion protein comprising a human CD80 fragment and a Fc domain, a polynucleotide (SEQ ID NO: 39) including a nucleotide sequence encoding a fusion protein comprising a signal peptide (SEQ ID NO: 1), a CD80 fragment (SEQ ID NO: 2), a linker-conjugated Ig hinge (SEQ ID NO: 3), and a Fc domain (SEQ ID NO: 4) in this order from N-terminus was synthesized through Invitrogen GeneArt Gene Synthesis service of ThermoFisher Scientific Inc., and cloned into a pcDNA3_4 vector. In addition, the vector was introduced into CHO cells (EXPI-CHO™) to express a fusion protein of SEQ ID NO: 40. After introducing the vector, the cells were cultured in an environment of 37°C, 125 RPM, and 8% CO_2 for 7 days, and then collected to purify a fusion protein dimer. The purified fusion protein dimer was named "hCD80-Fc."

Purification was performed using chromatography including MabSelect SuRe protein A resin. The fusion protein was bound under the condition of 25 mM Tris, 25 mM NaCl, and pH 7.4. Then, it was eluted with 100 mM NaCl and 100 mM acetic acid at pH 3. After putting 20% of 1 M Tris-HCl at pH 9 into a collection tube, the fusion protein was collected. The collected fusion protein was dialyzed into PBS buffer for 16 hours to change.

Then, absorbance at a wavelength of 280 nm over time was measured by using size exclusion chromatography with TSKgel G3000SWXL column (TOSOH Bioscience) to obtain a high concentration of fusion protein. At this time, the isolated and purified fusion protein was subjected to SDS-PAGE under the reducing (R) or non-reducing (NR) conditions, and stained with coomassie blue to confirm its purity (FIG. 2A). As a result, it was confirmed that the fusion protein forms a dimer. Also, the result analyzed using size exclusion chromatography is as shown in FIG.

2B.

Preparation example 1. Preparation of a natural killer cell culture medium

Natural killer cell culture media compositions were prepared by respectively adding substances corresponding to the adding conditions 1 to 4 of Table 5 to each basal culture medium having composition of Table 1 to Table 4 below.

[Table 1]

Basal culture medium 1				
Components	Manufacturer	Cat.#	Dose	Final concentration
CTS™ Immune Cell SR (Serum replacement)	Thermo	A2596102	0.5ml	5%
GLUTAMAX 100X	GIBCO	A12860-01	0.5ml	1X
rhIL-15	R&D systems	247-ILB/CF	adding immediately before use	66.7IU
rhiL-21	R&D systems	8879-IL/CF	adding immediately before use	0.341IU
anti-CD2/CD335 Beads	Miltenyi Biotec	130-094-483		
OKT3	Biolegend	317326		10ng/mL
AIM-V medium	GIBCO	0870112-DK	to 50ml	

[Table 2]

Basal culture medium 2				
Components	Manufacturer	Cat.#	Dose	Final concentration
Human AB serum	Sigma	H4522	2.5ml	5%
GLUTAMAX 100X	GIBCO	A12860-01	0.5ml	1X
rhIL-15	R&D systems	247-ILB/CF	adding immediately before use	66.7IU
rhIL-21	R&D systems	8879-IL/CF	adding immediately before use	0.341IU
anti-CD2/CD335 Beads	Miltenyi Biotec	130-094-483		
OKT3	Biolegend	317326		10ng/mL
AIM-V medium	GIBCO	0870112-DK	to 50ml	

[Table 3]

Basal culture medium 3				
Components	Manufacturer	Cat.#	Dose	Final concentration
Human AB serum	Sigma	H4522	2.5ml	5%
GLUTAMAX 100X	GIBCO	A12860-01	0.5ml	1X
rhIL-15	R&D systems	247-ILB/CF	adding immediately before use	66.7IU
rhIL-21	R&D systems	8879-IL/CF	adding immediately before use	0.341IU

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anti-CD2/CD335 Beads	Miltenyi Biotec	130-094-483		
OKT3	Biolegend	317326		10ng/mL
X-VIVO™ 15 Medium	Lonza	04-418Q	to 50ml	

[Table 4]

Basal culture medium 4				
Components	Manufacturer	Cat.#	Dose	Final concentration
Human AB serum	Sigma	H4522	2.5ml	5%
NK MACS supplement	Miltenyi Biotec	130-113-102	500 $\mu\ell$	1%
anti-CD2/CD335 beads	Miltenyi Biotec	130-094-483		
OKT3	Biolegend	317326		10ng/mL
NK MACS medium		130-112-968	to 50ml	

[Table 5]

Adding material				
Classification	Components	Manufacturer	Dose	Final concentration
Adding condition 1	GI-101	GI-Innovation	adding immediately before use	1.6nM / 50nM
Adding condition 2	GI-101WT	GI-Cell	adding immediately before use	1.6nM / 50nM
Adding condition 3	CD80-Fc+Fc-IL2WT	GI-Cell	adding immediately before use	CD80-Fc(1.6nM)+ Fc-IL2WT(1.6nM) / CD80-Fc(50nM)+ Fc-IL2WT(50nM)
Adding condition 4	CD80-Fc+Fc-IL2v2	GI-Cell	adding immediately before use	CD80-Fc(1.6nM)+ Fc-IL2v2(1.6nM) / CD80-Fc(50nM)+ Fc-IL2v2(50nM)

Example 1. Preparation of CD3(-)CD56(+) natural killer cells derived from peripheral blood mononuclear cells (PBMC)

5 In order to obtain CD3(-) cells, the number of PBMCs (peripheral blood mononuclear cells, Zen-Bio. Inc, NC 27709, USA, Cat#: SER-PBMC-200-F) was counted using an ADAM-MC2 automated cell counter (NanoEnTek, purchased from Cosmo Genetech Co., Ltd.). The PBMCs were transferred to a new tube, and then centrifuged at 300×g for 5 minutes at a temperature of 4°C. 0.5% (v/v) bovine serum
10 albumin (BSA) and EDTA at a concentration of 2 mM were included in PBS to prepare MACS buffer (pH 7.2). After centrifugation was completed, a cell pellet was treated with 80 µl of MACs buffer and 20 µl of CD3 magnetic beads (Miltenyi biotech, 130-050-101) per 1×10^7 cells to suspend, and then incubated at a

temperature of 4°C for 15 minutes. 10 ml of MACs buffer was added for washing and centrifuged at 300×g for 10 minutes at a temperature of 4°C, and then the cell pellet was resuspended in 0.5 ml of MACs buffer.

2 ml of MACs buffer was first flowed into the LD column (Miltenyi Biotec, Bergisch Gladbach, Germany, Cat#: 130-042-901), and then the cell suspension was flowed. Then, CD3(-) cells passing through the LD column were obtained. At this time, CD3(-) cells were obtained by flowing 2 ml of MACs buffer three times so that the cells remaining in the LD column could be sufficiently separated. The obtained CD3(-) cells were counted using a cell counter, and then placed in a new tube and centrifuged at 300×g for 5 minutes at a temperature of 4°C. Then, the supernatant was removed, and then 80 μl of MACs buffer and 20 μl of CD56 magnetic beads (Miltenyi biotech, Cat#: 130-050-401) were added per 1×10^7 cells, followed by incubation at a temperature of 4°C for 15 minutes. 10 ml of MACs buffer was added for washing and centrifuged at 300×g for 10 minutes at a temperature of 4°C, and then the cell pellet was resuspended in 0.5 ml of MACs buffer.

3 ml of MACs buffer was first flowed into the LS column (Miltenyi Biotec, Bergisch Gladbach, Germany, Cat#: 130-042-901), and then the cell suspension was flowed. At this time, 2 ml of MACs buffer was flowed three times so that the cells remaining in the LS column could be sufficiently separated. Then, after the LS column was separated from a magnet stand, 5 ml of MACs buffer was added, and pressure was applied with a piston to obtain CD3(-)CD56(+) natural killer cells. The obtained CD3(-)CD56(+) natural killer cells was placed in a new tube and centrifuged at 300×g for 5 minutes at a temperature of 4°C. After removing the supernatant, the cells were suspended in the basal culture media shown in Table 1 to Table 4 in consideration of the culture conditions. The number of suspended cells

was counted using a cell counter.

Example 2. Culture of CD3(-)CD56(+) natural killer cells derived from peripheral blood mononuclear cells (PBMC)

100 μL of CD335 (NKp46)-biotin and 100 μL of CD2-biotin included in a

5 NK Cell Activation/Expansion Kit (Cat#: 130-112-968) (Miltenyi Biotec, Bergisch Gladbach, Germany) were placed in a 1.5 mL microtube and mixed, and then 500 μL of Anti-Biotin MACSiBead Particles was added and mixed. Then, 300 μL of MACs buffer was added, and mixed at 2°C to 8°C for 2 hours using a microtube rotator. Considering the number of cells, 5 μL of NK activation beads per $1x10^6$ cells was

10 transferred to a new tube. 1 mL of PBS was added and centrifuged at 300×g for 5 minutes. After removing the supernatant, NK MACs medium (Cat#: 130-094-483) (Miltenyi Biotec, Bergisch Gladbach, Germany) to be used was added on the basis of 5 μL per 10^6 NK cells, and released beads, followed by inoculating into the CD3(-)CD56(+) natural killer cells isolated in Example 1.

15 Next, the prepared CD3(-)CD56(+) natural killer cells were suspended in a culture medium composition containing an additive prepared in Preparation example 1 so that the total number of cells was $2.5x10^5$, and seeded in a 48-well plate, followed by culturing under the condition of 37°C and 5% CO_2. Then, the number of cells was determined every 2 days to subculture in the order of a 48-well plate, a

20 24-well plate, a 12-well plate, a 6-well plate, and a 25T flask when the cells were confluent 80% or more of culture vessel (confluency), and finally all cells were harvested on day 21.

Example 3. Counting of cell number and comparison of cell viability

The total number of cells and viability of the cultured natural killer cells were

25 counted using a cell counter (ADAM-MC2) on days 5, 9, 11, 13, 15, 17, and 21. At

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this time, the number of cells were counted on the dates above as the cells reach 80% confluency which is a criterion for subculture because the proliferation rate of cells varies depending on a treated material and type of culture medium.

The results of comparing the total number of cells and viability of CD3-

5 CD56+ cells cultured under the conditions of the culture medium composition prepared in Preparation example 1 are shown in Tabled 6 to 13, and FIGS. 5A to 12B.

As a result, it was confirmed that all culture media compositions to which GI-101 prepared by Preparatory example 1 was added had the total number of natural killer cells greater than the control group (addition of CD80-Fc+Fc-IL2v2 or CD80-

10 Fc+Fc-IL2WT), regardless of the treatment concentration in four basal culture medium conditions (Tables 1 to 4) (FIGS. 5A, 5B, 7A, 7B, 9A, 9B, 11A and 11B).

In addition, even for cells viability, when GI-101 was added, all culture media compositions exhibited high viability regardless of the basal culture medium and the concentration (FIGS. 6A, 6B, 8A, 8B, 10A, 10B, 12A and 12B).

15 Based on the results, it was confirmed that GI-101 plays an important role in improving proliferation ability and viability of natural killer cells as compared with the control group (addition of CD80-Fc+Fc-IL2v2 or CD80-Fc+Fc-IL2WT), regardless of the basal culture medium and the concentration.

[Table 6]

TOTAL CELL NUMBER ($X10^5$)										
Culture condition	Conc.	Treated material	CD3-CD56+ cells							
			DAY0 (seeding)	DAY5	DAY9	DAY11	DAY13	DAY15	DAY17	DAY21
Table 1	50nM	GI-101	2.50	3.17	8.56	-	23.84	-	27.38	23.76
		GI-101WT	2.50	2.36	6.40	-	-	-	15.45	13.33
		CD80-Fc+Fc-IL2v2	2.50	2.62	6.61	-	16.16	-	21.93	13.19
		CD80-Fc+Fc-IL2WT	2.50	1.87	-	11.04	13.92	-	17.86	13.33
	1.6nM	GI-101	2.50	2.27	6.79	-	13.98	-	15.62	11.19
		GI-101WT	2.50	1.92	4.65	-	-	-	-	3.58

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		CD80-Fc+Fc-IL2v2	2.50	0.23	-	-	-	-	-	-
		CD80-Fc+Fc-IL2WT	2.50	1.76	-	-	6.96	-	6.64	4.46

[Table 7]

CELL VIABILITY (%)										
Culture condition	Conc.	Treated material	CD3-CD56+ cells							
			DAY0 (seeding)	DAY5	DAY9	DAY11	DAY13	DAY15	DAY17	DAY21
Table 1	50nM	GI-101	-	76.58	82.41	-	90.94	-	86.68	84.19
		GI-101WT	-	75.98	83.42	-	-	-	89.27	89.53
		CD80-Fc+Fc-IL2v2	-	78.18	81.73	-	90.57	-	88.76	89.64
		CD80-Fc+Fc-IL2WT	-	83.64	-	86.50	92.19	-	89.62	82.36
	1.6nM	GI-101	-	75.08	82.94	-	87.85	-	90.34	88.22
		GI-101WT	-	77.55	82.32	-	-	-	-	87.01
		CD80-Fc+Fc-IL2v2	-	49.77	-	-	-	-	-	-
		CD80-Fc+Fc-IL2WT	-	74.51	-	-	86.47	-	88.90	93.29

[Table 8]

TOTAL CELL NUMBER (X10^5)										
Culture condition	Conc.	Treated material	CD3-CD56+ cells							
			DAY0 (seeding)	DAY5	DAY9	DAY11	DAY13	DAY15	DAY17	DAY21
Table 2	50nM	GI-101	2.5	11.71	38.81	-	214.88	415.25	-	694.59
		GI-101WT	2.5	8.57	30.58	62.31	96.42	-	-	152.16
		CD80-Fc+Fc-IL2v2	2.5	8.12	-	-	90.54	122.27	-	153.14
		CD80-Fc+Fc-IL2WT	2.5	7.80	29.97	-	92.32	118.56	-	152.23
	1.6nM	GI-101	2.5	5.60	28.69	-	55.39	-	-	94.22
		GI-101WT	2.5	6.58	14.92	-	35.18	-	-	37.81
		CD80-Fc+Fc-IL2v2	2.5	6.02	19.85	-	56.26	-	-	75.83
		CD80-Fc+Fc-IL2WT	2.5	6.04	18.39	-	46.50	-	-	55.51

[Table 9]

CELL VIABILITY (%)										
Culture condition	Conc.	Treated material	CD3-CD56+ cells							
			DAY0 (seeding)	DAY5	DAY9	DAY11	DAY13	DAY15	DAY17	DAY21
Table 2	50nM	GI-101	-	93.31	95.64	-	96.47	96.58	-	93.21

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		GI-101WT	-	94.38	95.45	96.06	94.96	-	-	92.14
		CD80-Fc+Fc-IL2v2	-	93.32	-	-	97.01	95.96	-	91.73
		CD80-Fc+Fc-IL2WT	-	91.51	96.49	-	96.19	95.23	-	91.81
	1.6nM	GI-101	-	91.81	93.75	-	95.14	-	-	85.22
		GI-101WT	-	93.56	93.56	-	94.81	-	-	88.63
		CD80-Fc+Fc-IL2v2	-	93.57	94.00	-	94.36	-	-	89.53
		CD80-Fc+Fc-IL2WT	-	90.87	94.04	-	95.01	-	-	87.36

[Table 10]

TOTAL CELL NUMBER (X10^5)										
Culture condition	Conc.	Treated material	CD3-CD56+ cells							
			DAY0 (seeding)	DAY5	DAY9	DAY11	DAY13	DAY15	DAY17	DAY21
Table 3	50nM	GI-101	2.50	6.07	24.64	-	46.13	61.25	-	99.60
		GI-101WT	2.50	6.98	24.90	-	49.75	64.15	-	67.71
		CD80-Fc+Fc-IL2v2	2.50	4.11	-	21.60	-	-	-	56.02
		CD80-Fc+Fc-IL2WT	2.50	5.73	23.52	-	51.17	66.32	-	76.19
	1.6nM	GI-101	2.50	6.47	25.14	-	54.13	69.59	-	86.68
		GI-101WT	2.50	3.34	-	-	-	-	-	10.91
		CD80-Fc+Fc-IL2v2	2.50	1.07	-	-	-	-	-	-
		CD80-Fc+Fc-IL2WT	2.50	4.61	13.16	-	16.27	-	-	13.16

[Table 11]

CELL VIABILITY (%)										
Culture condition	Conc.	Treated material	CD3-CD56+ cells							
			DAY0 (seeding)	DAY5	DAY9	DAY11	DAY13	DAY15	DAY17	DAY21
Table 3	50nM	GI-101	-	89.72	90.65	-	90.59	88.11	-	82.08
		GI-101WT	-	90.74	92.95	-	93.20	90.52	-	87.38
		CD80-Fc+Fc-IL2v2	-	87.57	-	88.80	-	-	-	87.63
		CD80-Fc+Fc-IL2WT	-	88.60	93.44	-	95.13	94.99	-	91.38
	1.6nM	GI-101	-	90.07	92.11	-	92.64	90.80	-	85.62
		GI-101WT	-	85.76	-	-	-	-	-	75.98
		CD80-Fc+Fc-IL2v2	-	66.33	-	-	-	-	-	-
		CD80-Fc+Fc-	-	88.05	90.17	-	89.08	-	-	79.81

		IL2WT								

[Table 12]

TOTAL CELL NUMBER (X10^5)										
Culture condition	Conc.	Treated material	CD3-CD56+ cells							
			DAY0 (seeding)	DAY5	DAY9	DAY11	DAY13	DAY15	DAY17	DAY21
Table 4	50nM	GI-101	2.50	6.31	37.36	58.21	-	146.76	-	467.75
		GI-101WT	2.50	5.74	30.24	52.00	-	126.99	-	347.59
		CD80-Fc+Fc-IL2v2	2.50	6.96	31.20	51.52	-	130.65	-	285.63
		CD80-Fc+Fc-IL2WT	2.50	6.30	35.52	50.08	-	147.54	-	350.28
	1.6nM	GI-101	2.50	6.75	37.28	63.68	-	166.42	-	419.02
		GI-101WT	2.50	2.46	-	8.08	9.84	-	-	8.93
		CD80-Fc+Fc-IL2v2	2.50	0.73	-	-	-	-	-	-
		CD80-Fc+Fc-IL2WT	2.50	3.86	18.85	-	48.90	67.86	-	134.21

[Table 13]

CELL VIABILITY (%)										
Culture condition	Conc.	Treated material	CD3-CD56+ cells							
			DAY0 (seeding)	DAY5	DAY9	DAY11	DAY13	DAY15	DAY17	DAY21
Table 4	50nM	GI-101	-	94.72	96.93	97.24	-	96.20	-	96.04
		GI-101WT	-	94.90	96.79	96.11	-	63.11	-	64.73
		CD80-Fc+Fc-IL2v2	-	95.33	97.83	96.70	-	95.73	-	96.43
		CD80-Fc+Fc-IL2WT	-	95.99	97.86	97.52	-	97.09	-	96.39
	1.6nM	GI-101	-	94.75	97.72	96.89	-	94.67	-	95.00
		GI-101WT	-	89.00	-	88.06	-	-	-	88.22
		CD80-Fc+Fc-IL2v2	-	75.94	-	-	-	-	-	-
		CD80-Fc+Fc-IL2WT	-	91.40	96.61	-	-	96.49	-	95.99

II. Characterization of natural killer cells using natural killer cell culture composition

5 **Example 4. Measurement of the purity of natural killer cells**

CD3-CD56+ natural killer cells obtained from Example 2 were respectively centrifuged at 300×g condition for 5 minutes to remove the supernatant, and 1 ml of

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FACS buffer was added to release the pellet. Then, 3% (v/v) FBS, 10 mM EDTA, 20 mM HEPES, 10 μg/ml polymyxin B, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 mM sodium pyruvate were added to PBS to prepare FACS buffer, and 1 ml of prepared FACS buffer was added to resuspend the cell pellet. 5 Next, it was diluted with FACS buffer to 2×10^6 cells/ml using a cell counter.

100 μl of the diluted cell solution was added to each of a 5 ml FACS tube, and 100 μl of FACS buffer was further added thereto, followed by treatment with a PerCP-labeled anti-human CD3 antibody (PerCP human anti-CD3(Clone UCHT1)) and a PE/cy7-labeled anti-human CD56 antibody (PE/cy7 human anti-CD56(Clone 10 B159)). Then, after incubating at 4°C for 20 minutes, 200 μl of FACS buffer was added and centrifuged at 1,500 rpm for 3 minutes. The supernatant was removed, and 200 μl of FACS buffer was added to suspend, and then phenotype of the cells was determined using a flow cytometer (CYTEK® Aurora, Cytek, Fremont, CA, USA).

15 Information about antibodies used in the experiment is shown in Table 14. In addition, the purities of CD3-CD56+ natural killer cells cultured for 21 days under the conditions of the culture media compositions prepared in Preparation example 1 were measured and shown in FIGS. 13 to 16.

[Table 14]

	Target	Color	Clone	Producer	Cat#
NK marker	CD3	PerCP	UCHT1	BioLegend	300428
	CD56	APC/cy7	5.1H11	BioLegend	362512

20 **Example 5. Identification of activation and inhibition markers for natural killer cells**

CD3-CD56+ natural killer cells obtained from Example 2 were respectively centrifuged at 300×g condition for 5 minutes to remove the supernatant, and 1 ml of FACS buffer was added to release the pellet.

3% (v/v) FBS, 10 mM EDTA, 20 mM HEPES, 10 μg/ml polymyxin B, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 mM sodium pyruvate were added to PBS to prepare FACS buffer, and 1 ml of prepared FACS buffer was added to resuspend the cell pellet. Then, it was diluted with FACS buffer to 2×10^6 cells/ml
5 using a cell counter. 100 μl of the diluted cell solution was added each of a 5 ml FACS tube, and confirmed by using a Pe-CF594-labeled anti-human CD16 antibody (PE-CF594 human anti-CD16 (Clone 3G8)), APC-labeled anti-human DNAM1 antibody (APC human anti-DNAM1 (Clone 11A8)), BV605-labeled anti-human NKG2C antibody (BV605 human anti-NKG2C (Clone 134591)), BV650-labeled
10 anti-human NKG2D antibody (BV650 human anti-NKG2D(Clone 1D11)), BB515-labeled anti-human NKp46 antibody (BB515 human anti-NKp46 (Clone 9E2)), BV480-labeled anti-human NKp30 antibody (BV480 human anti-NKp30 (Clone p30-15)), PE-labeled anti-human PD-1 antibody (PE human anti-PD-1 (Clone EH12.2H7)), and APC-labeled anti-human NKG2A antibody (APC anti-human
15 NKG2A (Clone 131411)), using a flow cytometer. Then, after incubating at 4°C for 20 minutes, 100 μl of FACS buffer was added and centrifuged at 1,500 rpm for 3 minutes.

The supernatant was removed, and 200 μl of FACS buffer was added to suspend, and then phenotype of the cells was determined using a flow cytometer
20 (CYTEK® Aurora, Cytek, Fremont, CA, USA). Information of antibodies used in the experiment is shown in Table 15. The activation and inhibition markers for CD3-CD56+ natural killer cells cultured for 21 days under the conditions of the culture media compositions prepared in Preparation example 1 were identified and shown in FIGS. 17 to 24.

25 [Table 15]

<table>
<tr><th></th><th>Target</th><th>Color</th><th>Clone</th><th>Producer</th><th>Cat#</th></tr>
<tr><td rowspan="4">Activation marker</td><td>CD16</td><td>PE-CF594</td><td>3G8</td><td>BD</td><td>562293</td></tr>
<tr><td>DNAM1</td><td>APC</td><td>11A8</td><td>BioLegend</td><td>338312</td></tr>
<tr><td>NKp46</td><td>BB515</td><td>9E2</td><td>BD</td><td>564536</td></tr>
<tr><td>NKp30</td><td>BV480</td><td>p30-15</td><td>BD</td><td>746491</td></tr>
<tr><td rowspan="2">Inhibition marker</td><td>PD-1</td><td>PE</td><td>EH12.2H7</td><td>BioLegend</td><td>329906</td></tr>
<tr><td>NKG2A</td><td>APC</td><td>131411</td><td>R&D systems</td><td>FAB1059A-100</td></tr>
</table>

Example 6. Determination of granzyme B, perforin, interferon gamma secretory capacity of natural killer cells

In order to determine granzyme and perforin secretory capacity of CD3-CD56+ natural killer cells obtained from Example 2, an amount of expression of
5 granzyme B, perforin, and interferon gamma in the natural killer cells were measured by intracellular staining. The cultured natural killer cells were centrifuged at 300×g condition for 5 minutes and the supernatant was removed. Then, it was diluted with each culture composition to $2x10^6$ cells/ml using a cell counter.

200 μl of the prepared cells were dispensed into each well of a 96-well plate,
10 and then 1% (v/v) Stimulation Cocktail(1X) (Thermo Scientific, Waltham, MA, USA) was added and incubated at 37°C, CO_2 condition for 4 hours. Then, the plates were centrifuged at 300×g condition for 5 minutes and the supernatant was removed. Next, 3% (v/v) FBS, 10 mM EDTA, 20 mM HEPES, 10 μg/ml polymyxin B, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1 mM sodium pyruvate were added to
15 PBS to prepare FACS buffer, and 100 μl of prepared FACS buffer was added to resuspend the cell pellet. The supernatant was removed, and 100 μl of BD CYTOFIX/CYTOPERM™ buffer (perm/fixation buffer, BD science) was added for fixation and permeation and then suspended, followed by incubation at 4°C for 30 minutes. 100 μl of FACS buffer was further added and centrifuged at 1,500 rpm for
20 3 minutes.

A PE/cy7-labeled anti-human granzyme B antibody (PE/cy7 anti-human Granzyme B (Clone NGZB)), an APC-labeled anti-human perforin antibody (APC

anti-human Perforin (Clone B-D48)), and BV421-labeled anti-human interferon gamma antibody (BV421 anti-human IFN-gamma (Clone B27)) were treated. Then, after incubating at 4°C for 20 minutes, 100 μl of FACS buffer was added and centrifuged at 1,500 rpm for 3 minutes. After supernatant was removed and 200 μl

5 of FACS buffer (fixation buffer) was added to suspended, an amount of expression of the cells was determined using a flow cytometer.

Information of antibodies used in the experiment is shown in Table 16. The markers for CD3-CD56+ natural killer cells cultured for 21 days under the conditions of the culture media compositions prepared in Preparation example 1 were identified

10 and shown in FIGS. 25 to 28.

[Table 16]

	Target	Color	Clone	Producer	Cat#
Cytotoxicity	Perforin	APC	B-D48	BioLegend	353312
	IFN-γ	BV421	B27	BioLegend	506538
	GranzymeB (GB11)	PE/cy7	NGZB	ebioscience	25-8898-82

III. Analysis of cancer cell killing capacity of natural killer cells according to a culture composition

Example 8. Confirmation of degranulation ability and killing effect of

15 **natural killer cells against cancer cells**

Specifically, a K562 cancer cell line (American Type Culture Collection, ATCC) was diluted in PBS to number of cells shown in Table 17 below, and dispensed into each well.

[Table 17]

E : T	1 : 1	1 : 5	1 : 10
(E) NK cells	$2.5X10^5$ cells	$1X10^5$ cells	$1X10^5$ cells
(T) K562	$2.5X10^5$ cells	$5X10^5$ cells	$1X10^6$ cells

20 Specifically, a K562 cancer cell line was diluted with each culture composition to 1×10^7 cells/ml, and then dispensed according to the number of cells

specified in the above table for each well. Then, natural killer cells were also diluted with each culture composition to 5×10^6 cells/ml, and then dispensed according to the number of cells specified in the above table for each well, and centrifuged at 30×g condition for 3 minutes. Next, after culture at 37°C, 5% CO_2 condition for 4 hours, a
5 BV421-labeled anti-human CD3 antibody (BV421 human anti-CD3 (Clone UCHT1)), a PE-labeled anti-human CD16 antibody (PE human anti-CD16 (Clone 3G8)), a PE/cy7-labeled anti-human CD56 antibody (PE/cy7 human anti-CD56(Clone B159)), and a FITC-labeled anti-human CD107a antibody (FITC anti-human CD107a antibody (Clone H4A3)) were treated and incubated on ice for 20
10 minutes.

Then, 100 μL of FACS buffer was added and centrifuged at 1,300 rpm, 4°C condition for 5 minutes. After removing the supernatant, 7-AAD Viability Staining Solution was treated, and light was blocked to react at room temperature for 15 minutes. Then, 100 μL of FACS buffer was added and centrifuged at 1,300 rpm,
15 4°C condition for 5 minutes. 200 μL of FACS buffer was added again, and centrifuged at 1,300 rpm, 4°C condition for 5 minutes. After repeating the above process once more, the supernatant was removed, and 400 μL of FACS buffer was added, followed by analysis using a flow cytometer (CYTEL® Aurora, Cytek, Fremont, CA, USA).

20 The degranulation ability and killing effect of natural killer cells cultured for 21 days in a composition in which 50 nM of the additive in Table 5 was added to the basal culture medium in Tables 1 to 4, respectively, are shown in FIGS. 29 to 34.

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WHAT IS CLAIMED IS:

1. A composition for culturing a natural killer cell comprising as an active ingredient a fusion protein dimer comprising IL-2 or a variant thereof and CD80 or a
5 fragment thereof.

2. The composition of claim 1, wherein the IL-2 variant is obtained by substitution of the 38th and 42nd amino acids in the amino acid sequence of SEQ ID NO: 10.

10

3. The composition of claim 1, wherein the IL-2 variant consists of the amino acid sequence of SEQ ID NO: 6.

4. The composition of claim 1, wherein the fragment of CD80 consists of the
15 35th to 242nd amino acids in the amino acid sequence of SEQ ID NO: 11.

5. The composition of claim 1, wherein the fusion protein consists of the amino acid sequence of SEQ ID NO: 9.

20 6. A method for culturing a natural killer cell, which comprises:

i) isolating a cell that do not express CD3 from peripheral blood mononuclear cells (PBMCs);

ii) isolating a cell that express CD56 from the cell that do not express CD3, isolated in the above step; and

25 iii) culturing the isolated cell in the presence of a fusion protein dimer

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comprising IL-2 or a variant thereof and CD80 or a fragment thereof.

7. The method of claim 6, wherein the fusion protein dimer is treated at a concentration of 1 nM to 500 nM.

5

8. The method of claim 6, wherein the culture period in the culturing step is 5 days to 25 days.

9. A natural killer cell prepared by the method of claim 6.

10

10. The natural killer cell of claim 9, wherein the expression of CD16 and NKp46 is increased in the natural killer cell.

11. The natural killer cell of claim 9, wherein the expression of granzyme B and
15 perforin is increased in the natural killer cell.

12. A pharmaceutical composition for preventing or treating cancer comprising the natural killer cell of claim 9 as an active ingredient.

20 13. The pharmaceutical composition of claim 9, wherein the cancer is any one selected from the group consisting of gastric cancer, liver cancer, lung cancer, colorectal cancer, breast cancer, prostate cancer, ovarian cancer, pancreatic cancer, cervical cancer, thyroid cancer, larynx cancer, acute lymphoblastic leukemia, brain tumor, neuroblastoma, retinoblastoma, head and neck cancer, salivary cancer, and
25 lymphoma.

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14. A method for culturing a natural killer cell, which comprises:

i) isolating a cell that do not express CD3 from PBMCs; and

ii) culturing the isolated cell in the presence of a fusion protein dimer
5 comprising IL-2 or a variant thereof and CD80 or a fragment thereof.

15. A natural killer cell prepared by the method of claim 14.

16. A pharmaceutical composition for preventing or treating cancer including the
10 natural killer cell of claim 15 as an active ingredient.

17. The pharmaceutical composition of claim 16, wherein the cancer is any one selected from the group consisting of gastric cancer, liver cancer, lung cancer, colorectal cancer, breast cancer, prostate cancer, ovarian cancer, pancreatic cancer,
15 cervical cancer, thyroid cancer, larynx cancer, acute lymphoblastic leukemia, brain tumor, neuroblastoma, retinoblastoma, head and neck cancer, salivary cancer, and lymphoma.

18. A method for culturing a natural killer cell, which comprises:

20 i) isolating a cell that express CD56 from PBMCs; and

ii) culturing the isolated cell in the presence of a fusion protein dimer comprising IL-2 or a variant thereof and CD80 or a fragment thereof.

19. A natural killer cell prepared by the method of claim 18.

25

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20. A pharmaceutical composition for preventing or treating cancer including the natural killer cell of claim 19 as an active ingredient.

21. The pharmaceutical composition of claim 20, wherein the cancer is any one selected from the group consisting of gastric cancer, liver cancer, lung cancer, colorectal cancer, breast cancer, prostate cancer, ovarian cancer, pancreatic cancer, cervical cancer, thyroid cancer, larynx cancer, acute lymphoblastic leukemia, brain tumor, neuroblastoma, retinoblastoma, head and neck cancer, salivary cancer, and lymphoma.

22. A method for promoting the activity of a natural killer cell in PBMCs, which comprises culturing PBMCs in the presence of a fusion protein dimer comprising IL-2 or a variant thereof and CD80 or a fragment thereof.

23. A peripheral blood mononuclear cell prepared by the method of claim 22.

24. A pharmaceutical composition for preventing or treating cancer comprising the natural killer cell of claim 23 as an active ingredient.

25. The pharmaceutical composition of claim 24, wherein the cancer is any one selected from the group consisting of gastric cancer, liver cancer, lung cancer, colorectal cancer, breast cancer, prostate cancer, ovarian cancer, pancreatic cancer, cervical cancer, thyroid cancer, larynx cancer, acute lymphoblastic leukemia, brain tumor, neuroblastoma, retinoblastoma, head and neck cancer, salivary cancer, and lymphoma.

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FIG. 1A

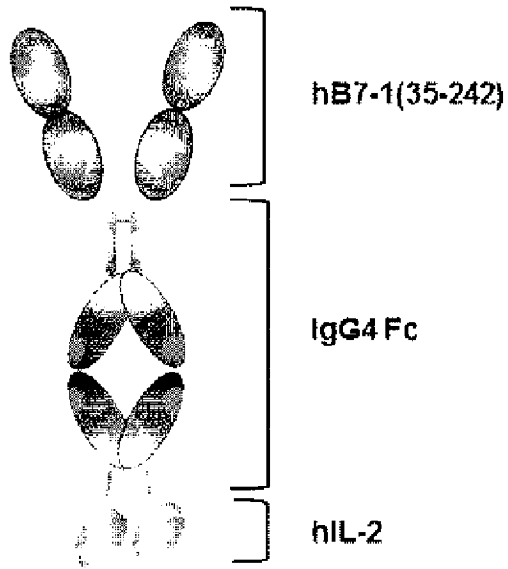

FIG. 1B

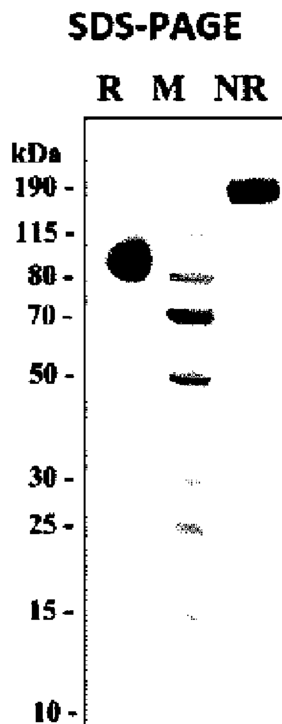

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FIG. 1C

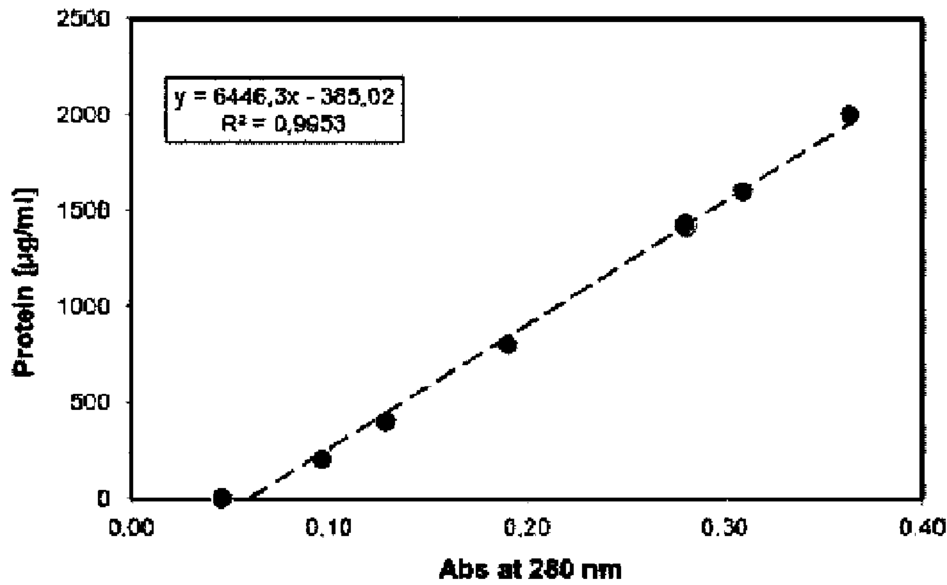

FIG. 1D

Anlytical size exclusion chromatography (SEC)

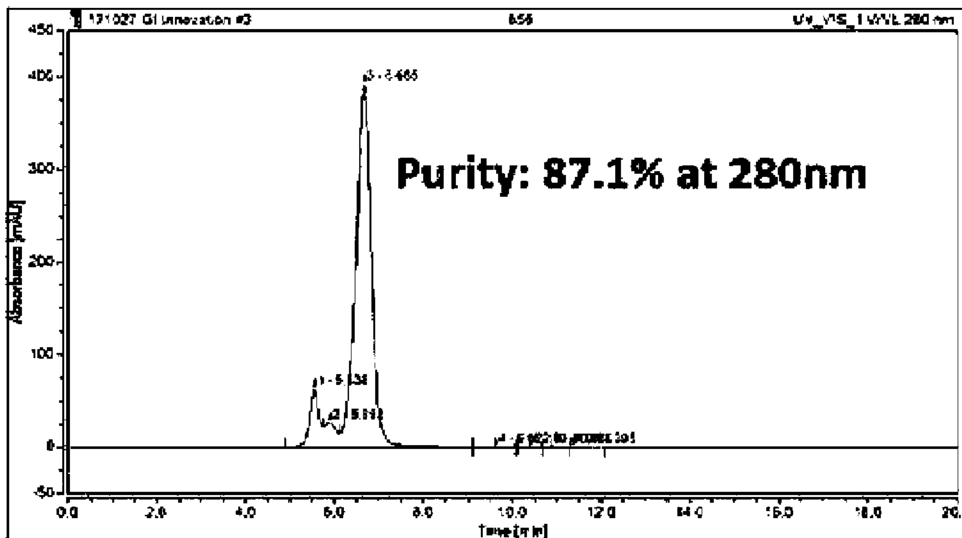

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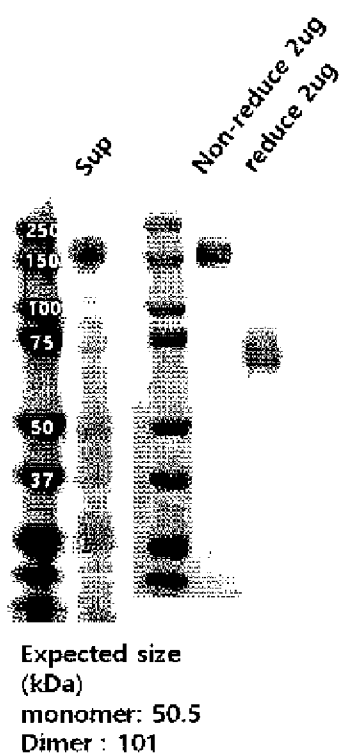
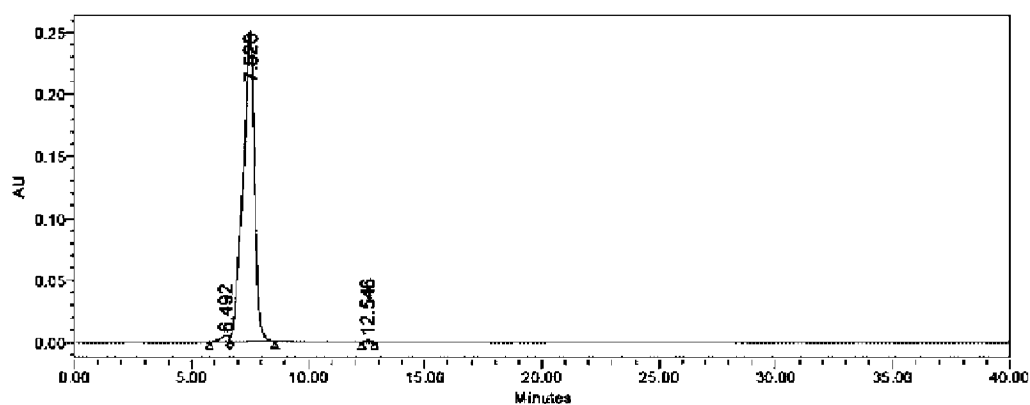
FIG. 2A



Expected size
(kDa)
monomer: 50.5
Dimer : 101

FIG. 2B



	RT	Area	% Area	Height
1	6.492	143663	1.66	5185
2	7.528	8497629	98.06	250509
3	12.546	24077	0.28	2006

FIG. 3A

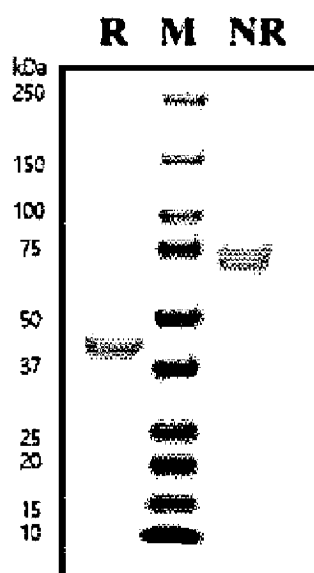
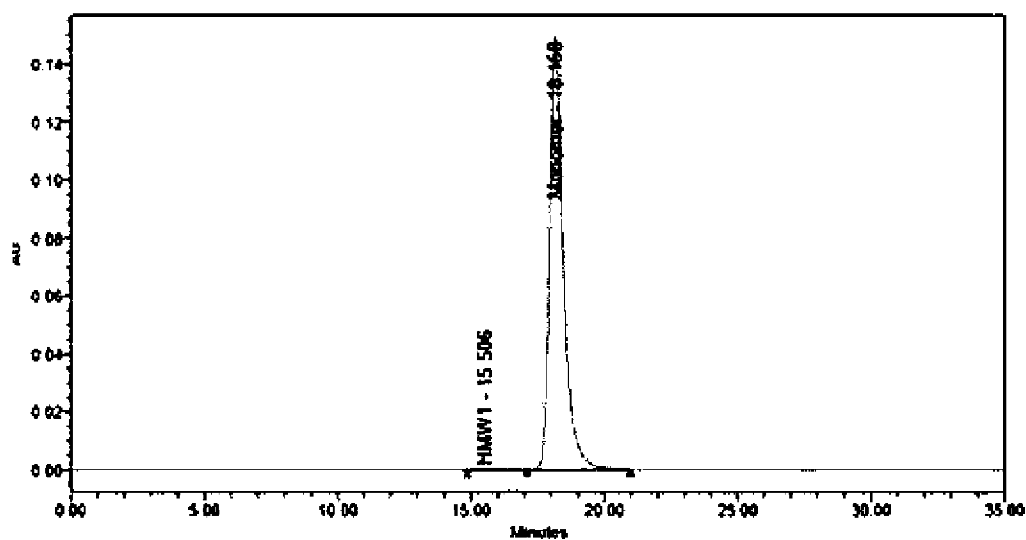


FIG. 3B



	Peak Name	RT	Area	% Area	Height
1	HMW1	15 506	28137	0.52	426
2	Monomer	18 158	5363386	99.48	148945

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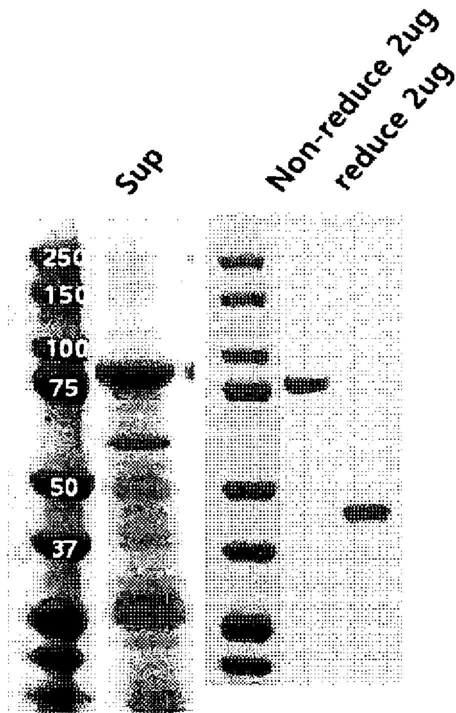
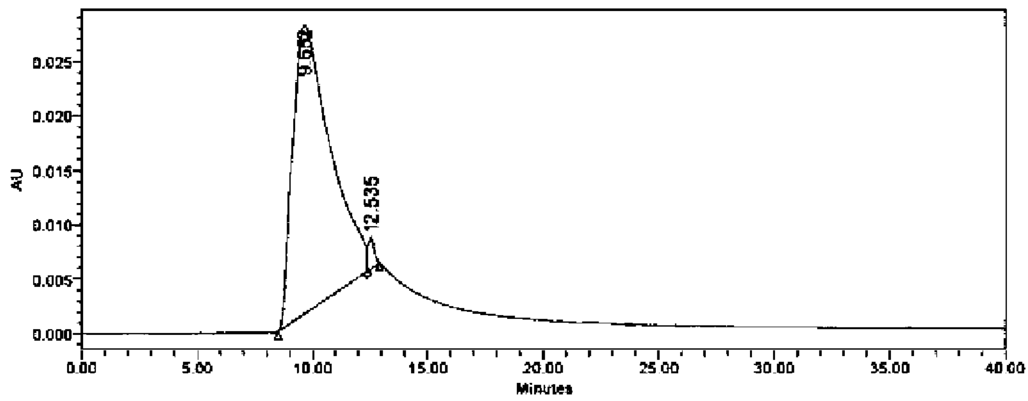
FIG. 3C



Expected size (kDa)
monomer: 41.3
Dimer : 82.6

FIG. 3D



	RT	Area	% Area	Height
1	9.652	2996084	98.41	26478
2	12.535	48430	1.59	2882

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FIG. 4A

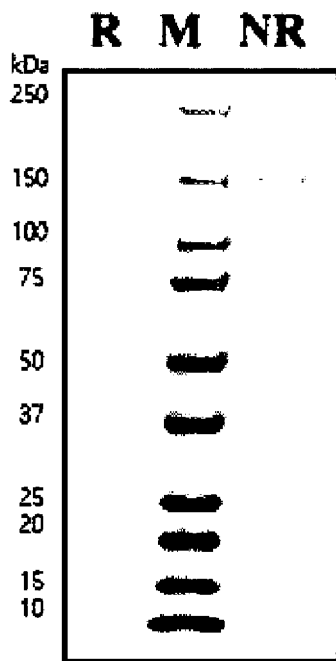
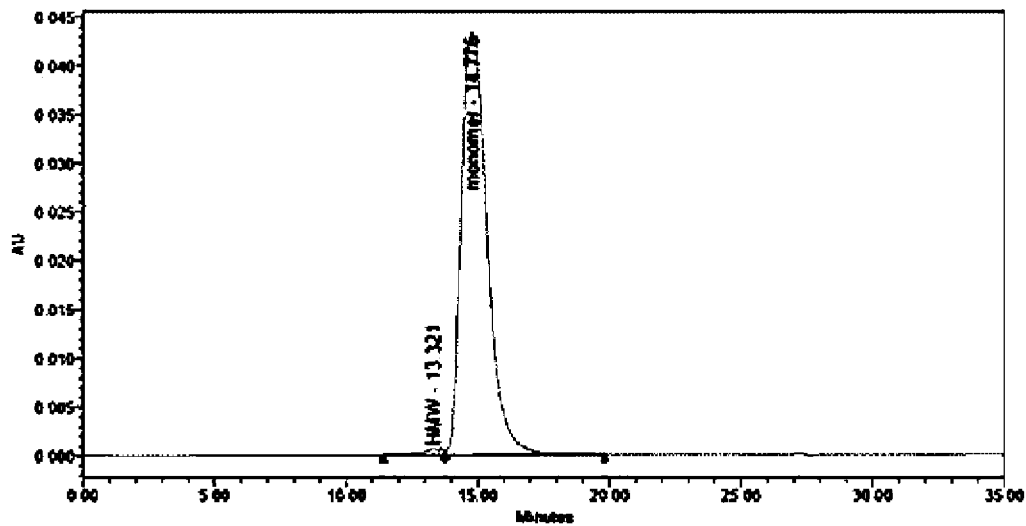


FIG. 4B



	Peak Name	RT	Area	% Area	Height
1	HMW	13.321	33675	1.13	625
2	monomer	14.775	2952831	98.87	43291

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FIG. 5A

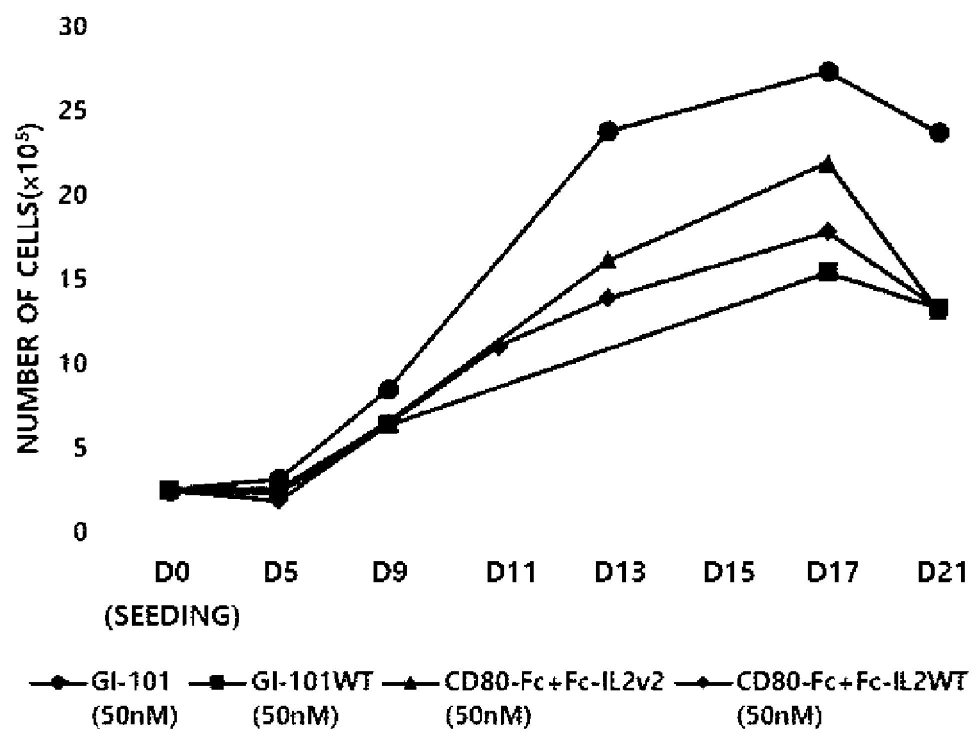

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FIG. 5B

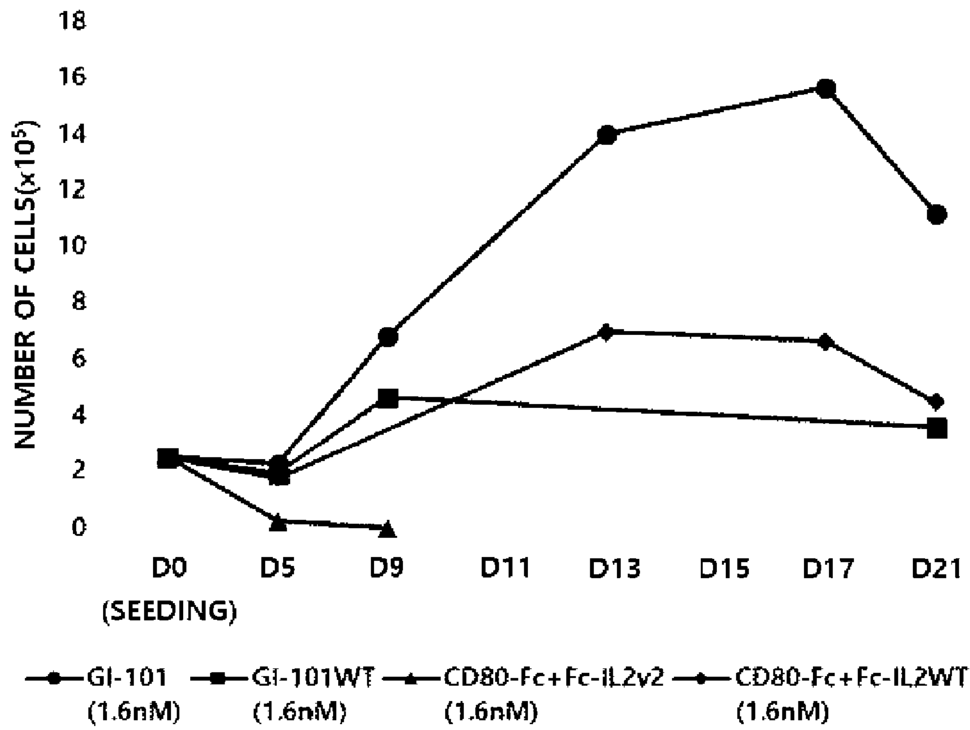

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FIG. 6A

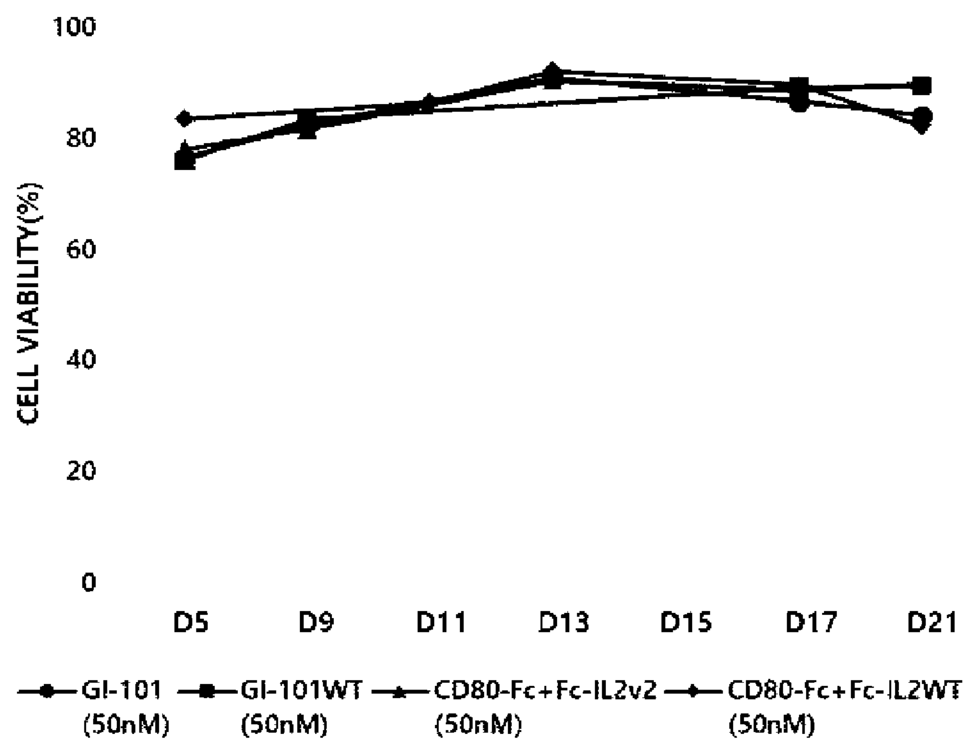

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FIG. 6B

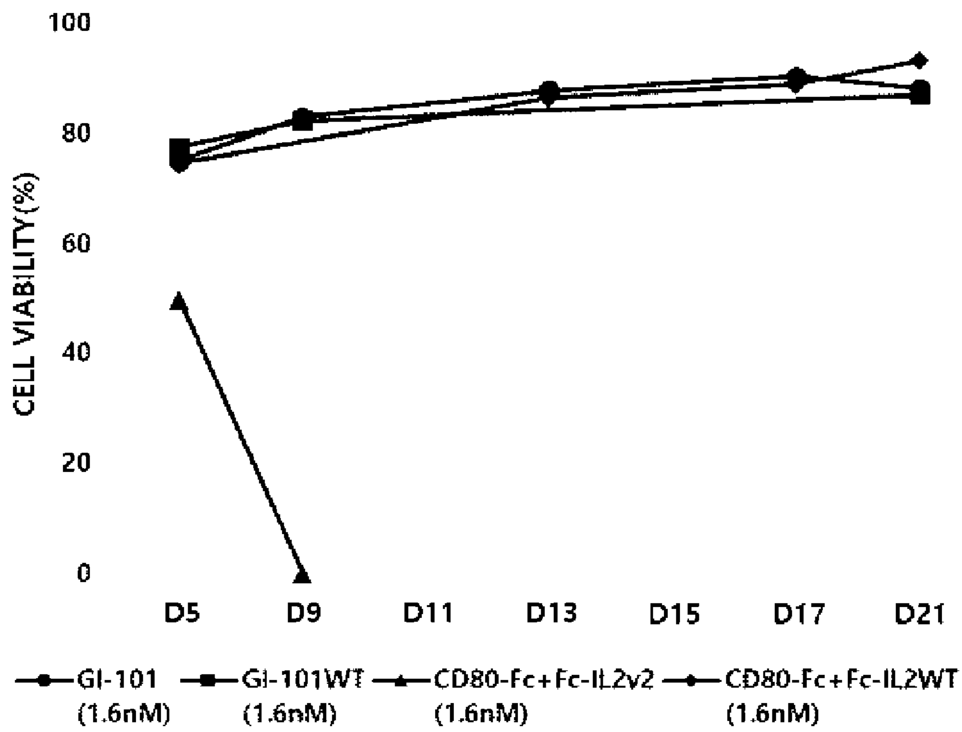

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FIG. 7A

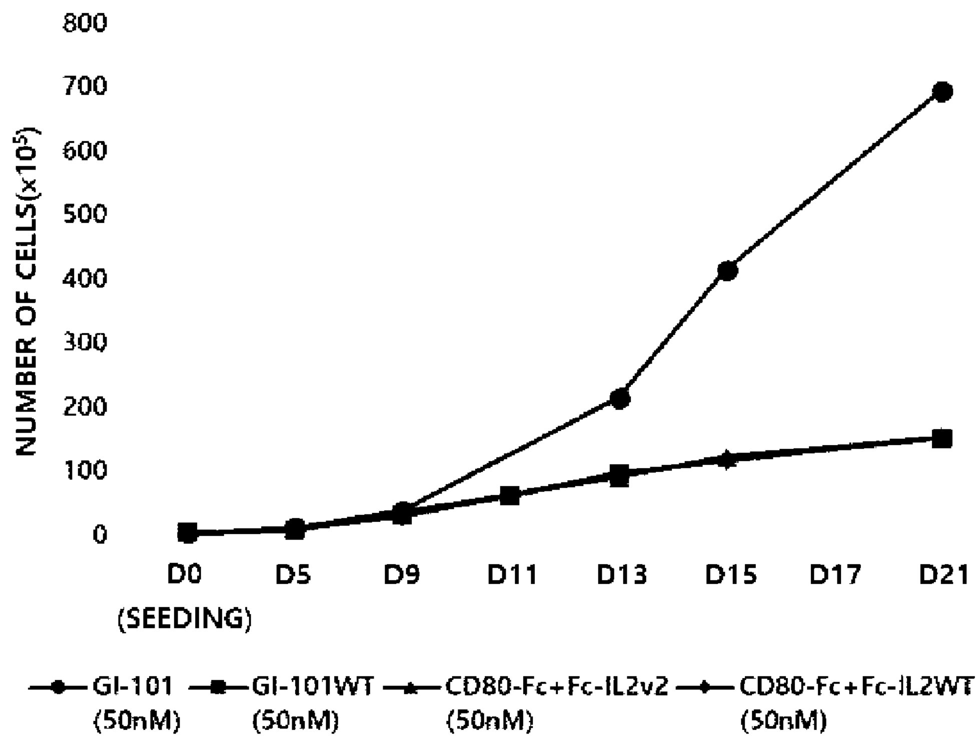

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FIG. 7B

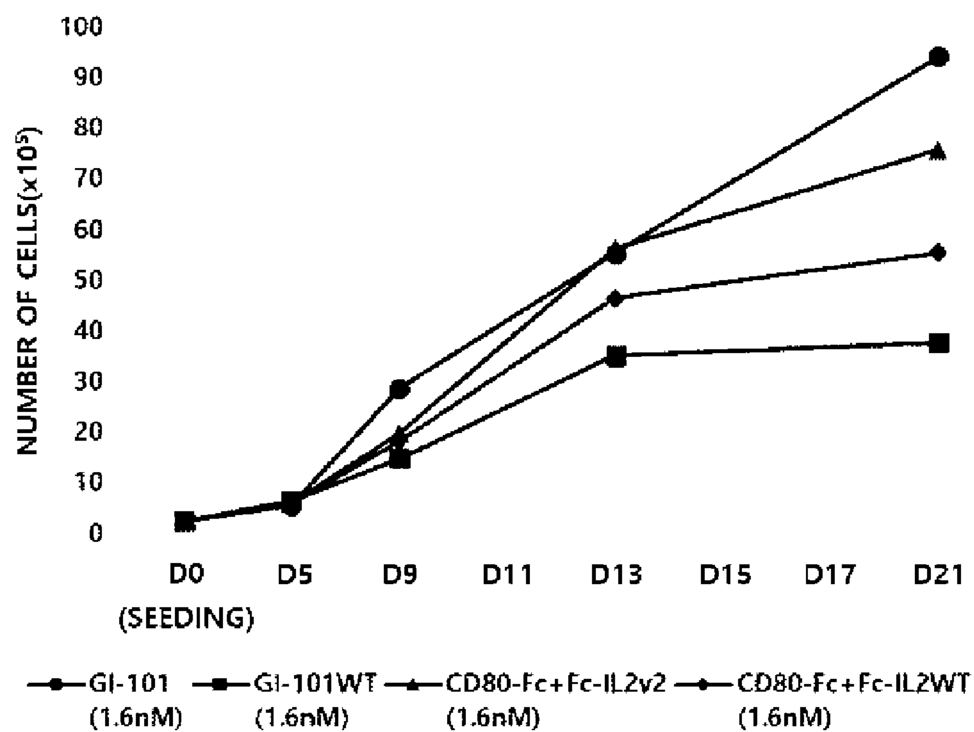

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FIG. 8A

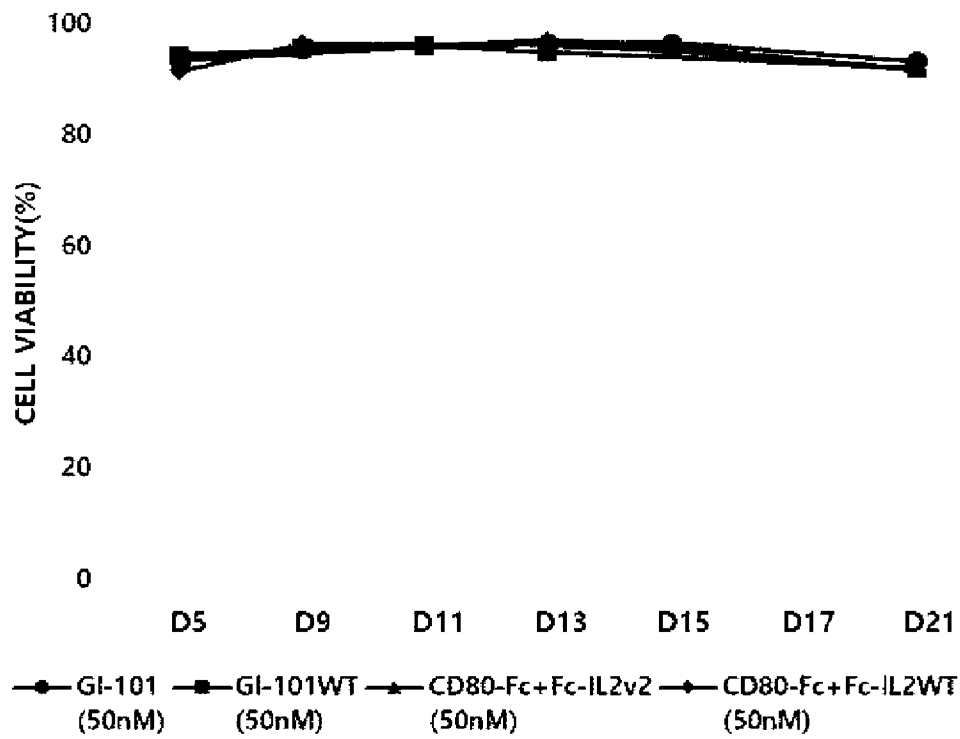

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FIG. 8B

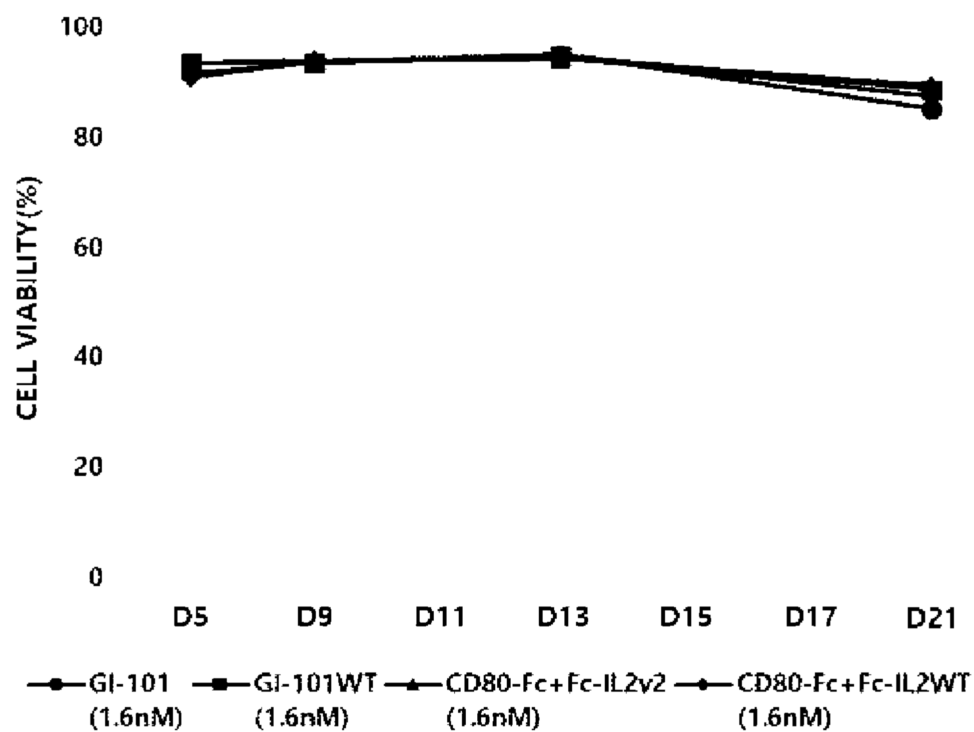

FIG. 9A

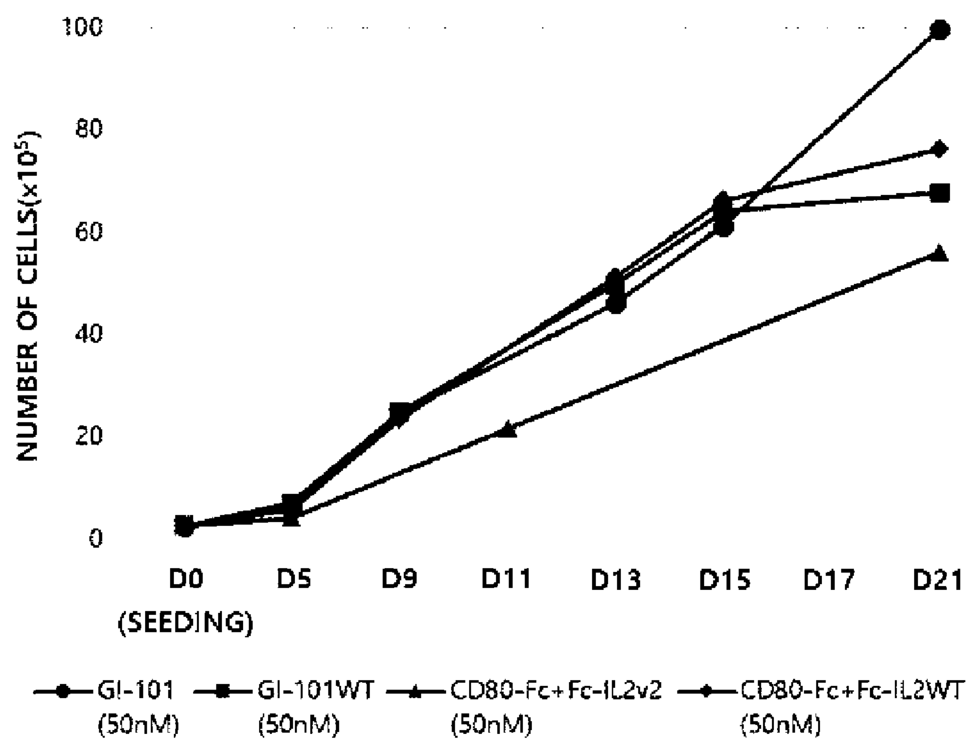

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FIG. 9B

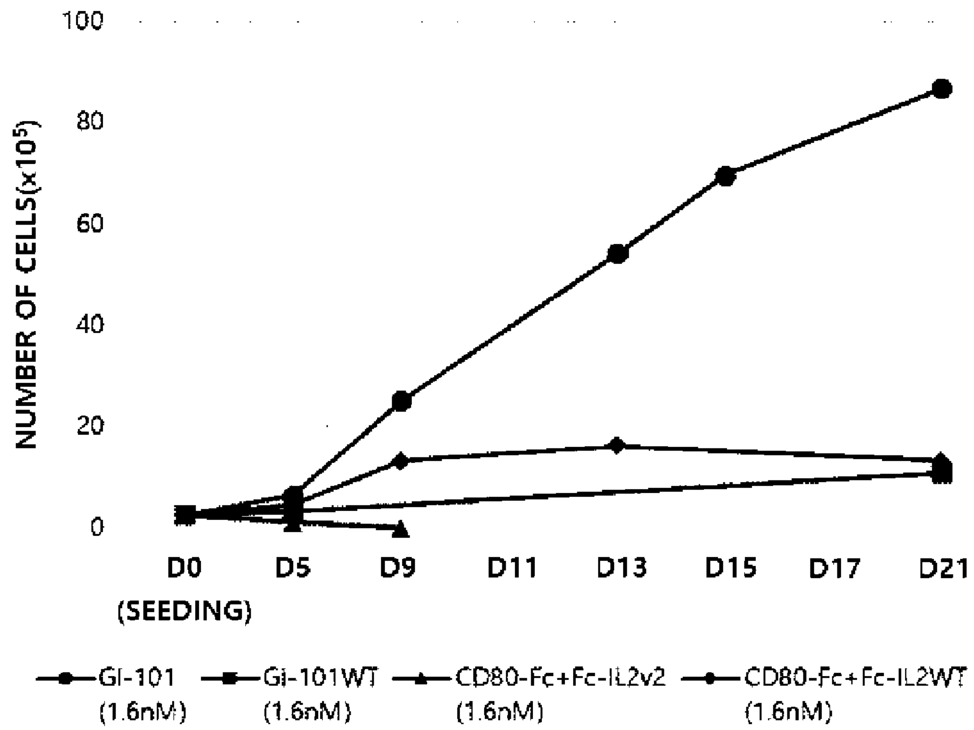

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FIG. 10A

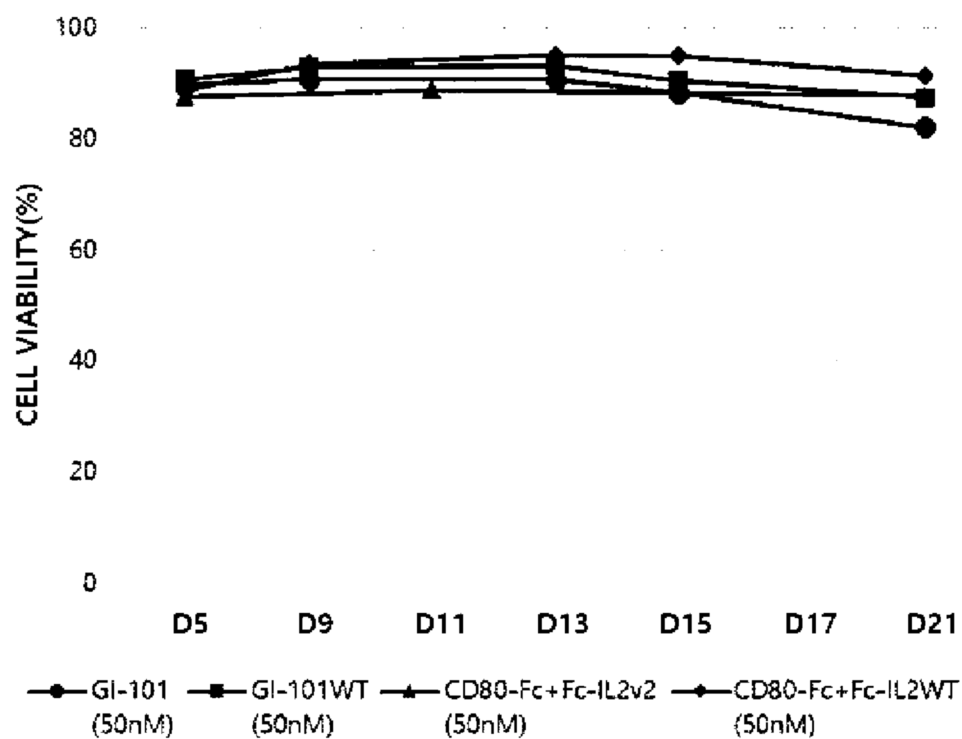

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FIG. 10B

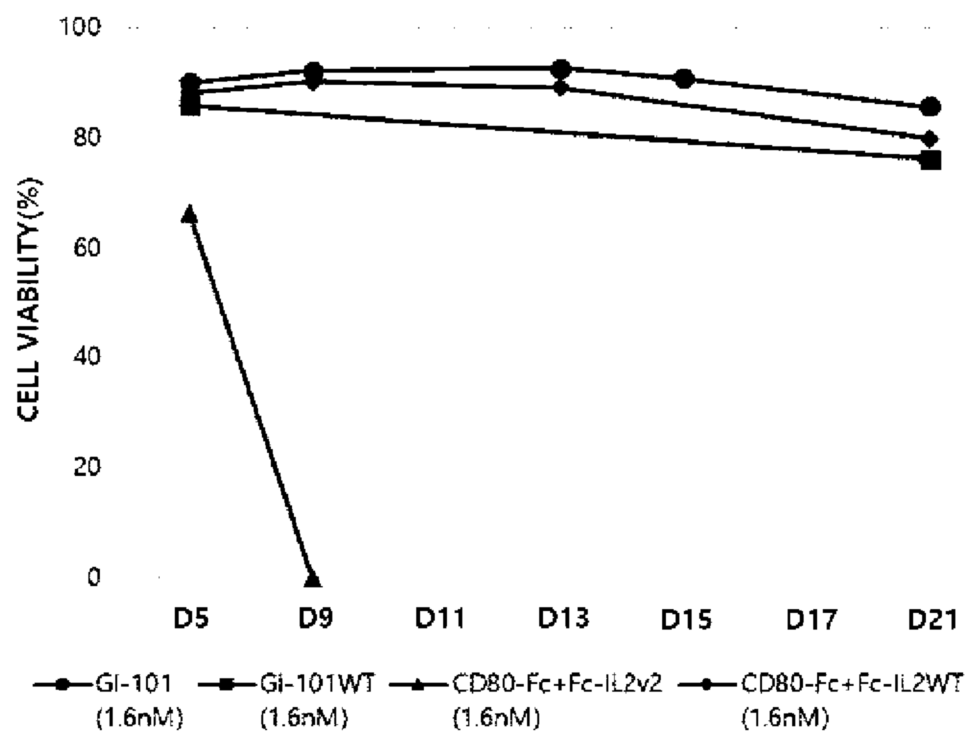

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FIG. 11A

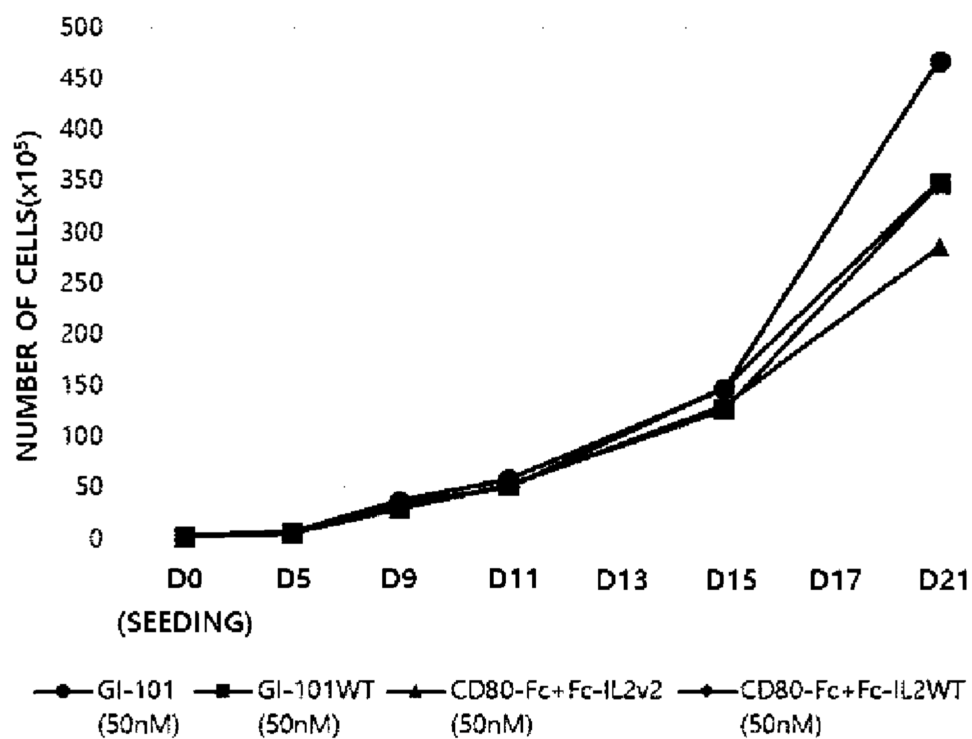

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FIG. 11B

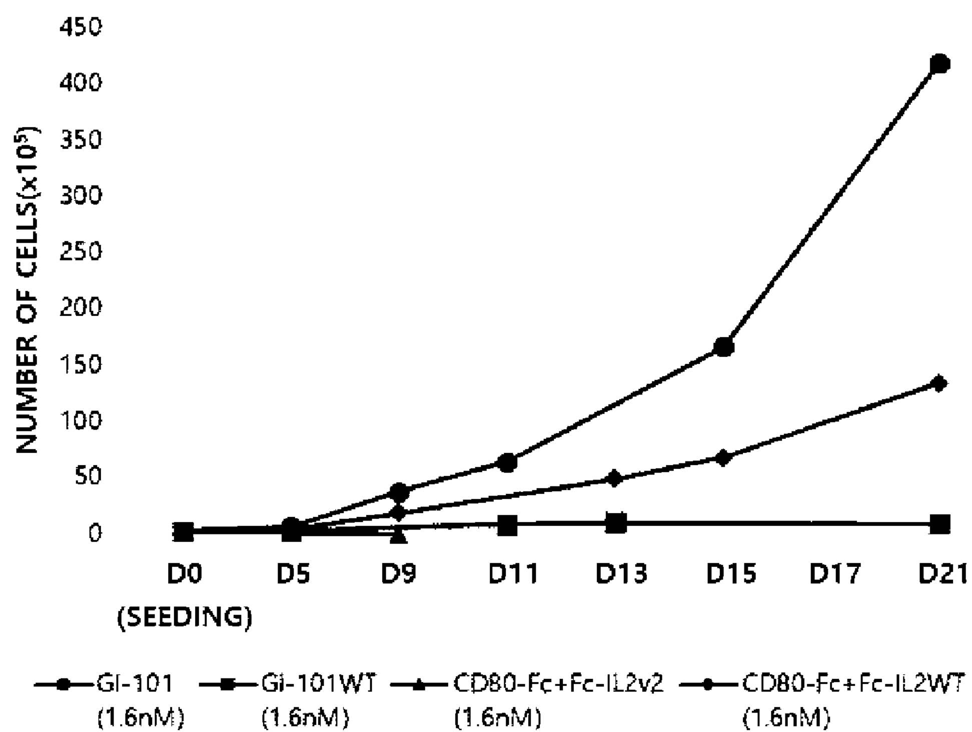

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FIG. 12A

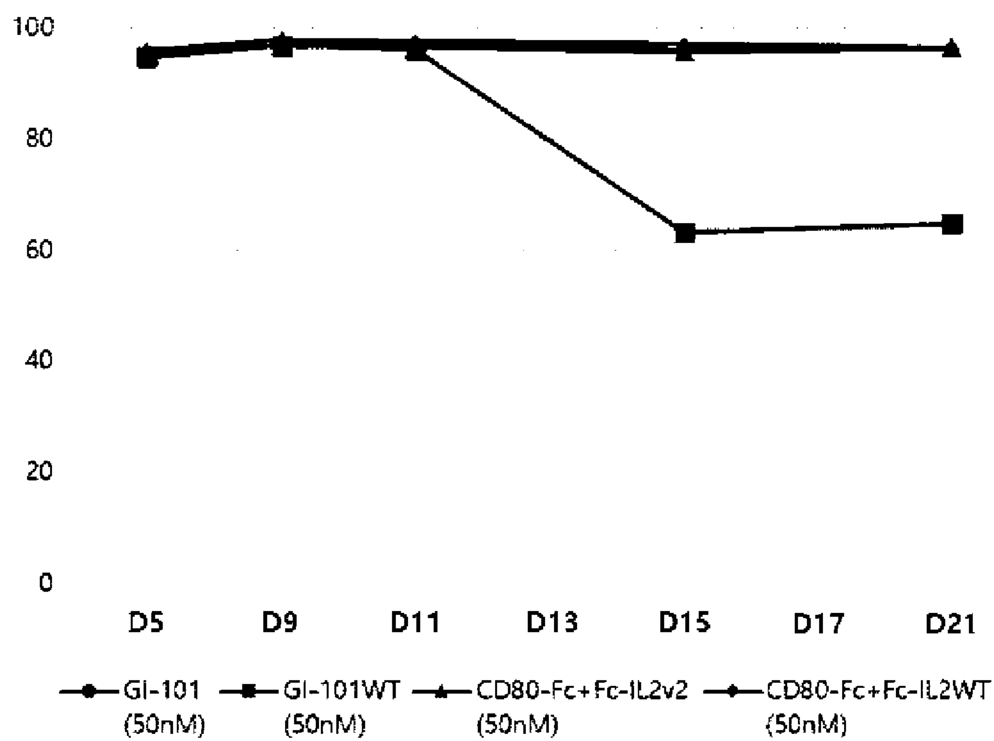

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FIG. 12B

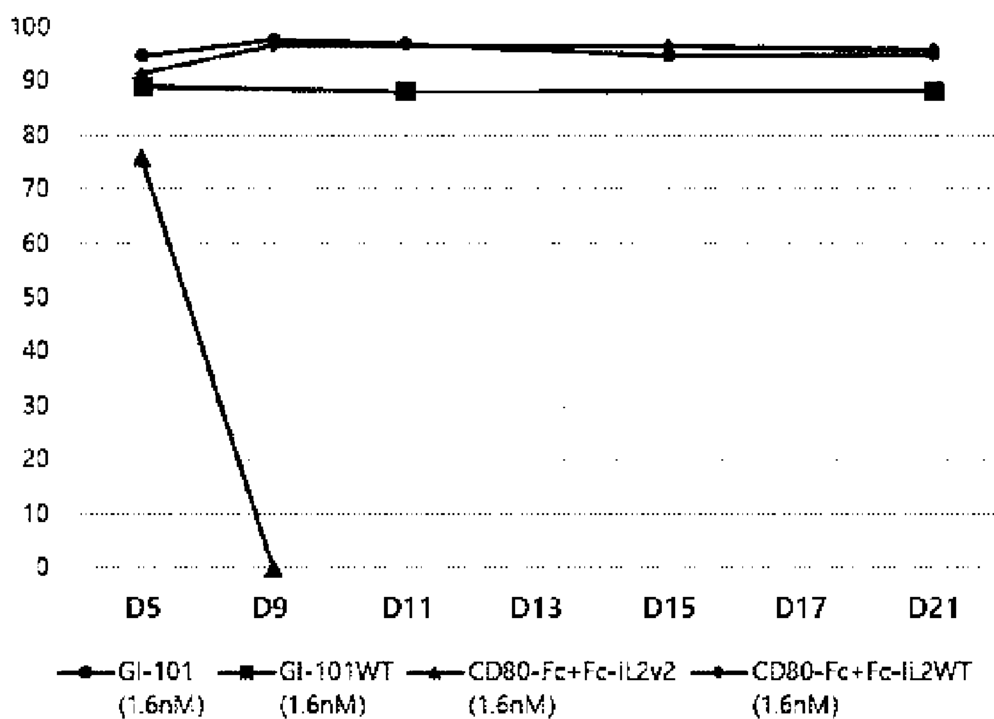

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FIG. 13

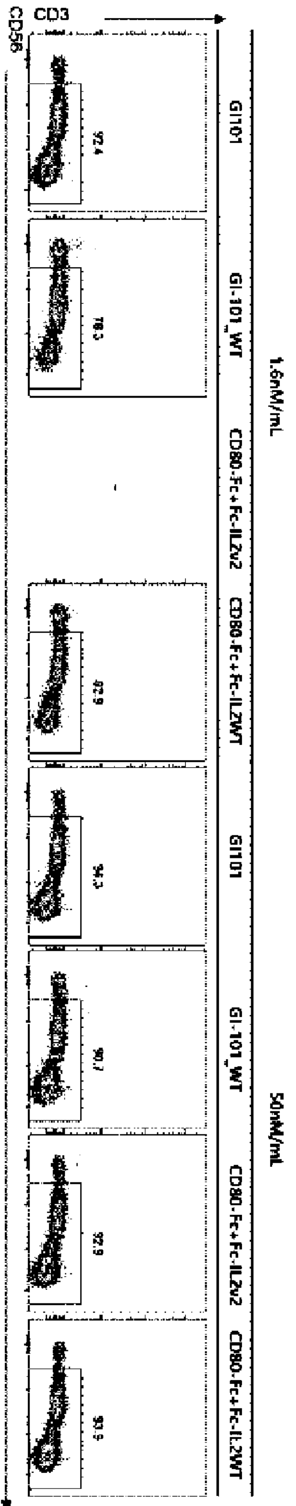

FIG. 14

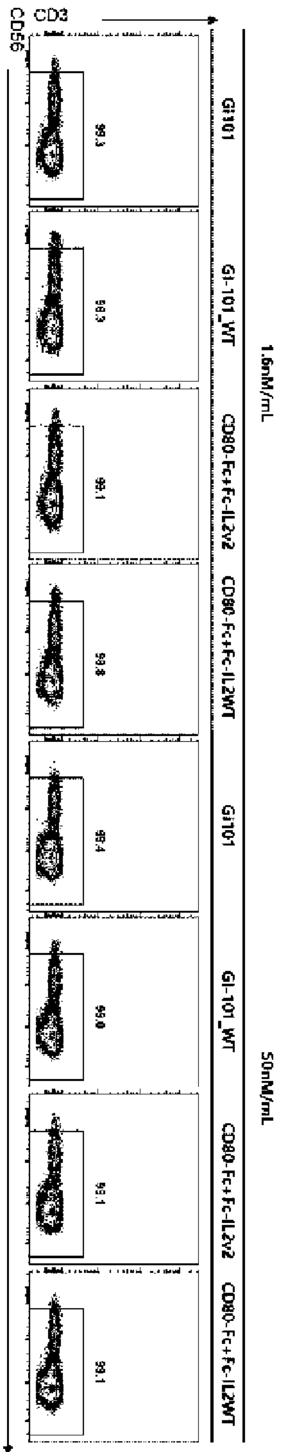

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FIG. 15

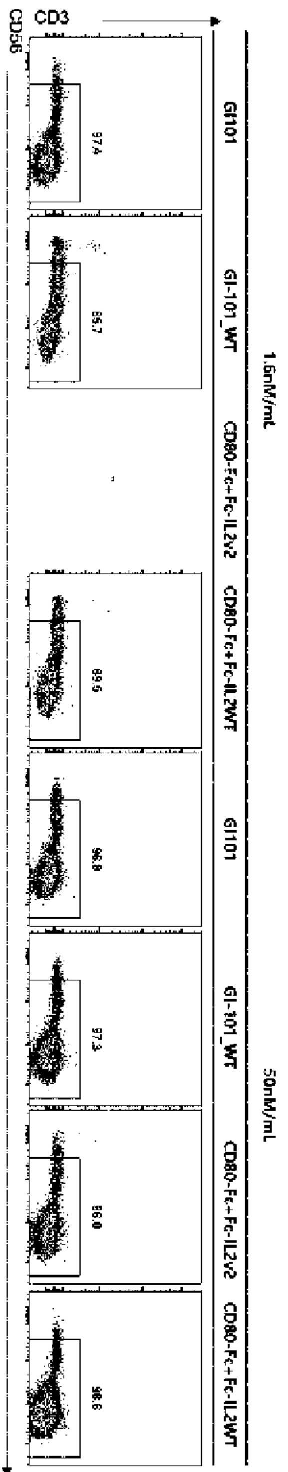

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FIG. 16

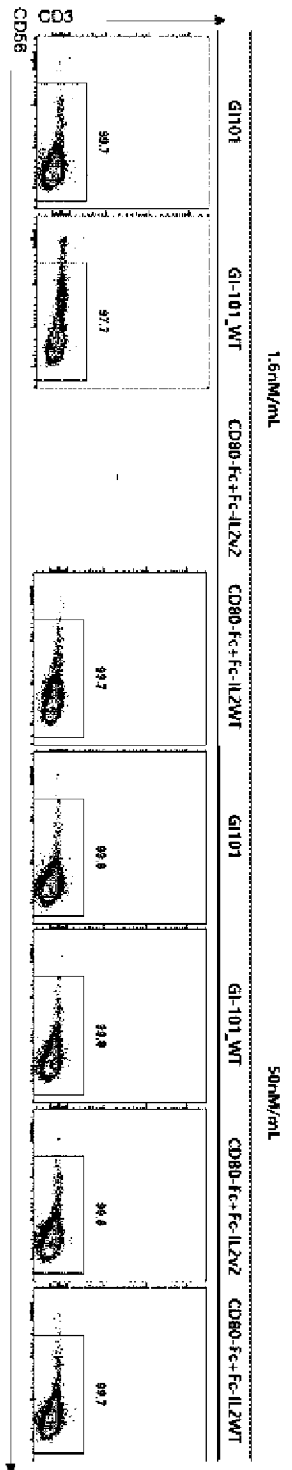

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FIG. 17

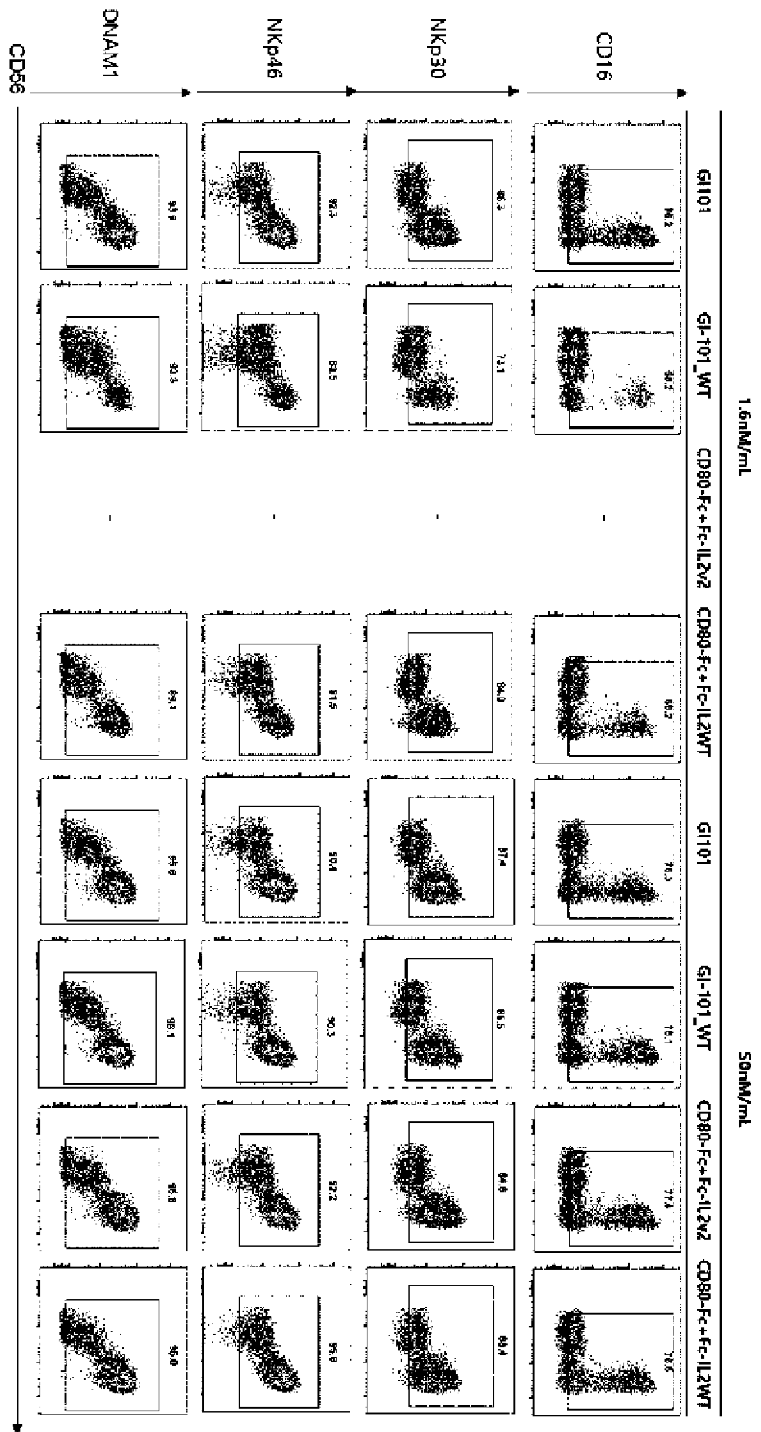

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FIG. 18

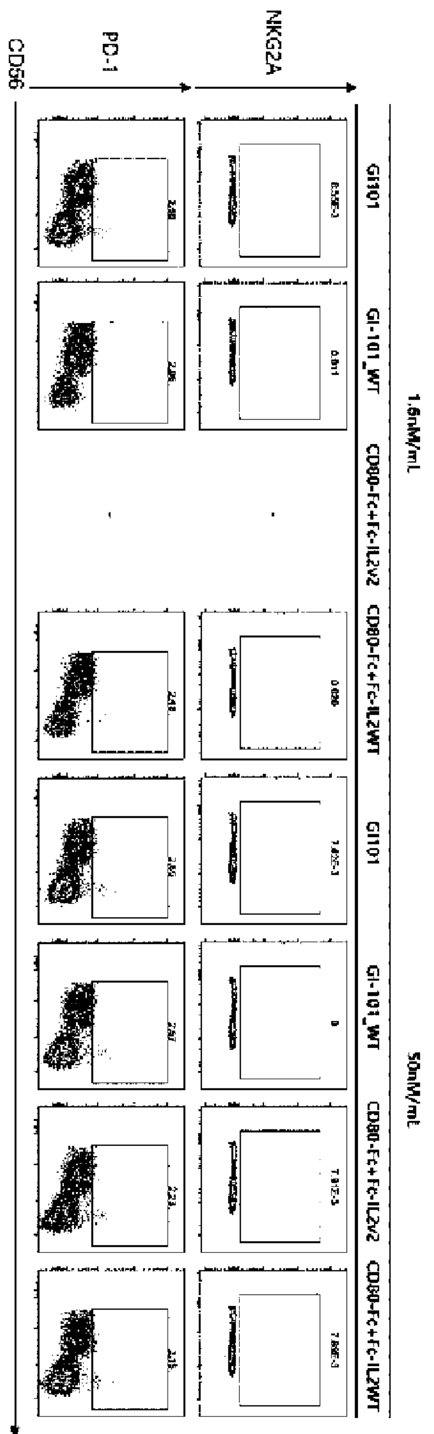

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FIG. 19

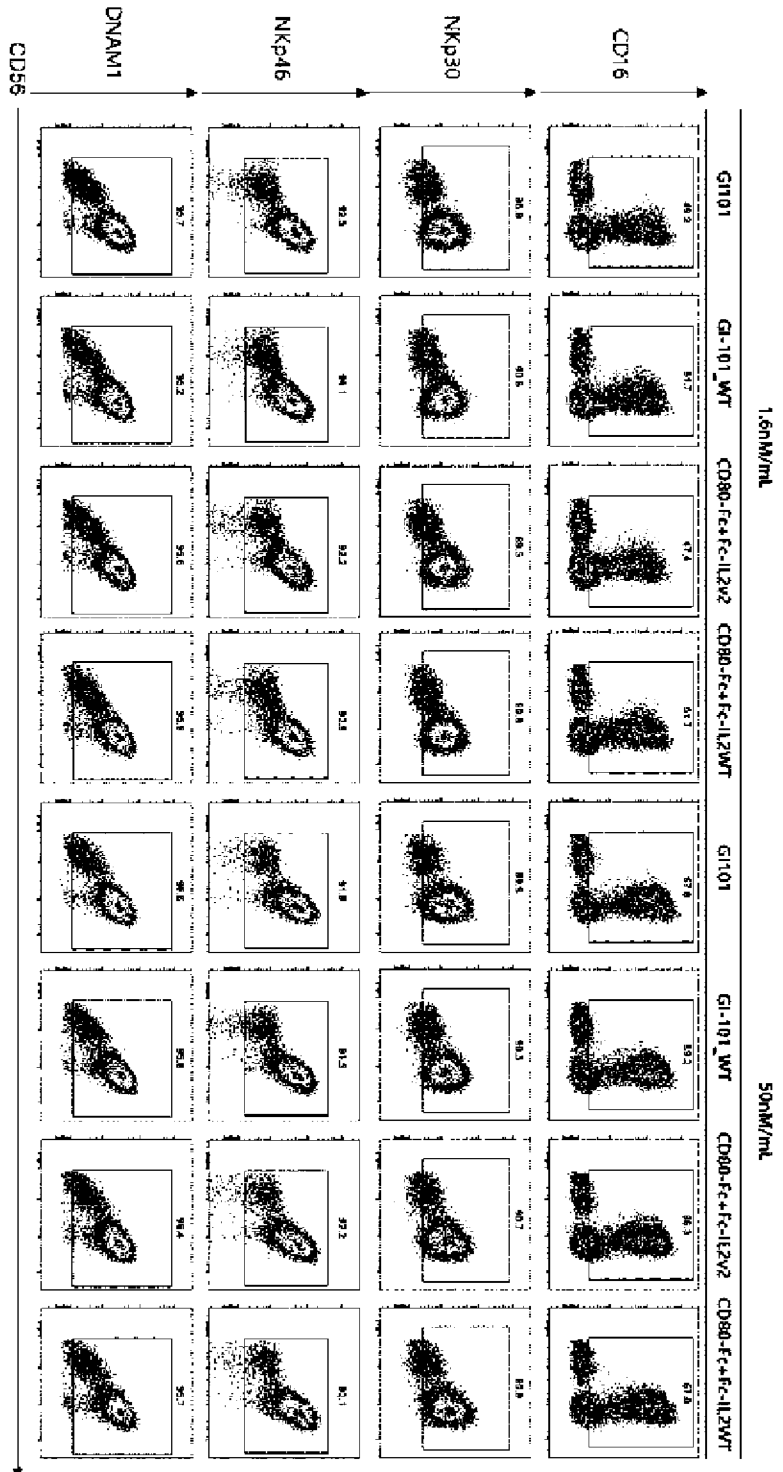

FIG. 20

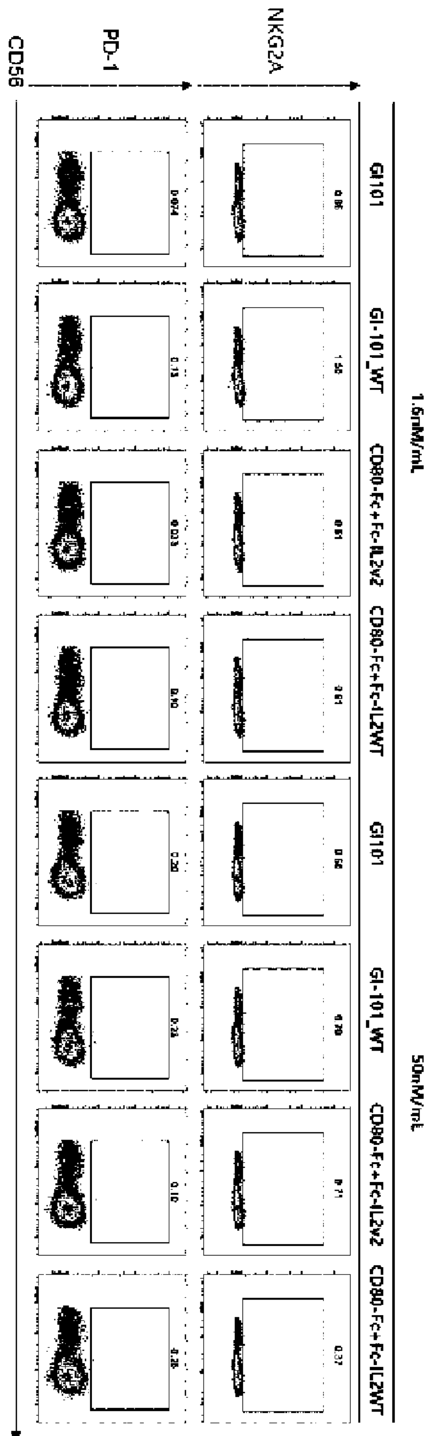

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FIG. 21

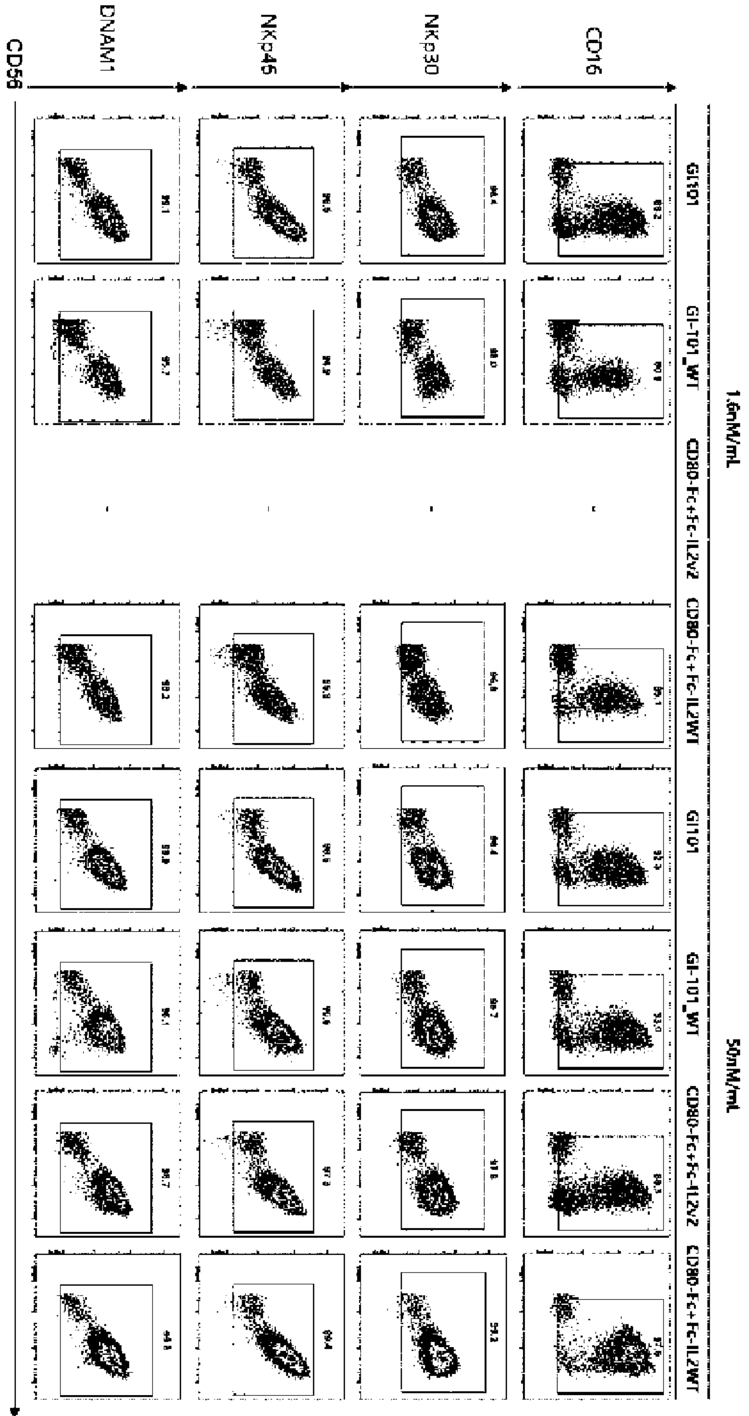

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FIG. 22

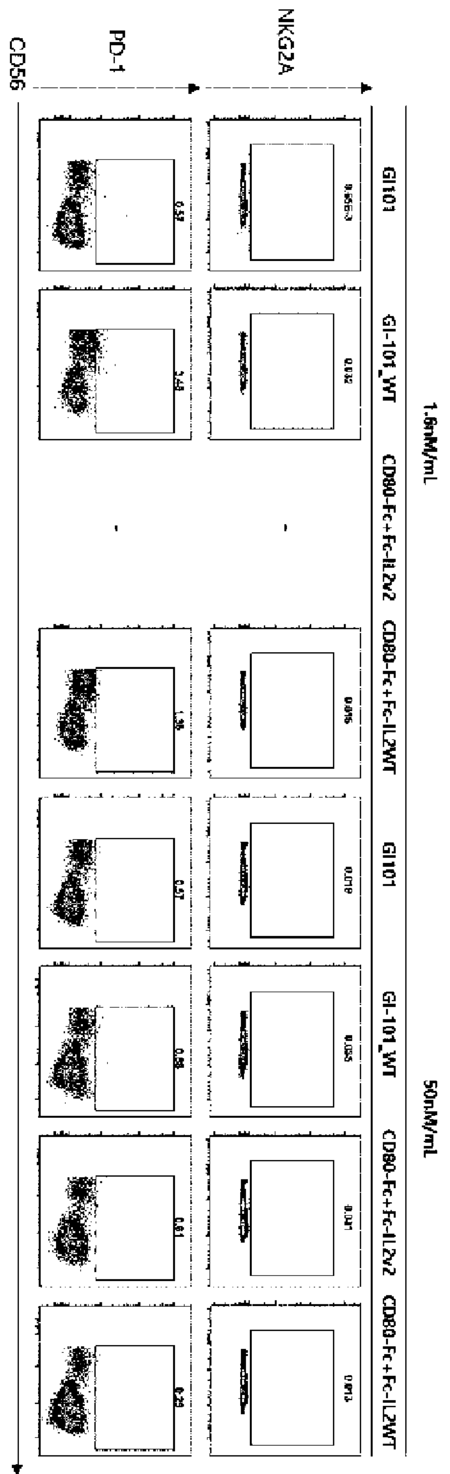

FIG. 23

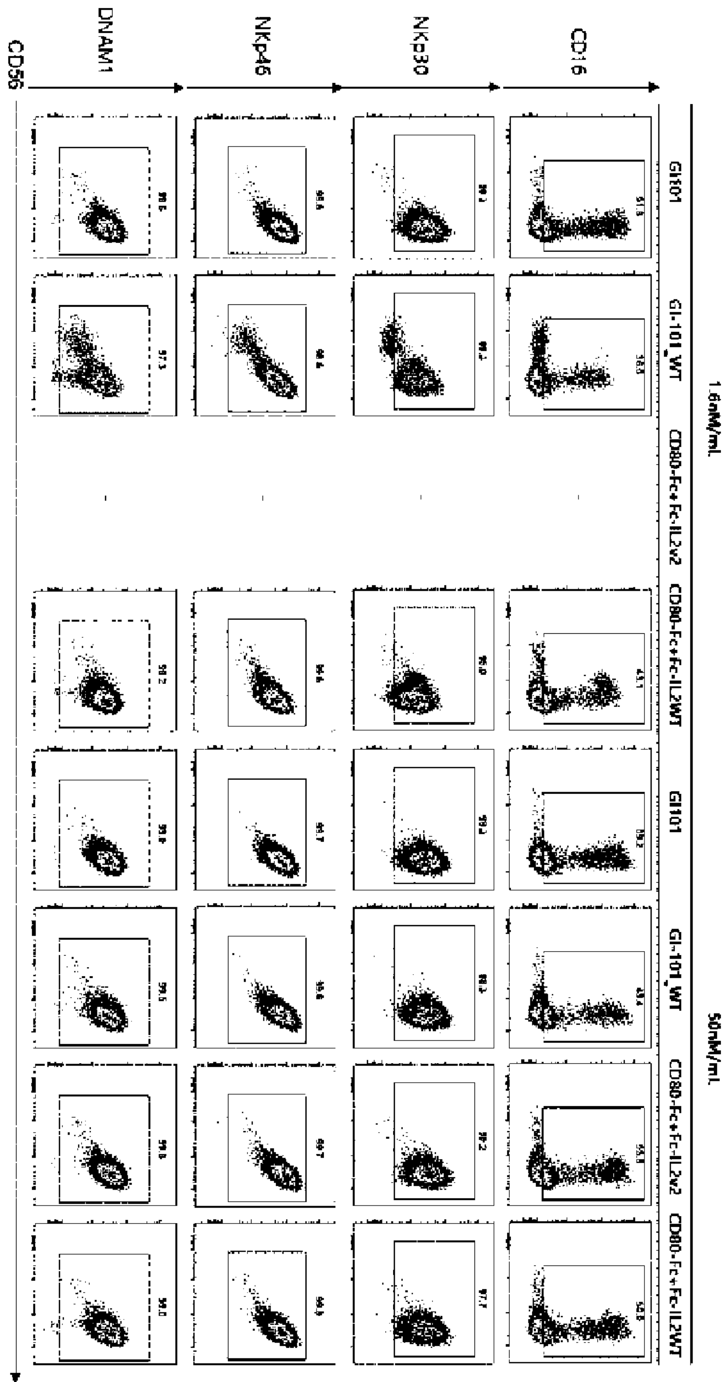

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FIG. 24

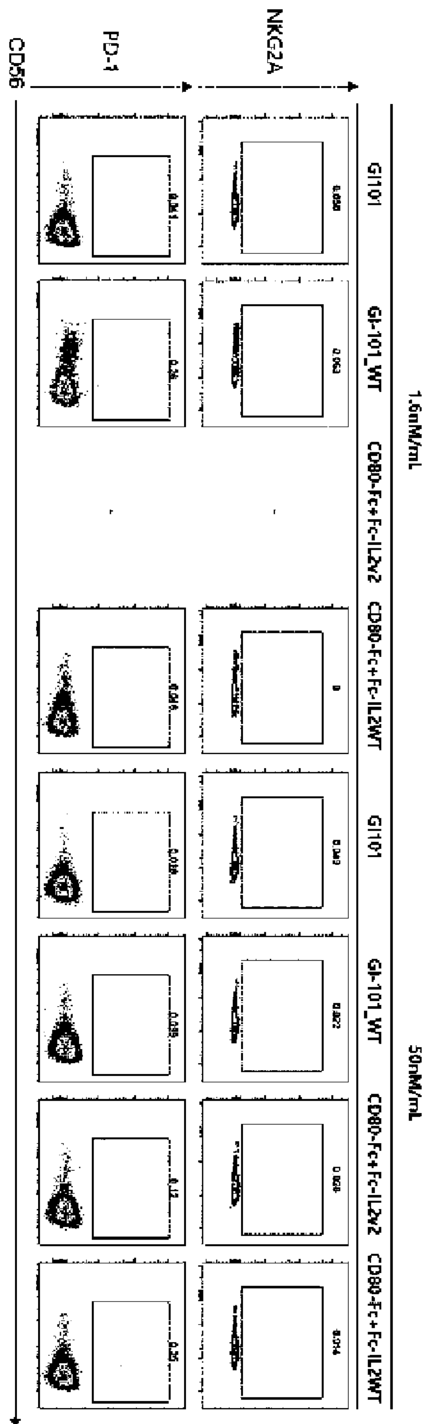

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FIG. 25

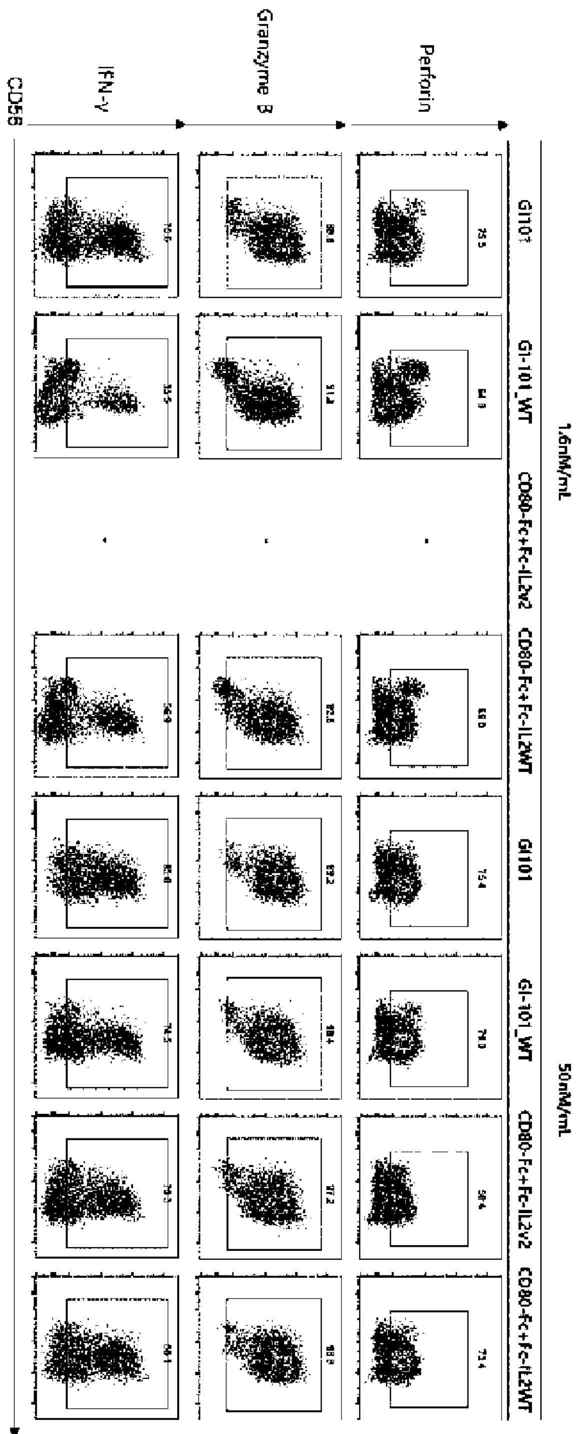

FIG. 26

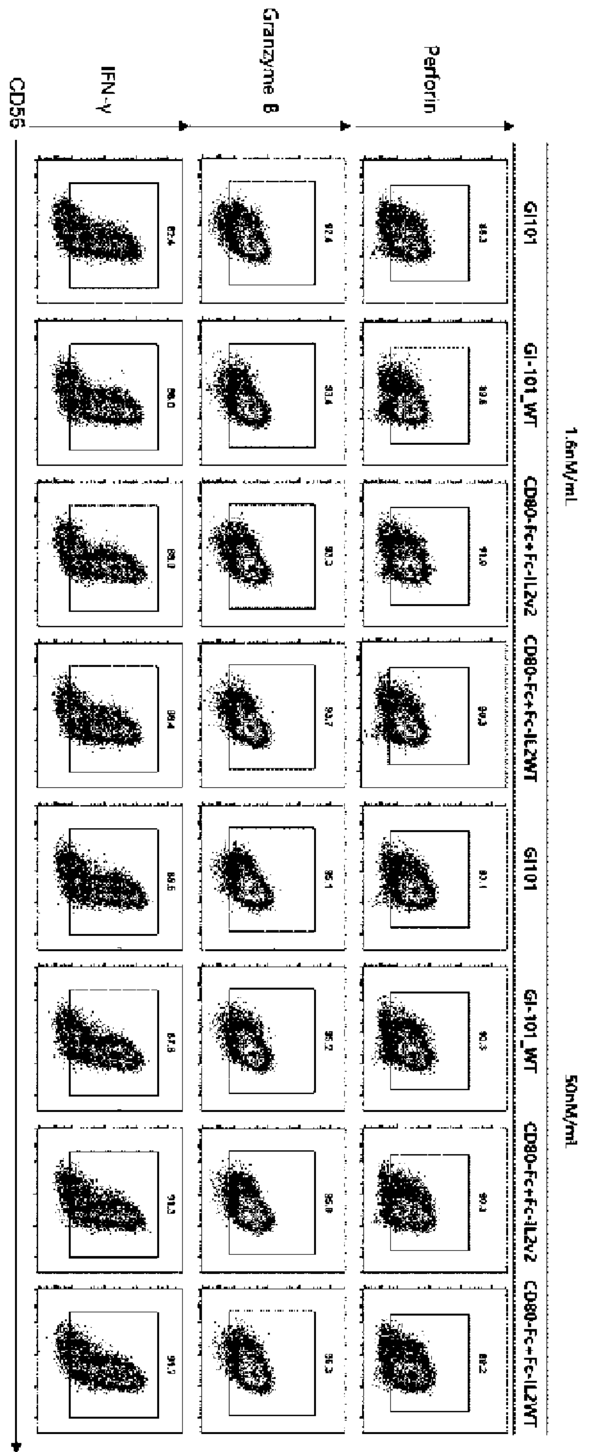

FIG. 27

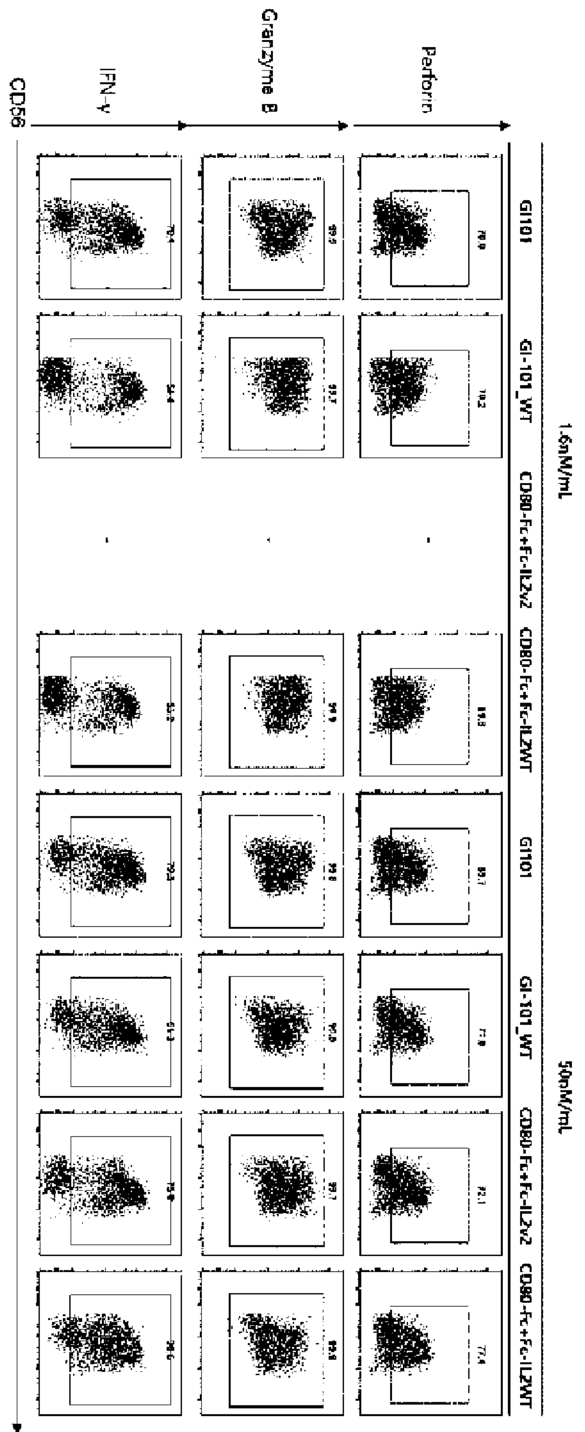

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FIG. 28

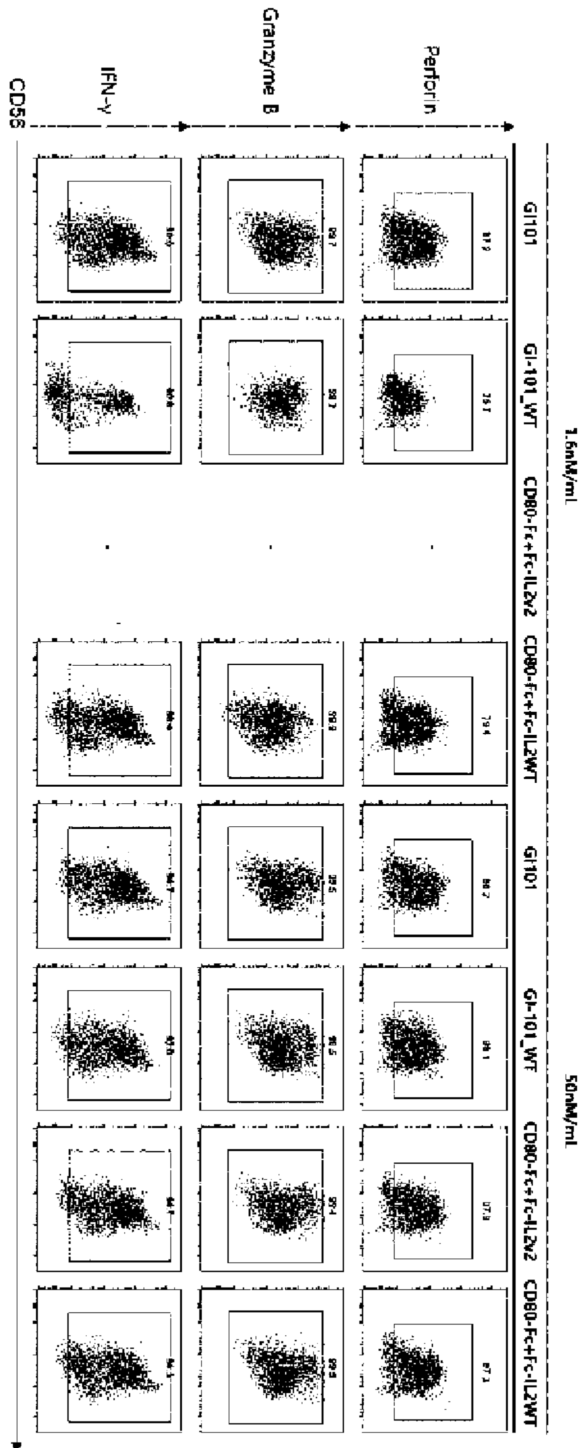

FIG. 29

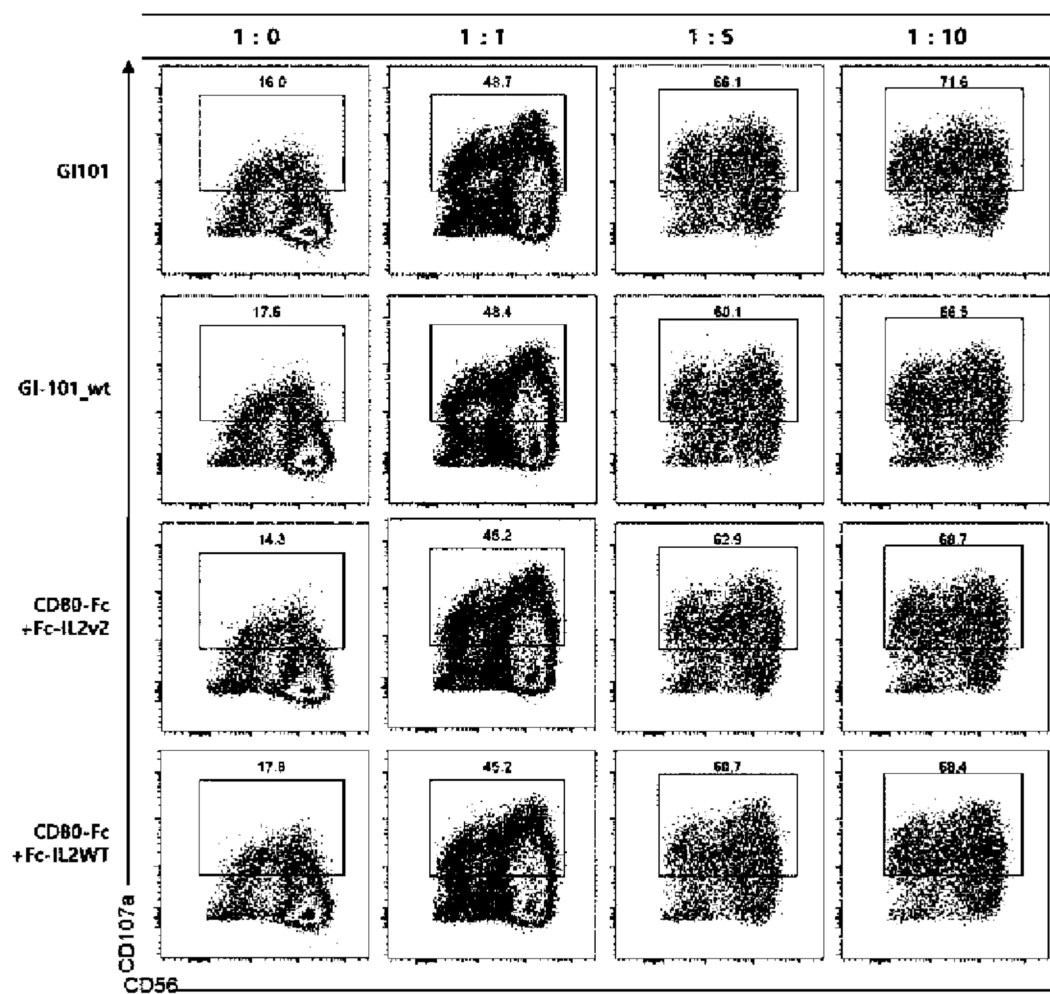

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FIG. 30

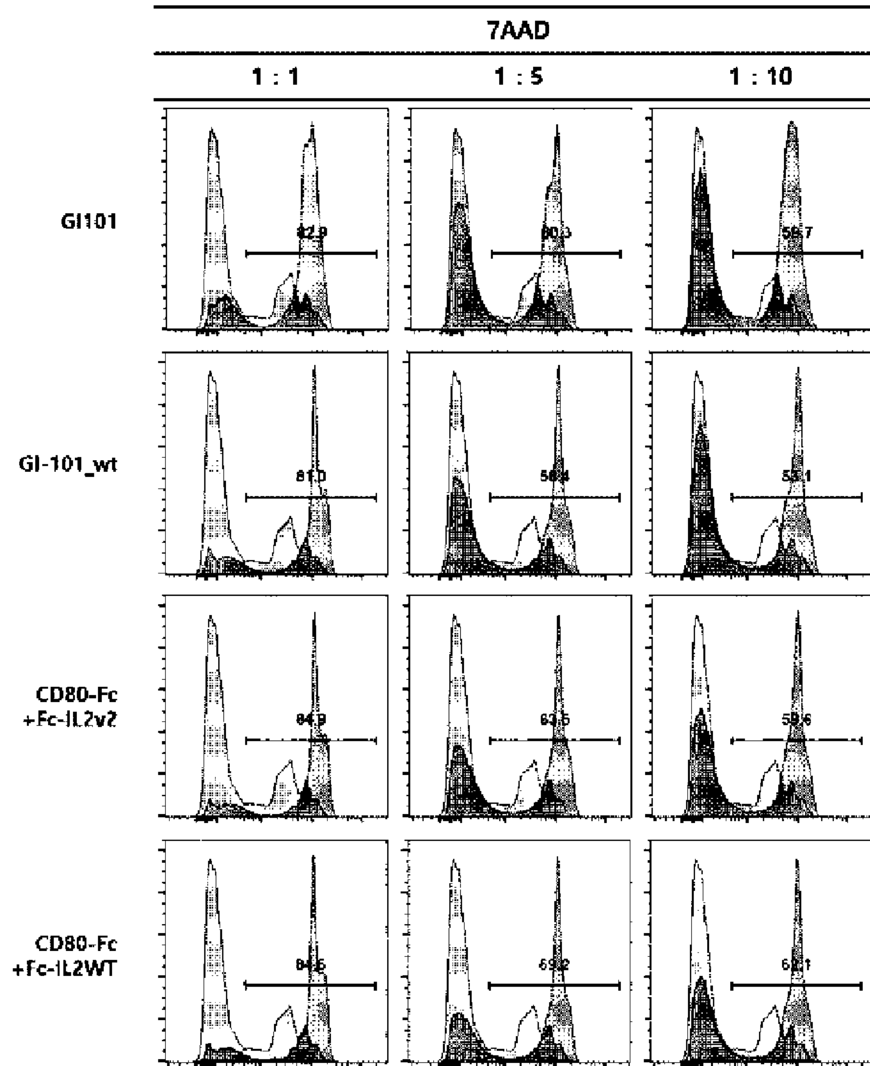

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FIG. 31

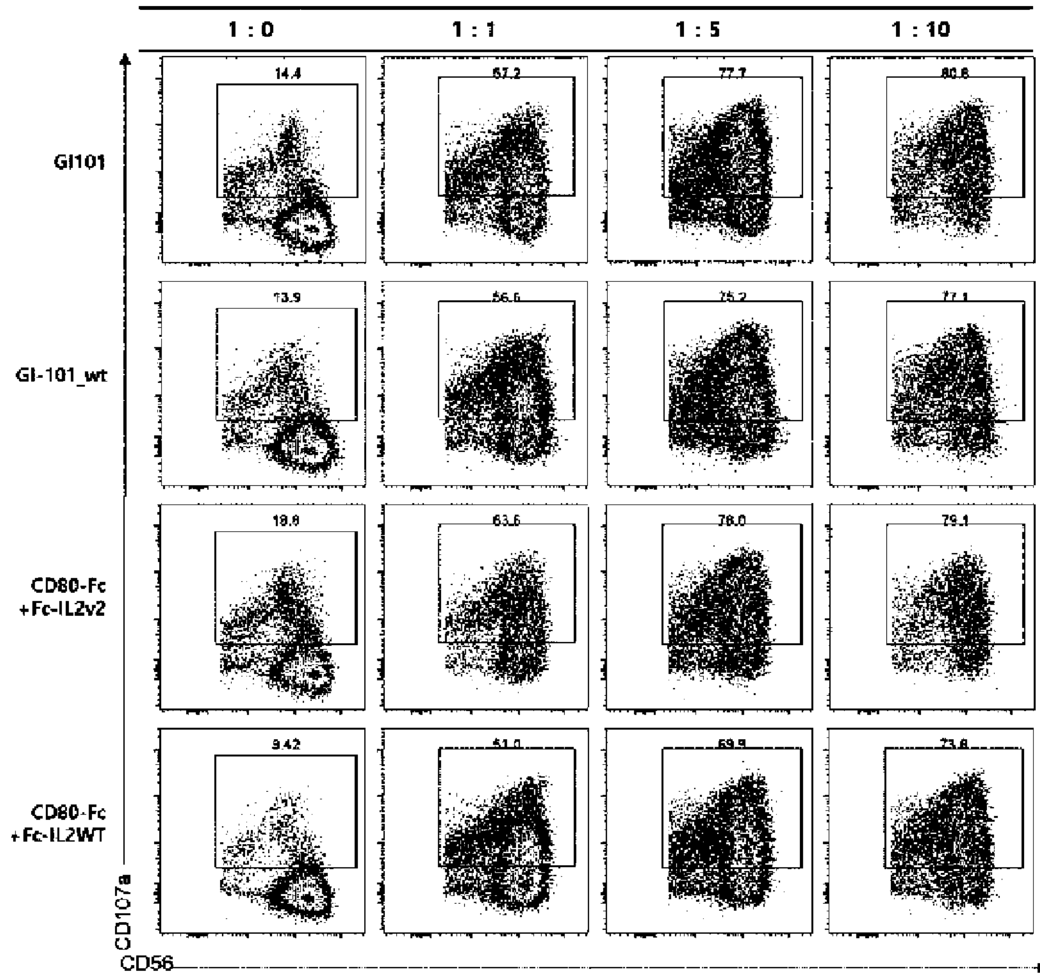

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FIG. 32

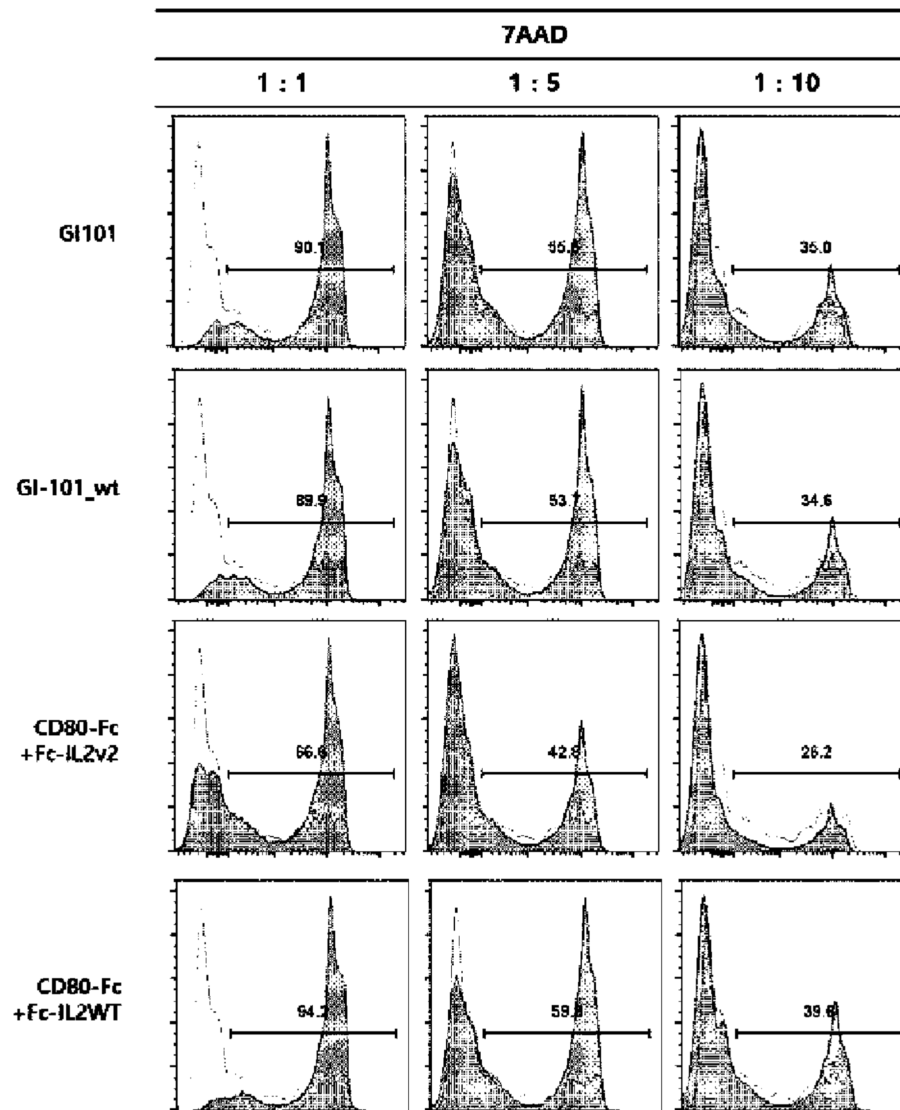

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FIG. 33

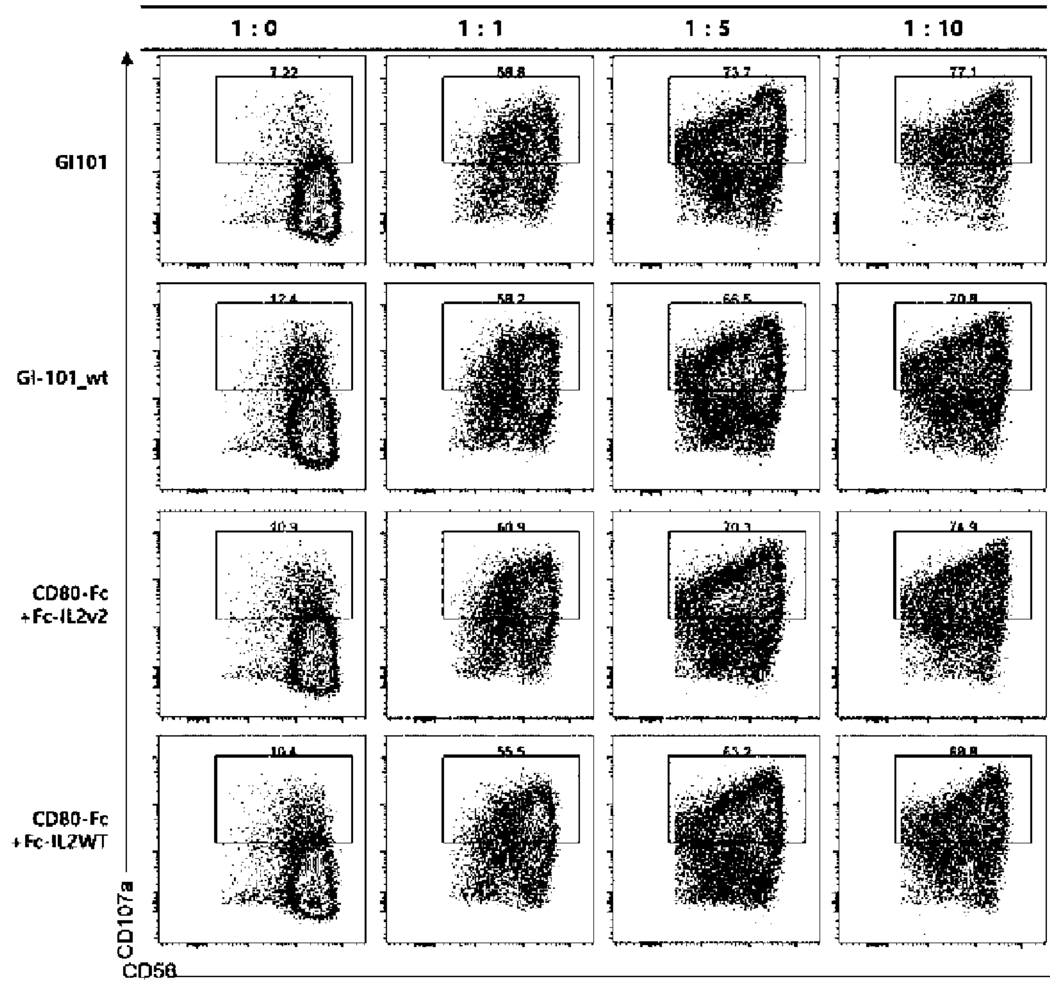
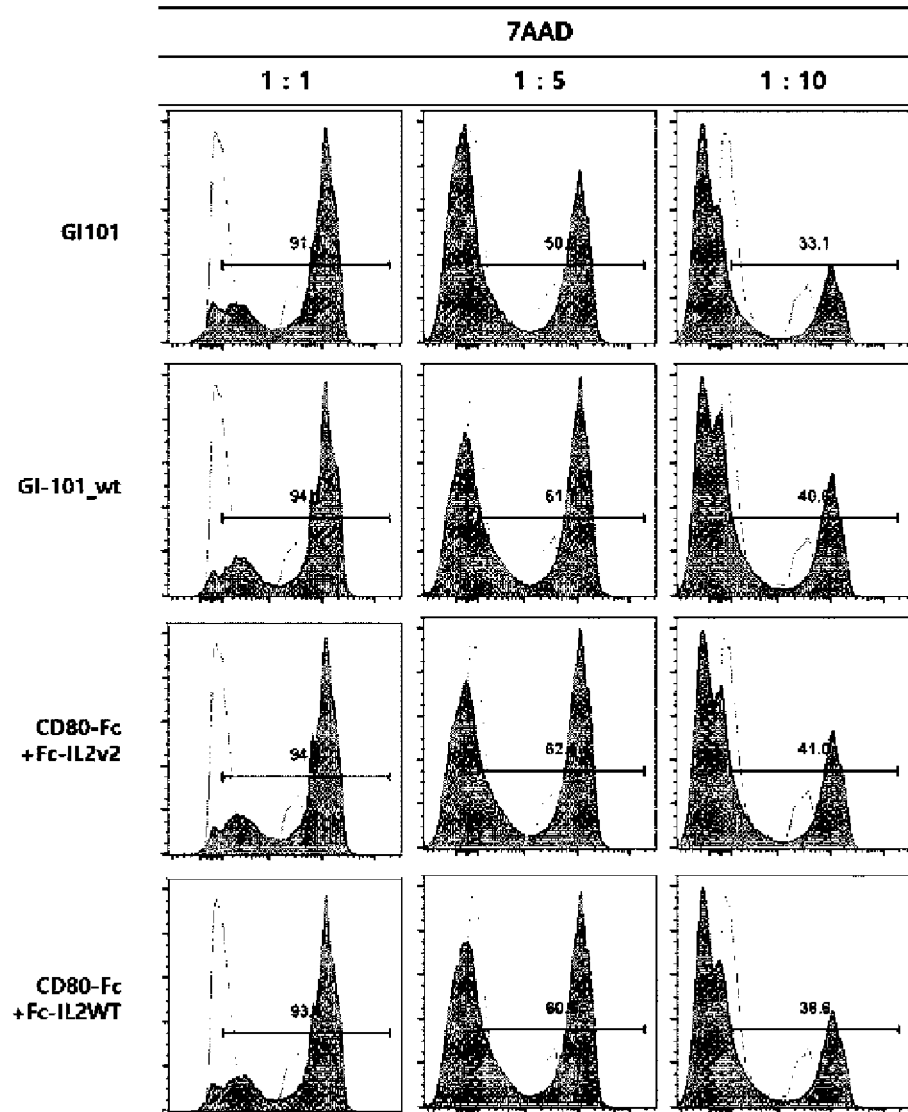


FIG. 34



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