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(54) Title: A COMPOSITION COMPRISING HUMANIZED ANTIBODY HBBK4 FOR THE TREATMENT OF CANCER AND
THE USE THEREOF

BBK-4-H

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m4B4-H          QVQLQQPQAEVLKPGASVKLSCKASGYTFSSYMMHWVRQRPGQVLEWIGIEINPGNGHTNYNEKFKSKATL
Human  VH1-46/J4 QVQLVQSGAEVVKPGASVKVSCKASGYTFSSYMMHWVRQAPGQGLEWMGLINEPSGSGSTSYAQKFGQRTVM
H4B4-H          QV*L**SGAEVKPGASVK*SKASGYTFSSYMMHWV*Q*PGQGLEWIGIEINPGNGHTNYNEKFKS**T*
H4B4-H-2        QVQLQQSGAEV*KEGASVK*SKASGYTFSSYMMHWV*CAPGQGLEWIGIEINPGNGHTNYNEKFKS**T*
          # # # #
m4B4-H          TVDKSSSTAYMQLSSLTSEDRAVYYCARSEFTTARAFAYWGQSTLVTVSS
Human  VH1-46/J4 TRDTSTSTVYMESSLRSEDRAVYYCAR          YFDYWGQSTLVTVSS
H4B4-H          TRDTSTSTVYMESSLRSED*AVYYCARSEFTTARAFAYWGQSTLVTVSS
H4B4-H-2        T*DTSTST*YM*LSSLRSED*AVYYCARSEFTTARAFAYWGQSTLVTVSS

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BBK-4-L

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m4B4-L          DIVMTQSQATQSVYTPQGVNLSLRASQTISDYLHWYQQKRPDQPKLLIKYASQSIGTSPRFSGSGSGSDPTLS
Human  A14/J2    DIVMTQSPAFLSVTPGKVTITCOASEGIGNLYHWYQQKRPDQPKLLIKYASQSIGTSPRFSGSGSGSDPTPTT
H4B4-L          D**MTQSPA*LSVTPGKVT**CRASQTISDYLHWYQQK*D**P*LLIKYASQSIG*SPRFSGSGSG*DPTPT
H4B4-L-2        D*VMTQSPAFLSVTPGKVT*TCRASQTISDYLHWYQQKPDQ*PKLLIKYASQSIG*SPRFSGSGSGSDPT**
          # *# *# # @
m4B4-L          ISSLEAEDAATYYCQDSKHPPTFGGQTKLEIK
Human  A14/J2    ISSLEAEDAATYYCQDSKHPPTFGGQTKLEIK
H4B4-L          ISSLEAEDAATYYCQDSKHPPTFG*GTKLEIK
H4B4-L-2        ISS*EAED**YYCQDSKHPPTFGGQTKLEIK

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- Underlined: residues for antigen contact (m)
- *: canonical residues (m)
- #: residues at the domain of interface (m)
- @: unusual sequence (h)
- Solvent-exposed residues (h):
H chain: 11-18, 68-85 (Kabat sequence)
L chain: 15-20, 71-85 (Kabat sequence)

(57) Abstract: The present invention is related to a pharmaceutical composition comprising humanized anti- 4- IBB antibody (HBBK4) for treating cancer by inducing increase of CD1 1+CD8+ T cell and IFN-γ, and inhibiting proliferation of cancer cells, together with a pharmaceutically acceptable carrier and the use. Accordingly, it can be useful in the prevention or treatment of cancer without adverse response.



For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

Description

A COMPOSITION COMPRISING HUMANIZED ANTIBODY HBBK4 FOR THE TREATMENT OF CANCER AND THE USE THEREOF

Technical Field

- [1] The present invention relates to a pharmaceutical composition comprising humanized antibody HBBK4 for treating or preventing cancer disease and the use thereof.

[2]

Background Art

- [3] Cancer cells express cancer cell-specific antigen resulting from the inductions of over-expression and mutation for specific proteins, which are removed due to the auto immune reaction of patient (Nagorsen D. et al., *Clin. Cancer Res.* 9, pp4296-4303, 2003; Yee C et al., *J. Exp. Med.* 192, pp1637-1644, 2000; Castelli C et al., *J. Cell Physiol.*, 182, pp323-331, 2000). Those immune reactions against cancer cells are represented by antigen-presenting cell (APC) such as dendritic cell which is the same with normal immune reaction and the cancer cells are removed by activation of appropriate T cell and B cell with APC (Jay A. et al., *J. Clin. Invest.* 113, pp1515-1525, 2004). Cancer cells cause anergy or tolerance through various mechanisms for examples, the decrease of MHC molecules, inhibition of co-stimulatory molecule expression, induction of immunosuppressive substance such as TGF- β and IL-10, infiltration of CD4+CD25+ regulatory T cell in cancer tissues etc, and try to protecting from the anti-cancer immune reaction (Smyth MJ et al., *Nat. Immunol.* 2, pp293-299, 2001; Dunn GP et al., *Immunity* 21, pp137-148, 2004).
- [4] The anti-cancer immune reactions depend on cell-mediated immunity. In particular, CD8+ T cells (CTLs) are critical to remove cancers, and keep the immunologic memory (Maeurer MJ et al., *Int. Rev. Immunol.* 14, pp97-132, 1997).
- [5] There need two kinds of signals to induce T cell activation (Mondino A et al., *J. Leukoc. Biol.* 55, pp805-815, 1994), i.e., first signal, namely antigen specific signal, is carried out by TCR and the second signal is carried out by co-stimulatory molecules. Ig (immunoglobulin) family and TNFR family are well known as co-stimulatory molecules. Since the immune agonistic and antagonistic roles of those molecules were publicly known, various treatment methods using thereby have been tried till now. At present, there have been reported that 4-1BB (CD137), CTLA-4, CD28, LIGHT, PD-1, CD27 etc showed potent anti-cancer activity among them (Ye Z et al., *Nat. Med.* 8, pp343-348, 2002; Phan GQ et al., *Proc. Natl. Acad. Sci. USA*, 100, pp8372-8377,

2003; Li Q et al., *J. Immunother.*, 25, pp304-313, 2002; Yu P et al., *Nat. Immunol.*, 5, pp141-149, 2004; Iwai Y et al., *Int. Immunol.*, 17, pp133-144, 2005; Arens R et al., *J. Exp. Med.*, 199, pp1595-1605, 2004).

[6] There have been reported that 4-1BB induces the expression of CTL and IFN- γ , and agonistic anti-4-1BB mAb on cancer treatment have been tried by many researchers (Shuford WW. et al, *J. Exp. Med.*, 186, pp47-55, 1997).

[7] CTLA-4 has been reported to limit the immune reaction and the inhibition of CTLA-4 signal transfer expressed by activated T cells prevents the proliferation of cancer cell due to maximized activation of T cell (Phan GQ et al., *Proc. Natl. Acad. Sci. USA*, 100, pp8372-8377, 2003).

[8]

[9] The present inventors have been tried to discover the function of anti-4-1BB antibody and finally found that the treatment using by agonistic anti-4-1BB antibody and antagonistic anti-CTLA-4 antibody increases cancer cell-specific CD8+ T cell, which induces the production of CD8+ T cell expressing CD11c molecule resulting in infiltrating into the tumor differently from other co-stimulatory molecules. The inhibition of cancer cell proliferation through the administration of agonistic anti-4-1BB antibody is caused by the abundant infiltration of CD11c+CD8+ T cells in tumor and highly expressed IFN- γ .

[10] Accordingly, the present invention has been completed by confirming that the treating effect of humanized anti-4-1BB antibody and/or antagonistic anti-CTLA-4 antibody on cancer is caused by increased CD11c+CD8+ T cell resulting in potent inhibiting effect on cancer by the intensification of CTL function and IFN- γ expression and further the effect is reinforced by the inhibition of CTLA-4 signal transfer.

[11] These and other objects of the present invention will become apparent from the detailed disclosure of the present invention provided hereinafter.

[12]

Disclosure of Invention

Technical Problem

[13] According to one aspect of present invention, the present invention provides a pharmaceutical composition comprising humanized anti-4-1BB antibody (HBBK4) for treating or preventing cancer disease through the induction of CD11+CD8+ T cell and IFN- γ expression increase as an effective ingredient, together with a pharmaceutically acceptable carrier.

[14]

[15] The present invention provides an immunotherapeutic method for treating or preventing cancer disease comprising administering to mammal an effective amount of

humanized anti-4-1BB antibody (HBBK4) as an effective ingredient, together with a pharmaceutically acceptable carrier thereof.

[16]

[17] The present invention also provides a use of humanized anti-4-1BB antibody (HBBK4) for the preparation of therapeutic agent for treating or preventing cancer disease in a mammal including human in need thereof.

[18]

Technical Solution

[19] Accordingly, it is an object of the present invention to provide a pharmaceutical composition comprising humanized anti-4-1BB antibody (hereinafter designated as HBBK4) for treating or preventing cancer disease through the induction of CD11+CD8+ T cell increase as an effective ingredient, together with a pharmaceutically acceptable carrier.

[20]

[21] In accordance with another aspect of the present invention, there is also provided a use of humanized anti-4-1BB antibody (HBBK4) for the preparation of therapeutic agent for treating and preventing cancer disease in a mammal including human in need thereof.

[22]

[23] In accordance with the other aspect of the present invention, there is also provided immunotherapeutic method for treating or preventing cancer disease comprising administering to mammal in an effective amount of humanized anti-4-1BB antibody (HBBK4) as an effective ingredient, together with a pharmaceutically acceptable carrier thereof.

[24]

[25] In accordance with the other aspect of the present invention, there is also provided a pharmaceutical composition comprising the combined of humanized anti-4-1BB antibody and anti-CTLA-4 antibody for treating or preventing cancer disease through the synergic inhibition effect on cancer cell proliferation as an effective ingredient, together with a pharmaceutically acceptable carrier.

[26]

[27] In accordance with another aspect of the present invention, there is also provided a use of the combined mixture humanized anti-4-1BB antibody and anti-CTLA-4 antibody for the preparation of therapeutic agent for treating and preventing cancer disease in a mammal including human in need thereof.

[28]

[29] In accordance with the other aspect of the present invention, there is also provided

immunotherapeutic method for treating or preventing cancer disease comprising administering to mammal in an effective amount of the combined mixture humanized anti-4-1BB antibody and anti-CTLA-4 antibody as an effective ingredient, together with a pharmaceutically acceptable carrier thereof.

[30]

[31] The pharmaceutical composition for treating or preventing cancer disease of the present invention could contain about 0.01 to 80 w/w%, preferably 0.1 to 50 w/w% of the above-described humanized anti-4-1BB antibody (HBBK4) of the present invention based on the total weight of the composition

[32]

[33] The term "humanized anti-4-1BB antibody (HBBK4)" disclosed herein can comprises nucleotide sequence of SEQ ID NO. 59, SEQ ID NO. 61 or SEQ ID NO. 63 and also comprises amino acid sequence of SEQ ID NO. 60, SEQ ID NO. 62 or SEQ ID NO. 64.

[34]

[35] The term "cancer disease" disclosed herein comprise lung cancer, arsenic cellular lung cancer, colon cancer, bone cancer, pancreatic cancer, skin cancer, cephalic or cervical cancer, skin or endophthalmic melanoma, hystero carcinoma, ovarian cancer, rectal cancer, stomach cancer, perianal cancer, colonic cancer, breast cancer, endometrioma, cervical carcinoma, vaginal carcinoma, vulvul carcinoma, Hodgkin's disease, esophageal cancer, enteric cancer, endocrine gland cancer, thyroid cancer, parathyroid cancer, adrenal cancer, smooth tissue sarcoma, urethral cancer, penile cancer, prostatic cancer, chronic or acute leukemia, lymphocytoma, cystic cancer, nephritic or hydrouretic cancer, renal cell carcinoma, renal pelvic carcinoma, CNS tumor, primary CNS lymphoma, spinal medulla tumor, brain stem neuroglioma, hypophyseal adenomatosis and the like.

[36]

[37] Hereinafter, the present invention is described in detail.

[38]

[39] For the present invention, the humanized anti-4-1BB antibody (HBBK4) disclosed herein can be prepared by following procedure;

[40]

For example, HBBK4 of the present invention can be prepared by the procedure comprising the steps; preparing agonistic anti-4-1BB antibody (BBK4) using by hybridoma cell line at 1st step; cloning of BBK4 gene at 2nd step; humanizing VH and VL of BBK4 at 3rd step; selecting humanized BBK4 (HBBK4) clone through the function analysis of humanized BBK4 (HBBK4) ScFv at last step to obtain HBBK4 of the present invention.

[41]

[42] Therefore, the inventive HBBK4 prepared by methods described above, can be used as an active ingredient in preparing a pharmaceutical composition to prevent and treat cancer disease.

[43]

[44] Furthermore, combined mixture of HBBK4 prepared by methods described above and anti-CTLA-4 antibody, can be used as an active ingredient in preparing a pharmaceutical composition to prevent and treat cancer disease.

[45]

[46] The inventive composition may additionally comprise appropriate carriers, adjuvants or diluents, conventionally used in the art. The appropriate carriers, adjuvants or diluents is not limited to a specific material, and can be chosen, according to the usage and application method. Appropriate diluents are listed in the written text of Remington's Pharmaceutical Science (Mack Publishing Co., Easton PA).

[47]

[48] The inventive HBBK4 antibody can be used independently or in combination with well-known cancer drugs such as taxol, cyclophosphamide, doxorubicin and the like. .

[49]

[50] Hereinafter, the following formulation methods and excipients are merely exemplary and in no way limit the invention.

[51]

[52] The composition according to the present invention can be provided as a pharmaceutical composition containing pharmaceutically acceptable carriers, adjuvants or diluents, e.g., lactose, dextrose, sucrose, sorbitol, mannitol, xylitol, erythritol, maltitol, starches, acacia rubber, alginate, gelatin, calcium phosphate, calcium silicate, cellulose, methyl cellulose, polyvinyl pyrrolidone, water, methylhydroxy benzoate, propylhydroxy benzoate, talc, magnesium stearate and mineral oil. The formulations may additionally include fillers, anti-agglutinating agents, lubricating agents, wetting agents, flavoring agents, emulsifiers, preservatives and the like. The compositions of the invention may be formulated so as to provide quick, sustained or delayed release of the active ingredient after their administration to a patient by employing any of the procedures well known in the art.

[53]

[54] For example, the composition of the present invention can be dissolved in oils, propylene glycol or other solvents which are commonly used to produce an injection. Suitable examples of the carriers include physiological saline, polyethylene glycol, ethanol, vegetable oils, isopropyl myristate, etc., but are not limited to them. For topical administration, the compounds of the present invention can be formulated in the form of ointments and creams.

[55]

[56] Pharmaceutical formulations containing inventive composition may be prepared in any form, such as oral dosage form (powder, tablet, capsule, soft capsule, aqueous medicine, syrup, elixirs pill, powder, sachet, granule), or topical preparation (cream, ointment, lotion, gel, balm, patch, paste, spray solution, aerosol and the like), or injectable preparation (solution, suspension, emulsion).

[57]

[58] The composition of the present invention in pharmaceutical dosage forms may be used in the form of their pharmaceutically acceptable salts, and also may be used alone or in appropriate association, as well as in combination with other pharmaceutically active compounds.

[59]

[60] The desirable dose of the inventive composition varies depending on the condition and the weight of the subject, severity, drug form, route and period of administration, and may be chosen by those skilled in the art. However, in order to obtain desirable effects, it is generally recommended to administer at the amount ranging 10 μ /kg, preferably, 0.1 to 1000 μ /kg by weight/day of the inventive extract or composition of the present invention. The dose may be administered in single or divided into several times per day.

[61]

[62] In terms of composition, the inventive composition should be present between 0.01 to 80% by weight, preferably 0.5 to 50% by weight based on the total weight of the composition.

[63]

[64] The pharmaceutical composition of present invention can be administered to a subject animal such as mammals (rat, mouse, domestic animals or human) *via* various routes. All modes of administration are contemplated, for example, administration can be made orally, rectally or by intravenous, intramuscular, subcutaneous, intra-cutaneous, intrathecal, epidural or intracerebroventricular injection.

[65]

[66] It will be apparent to those skilled in the art that various modifications and variations can be made in the compositions, use and preparations of the present invention without departing from the spirit or scope of the invention.

[67]

[68] The present invention is more specifically explained by the following figures and examples. However, it should be understood that the present invention is not limited to these examples in any manner.

[69]

Advantageous Effects

[70] The inventive HBBK4 antibody of the present invention showed inhibition effect on cancer cell proliferation caused by increase of CD11c+CD8+ T cell population and IFN- γ expression. Accordingly, it can be useful in the prevention or treatment of cancer diseases without adverse response.

[71]

Brief Description of the Drawings

[72] The above and other objects, features and other advantages of the present invention will more clearly understood from the following detailed description taken in conjunction with the accompanying drawings, in which;

[73]

[74] Fig.1. presents a humanizing design of agonistic anti-4-1BB antibody; Fig.2. presents a primer design for producing humanized ScFv of BBK4;

[75] Fig.3. depicts the purification procedure for purifying humanized BBK4(HBBK4) ScFv using by anti-E tag sepharose;

[76] Fig.4. depicts a flow cytometry analysis of HBBK4;

[77] Fig.5. depicts the result of competitive ELISA method;

[78] Fig.6. depicts the result of western blotting using by H2L2 (lane 1: HBBK4-75/pcDNA transfectant, lane 2: HBBK4-75/pD18 transfectant, lane 3: 293 EBNA-negative control);

[79] Fig.7. shows a nucleotide sequence of HBBK4-75G1;

[80] Fig.8. shows an amino acid sequence of HBBK4-75G1;

[81] Fig.9. shows a nucleotide sequence of HBBK4-75G4;

[82] Fig.10. represents an amino acid sequence of HBBK4-75G4;

[83] Fig.11. represents a nucleotide sequence of HBBK4-75L;

[84] Fig.12. represents an amino acid sequence of HBBK4-75L;

[85] Fig.13. represents the increasing effect of HBBK on the T cell proliferation;

[86] Fig.14. depicts the change of tumor size in multi-therapy of melanoma using by combined mixture of agonistic anti-4-1BB and antagonistic anti-CTLA-4 antibody;

[87] Fig.15. depicts the change survival rate of rat in multi-therapy of melanoma using by combined mixture of agonistic anti-4-1BB and antagonistic anti-CTLA-4 antibody;

[88] Fig.16. depicts the analysis of flowcytometry stained with anti-CD4-FITC+anti-CD8-PE;

[89] Fig.17. depicts the analysis of flowcytometry stained with anti-CD8-FITC+anti-IFN- γ -PE;

[90] Fig.18. depicts the analysis of flowcytometry stained with anti-CD8-FITC+anti-CD11c-PE;

- [91] Fig.19. shows the infiltration of CD11c+CD8+ T cell into tumor tissue resulting from the administration of agonistic anti-4-1BB, of which cells are stained with anti-CD4-FITC+anti-CD8-PE;
- [92] Fig.20. shows the infiltration of CD11c+CD8+ T cell into tumor tissue resulting from the administration of agonistic anti-4-1BB, of which cells are stained with anti-CD8-FITC+anti-CD11c-PE;
- [93] Fig.21. shows the infiltration of CD11c+CD8+ T cell into tumor tissue resulting from the administration of agonistic anti-4-1BB, of which cells are stained with anti-CD3-FITC+anti-NK1.1-PE;
- [94] Fig.22. shows the result of calculating an absolute number of CD4+/CD8+ T cells among the recovered cells from each tumor tissue in flow cytometry analysis,
- [95] Fig.23. shows the level and distribution of gene expression of CD11c+CD8+ T cell.

[96]

Best Mode for Carrying Out the Invention

- [97] It will be apparent to those skilled in the art that various modifications and variations can be made in the composition, use and preparations of the present invention without departing from the spirit or scope of the invention.

[98]

- [99] The present invention is more specifically explained by the following examples. However, it should be understood that the present invention is not limited to these examples in any manner.

[100]

EXAMPLES

- [101]
- [102] The following Example and Experimental Examples are intended to further illustrate the invention without limiting its scope.

[103]

Example 1. Preparation of agonistic anti-human 4-1BB (BBK4)

1-1. Preparation of human 4-1BB protein

- [104]
- [105]
- [106] pGEX3 expression vector was prepared by combining cDNA coding human 4-1BB molecule with DNA (Pharmacia LKB Biotechnology) coding the binding site of glutathion-S-transferase (GST).

- [107] After transforming E. Coli with the above vector, it was cultured under the proper condition to induce the expression of 4-1BB-GST fusion protein, and then 4-1BB-GST fusion protein was obtained by performing further separation and purification using by glutathion sepharose-4B column (Pharmacia LKB Biotechnology).

[108]

[109] **1-2. Immunity**

[110] 20 μ l of 4-1BB-GST and 0.1 μ l of Freund complete adjuvant was mixed and administered into the abdominal cavity of Balb/c mice aged from 4 to 8 weeks to induce minimal immune response. The mixture of 20 μ l of 4-1BB-GST and Freund incomplete adjuvant was administered into the abdominal cavity of Balb/c mice twice at the interval of three weeks to induce immunity.

[111] Three days after the end of immunity, the titer of antibody was determined according to enzyme immuno assay (EIA) by collecting small quantity of blood sample from tail, and the spleen cell obtained by delivered spleen was used to cell fusion when the immune value reached at specific level.

[112]

[113] **1-3. cell fusion**

[114] The spleen cell obtained from immunized mouse was mixed with SP2/0-Ag14 myeloma cell (ATCC CRL 1581) with the mixed ratio of 1:2, and fused by using polyethylene glycol 4000 (Gibco). Following experiment was performed by the procedure disclosed in the literature (Mishell and Shiigi, *Selected Methods in Cellular Immunology*. W. H. Freeman Company, 1980)

[115]

[116] To select hybridoma cells, the fused cell mixture was suspended in HAT medium, i.e., supplemented RPMI medium with 15% Fetal Calf Serum, 0.1mM hypoxanthine, 0.4 μ M aminopterin and 16 μ M thymidine, poured into 96 well micro culture tray in a concentration of 3×10^7 cells/ml and incubated with cell culture supplied with 5% CO₂ gas at 37 °C. Since both of the myeloma cell and other non-fused spleen cell could not grow in HAT medium, only already fused cells had been regarded as being grown in HAT medium. After each 6, 7 and 8 days, half of the culture medium was replaced with new culture medium. The antibody containing supernatant solution was collected from 96 well micro-culture plate when the cells were sufficiently grown with being examined by reverse phase microscope to perform ELISA method.

[117]

[118] **1-4. selection of hybridoma cell line**

[119] The concentration of 4-1BB-GST was adjusted to 200 ng/0.1ml by diluting with carbonate buffer solution (pH 9.6) and it was coated on ELISA plate. The completely coated plates were washed with phosphate buffered saline (PBS) solution and PBS containing 1% FBS albumin was thereto to let alone for 1 hour. The plates were washed with PBS several times and incubated with supernatant solution prepared in example 1-3 for 1 hr. After the incubation, the plates were washed with PBS several times and 0.1 ml of diluted alkaline phosphatase binding goat anti-mouse immunoglobulin (x1000, Southern Biotech) was added thereto. After one hour, the plates

was washed with PBS several times and 0.1ml of substrate solution prepared by dissolving p-nitrophenyl sodium monophosphate in carbonate buffer solution into 1mg/ml (pH 9.6) was added thereto to store at room temperature for 10 mins. Through determining the reaction using by auto-micro plate recorder, the hybridoma cell line producing 4-1BB specific-binding antibody, which is designated as BBK-4 hereinafter.

[120]

[121]

1-5. Preparation of antibody using by hybridoma cell line

[122]

The BBK-4 cell line (1×10^7) prepared in example 1-4 was proliferated in the medium and intraperitoneally administrated into Balb/c mice pretreated with 0.5ml of pristane (2,6,10,14-tetramethylpentadecane, Sigma) to collect ascites fluid. The fluid was subjected to affinity column chromatography using by protein-G sepharose column (Pharmacia) to isolate monoclonal antibody of the present invention of which producing rate of antibody is 1mg/ml ascites fluid.

[123]

At the result of determination using by antibody analysis kit, the antibody of the present invention is determined to IgG1 having isotype of kappa light chain.

[124]

[125]

Example 2. Gene cloning of agonistic anti-human 4-1BB (BBK4)

[126]

2-1. mRNA of BBK-4 clone

[127]

Total RNA was extracted from 2×10^8 cells of BBK-4 clone. The cell pellet was treated with TriPure Isolation Reagent (Sigma) and subjected to extraction with Phenol/chloroform one time. The supernatant was treated with isopropanol to precipitate RNA. The RNA was washed with 75% ethanol twice, dissolved in DEPC'd water to obtain total RNA. The extraction of mRNA was performed by Dynalbeads mRNA purification kit (DYNAL).

[128]

[129]

2-2. cDNA library preparation of BBK-4 clone

[130]

ZAP-cDNA synthesis kit and ZAP-cDNA Gigapack III Gold Cloning (STRATAGENE) were used to prepare cDNA library of BBK-4.

[131]

[132]

2-2-1. First Strand Synthesis

[133]

The extracted mRNA prepared in example 2-1 was used to synthesis first strand of BBK4. The mixture of 5 μ l of mRNA, linker primer, RNase H-reverse transcriptase, methyl nucleotide mixture and RNase inhibitor was reacted at 37 °C for one hour. Linker primer has Xho I site and sequence of SEQ ID NO. 1.

[134]

[135]

2-2-2. Second Strand Synthesis

[136]

The RNase H and DNA polymerase I was used to synthesis second strand and the mixture was reacted at 16 °C for 2.5 hours. After the reaction, the reactant was

subjected to phenol/chloroform extraction and concentrated using by ethanol. The concentrates were washed with 70% ethanol, dissolved in water and the protein was removed by Utrafree-Probind filter (Sigma).

[137]

[138] **2-2-3. Blunting the cDNA Termini**

[139] Both 3'-end and 5'-end of cDNA were blunted with Klenow fragment and dNTP. The protein was removed by Utrafree-Probind filter (Sigma).

[140]

[141] **2-2-4. Ligation EcoRI Adaptors**

[142] Both of rATP and T4 DNA ligase were added thereto. The solution was reacted at 4 °C for 12 hours and heated to inactivate the enzyme at 70 °C for 30 minutes.

[143]

[144] **2-2-5. Kinasing the EcoRI Ends**

[145] Both of rATP and T4 polynucleotide kinase were added thereto. The solution was reacted at 37 °C for 30 minutes and heated to inactivate the enzyme at 70 °C for 30 minutes.

[146]

[147] **2-2-6. Xho I Digestion**

[148] Digestion was performed using by Xho I and the protein was removed by Utrafree-Probind filter (Sigma).

[149]

[150] **2-2-7. Ligating cDNA into Uni-ZAP XR Vector Arms**

[151] The amount of prepared cDNA was determined by using Ethidium bromide. The mixture of 100 ng cDNA and 125 μ Uni-ZAP XR vector was reacted at 12 °C for 12 hours using by rATP and T4 DNA ligase.

[152]

[153] **2-2-8. Packaging**

[154] At the beginning of melting packaging extract which was stored at -80 °C, 2 μ of ligated DNA was added thereto carefully in order not to form bubble and mixed with pipette. The solution was reacted for 2 hours and mixed with 500 μ of SM buffer solution containing 100 mM NaCl, 10 mM $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 50 mM Tris-HCl (pH 7.5) and 0.01 % gelatin and 20 μ of chloroform. The solution was centrifuged and the supernatant was transferred to new tube.

[155]

[156] **2-2-9. Titering the library**

[157] XL-1 Blue MRF was used as a host cell and the single colony prepared by inoculating on LB/tetracycline plate (60 μ /ml) was inoculated into LB medium containing 10mM MgSO_4 and 0.2% maltose. The colony was cultured with vibrating at the speed

200rpm in order not to exceed 1.0 of OD₆₀₀ at 37 °C. The culture was centrifuged for 10 mins at the speed of 500xg to remove the medium and 10mM MgSO₄.7H₂O was added to the cell pellet to the extent the OD₆₀₀ reaches to 0.5. The prepared host cell was stored at 4 °C to let the cell alive for 48 hours. 1 μl of diluted packaged reaction to 1/10 was added to 200 μl of host cell and attached to the host cell at 37 °C for 15 minutes. 3μl of cooled top agar at 48 °C was added thereto, subjected to vortexing and poured to preheated LB agar plate. The cells were grown at 37 °C for 10 hours and the cell number was calculated by counting the number of plaques (pfu/ml). It has been confirmed that final counted cell number was 1.9 x 10⁶ pfu.

[158]

[159] **2-3. Screening of heavy chain gene and kappa chain gene**

[160] The above cells were subjected to plate in order to form about 200-300 plaques per 100mm and transferred to nylon membrane to make UV cross linking. Hybridization was performed using by the membrane. Each completed fragment cloned by PCR was used as a probe for screening heavy chain and kappa chain. ECL direct nucleic acid labeling and detection kit was used to hybridization. The clones were transformed into plasmids through *in vivo* removal system using the Exassit/SOLR and the sequence was determined by sequencing method.

[161]

[162] **Example 3. VH and VL humanization of agonistic anti-human 4-1BB (BBK4)**

[163] **3-1. Selection of human homolog VH and VL of BBK-4 clone**

[164] The similar one among human antibody genes to the V region sequence of clone BBK-4 coding mouse protein was selected through data base search system. The sequence comparing with BBK-4 sequence was used for preparing PCR template and primer necessary to prepare combinatorial antibody library.

[165]

[166] **3-2. Design for humanization of BBK-4 gene**

[167] The CDR and FR part region in heavy and light chain of BBK-4 was determined and the standard to determine whether the region was changed to human amino acid residue or remained not was followed pursuant to the rule disclosed in Table 1.

[168] CDR region, i.e., the directly linked region with antigen among lots of part sequences of H and L chain was used in mouse sequence and canonical region was used in mouse sequence. The sequence positioned at interface between H and L chain was used in mouse sequence although it existed at FR region and specific sequence was used in human sequence. The solvent exposed residue was used in mouse sequence and the humanized nucleotide sequence was determined based on the protocol (See Fig. 1).

[169]

[170] Table 1

Mouse residue	Comments	Decision
CDR	According to Ig Blast(http://www.ncbi.nlm.nih.gov)	Mouse
Canonical residues	Chothia canonical assignment(http://www.cryst.bbk.ac.uk/~ubcg07s/)	Mouse
Interchain packing residues		Mouse
N-glycosylation sites		Human
Unusual framework residues	Any residues within 5-6 Å of any CDR residues	Mouse
	All the other residues	Human
Solvent-exposed residues	Any residues of CDR, canonical, interchain	Mouse
	All the other residues(http://imgt.cines.fr:8104/textes/IMGT repertoire.html)	Human

[171]

[172] **3-3. Primer preparation to make combinatorial antibody library**

[173] To prepare insertion for preparing combinatorial antibody library, the combinatorial primer was prepared based on amino acid sequence obtained by comparing the heavy and light chain sequences of BBK-4 clone with human template. The primer sequences were shown as SEQ ID NO. 2 - SEQ. ID NO. 18 and in Table 2.

[174]

[175] Table 2

primer	Sequence
HB BK4-1U	5'-actgcggcccagccggccatggcccaggtgcagctgcagcagctctggggctgaagtarwaaagcctggggc-3'
HB BK4-1D	5'-agtagctgctgaaggtgtagccagaagccttgcaggaaascctcactgaagcccaggcctttwyacttc-3'
HB BK4-2U	5'-tacaccttcagcagctactggatgcactgggtgargcaggc acctggacaaggccttgagtggattggag-3'
HB BK4-2D	5'-tgctcttgaacttctcattgtagttatgatgac cgttgcaggattaatctctccaatccactcaaggcc-3'
HB BK4-3U	5'-aatgagaagttcaagagcarggyaactmtgactskggacacctctac aagcacagyatacatgsaactca-3'
HB BK4-3D	5'-taaaagatcttgacagtaataagaccgcggwgtcctcagaccgcaggctgctgagttscatgtatctgt-3'
HB BK4-4U	5'-tactgtgcaagatctttactacggcacggcggtttgcttactggggcc aagggacccctcgtgaccgtct-3'
HB BK4-4D	5'-ctgagccggccggccgctgagccggccgcgcctgagccggccggccgctgaggagacggtcacgagggtccc-3'
HB BK4-5U	5'-tcaggcggcggcggtcagacrttgtgatgactcagtcctcagcccttctgtgactccaggagaga-3'
HB BK4-5D	5'-agtgtaaagtagtcgctaatagtctggctggccctgcaagtaakagtcactttctctctggagtcacaga-3'
HB BK4-6U	5'-attagcgactactttacacttggtatcaacaaaaacccgataakctccc aaacttctcatcaaatatgctt-3'
HB BK4-6D	5'-tccttgatccactgccactgaacctggagggaaycccagagatggattgggaagcatatttgatgagaag-3'
HB BK4-7U	5'-agtggcagtgatcagggactgatttctactytastatctcgtcgtcggagcagaagatgytgsgrgt-3'
HB BK4-7D	5'-tagttccttgaccgaaagtgggggaaagctgtgaccatcttgacagtaatacrylicscarcatcttctgc-3'
HB BK4-8D	5'-gagtcattctcgaactgcggccgcttgatctcagtttagttccttgaccgaaagt-3'
HB BK4-SfiI-U	5'- actgcggcccagccggccat-3'
HB BK4-NotI-D	5'- ttagttccttgaccgaaagt-3'

[176]

[177] The PCR for Scfv region amplifying was performed using by primer prepared according to Table 2. The PCR was performed as follows; pre-denaturation at 94 °C for 5 minutes, 1 cycle consists of denaturation at 94 °C for 30 sec, annealing at 59 °C for 1 minute and elongation at 72 °C for 30 second were repeated for 35 and post-elongation at 72 °C for 7 minutes. Resulting PCR products were isolated by QIAGEN Gel extraction kit.

[178]

[179] **3-4. Addition of restriction enzyme site to ligating ScFv**

[180] The restriction enzyme site was shown in Table 3 and as SEQ. ID NO. 19 to SEQ. ID NO. 20.

[181]

[182] Table 3

	Restriction enzyme site
HB BK4 SfiI-U	5'- actgcggcccagccggccat-3'
HB BK4 NotI-D	5'- ttagttccttgaccgaaagt-3'

[183]

[184] The enzyme site was added to 5'-, 3'- ends of ScFv using by HB BK4 SfiI-U and

HBBK4 NotI-D through PCR method and Resulting PCR products were isolated by QIAGEN Gel extraction kit.

[185]

[186] **3-5. Digestion and purification of insert**

[187] To perform Sfi I digestion reaction, 85 μ l of the mixture solution containing 8.5 μ l of 10x buffer solution (Roche), 2 μ l of SfiI (10U/ μ l, Roche), appropriate volume of purified ScFv product (1 μ l) and distilled water was added to 85 μ l of mineral oil and reacted together at 50 °C for 4 hours.

[188]

[189] To perform Not I digestion reaction, 15 μ l of the mixture solution containing 3.6 μ l of 3 M NaCl, 1.5 μ l of 10x buffer solution (Roche), 4 μ l of Not I (10 U/ μ l, Roche) and appropriate volume of distilled water was mixed thoroughly and reacted together at 37 °C for 4 hours. The reacted solution was subjected to phenol/chloroform extraction and isolation with Spin-column purification method.

[190]

[191] **3-6. Quantification of ScFv and ligation to pCANTAB 5E vector**

[192] The amount of ScFv was determined by electrophoresis: 25ng of isolated ScFv were subjected to electrophoresis along with both 12.5 ng and 25 ng ScFv markers, and developed on 0.75 cm width of 1% agarose gel to compare therewith.

[193] The insert was used in further ligation process and 50 μ l of ligation mixture comprising 5 μ l of 10x OPA buffer solution, 5 μ l of pCANTAB 5E (50 ng/ μ l), 5 μ l of 10 mM ATP, 2 μ l of T4 DNA ligase (4 U/ μ l), 50 μ l of distilled water and appropriate volume of BBK-4 ScFv gene fragment (150 ng) was reacted together at 16 °C for 1 hour. The solution was then reacted at 70 °C for 10 minutes to inactivate ligase and cooled in the ice for 5 minutes.

[194]

[195] **3-7. Confirmation of transformation and library size**

[196] The product prepared in above-described ligation reactant was added to 1ml of suitable TG1 cell and left alone in ice for 45 minutes. The mixture was incubated at 42 °C for 2 minutes and cooled in ice. 900 μ l of LBG was added to 100 μ l of the cooled solution and subjected to suspension incubation at the speed of 250 rpm. Those transformed cells were diluted and plating to obtain 6×10^6 cfu of cell library.

[197]

[198] **3-8. Transformation into BBK-4 recombinant phage antibody library**

[199] 900 μ l of the cell library prepared in above-described step was added to 9.1 μ l of 2x YT-G medium and subjected to suspension culture at the speed of 250 rpm at 37 °C for 1 hour. The supernatant was removed by centrifuging at the speed of 1000x g for 10 minutes to precipitate the cells. 10 μ l of 2x YT-AK medium was added to the pre-

precipitated cells and subjected to overnight suspension culture at the speed of 250 rpm at 37 °C. The suspension was centrifuged culture at the speed of 1000 xg for 20 minutes to precipitate the cell again. The supernatant was filtered with 0.45 µ of filter paper and transferred to 50 µ of sterilized conical tube to store at 4 °C until it is subjected to panning.

[200]

[201] **3-9. PEG precipitation of recombinant phage**

[202] 2 µ of PEG/NaCl was added to BBK-4 recombinant phage antibody library, mixed with together thoroughly and incubated in ice for 60 minutes. The suspension was subjected to centrifugation at the speed of 1000 xg at 4 °C for 20 minutes to precipitate the cell and remove the supernatant. 16 µ of 2x YT medium was added to the pre-precipitates and mixed with together. The supernatant was filtered with 0.45 µ of filter paper and subjected to panning.

[203]

[204] **3-10. Panning for selecting antibody-positive recombinant phage antibody**

[205] 4-1BB was diluted to 10 µ/µ with 0.05 M carbonate buffer solution (pH 9.6) and the solution was coated on 25 flasks at room temperature for 1 or 2 hours. The flask was washed with PBS solution three times, filled with blocking buffer solution to store at room temperature for one hour and washed again with PBS three times. 16 µ of pre-precipitated recombinant phage with PEG was diluted with 14 µ of blocking buffer solution containing 0.01% sodium azide and reacted at room temperature for 15 minutes. 20 µ of diluted recombinant phage was poured to flask and reacted at 37 °C for 2 hours. The flask was washed with 50 µ of PBS solution 20 times and washed again with 50 µ of 0.1% PBS-T solution 20 times.

[206] 10 µ of grown-staged TG1 cell was poured in flask and incubated at 37 °C for 1 hour. 100 µ of the incubates was diluted to various concentration i.e., 1:10, 1:100, 1:1000 and subjected to plating on SOBAG plates by 100 µ per plate. The flask was subjected to overnight culture at 30 °C and the cfu of each plate was estimated. It has been confirmed that the library size was 9×10^4 cfu.

[207]

[208] **3-11. Selection of monoclonal for screening**

[209] 880 colonies obtained after panning was inoculated into 96 well plates containing 200 µ of 2X YT-AG medium and subjected to overnight culture at the speed of 250 rpm at 30 °C. 50 µ of 2x YT-AG medium containing M13K07 (2.5×10^9 pfu) was distributed to new 96 well plates for 200 µ/well plate. The overnight incubates was inoculated into the plates by 40 µ/plate. The inoculated plates were subjected to suspension culture medium culture at the speed of 250 rpm at 37 °C and centrifugation at the speed of 1500 xg for 20 minutes. The supernatant was further screened by ELISA method.

[210]

[211] **3-12. Screening through ELISA method**

[212] 4-1BB was diluted with 0.05 M carbonate buffer solution (pH 9.6) to 2 μ g/ml and coated on ELISA plate by 100 μ l/plate at room temperature for 1-2 hour. The plates were washed with PBS containing 0.01% tween 20 (PBS-T) three times. Each 200 μ l of blocking buffer solution was added to each well, reacted at room temperature for one hour and the plate was washed with PBS-T buffer solution three times. 100 μ l of recombinant phage antibody supernatant was added to the prepared plates and the equivalent amount of blocking buffer solution was mixed therewith to react at room temperature for 30 minutes. 200 μ l of diluted recombinant phage antibody supernatant was added to antigen-coated wells and reacted at room temperature for 1 hour. After the reaction, the plates were washed with 0.01% PBS-T solution five times and diluted HRP/anti-M13 monoclonal conjugate with blocking buffer solution to 1:5000 by 200 μ l/well to react at room temperature for 1 hour. The plates were washed with 0.01% PBS-T solution six times and 1X ABTS solution was added to each well by 200 μ l/well to react at room temperature for 20 minutes. The clone showing more than 0.5 of absorbance has been regarded as positive clones through determining test of absorbance at 405 nm.

[213]

[214] At the result of the screening, 162 positive clones were obtained and the nucleotide sequence of each clone was identified by sequencing analysis. Through the examination of overlapped sequences among the sequence, the sequences were divided into 54 groups having different sequence.

[215]

[216] **Example 4. Selection of HBBK4 clone through the functional analysis on humanized BBK4 (HBBK4) ScFv**

[217]

[218] **4-1. Purification of BBK4 ScFv**

[219] Each ScFv was purified using anti-E-Taq Sepharose column and the result was shown in Fig. 3.

[220]

[221] **4-2. Flow cytometry using HBBK4 ScFv**

[222] After the HBBK4 ScFv clones determined by ELISA screening, the clones were treated to Jurkat 8-1 cells expressing human 4-1BB to analyze the flow cytometry and the binding affinity of HBBK4 with human 4-1BB was identified. 2 μ g of HBBK4 was added to 2×10^5 Jurkat 8-1 cells, reserved at 4 °C for 30 minutes and washed with PBS solution containing 0.1% BSA. Anti-human IgG-FITC was treated with secondary antibody to detect HBBK4. The stained cells were analyzed by flow cytometry

analyzer (FACScan; BD Bioscience). Fig. 4 showed that several clones among HBBK4 clones recognize human 4-1BB similar to mouse BBK4.

[223]

[224] **4-3. Competitive ELISA**

[225] To identify each clone has original epitope such as mouse BBK4, following competitive ELISA adopted 4-1BB-GST as an antigen and the decreased ratio of HBBK4 ScFv binding affinity with the increased concentration of mouse BBK4 antibody prior to humanization was showed by the inhibition percentage represented by following Math Figure 1.

[226]

[227] MathFigure 1

$$InhibitionRatio(\%) = \frac{100 \times (non-competitive OD_{405} - competitive OD_{405})}{non-competitive OD_{405}}$$

[228]

[229] As shown in Fig. 5, the inhibition ratio (%) was increased with the concentration of mouse BBK4 in a dose dependent manner therefore it has been proved that HBBK4 ScFv has similar epitope to that in original antibody, which strongly suggested that HBBK4 ScFv shows similar biological effect to that in mouse BBK4.

[230]

[231] **4-4. Linkage to constant region of humanized ScFv**

[232] HBBK4 ScFv has VH, linker and VL regions linked together. To reform HBBK4 ScFv to original structure of antibody, i.e., H2L2 structure, leader sequence was linked to the VH region of BBK4 and the constant region of human IgG1 or IgG4 was linked thereto respectively. Both leader sequence and human kappa chain sequence were linked to kappa chain and the linkage of each fragment was performed through PCR method.

[233]

[234] **4-5. ELISA using by H2L2**

[235] The completely humanized sequence to constant region was subcloned to pcDNA, a eukaryote expressing vectors, transfected into 293EBNA cells and serial analyses i.e., ELISA and western blotting analysis were performed using by the cell medium. 4-1BB-GST was used as an antigen for ELISA and each 50 numbers of transfectant medium was used therein. It has been confirmed that clone 75 showed highest expression rate among 50 clones which showed about 15 times higher than that of negative control as can be seen the result of ELISA method in Table 4.

[236]

[237] Table 4

Sample	Absorbance
HBBK4-75/pcDNA transfected 293 EBNA cultured supernatant	1.935
293 EBNA cultured supernatant	0.126

[238]

[239] Western blotting was performed using the antibody produced by selected clone 75 and it has been confirmed that completely humanized antibody HBBK4-75 clone showed potent affinity with human 4-1BB as shown in Fig. 6.

[240]

[241] **Example 5. Humanization of agonistic anti-human 4-1BB (BBK4).**

[242] To apply the cancer treating method using by agonistic anti-4-1BB antibody to human body, the humanized antibody which minimize the adverse activity of antibody itself is necessary. The human antibody mostly similar to the heavy chain/light chain amino acids sequence of mouse BBK4 clone, namely agonistic anti-human 4-1BB antibody was screened and compared to perform humanization. Finally selected humanized agonistic anti-human 4-1BB antibody (HBBK4) was fused with two types of constant region, i.e., human IgG or IgG 4, which is designated as HBBK4-75G1 and HBBK4-75G4 respectively. The nucleotide sequence and amino acid sequence of finally synthesized HBBK4 antibody were shown in Fig 7-12 and SEQ. ID NO. 59 to SEQ. ID NO. 64.

[243]

[244] **Reference Example 1. Animal model test using agonistic anti-mouse 4-1BB antibody.**

[245]

[246] **1-1. Antibody**

[247] Both of agonistic anti-rat 4-1BB and antagonistic anti-CTLA-4 antibody were isolated from rat ascites with protein G-column. Rat IgG used as a positive control was purchased from Sigma Co. and other antibodies used in cytometry analysis, i.e., anti-CD8-PE (53-6.7), anti-CD4-FITC (GK1.5), anti-CD8-FITC (53-6.7), anti-IFN- γ -PE (XMG1.2), anti-CD11c-PE (HL3), anti-NK1.1-PE (PK136) were from BD PharMingen Co.

[248]

[249] **1-2. Animal model and Antibody treatment**

[250] B16-F10 melanoma cancer cell line, a B16 melanoma mutant firstly reported by Hart (Francia G et al., *Cancer Res.* 56, 3855-3858, 1996) shows low antigenecity. 4×10^5 B16-F10 melanoma cells were subcutaneously injected to the back of mice to occur tumor. 200 μ l of each agonistic anti-4-1BB (3H3 clone) and antagonistic anti-CTLA-4

antibody (9H10 clone) was injected into the rats with intraperitoneal administration of cancer cell line at five times per every 4 days for together or respectively. The size of tumor was determined at every 4 days according to the following Math Figure 2 and the survival rate of tumor-bearing mice was observed everyday.

[251]

[252] MathFigure 2

$$TheSizeOfTumor = (width \times length \times height) \times \left(\frac{\pi}{6} \right)$$

[253]

[254] **1-3. Isolation of infiltrated immune cells from tumor tissue**

[255] The tumor tissue was isolated from agonistic anti-4-1BB, antagonistic anti-CTLA-4 or rat IgG-treated mice 18 days after the treatment. The isolated tumor tissue was mixed with HBSS to form single cell suspension and subjected to centrifugation by loading on upper surface of 63%/36% percoll gradient at the speed of 500xg at 45 minutes. After the centrifugation, white immune cell formed between 63% and 36% percoll was recovered and washed with RPMI 1640 twice to be used in following experiment.

[256]

[257] **1-4. Cell staining for flow cytometry**

[258] 1 μ l of 2.4G2 Fc blocker was added to the isolated immune cells from draining lymph node or tumor tissue and stored at 4 °C for 10 minutes. The cells were stained with anti-CD4-FITC+anti-CD8-PE and anti-CD8-FITC+anti-CD11c-PE, washed with PBS containing 0.1% BSA and analyzed with FACScan (BD Bioscience). The cells were surface-stained with anti-CD8-FITC for 30 minutes, fixed and permeabilized with 500 μ l of Cytofix/Perm (BD PharMingen) for 20 minutes at room temperature, and washed with PermWash buffer (BD PharMingen) containing saponin. Above cells were intracellularly stained with anti-IFN- γ -PE for 30 minutes and analyzed with FACScan (BD Bioscience).

[259]

[260] **1-5. Counting the number of infiltrated CD4+ and CD8+ T cell in tumor tissue**

[261] As described in the above, the infiltrated immune cells in tumor tissue were isolated using percoll and the cell number was counted by hemacytometer. The number of infiltrated CD4+ and CD8+ T cells in tumor tissue was calculated by following Math Figure 3.

[262]

[263] MathFigure 3

$$NmberOfCD4+andCD8+TcellInTumorTissue =$$

[264]

$$\frac{(\text{Number Of Total Immune Cell Infiltrated In Tumor Cell}) \times \text{Ratio Of CD4+ or CD8+ T cell}(\%) }{100}$$

[265]

[266] **1-6. Isolation of CD8c+CD11c+ T cell**

[267] CD8+ T cell and CD11c+CD8+ T cell were isolated from the delivered draining lymph node from arthritis-induced mice treated with agonistic anti-4-1BB or rat IgG respectively and the cells were isolated by MACS magnetic isolation system. The cells were incubated with suitable microbead-conjugated antibody to remove CD4+, F4/80+, CD40+, B220+ and DX5+, and further incubated with CD8-microbeads to isolate CD8+ T cell. The isolated CD8+ T cells were treated with CD11c-microbead and both of CD8+CD11c+ and CD8+CD11c- were further isolated therefrom using by LS isolation column (Miltenyi Biotech. Co. Ltd). Each isolated cell showed high purity of 96-98%.

[268]

[269] **1-7. Microarray and RT-PCR**

[270] CD8+ T, CD11c-CD8+ T, and CD11c+CD8+ T cells were isolated from the lymph node of B16-F10 tumor model 15 days after the tumor injection, and total RNA was extracted from each cells.

[271] The extracted RNA was synthesized to cDNA using by in vitro reverse transcriptase and PCR was performed for each 0.5 μM primer using by 0.5 μ of cDNA. The expression level of each gene was compared using by gene-specific primers disclosed in Table 5, and SEQ. ID 21 to SEQ. ID 58.

[272]

[273] Table 5

Name	Orientaion	Primer	Name	Orientaion	Primer
AnnexinA4	Sense	aatcaaccagacataaccagc	Tim-3	Sense	atccagcagataccagctaa
	Anti-sense	tcttcaagcttcagatgt		Anti-sense	tccattgttattggaggg
Klrg1	Sense	ctttgcaatggtggcttt	IL-4 ig 1	Sense	gtatcttcactttccgggat
	Anti-sense	ctccagccatcaatgttc		Anti-sense	gaggtagaagaagccctcc
CD68 Ag	Sense	gcatactctgtttgaatccc	pleckstrin	Sense	actgaatctggagaaggaca
	Anti-sense	ccttagagagagcaggtcaa		Anti-sense	ttcagtaaacatc cctgctt
THRI13	Sense	gtgatgaccaccgtactctt	TTP2	Sense	aaagcaaagcaaatgaagag
	Anti-sense	gcattgactactcggatagc		Anti-sense	tcagtggaggaatggtaatc
NKG2	Sense	cgattcaccctaacacatt	LAG3	Sense	gtctccatcacgtacaacct
	Anti-sense	gctggaattttgagacaaac		Anti-sense	cacaaatcttctcttcag
TGF- β 1	Sense	ttgacgtcactggagttgta	Klrg2-A1	Sense	tctccagagaaactcattg
	Anti-sense	aatagttggtatccagggtc		Anti-sense	tacagtitttgaaatgcag
PRP1	Sense	cagcattaccacaagaatga	CD27	Sense	gctgaatctcacagttcctc
	Anti-sense	cccacattccagaagattta		Anti-sense	ccagtgtcacctggatag
IL-1R II	Sense	cgatgcaggctattacagat	Perforin	Sense	gtcacgtcgaagtacttg
	Anti-sense	atcaaaaaatcagcgacactt		Anti-sense	atggctgatagcctgtct
Granzyme B	Sense	cccaggcgcgaatgcta	IFN- γ	Sense	aacgtcacacactgcac
	Anti-sense	ccaggataagaaactcga		Anti-sense	gccgtggcagtaacagcc
GAPDH	Sense	gaacgggaagcttgcat			
	Anti-sense	ctaagcagttggtgtgc			

[274]

[275] **Experimental Example 1. Characteristic of humanized agonistic anti-human 4-1BB (HBBK4).**

[276] To identify the characteristic of HBBK4 antibody, T cell was isolated from human peripheral blood. The isolated cells were treated with anti-CD3 mAb (OKT3) to induce T cell activation and humanized anti-4-1BB antibody (HBBK4-75) in a dose dependent manner to determine the proliferation rate of the cell. The activity of T cell induced by OKT3 was increased in a dose dependent manner whereas the groups treated with HBBK4-75 or anti-CD28 antibody only did not induce T cell activation. (Fig. 7).

[277] Accordingly, it has been confirmed that finally prepared humanized anti-human 4-1BB antibody, HBBK4-75 recognizes as well as co-stimulates human 4-1BB as an agonistic antibody.

[278]

[279] **Experimental Example 2. Combined cancer therapy using agonistic anti-4-1BB and antagonistic anti-CTLA-4 antibody.**

- [280] To predict the treating activity of agonistic HBBK4 in Clinical study, animal model test using B16-F10 melanoma tumor cell injected animal model performed using by rat agonistic anti-4-1BB antibody together with antagonistic anti-CTLA-4 antibody well known to show potent anti-cancer activity to maximize their therapeutic efficacy and enhance T cell response.
- [281] To determine each and combined anti-cancer therapeutic activity of agonistic anti-4-1BB and/or antagonistic anti-CTLA-4 antibody, 4×10^5 B16-F10 melanoma cells were subcutaneously injected into the back of six-weeks old C57BL/6 mice together with 200 μ l of each antibody five times every four days and rat IgG antibody was treated as a control group. The tumor size in treatment group with only agonistic anti-4-1BB antibody was a rather smaller than that of control group and that in treatment group with only antagonistic anti-CTLA-4 antibody was similar to that in control group. On the contrary, the group treated with both of them showed slow cancer growth till 20th days after the test and the size of tumor was decreased slowly after then (Fig. 14). At 35th day after the injection of tumor cells, the survival rate of each group showed 80% in treatment group with agonistic anti-4-1BB antibody, 40% in treatment group with antagonistic anti-CTLA-4 antibody, 100% in the treatment with both of them while the lethal rate in control group showed 100% in 30th day (Fig. 15).
- [282]
- [283] **Experimental Example 3. CD8+ T cell increase and CD11c+CD8+ T cell production stimulated by 4-1BB.**
- [284] To analyze the combined treating activity of agonistic anti-4-1BB and antagonistic anti-CTLA-4 antibody, inguinal LN, i.e., draining lymph node was isolated from the mice in each group to investigate the proportion of each cell population. The ratio of CD8+ T cells necessary in removing tumor cell were increased to twice and the IFN- γ expression rate of CD8+ T cells were increased to more than five times when agonistic anti-4-1BB antibody was treated (Fig. 16 and Fig. 17). Both of the ratio of CD8+ T cells and IFN- γ expression rate showed no significant difference from control group when antagonistic anti-CTLA-4 antibody was treated, however both of CD4+ and CD8+ T cell showed increasing tendency (Fig. 16 and 17). The number of CD8+ T cells in treatment group with both of them were increased with similar to that in treatment group with anti-4-1BB, however the IFN- γ expression rate of CD8+ T cell was sharply increased to the extent about 30% among total CD8+ T cells expressed IFN- γ (Fig. 16 and Fig. 17).
- [285] There has been reported that the administration of agonistic anti-4-1BB antibody could treat and inhibit collagen induced arthritis (CIA) disease in mice, of which activity is mediated by CD11c+CD8+ T cell (Seo SK et al., *Nat. Med.*, 10, pp1088-1094, 2004). Based on those result, the present inventors have studied to

confirm whether the cancer cell therapy using by agonistic anti-4-1BB antibody produces the same cell population or not and finally confirmed the presence of CD11c+CD8+ T cell by staining the isolated cells from draining lymph node with fluorescence-conjugated anti-CD11c and anti-CD8 antibody. Identical cell groups were formed when anti-4-1BB antibody was administrated in animal tumor model and 36% of CD8+ cells were CD11c+CD8+ T cells. The ratio of identical cell groups was further increased to the extent that 50% CD8+ T cell expressed CD11c in case of combined therapy (Fig. 18).

[286]

[287] Those results showed that the treatment of agonistic anti-4-1BB antibody increased population of CD8+ T cells and expression of IFN- γ as well as anti-CTLA-4 antibody maximized those activity more. Additionally, it is suggested that CD11c+CD8+ T cell is involved in the cancer therapy using anti-4-1BB antibody.

[288]

[289] **Experimental Example 4. Infiltration of CD11c+CD8+ T cell into tumor by agonistic anti-4-1BB.**

[290] To confirm whether the increase of CD8+ T cells and IFN- γ as well as the formation of CD11c+CD8+T cells are involved with the proliferation of cancer cell directly or not, the proportion and characteristic of each cell group were analyzed by isolating immune cell from tumor. The B16-F10 melanoma cells together with 200 μ l of each antibody, i.e., rat IgG, anti-4-1BB, anti-CTLA-4 or anti-4-1BB + anti-CTLA-4 were injected subcutaneously into the rats every four days. The tumor isolated at 18th day after the injection of cancer cells was centrifuged by 36%/63% percoll. After investigation of the ratio of CD4+/CD8+ T cell among the cells, less than 5% CD4+/CD8+ T cells presented in control group treated with only rat IgG while the infiltration ratio of CD8+ T cells and CD4+ T cells in test group treated with anti-4-1BB were increased to 62% and 13%. The treatment group with antagonistic anti-CTLA-4 antibody showed similar level to that of control group and the treatment group with both of them showed similar level to that in treatment group with anti-4-1BB antibody (Fig. 19). It has been confirmed that the CD11c+CD8+ T cell found in draining lymph node was also found in tumor and more than 90% CD8+ T cell infiltrated into tumor was CD11c+CD8+ T cell (Fig. 20). NK cell was also involved with cancer cell removal.

[291] Additionally, we have studied to determine the ratio of NK cell in tumor to confirm whether those cell groups are involved in the cancer inhibiting process by agonistic anti-4-1BB antibody or not and found that the increase of NK cell in any test groups did not show (Fig. 21).

[292] The number of cells infiltrated into tumor in treatment groups with agonistic anti-

4-1BB and both of them was increased to about twice and 2.5 times respectively (Fig. 22). The number of CD8+ T cell in treatment group with anti-4-1BB and both of them was increased to about 10 times and 15 times respectively when the number of CD4+/CD8+ T cell in tumor was calculated from the flow cytometry analysis. The number of CD4+ T cell in treatment group with anti-4-1BB was a rather increased whereas that in treatment group with both of them was increased to 4 times (Fig. 22).

[293]

[294] Those results suggested that the treatment of agonistic anti-4-1BB antibody led to the differentiation and proliferation of CD11c+CD8+ T cells, and the cells were infiltrated into tumor and caused the inhibition of cancer cell proliferation. Anti-CTLA-4 antibody maximizes the anti-cancer potency of anti-4-1BB antibody and NK cell was not involved with those processes.

[295]

[296] **Experimental Example 5. Gene expression distribution of CD11c+CD8+ T cell.**

[297] To analyze the characteristics of CD11c+CD8+ T cell formed by agonistic anti-4-1BB antibody and confirm which gene expression effects on cancer cell proliferation. The gene expression of CD11c+CD8+ T cell and CD8+ T cell of anti-4-1BB- or rat IgG-treated mice was compared. There have been reported that the treatment of agonistic anti-4-1BB results in inhibiting autoimmune disease and the treatment activity is mediated by CD11c+CD8+ T cell. Accordingly, DNA array was performed using by the cells isolated from CIA (Collagen induced arthritis) model, i.e., CD11c+CD8+ T cell in anti-4-1BB antibody-treated mice and CD8+ T cell in rat IgG-treated mice and the gene expression of CD11c+CD8+ T cell formed in cancer model was determined based on such result.

[298] Collagen was administrated to mice induce arthritis and 200 μ l of each rat IgG or anti-4-1BB antibody was intraperitoneally injected five times every two days simultaneously. The draining lymph nodes of each mice, i.e., inguinal and iliac LN were delivered to isolate CD11c+CD8+ T cell in anti-4-1BB antibody-treated mice and CD8+ T cell in rat IgG-treated mice at 14th day after final administration of antibody. RNA was extracted from such cells and the gene expression difference between two cells was analyzed using by Affimatrix microarray system. CD11c+CD8+ T cell formed by 4-1BB stimulus showed high expression at about 220 gene and the gene expression involved with T cell effector function, apoptosis, anti-apoptosis and cell adhesion and migration in CD11c+CD8+ T cell was increased as shown in Table 6.

[299]

[300] Collagen was administrated to rats induce arthritis and 200 μ l of each rat IgG or anti-4-1BB antibody was intraperitoneally injected five times every two days simul-

taneously. The draining lymph nodes of each rat, i.e., inguinal and iliac LN was delivered to isolate CD11c+CD8+ T cell in anti-4-1BB antibody treated rat and CD8+ T cell in rat IgG treated rat CD8+ T cell at 12th day after collagen immunization. RNA was extracted from such cells and the gene expression difference between two cells was analyzed using by Affimatrix microarray system. The genes showing relatively high expression among the gene expression involved with T cell effector function, apoptosis, anti-apoptosis and cell adhesion and migration in CD11c+CD8+ T cell was shown in Table 6.

[301]

[302] Table 6

gene	fold	gene	fold
T cell effector functions		hyaluronan receptor RHAMM V5	1.34
annexin A4	3.2	lymphocyte-activation gene 3	1.25
interleukin-four induced gene 1	2.87	B lymphocyte induced maturation protein	1.25
Scyb9	2.78	Mac-2 antigen	1.2
small inducible cytokine A3	2.66	lectin, galactose binding soluble 1	1.19
Klrg1	2.47	CD9 antigen	1.16
interleukin 10 receptor, alpha	2.45	RHAMM	1.15
T cell immunoglobulin mucin-3	2.38	CD38 antigen	1.13
peptidoglycan recognition protein	2.32	B-cell CLL lymphoma 7C	1.05
interferon g	2.3		
CD68 antigen	2.3	Apoptosis	
natural killer cell protein group 2-A1	2.27	Fas antigen ligand	3.03
pleckstrin	2.12	calseculin	2.76
annexin A2	2.07	granzyme K	2.58
MHC class II antigen IE alpha (H2-Ea)	2.05	granzyme B	2.2
CD27L	2	Caspase 3	1.93
natural killer cell protein group 2-A2	1.95	hemopoietic-specific early-response protein	1.84
MHC class II H2-IA-beta chain	1.94	programmed cell death 1	1.53
NKG2	1.92	caspase 1	1.42
Ctla2a	1.88		
Ctla2b	1.87	Anti-apoptosis	
MHC class II H2-IA-beta chain	1.84	serine protease inhibitor 12	2.07
4-1BB	1.77	serine protease inhibitor 6	1.92
pleckstrin 1 small isoform	1.76	baculoviral IAP repeat-containing 5	1.66
transmembrane 7 superfamily member 1	1.72	cystatin C	1.62
neurocalcin delta	1.68		
metallopeptidase	1.67	Cell adhesion and migration	
lymphotactin	1.6	CD11c	2.67
thyroid hormone receptor interactor 13	1.55	MCP-1	2.49
C1qc	1.49	chemokine (C-C) receptor 2	2.38
interleukin 1 receptor, type II	1.44	C-C chemokine receptor 5	2.07
CD74	1.4	MIP-1 alpha receptor	2.03
CD38 antigen	1.38	catenin alpha 1	1.23
serine/threonine kinase 5	1.37	chemokine-like factor super family 7	1.09
Bcal	1.35	CD166	1.07

[303]

[304] Simultaneously with the injection of B16-F16 melanoma cell line, 200 μ l of anti-4-1BB or rat IgG was injected to the mice five times every two days and the draining lymph nodes of each mice, i.e., inguinal LN was delivered to isolate CD11c+CD8+ T cell and CD8+ T cell at 4th day after final administration of antibody. RNA was extracted from the isolated cells to synthesize cDNA and the increased gene expression shown in Table 6 was analyzed. The gene expression of Tim3, IL4, IL-1R II, THRI13, LAG3, pleckstrin, TIP2, K1rg2-A1, PRP1, Annexin A4, TGF- β , K1rg1, CD68Ag, NKG2, IFN- γ , perforin, granzyme B was determined by RT-PCR and most of gene expression excluding PRP1 and perforin was found to be increased (Fig. 23). Those results indicated there were no significant differences of CD11c+CD8+ T cells formed by anti-4-1BB treatment in between the autoimmune disease model and the cancer model. CD11c+CD8+ T cells isolated from cancer model showed high level of gene expression in respect to IFN- γ and granzyme B necessary in removal of cancer cell, which showed that those cell groups are effective in cancer cell removal. The expression of K1rg1 showing differentiated status reinforced with CTL effector function rather than T cell proliferation showed high level in CD11c+CD8+ T cell and besides the K1rg1, the expression of annexin A4 involved in anti-apoptosis in T cell, CD27 and THRI13, a co-stimulating molecules of T cell, Tim-3 gene and LAG-3 gene involved with the regulation of T cell function were found to be highly increased. Those results suggest that CD11c+CD8+ T cells are not a cell showing CTL function simply but a cell which could control immune response.

[305]

[306] Hereinafter, the formulating methods and kinds of excipients will be described, but the present invention is not limited to them. The representative preparation examples were described as follows.

[307]

[308] Preparation of injection

[309] HBBK4 100mg

[310] Sodium metabisulfite 3.0mg

[311] Methyl paraben 0.8mg

[312] Propyl paraben 0.1mg

[313] Distilled water for injection optimum amount

[314] Injection preparation was prepared by dissolving active component, controlling pH to about 7.5 and then filling all the components in 2 μ l ampule and sterilizing by conventional injection preparation method.

[315]

[316] Preparation of powder

[317] HBBK4 500mg
[318] Corn Starch 100mg
[319] Lactose 100mg
[320] Talc 10mg
[321] Powder preparation was prepared by mixing above components and filling sealed package.

[322]

[323] Preparation of tablet

[324] HBBK4 200mg
[325] Corn Starch 100mg
[326] Lactose 100mg
[327] Magnesium stearate optimum amount
[328] Tablet preparation was prepared by mixing above components and entabletting.

[329]

[330] Preparation of capsule

[331] HBBK4 100mg
[332] Lactose 50mg
[333] Corn starch 50mg
[334] Talc 2mg
[335] Magnesium stearate optimum amount
[336] Tablet preparation was prepared by mixing above components and filling gelatin capsule by conventional gelatin preparation method.

[337]

[338] Preparation of liquid

[339] HBBK4 1000mg
[340] Sugar 20g
[341] Polysaccharide 20g
[342] Lemon flavor 20g
[343] Liquid preparation was prepared by dissolving active component, and then filling all the components in 1000ml ample and sterilizing by conventional liquid preparation method.

[344]

[345] The invention being thus described, it will be obvious that the same may be varied in many ways. Such variations are not to be regarded as a departure from the spirit and scope of the present invention, and all such modifications as would be obvious to one skilled in the art are intended to be included within the scope of the following claims.

[346]

Industrial Applicability

- [347] As described in the present invention, the composition comprising inventive HBBK4 antibody of the present invention showed inhibition effect on cancer cell proliferation caused by increase of CD11c+CD8+ T cell population and IFN- γ expression. Accordingly, it can be useful in the prevention or treatment of cancer diseases and in providing an immune therapy of cancer disease without adverse response.

[348]

Sequence Listing

- [349] SEQ ID NO. 1: 5'-CTCGAGTTTT TTTTTTTT-3' is Linker primer containing XhoI site, SEQ ID NO. 2: 5'-CTG CAG CAC GGT ATA GGT GA-3' is HBBK4-1U which is BBK4 ScFv combinational primer, SEQ ID NO. 3: 5'-AGTAGCTGCT GAAGGTGTAG CCAGAAGCCT TGCAGGAAAS CTTCCTGAA GCCCCAGGCT TTWYTA CTTC-3' is HBBK4-1D which is BBK4 ScFv combinational primer, SEQ ID NO. 4: 5'-TACACCTTCA GCAGCTACTG GATGCACTGG GTGARGCAGG CACCTGGACA AGGCCTTGAG TGGATTGGA-3' is HBBK4-2U which is BBK4 ScFv combinational primer, SEQ ID NO. 5: 5'-TGCTCTTGAA CTTCTCATTG TAGTTAGTAT GACCGTTGCC AGGATTAATC TCTCCAATCC ACTCAAGGCC-3' is HBBK4-2D which is BBK4 ScFv combinational primer, SEQ ID NO. 6: 5'-AATGAGAAGT TCAAGAGCAR GGYAACTMTG ACTSKGGACA CCTCTACAAG CACAGYATAC ATGSAACTCA-3' is HBBK4-3U which is BBK4 ScFv combinational primer, SEQ ID NO. 7: 5'-TAAAAGATCT TGCACAGTAA TAGACCGCGG WGTCTCAGA CCGCAGGCTG CTGAGTTSCA TGTATRCTGT-3' is HBBK4-3D which is BBK4 ScFv combinational primer, SEQ ID NO. 8: 5'-TACTGTGCAA GATCTTTTAC TACGGCACGG GCGTTTGCTT ACTGGGGCCA AGGGACCCTC GTGACCGTCT-3' is HBBK4-4U which is BBK4 ScFv combinational primer, SEQ ID NO. 9: 5'-CTGAGCCGCC GCCGCCTGAG CCGCCGCCGC CTGAGCCGCC GCCGCCTGAG GAGACGGTCA CGAGGGTCCC-3' is HBBK4-4D which is BBK4 ScFv combinational primer, SEQ ID NO. 10: 5'-TCAGGCGGCG GCGGCTCAGA CRTTGATG ACTCAGTCTC CAGCCTTCTT ATCTGTGACT CCAGGAGAG-3' is HBBK4-5U which is BBK4 ScFv combinational primer, SEQ ID NO. 11: 5'-AGTGTAAGTA GTCGCTAATA GTCTGGCTGG CCCTGCAAGT AAKAGTCACT TTCTCTCCTG GAGTCACAGA-3' is HBBK4-5D which is BBK4 ScFv combinational primer, SEQ ID NO. 12: 5'-ATTAGCGACT ACTTACACTG GTATCAACAA AAACCCGATC AAKCTCCCAA ACTTCTCATC AAATATGCTT-3' is HBBK4-6U which is BBK4 ScFv combinational primer, SEQ ID NO. 13: 5'-TCCCTGATCC ACTGCCACTG AACCTGGAGG GAAYCCCAGA

GATGGATTGG GAAGCATATT TGATGAGAAG-3' is HBBK4-6D which is BBK4 ScFv combinational primer, SEQ ID NO. 14: 5'-AGTGGCAGTG GATCAGGGAC TGATTTCACT YTTASTATCT CGTCGSTCGA GGCAGAAAGAT GYTGSGRYGT-3' is HBBK4-7U which is BBK4 ScFv combinational primer, SEQ ID NO. 15: 5'-TAGTTCCTTG ACCGAAAGTT GGGGGAAAGC TGTGACCATC TTGACAGTAA TACRYCSCAR CATCTTCTGC-3' is HBBK4-7D which is BBK4 ScFv combinational primer, SEQ ID NO. 16: 5'-GAGTCATTCT CGACTTGCGG CCGCTTTGAT CTCGAGTTTA GTTCCTTGAC CGAAAGT-3' is HBBK4-8D which is BBK4 ScFv combinational primer, SEQ ID NO. 17: 5'-ACTGCGGCCC AGCCGGCCAT-3' is HBBK4 SfiI-U which is BBK4 ScFv combinational primer, SEQ ID NO. 18: 5'-TTAGTTCCTT GACCGAAAGT-3' is HBBK4 NotI-D which is BBK4 ScFv combinational primer, SEQ ID NO. 19: 5'-ACTGCGGCCC AGCCGGCCAT-3' is recognition site for restriction enzyme SfiI, SEQ ID NO. 20: 5'-TTAGTTCCTT GACCGAAAGT-3' recognition site for restriction enzyme NotI, SEQ ID NO. 21: 5'-AATCAACCAG ACATACCAGC-3' is forward primer for Annexin4, SEQ ID NO. 22: 5'-TCTTCAAAGC TTCCAGATGT-3' is reverse primer for Annexin4, SEQ ID NO. 23: 5'-CTTTGCAATG GTGGCTTT-3' is forward primer for Klrg1, SEQ ID NO. 24: 5'-CTCCAGCCAT CAATGTT-3' is reverse primer for Klrg1, SEQ ID NO. 25: 5'-GCATATCTGT TTTGAATCC-3' is forward primer for CD68 Ag, SEQ ID NO. 26: 5'-CCTTAGAGAG AGCAGGTCA-3' is reverse primer for CD68 Ag, SEQ ID NO. 27: 5'-GTGATGACCA CCGTACTCTT-3' is forward primer for THRI13, SEQ ID NO. 28: 5'-GCATTGACTA CTCGGATAGC-3' is reverse primer for THRI13, SEQ ID NO. 29: 5'-CGATTACCCC TTAACACATT-3' is forward primer for NKG2, SEQ ID NO. 30: 5'-GCTGGAATTT TGAGACAAAC-3' is reverse primer for NKG2, SEQ ID NO. 31: 5'-TTGACGTCAC TGGAGTTGTA-3' is forward primer for TGF-BETA1, SEQ ID NO. 32: 5'-AATAGTTGGT ATCCAGGGCT-3' is reverse primer for TGF-BETA1, SEQ ID NO. 33: 5'-CAGCATTACC ACAAGAATGA-3' is forward primer for PRP1, SEQ ID NO. 34: 5'-CCCACATTCC AGAAGATTTA-3' is reverse primer for PRP1, SEQ ID NO. 35: 5'-CGATGCAGGC TATTACAGA-3' is forward primer for IL-1R II, SEQ ID NO. 36: 5'-ATCAAAAATC AGCGACACT-3' is reverse primer for IL-1R II, SEQ ID NO. 37: 5'-CCCAGGCGCA ATGCTAA-3' is forward primer for GRANZYME B, SEQ ID NO. 38: 5'-CCAGGATAAG AAACCTCG-3' is reverse primer for GRANZYME B, SEQ ID NO. 39: 5'-GAACGGGAAG CTTGTCA-3' is forward primer for GAPDH, SEQ ID NO. 40: 5'-CTAAGCAGTT GGTGGTGC-3' is reverse primer for GAPDH, SEQ ID NO. 41: 5'-ATCCAGCAGA TACCAGCTA-3' is forward primer for TIM-3, SEQ ID NO. 42: 5'-TCCATTGTTA TTATGGAGG-3' is reverse primer for TIM-3, SEQ ID NO. 43: 5'-GTATCTTCAC TTTCCGGGA-3' is forward primer for IL-4 IG 1,

SEQ ID NO. 44: 5'-GAGGTAGAAG AAGCCCTC-3' is reverse primer for IL-4 IG 1, SEQ ID NO. 45: 5'-ACTGAATCTG GAGAAGGAC-3' is forward primer for PLECKSTRIN, SEQ ID NO. 46: 5'-TTCAGTAAAC ATCCCTGCT-3' is reverse primer for PLECKSTRIN, SEQ ID NO. 47: 5'-AAAGCAAAGC AAATGAAGA-3' is forward primer for TIP2, SEQ ID NO. 48: 5'-TCAGTGGAGG AATGGTAAT-3' is reverse primer for TIP2, SEQ ID NO. 49: 5'-GTCTCCATCA CGTACAACC-3' is forward primer for LAG3, SEQ ID NO. 50: 5'-CACAAATCTT TCCTTTCCA-3' is reverse primer for LAG3, SEQ ID NO. 51: 5'-TCCTCCAGAG AAATCATT-3' is forward primer for KLRG2-A1, SEQ ID NO. 52: 5'-TACAGTTTTT GGAAATGCA-3' is reverse primer for KLRG2-A1, SEQ ID NO. 53: 5'-GCTGAATCTC ACAGTTCCT-3' is forward primer for CD27, SEQ ID NO. 54: 5'-CCAGTGTACAC CTGGATAT-3' is reverse primer for CD27, SEQ ID NO. 55: 5'-GTCACGTCGA AGTACTT-3' is forward primer for PERFORIN, SEQ ID NO. 56: 5'-ATGGCTGATA GCCTGTC-3' is reverse primer for PERFORIN, SEQ ID NO. 57: 5'-AACGCTACAC ACTGCAT-3' is forward primer for IFN-GAMMA, SEQ ID NO. 58: 5'-GCCGTGGCAG TAACAGC-3' is reverse primer for IFN-GAMMA, SEQ ID NO. 59: 5'-gatctcacca tgggatggag ctgtatcatc ctcttcttgg tagcaacagc tacaggtgtc cactcccagg tgcagctgca gcagtctggg gctgaagtaa taaagcctgg ggettcagtg aagctttcct gcaaggcttc tggctacacc ttcagcagct actggatgca ctgggtgagg caggcacctg gacaaggcct ttagtggatt ggagagatta atcctggcaa cggtcatact aactacaatg agaagtcaa gacgagggca actctgactg gggacacctc tacaagcaca gtatacatgg aactcagcag cctgcggtct gaggacaccg cggtctatta ctgtgcaaga tcttttacta cggcacgggc gtttcttac tggggccaag ggacctcgt gacctctcc tcagttcca ccaagggccc atcggtcttc ccctggcac cctctccaa aagcacctct gggggcacag cggccctggg ctgcctggtc aaggactact tcccgaacc ggtgacggtg tctggaact caggcgccct gaccagcggc gtgcacacct tcccggctgt cctacagtcc tcaggactct actccctcag cagcgtggtg accgtgcct ccagcagctt gggcacccag acctacatct gcaacgtgaa tcacaagccc agcaacacca aggtggacaa aaaaagtttg agtcccaaat ctgtgacaa aactcatact tgcccccggt gccccgctcc ggaactcctg gggggaccgt cagtcttctt ctcccccca aaaccaagg acacctcat gatctcccg accctgagg tcacatgcgt ggtggtggac gtgagccacg aagacctga ggtcaagtc aactggtacg tggacggcgt ggaggtgcat aatgccaaga caaagccgcg ggaggagcag tacaacagca cgtacctgt ggtcagegtc ctcacctcc tgcaccagga ctggctgaat ggcaaggagt acaagtcaa ggtctccaac aaagccctcc cagccccat cgagaaaacc atctcaaag ccaaagggca gccccgagaa ccacaggtgt acacctgcc cccatcccg gaggagatga ccaagaacca ggtcagcctg acctgcctgg tcaaaggett ctatccagc gacattgccg tggagtggga gagcaatggg cagccggaga acaactaaa gaccacgct cccgtgctgg actccgacgg ctcttcttc ctctatagca agtcacctg ggacaagagc aggtggcagc aggggaacgt cttctcatgc tccgtgatgc atgaggetct gcacaaccac tacacgcaga agagctctc cctgtccccg ggtaaatga-3' is base sequence of HBBK4-75G1, SEQ ID NO. 60: 5'-DLTMGWSCII LFLVATATGV HSQVQLQQSG AEVIKPGASV KLSCKASGYT FSSYWMHWVR

QAPGQGLEWI GEINPGNGHT NYNEKFKSRA TLTGDTSTST VYMELSSLR
 EDTAVYYCAR SFTTARAFAY WGQGLTVTVS SASTKGPSVF PLAPSSKSTS
 GGTAALGCLV KDYFPEPVTV SWNSGALTSG VHTFPAVLQS SGLYSLSSVV
 TVPSSSLGTQ TYICNVNHKP SNTKVDKKS L SPKSCDKTHT CPPCPAPELL
 GGPSVFLFPP KPKDTLMISR TPEVTCVVVD VSHEDPEVKF NWYVDGVEVH
 NAKTKPREEQ YNSTYRVVSV LTVLHQDWLN GKEYKCKVSN KALPAPIEKT
 ISKAKGQPRE PQVYTLPPSR EEMTKNQVSL TCLVKGFYPS DIAVEWESNG
 QPENNYKTTP PVLDSGDSFF LYSKLTVDKS RWQQGNVFSC SVMHEALHNH
 YTQKSLSLSP GK-3' is amino acid sequence of HBBK4-75G1, SEQ ID NO. 61:

5'-gatctacca tgggatggag ctgtatcatc ctctcttgg tagcaacagc tacaggtgtc cactcccagg
 tgcagctgca gcagctctggg gctgaagtaa taaagcctgg ggcttcagtg aagctttcct gcaaggcttc
 tggctacacc ttacagcagct actggatgca ctgggtgagg caggcacctg gacaaggcct tgagtggatt
 ggagagatta atcctggcaa cggtcactact aactacaatg agaagttcaa gacgagggca actctgactg
 gggacacctc tacaagcaca gtatacatgg aactcagcag cctgcggtct gaggacaccg cgtctatta
 ctgtgcaaga tcttttacta cggcacgggc gtttcttac tggggccaag ggacctcgt gacctctcc teagttcca
 ccaagggccc atccgtcttc cccctggcgc cctgtccag gacacctcc gagagcacag ccgcctggg
 ctgcctggtc aaggactact tccccgaacc ggtgacgggtg tcgtggaact caggcgccct gaccagcggc
 gtgcacacct tcccggctgt cctacagtcc tcaggactct actccctcag cagcgtggtg accgtgcct
 ccagcagctt gggcacgaag acctacacct gcaacgtaga tcacaagccc agcaacacca aggtggacaa
 gagagttgag tccaaatatg gtccccatg cccatcatgc ccagcacctg agttctctggg gggaccatca
 gtctctctgt tcccccaaa acccaaggac actctcatga tctccggac ccctgaggtc acgtgcgtgg
 tgggtggacgt gagccaggaa gaccccgagg tccagttcaa ctggtacgtg gatggcgtgg aggtgcataa
 tgccaagaca aagccgcggg aggagcagtt caacagcacg taccgtgtgg tcagcgtcct caccgtctg
 caccaggact ggctgaacgg caaggagtac aagtgaagg tctccaaca aggcctccg tctccatcg
 agaaaacat ctccaaagcc aaagggcagc cccgagagcc acaggtgtac acctgcccc catcccagga
 ggagatgacc aagaaccagg tcagcctgac ctgcctggtc aaaggcttct accccagcga catgcctgt
 gagtgggaga gcaatgggca gccggagaac aactacaaga ccacgcctcc cgtgtgtgac tccgacggct
 ccttctctct ctacagcagg ctaaccgtgg acaagagcag gtggcaggag gggaatgtct tctcatgctc
 cgtgatgcat gaggtctctg acaaccacta cacacagaag agcctctccc tgtctctggg taaatga-3' is base
 sequence of HBBK4-75G4, SEQ ID NO. 62: 5'-MGWSCILFL VATATGVHSQ
 VQLQQSGAEV IKPGASVKLS CKASGYTFSS YWMHWVRQAP GQGLEWIGE
 NPGNGHTNYN EKFKSRATLT GDTSTSTVYM ELSSLRSEDV AVYYCARSFT
 TARAFAYWGQ GTLVTVSSAS TKGPSVFPLA PCSRSTSEST AALGCLVKDY
 FPEPVTVSWN SGALTSGVHT FPAVLQSSGL YSLSSVVTVP SSSLGKTYT
 CNVDHKPSNT KVDKRVESKY GPPCPSCPAP EFLGGPSVFL FPPKPKDTLM
 ISRTPEVTCV VVDVSQEDPE VQFNWYVDGV EVHNAKTKPR EEQFNSTYRV
 VSVLTVLHQD WLNGKEYKCK VSNKGLPSSI EKTISKAKGQ PREPQVYTL
 PSQEEMTKNQ VSLTCLVKGF YPSDIAVEWE SNGQPENNYK TTPPVLDSDG

SFFLYSRLTV DKSRWQEGNV FSCSVMHEAL HNHYTQKSLS LSLGK-3' is amino acid sequence of HBBK4-75G4, SEQ ID NO. 63: 5'-gcctggacat gatgaggttc tetgetcagt tccttgggtct cctgttgctc tgttttcaag gtaccagatg tgacattgtg atgactcagt ctccagcctt cttatctgtg actccaggag agaaagtgc tattacttgc agggccagcc agactattag cgactactta cactggtatc aacaaaaacc cgatcaagct cccaaacttc tcatcaaata tgcctcccaa tccatctctg ggattccctc cagggtcagt ggcagtggtat cagggactga ttctactttt actatctcgt cgctcgaggc agaagatgct gcgacgtatt actgtcaaga tggtcacagc ttcccccaa ctttcgggtc aggaactaaa ctcgagatca aaactgtggc tgcaccatct gtcttcatct tcccgccatc tgatgagcag ttgaaatctg gaactgcctc tgttgtgtgc ctgctgaata acttctatcc cagagaggcc aaagtacagt ggaaggtgga taacgccctc caatcgggta actcccagga gagtgtcaca gagcaggaca gcaaggacag cacctacagc ctgagcagca ccctgacgct gagcaaagca gactacgaga aacacaaagt ctacgcctgc gaagtcaccc atcagggcct gaggtcgccc gtcacaaaga gcttcaacag gggagagtgt tag-3' is base sequence of HBBK4-75L, SEQ ID NO. 64: 5'-LDMMRFSAQF LGLLLLCFQG TRCDIVMTQS PAFLSVTPGE KVTITCRASQ TISDYLHWYQ QKPDQAPKLL IKYASQSISG IPSRFSGSGS GTDFTFTISS LEAEDAATYY CQDGHSPPT FGQGTKLEIK TVAAPSVFIF PPSDEQLKSG TASVVCLLNN FYPREAKVQW KVDNALQSGN SQESVTEQDS KDSTYLSST LTLSKADYEK HKVYACEVTH QGLSSPVTKS FNRGEC-3' is amino acid sequence of HBBK4-75L.

Claims

- [1] A pharmaceutical composition comprising humanized anti-4-1BB antibody (HBBK4) for treating or preventing cancer disease through the induction of CD11+CD8+ T cell increase as an effective ingredient, together with a pharmaceutically acceptable carrier.
- [2] A pharmaceutical composition comprising the combined mixture of HBBK4 and anti-CTLA-4 antibody for treating or preventing cancer disease through the synergic inhibition effect on cancer cell proliferation as an effective ingredient, together with a pharmaceutically acceptable carrier.
- [3] The pharmaceutical composition according to claim 1 or claim 2 wherein said composition contains the humanized anti-4-1BB antibody (HBBK4) between 0.1 to 50% by weight based on the total weight of composition.
- [4] The pharmaceutical composition according to claim 1 or claim 2 wherein said humanized anti-4-1BB antibody (HBBK4) comprises nucleotide sequence of SEQ ID NO. 59.
- [5] The pharmaceutical composition according to claim 1 or claim 2 wherein said humanized anti-4-1BB antibody (HBBK4) comprises nucleotide sequence of SEQ ID NO. 61.
- [6] The pharmaceutical composition according to claim 1 or claim 2 wherein said humanized anti-4-1BB antibody (HBBK4) comprises nucleotide sequence of SEQ ID NO. 63.
- [7] The pharmaceutical composition according to claim 1 or claim 2 wherein said humanized anti-4-1BB antibody (HBBK4) comprises amino acid sequence of SEQ ID NO. 60.
- [8] The pharmaceutical composition according to claim 1 or claim 2 wherein said humanized anti-4-1BB antibody (HBBK4) comprises amino acid sequence of SEQ ID NO. 62.
- [9] The pharmaceutical composition according to claim 1 or claim 2 wherein said humanized anti-4-1BB antibody (HBBK4) comprises amino acid sequence of SEQ ID NO. 64.
- [10] A use of humanized anti-4-1BB antibody (HBBK4) for the preparation of therapeutic agent for treating and preventing cancer disease in a mammal including human in need thereof.
- [11] A use of the combined mixture of humanized anti-4-1BB antibody (HBBK4) and anti-CTLA-4 antibody for the preparation of therapeutic agent for treating and preventing cancer disease in a mammal including human in need thereof.

[Fig. 1]

BBK-4-H

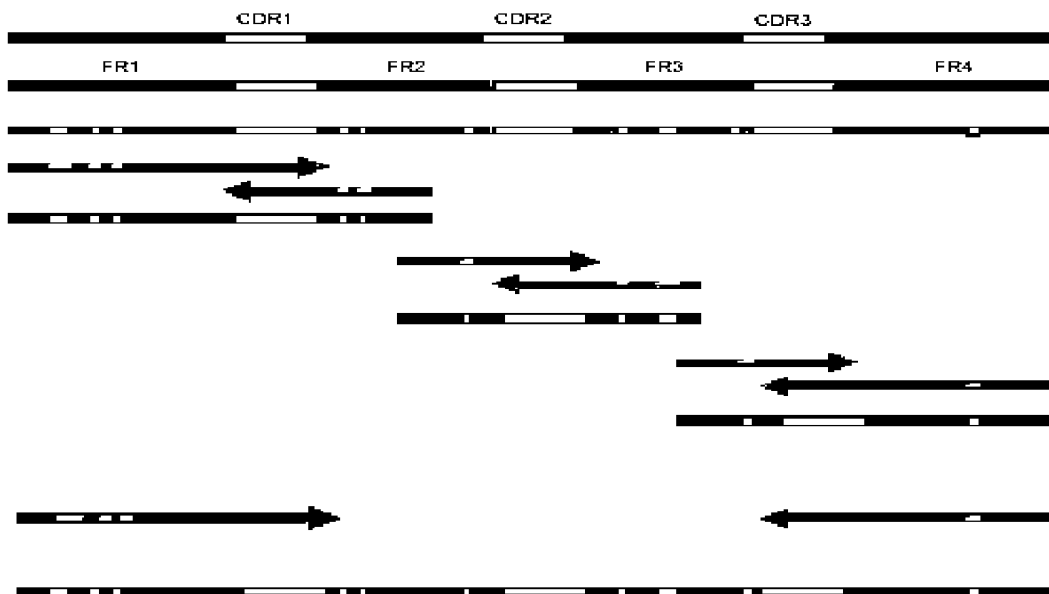
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m4B4-H	<u>QVQLQQP</u> <u>GAE</u> <u>LVKPGAS</u> <u>VKL</u> <u>SCKAS</u> <u>GYTF</u> <u>SSYMM</u> <u>HWVKOR</u> <u>PGQVLE</u> <u>WIGE</u> <u>INP</u> <u>GN</u> <u>GHTN</u> <u>YNEK</u> <u>FKSKA</u> <u>TL</u>			
Human VH1-46/J4	QVQLVQSGAEVKKPGASVKVSCKASGYTFTSYMMHWVRQAPGQGLEWMGIINPSSGGSTSYAQKFQGRVTM			
H4B4-H	QV*L**SGAEVKKPGASVK*SCKASGYTFSSYMMHWV*Q*PGQGLEWIGEINPGNHTNYNEKFKS**TM			
H4B4-H-2	QVQLQQSGAEV*KPGASVK*SCKASGYTFSSYMMHWV*QAPGQGLEWIGEINPGNHTNYNEKFKS**T*			
		# # #	#	
m4B4-H	<u>TVDR</u> <u>SS</u> <u>STAY</u> <u>ML</u> <u>SSL</u> <u>TS</u> <u>ED</u> <u>SA</u> <u>VY</u> <u>CA</u> <u>RS</u> <u>FT</u> <u>TAR</u> <u>AF</u> <u>Y</u> <u>W</u> <u>G</u> <u>Q</u> <u>G</u> <u>T</u> <u>L</u> <u>V</u> <u>T</u> <u>V</u> <u>S</u> <u>S</u>			
Human VH1-46/J4	TRDTSTSTVYMEISSLRSEDAVYYCAR YFDYWGQGTIVTVSS			
H4B4-H	TRDTSTSTVYMEISSLRSED*AVYYCARSFTTARAFAYWGQGTIVTVSS			
H4B4-H-2	T*DTSTST*YM*LSSLRSED*AVYYCARSFTTARAFAYWGQGTIVTVSS			

BBK-4-L

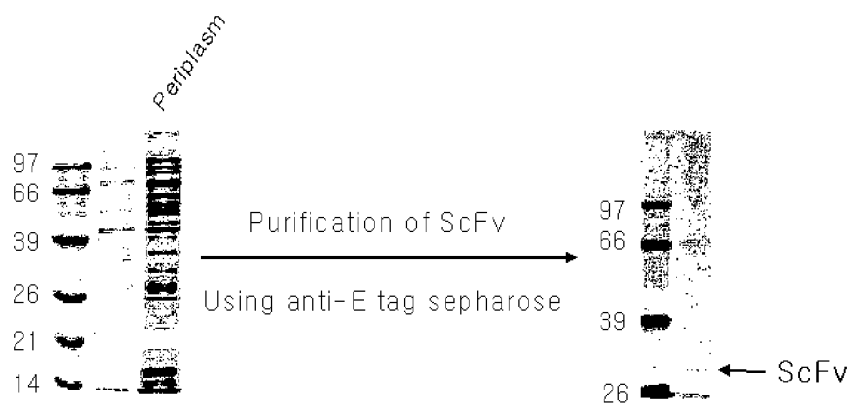
	@	*	*	#	#	#	#
m4B4-L	<u>DIV</u> <u>MTQ</u> <u>SQA</u> <u>TQ</u> <u>SV</u> <u>TF</u> <u>G</u> <u>D</u> <u>R</u> <u>V</u> <u>S</u> <u>L</u> <u>S</u> <u>C</u> <u>R</u> <u>A</u> <u>S</u> <u>Q</u> <u>T</u> <u>I</u> <u>S</u> <u>D</u> <u>Y</u> <u>L</u> <u>H</u> <u>W</u> <u>Y</u> <u>Q</u> <u>Q</u> <u>K</u> <u>S</u> <u>H</u> <u>E</u> <u>S</u> <u>P</u> <u>K</u> <u>L</u> <u>L</u> <u>I</u> <u>K</u> <u>Y</u> <u>A</u> <u>S</u> <u>Q</u> <u>S</u> <u>I</u> <u>S</u> <u>G</u> <u>I</u> <u>P</u> <u>S</u> <u>R</u> <u>F</u> <u>S</u> <u>G</u> <u>S</u> <u>G</u> <u>S</u> <u>G</u> <u>D</u> <u>F</u> <u>T</u> <u>L</u> <u>S</u>						
Human A14/J2	DVVMTQSPAPFLSVTPGEKVTITCQASEGIGNYLWYQQKPDQAPKLLIKYASQSIGVPSRFSGSGSGDFTFT						
H4B4-L	D**MTQSPA*LSVTPGEKVT**CRASQTISDYLHWYQK*D**P*LLIKYASQSIG*PSRFSGSGSG*DFTFT						
H4B4-L-2	D*VMTQSPAPFLSVTPGEKVT*TCRASQTISDYLHWYQKPDQ*PKLLIKYASQSIG*PSRFSGSGSGDFT**						
		#	*	#	*	#	@
m4B4-L	<u>INS</u> <u>V</u> <u>E</u> <u>P</u> <u>E</u> <u>D</u> <u>V</u> <u>G</u> <u>V</u> <u>Y</u> <u>C</u> <u>Q</u> <u>D</u> <u>G</u> <u>H</u> <u>S</u> <u>F</u> <u>P</u> <u>P</u> <u>T</u> <u>F</u> <u>G</u> <u>G</u> <u>T</u> <u>K</u> <u>L</u> <u>E</u> <u>I</u> <u>K</u>						
Human A14/J2	ISSLEAEDAATYYCQGNKHPWTFGQGTKLEIK						
H4B4-L	ISSLEAEDAATYYCQDGHSFPPTFG*GTKLEIK						
H4B4-L-2	ISS*EAED***YYCQDGHSFPPTFGQGTKLEIK						

- Underlined: residues for antigen contact (m)
- *: canonical residues (m)
- #: residues at the domain of interface (m)
- @: unusual sequence (h)
- **Solvent-exposed residues** (h):
 - H chain: 11-18, 68-85 (Kabat sequence)
 - L chain: 15-20, 71-85 (Kabat sequence)

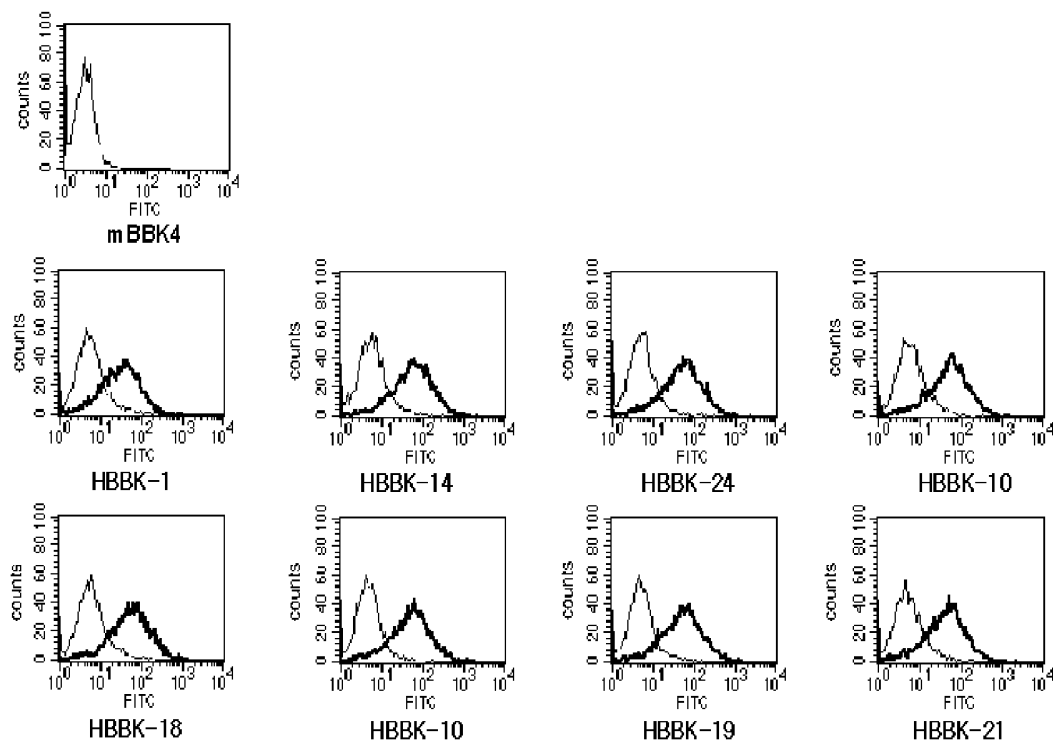
[Fig. 2]



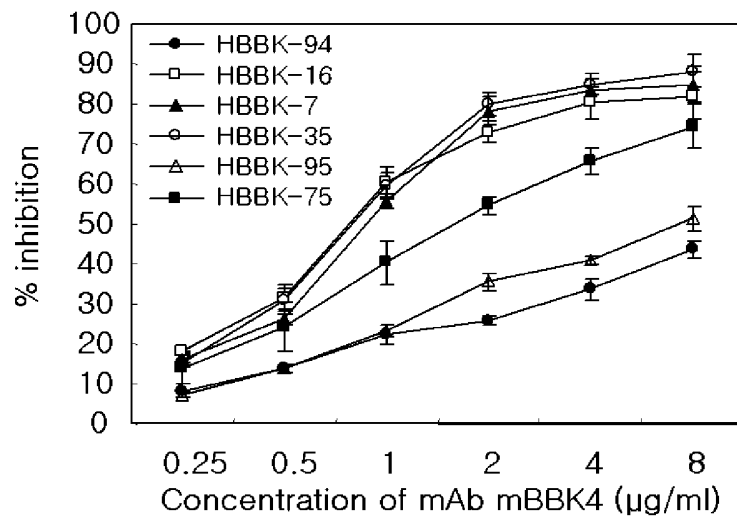
[Fig. 3]



[Fig. 4]



[Fig. 5]





lane 1: HBBK4-75/pcDNA transfectant
 lane 2: HBBK4-75/pcDNA18 transfectant
 lane 3: 293 EBNA-negative control

[Fig. 7]

Nucleotide sequence of HBBK4-75G1

```

gatctcaccATGGATGGAGCTGTATCATCCTCTTCTTGGTAGCAACAGCTACAGGTGTCCACTCCCAGGTGCAGCTGCAGCAGT
CTGGGGCTGAAGTAATAAAGCCTGGGGCTTCAGTGAAGCTTCTGCAAGGCTTCTGGCTACACCTTCAGCAGCTACTGGATGCA
CTGGGTGAGGCAGGCACCTGGACAAGGCCTTGAGTGGATTGGAGAGATTAATCCTGGCAACGGTCATACTAACTACAATGAGAAG
TTCAAGAGCAGGGCAACTCTGACTGGGGACACCTCTACAAGCACAGTATACATGGAACCTCAGCAGCCTGOGGTCTGAGGACACCG
CGGTCTATTACTGTGCAAGATCTTTACTACGGCACGGGCGTTTGCTTACTGGGGCCAAGGGACCOCTCGTGACCGTCTCCTCAGC
TTCCACCAAGGGCCCATCGGTCTTCCCCCTGGCACCOCTCCTCCAAAAGCACTCTGGGGGCACAGCGGCCCTGGGCTGCCTGGTC
AAGGACTACTTCCCGAACCGGTGACGGTGTCTGGAACCTCAGGCGCOCTGACCAGCGCGGTGCACACCTTCCCGGCTGTCTAC
AGTCCTCAGGACTCTACTCCCTCAGCAGCGTGGTGACCGTGCCCTCCAGCAGCTTGGGCACCCAGACCTACATCTGCAACGTGAA
TCACAAGCCCAGCAACACCAAGGTGGACAAAAAAGTTTGAAGTCCCAAATCTTGTGACAAAACCTCATACTTGCCCGCCGTGCCCG
GCTCCGGAACCTCTGGGGGACCGTCAGTCTTCTCTTCCCCCAAAACCCAGGACACCCCTCATGATCTCCCGGACCCCTGAGG
TCACATGCGTGGTGGTGGACGTGAGCCACGAAGACCCCTGAGGTCAAGTCAACTGGTACGTGGACGGCGTGGAGGTGCATAATGC
CAAGACAAAGCCGCGGGAGGAGCAGTACAACAGCACGTACCGTGTGGTCAGCGTCCTCACCGTCTGCAACAGGACTGGCTGAAT
GGCAAGGAGTACAAGTGCAAGGTCTCCAACAAAGCCCTCCAGCCCCCATCGAGAAAACCATCTCCAAAGCCAAAGGGCAGCCCC
GAGAACCAAGGTGTACACCTGCCCCCATCCCGGGAGGAGATGACCAAGAACCAGGTGAGCCTGACCTGCCTGGTCAAAGGCTT
CTATCCAGCGACATTGCGGTGGAGTGGGAGAGCAATGGGCGCCGGAGAACAACTACAAGACCAGCCTCCCGTGCTGGACTCC
GACGGCTCCTTCTTCTCTATAGCAAGCTCACCGTGGACAAGAGCAGGTGGCAGCAGGGGAACGTCTTCTCATGCTCCGTGATGC
ATGAGGCTCTGCACAACCACTACACGCAGAAGAGCCTCTCCCTGTCCCGGGTAAATGA
  
```

[Fig. 8]

Amino acid sequence of HBBK4-75G1

```

DLTMGWSCIILFLVATATGVHSQVQLQQSGAEVVKPGASVKLSCKASGYTFSSYWMHWVRQAPGQGLEWIGELNPGNGHTNYNEK
FKSRATLTGDTSTSTVYMELSSLRSEDTAVYYCARSFTTARAFAFWGQGLVTVSSASTKGPSVFPLAPSSKSTSGGTAALGCLV
KDYFPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVTPSSSLGTQTYICNVNHKPSNTKVDKKSLSPKSCDKTHTCPPCP
APPELLGGPSVFLFPPPKPDTLMI SRTPEVTCVVVDVSHEDPEVKFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLN
GKEYKCKVSNKALPAPI EKTISKAKGQPREPQVYTLPPSREEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSD
DGSFFLYSKLTVDKSRWQQGNVFSQSVMEALHNHYTQKSLSLSPGK
  
```

[Fig. 9]

Nucleotide sequence of HBBK4-75G4

gatctaccATGGGATGGAGCTGTATCATCTCTTCTTGGTAGCAACAGCTACAGGTGTCCACTCCAGGTGCAGCTGCAGCAGT
 CTGGGGCTGAAGTAATAAAGCCTGGGGCTTCAGTGAAGCTTCTGCAAGGCTTCTGGCTACACCTTCAGCAGCTACTGGATGCA
 CTGGGTGAGGCAGGCACCTGGACAAGGCTTGAGTGGATTGGAGAGATTAATCCTGGCAA CGGTCACTAACTACAATGAGAAG
 TTCAAGAGCAGGGCAACTCTGACTGGGACACCTCTACAAGCAGTATACATGGAAGTCAGCAGCCTGCGGTCTGAGGACACCG
 CCGTCTATTACTGTGCAAGATCTTTTACTACGGCA CGGGCGTTTGCTTACTGGGGCCAAGGGA CCCTCGTGACCGTCTCCTCAgc
 ttccaccaaggggcccatccgtcttccccctggcgccctgctccaggagcaacctccgagagcacagccgccccctgggctgcctggtc
 aaggactacttccccgaaccggtagcgggtgctgtggaactcaggcgccctgaccagcggcggtgcacaccttccccggtgtcctac
 agtcttcaggactctactccctcagcagcgtggtagccgtgccccccagcagcttgggcacgaagaacctacacctgcaacgtaga
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 ttctctacagcaggctaacgtggacaagagcagggtggcaggaggaggaaatgtcttctcatgctccgtgatgcattgaggctctgc
 acaaccactacacacagaagagcctctcccgtctctgggtaaatga

[Fig. 10]

Amino acid sequence of HBBK4-75G4

MGWSC I LFLVATATGVHSQVQLQQSGAEVI KPGASVKLSCKASGYTFSSYWMHWVRQAPGQGLEWIGE INPGNGHTNYNEKFKS
 RATLTGDTSTSTVYMELSSLRSEDAVYYCARSF TTAARAFAYWQGTLTVTVSSASTKGPSVFPLAPCSRSTSESTAALGCLVKDY
 FPEPVTVSWNSGALTSGVHTFPAVLQSSGLYSLSSVTVPSSSLGKTYTNCVDHKPSNTKVDKRVESKYGP PPSCPAPEFLGG
 PSVFLFPPKPKDTLMISRTPEVTCVVVDVSDQEDPEVFQFNWYVDGVEVHNAKTKPREEQFNSTYRVVSVLTVLHQDWLNGKEYKCK
 VSNKGLPSSIEKTI SKAKGQPREPQVYTLPPSQEEMTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTPPVLDSDGSFFLY
 SRLTVDKSRWQEGNVFSCSVMHEALHNHYTQKSLSLSLGK

[Fig. 11]

Nucleotide sequence of HBBK4-75L

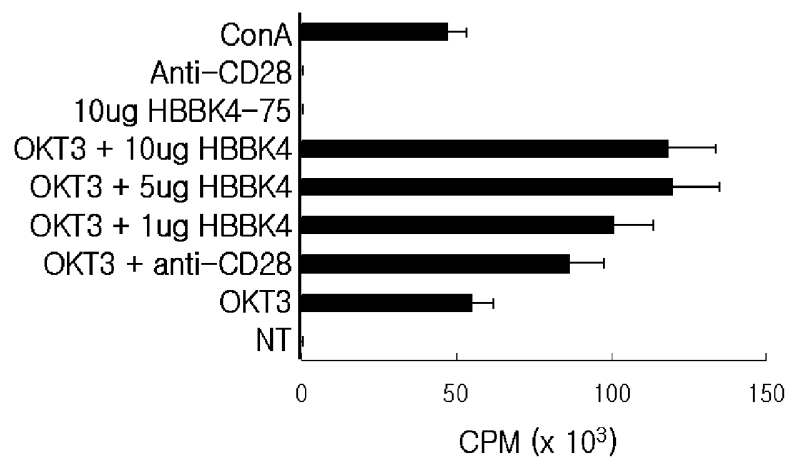
gcctggacatgATGAGGTTCTCTGCTCAGTTCCTTGGTCTCCTGTTGCTCTGTTTTCAAGGTACCAGATGTGACATTGTGATGAC
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 TCAGTGGCAGTGGATCAGGGACTGATTTCACTTTTACTATCTCGTCGCTCGAGGCAGAAAGATGCTGCGAOGTATTACTGTCAAGA
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 cagcacccctgacgctgagcaaaagcagactacgagaaacacaaaagctacgcctgcgaagtcacccaacaggccctgagttcgccc
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[Fig. 12]

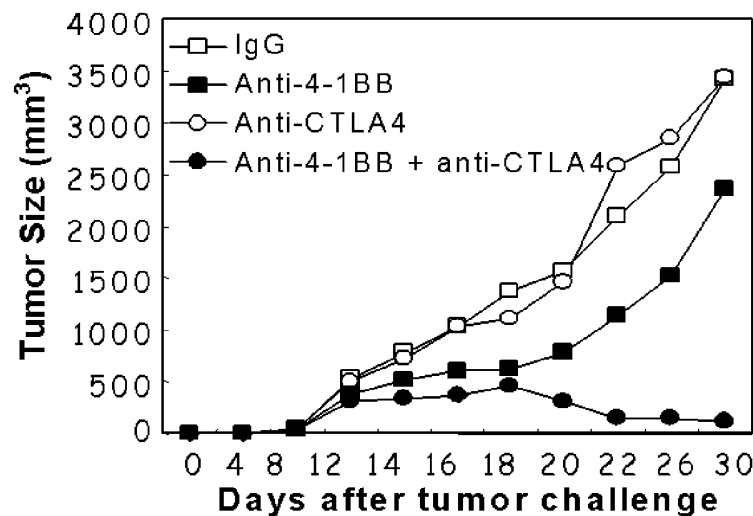
Amino acid sequence of HBBK4-75L

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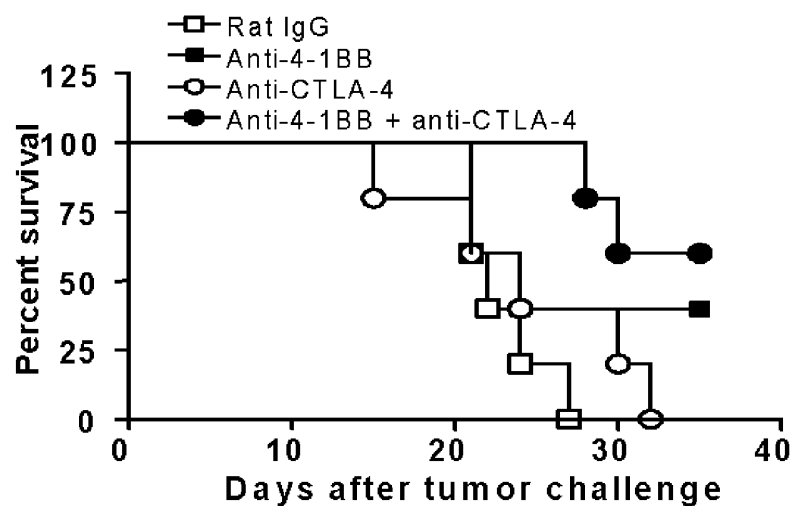
[Fig. 13]



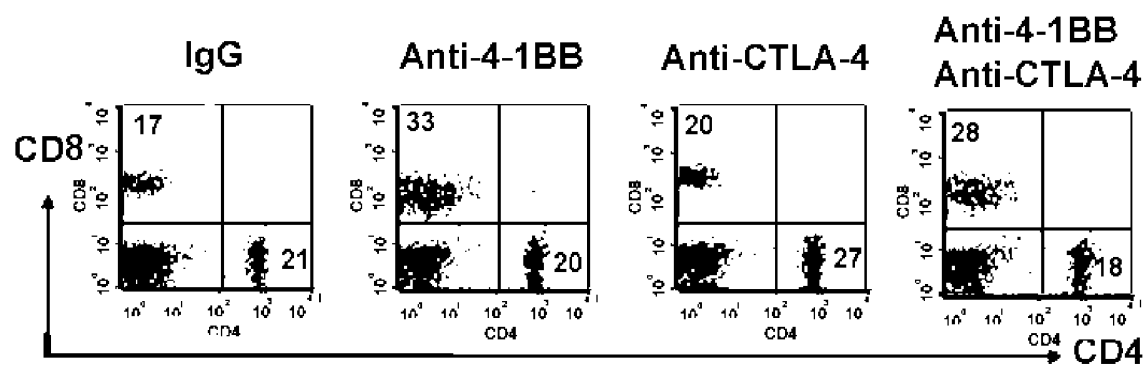
[Fig. 14]



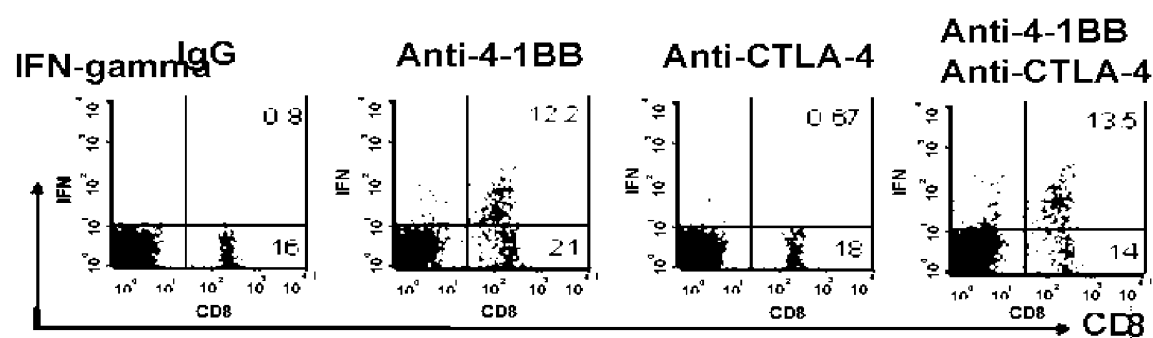
[Fig. 15]



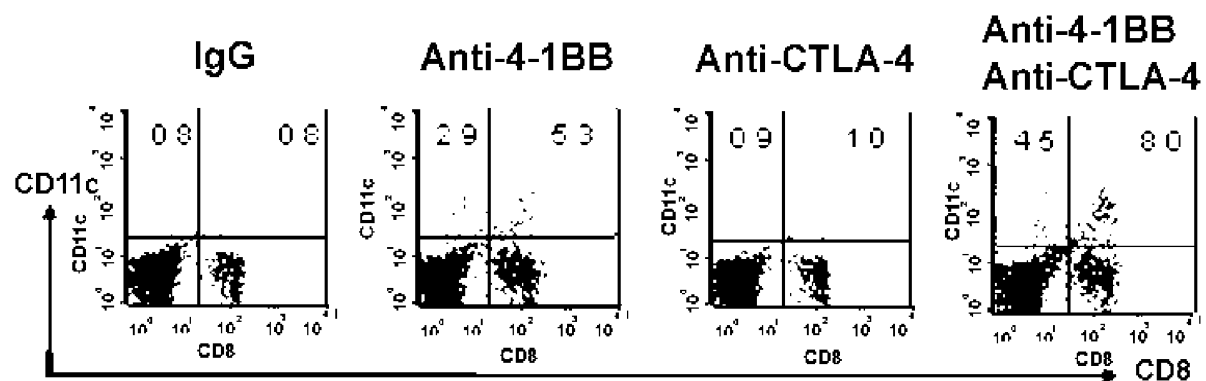
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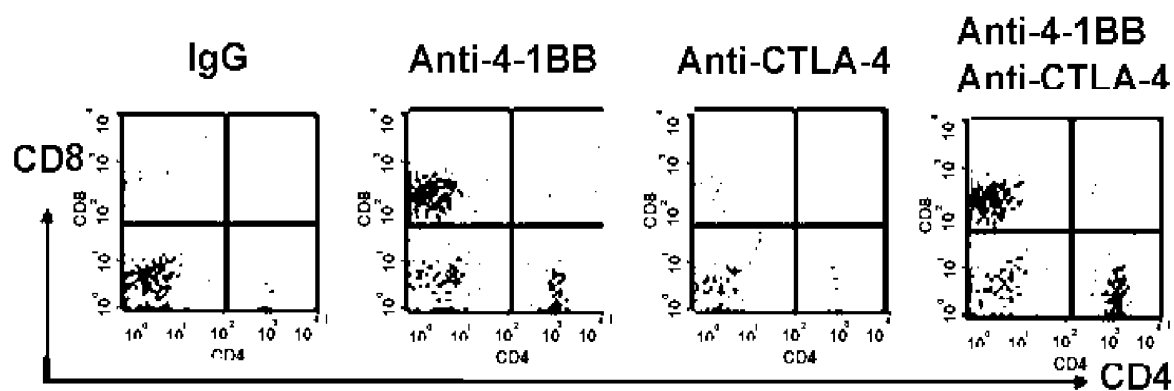
[Fig. 17]



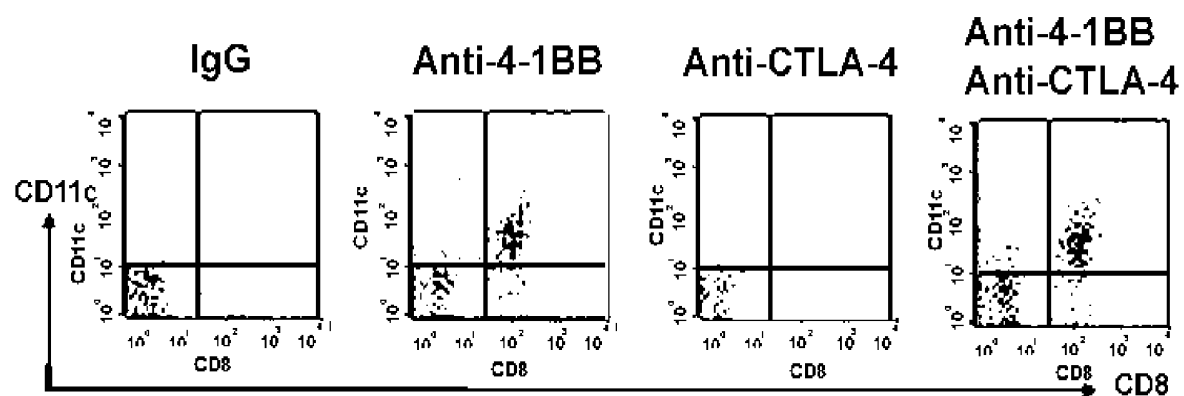
[Fig. 18]



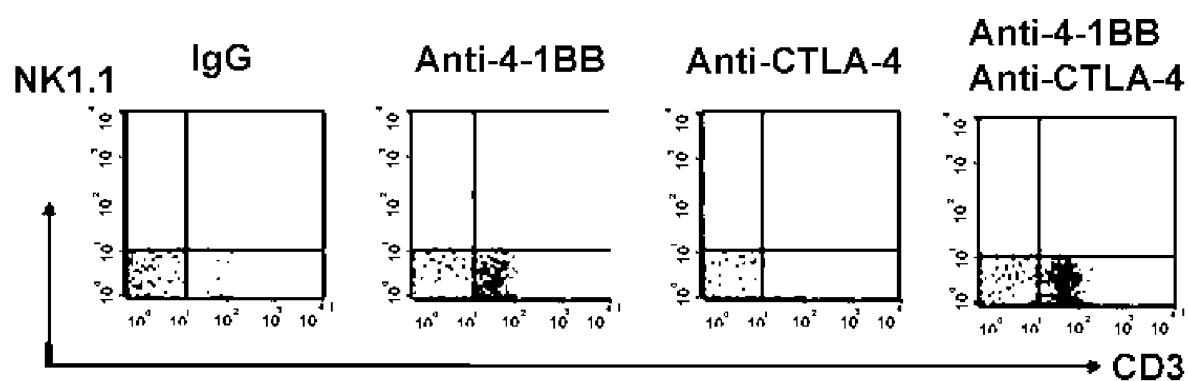
[Fig. 19]



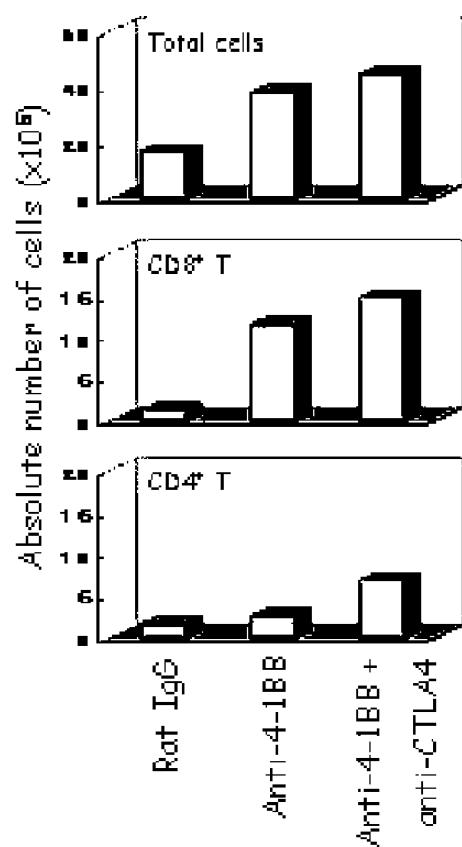
[Fig. 20]



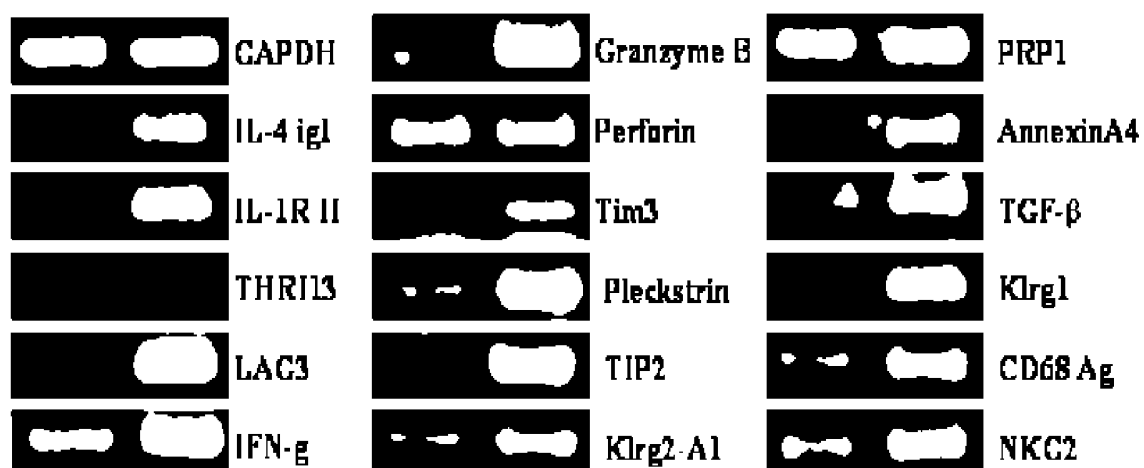
[Fig. 21]



[Fig. 22]



[Fig. 23]



INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2006/001963**A. CLASSIFICATION OF SUBJECT MATTER***A61K 39/395(2006.01)i*

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 8 A61K 39/395

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Korean Patents and Applications for Inventions since 1975

Korean Utility Models and Applications for Utility Models since 1975

Japanese Utility Models and Applications for Utility Models since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

eKIPASS, WPI, USPTO, PAJ, NCBI, CAPLUS(STN), INSPECT "4-1BB, CPLK, cancer."

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	SON JH et al. 'Humanization of agonistic anti-human 4-1BB monoclonal antibody using a phage-displayed combinatorial library' In: J. Immunol Methods 2004, Vol.286 No. 1-2, pp. 187-201 see abstract, Fig. 2, p. 192 right column	1, 10
X	MURILLO O et al. 'Potentiation of Therapeutic Immune Responses against Malignancies with Monoclonal Antibodies' In Clinical Cancer Research(Review) 2003, Vol.9, pp. 5454-5464 see abstract, pp. 5456-5457	2-9, 11



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search

25 AUGUST 2006 (25.08.2006)

Date of mailing of the international search report

28 AUGUST 2006 (28.08.2006)

Name and mailing address of the ISA/KR

Korean Intellectual Property Office
920 Dunsan-dong, Seo-gu, Daejeon 302-701,
Republic of Korea

Facsimile No. 82-42-472-7140

Authorized officer

LIM, Hea Joon

Telephone No. 82-42-481-5600



INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2006/001963

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.b of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

a. type of material



a sequence listing



table(s) related to the sequence listing

b. format of material



on paper



in electronic form

c. time of filing/furnishing



contained in the international application as filed



filed together with the international application in electronic form



furnished subsequently to this Authority for the purposes of search

2. ☐ In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR2006/001963

Patent document
cited in search reportPublication
datePatent family
member(s)Publication
date

none