METHOD FOR CULTURING CORD BLOOD-DERIVED NATURAL KILLER CELLS USING TRANSFORMED T CELLS

Technical Field

The present invention relates to a method for culturing cord blood-derived natural killer cells using transformed T cells.

Background Art

Immunotherapies aimed at treating cancer patients and preventing the recurrence of cancer in patients using their own immunity have been developed. Particularly, immunotherapies using natural killer cells that can be mass produced and frozen have also been investigated. Natural killer cells are lymphoid cells that account for about 15% of all peripheral blood lymphocytes and play a key role in the innate immune response.

Specifically, natural killer cells activate dendritic cells and induce cytotoxic T lymphocytes (CTLs) to specifically respond to tumors, eliminating tumor cells. Natural killer cells directly kill malignant tumors such as sarcoma, myeloma, carcinoma, lymphoma, and leukemia. However, the majority of natural killer cells in healthy subjects exist in an inactive state and need to be activated to eliminate tumors. The immune escape mechanism of cancer cells leads to functional defects of natural killer cells in cancer patients.

Thus, the activation of natural killer cells is very important for their use in therapeutic applications. Due to the limited number of natural killer cells in the body, it is essential to develop a technique for large-scale proliferation and freezing of natural killer cells from the blood of healthy subjects or patients.

Ex vivo expansion methods are used to proliferate natural killer cells on a large scale. In addition, research has been conducted on methods for mass culture of natural killer cells using peripheral blood lymphocytes (PBMCs), cord blood (CB) or human-induced pluripotent stem cells as raw materials.

Particularly, cord blood can be obtained from the umbilical cord discarded after

childbirth by simple treatment, unlike bone marrow. Due to vitalization of the cord blood storage industry and ease of donor acquisition, methods for culturing natural killer cells from cord blood have been actively investigated.

Specifically, *ex vivo* expansion of cord blood-derived natural killer cells is based on the proliferation of natural killer cells from mononuclear cells (MNCs) as seed cells or the proliferation of natural killer cells from hematopoietic progenitor cells (CD34+ cells) as seed cells. The approach of using mononuclear cells as seed cells involves the use of interleukin-2 (IL-2), interleukin-15 (IL-15), and FLT-3L alone or in combination to help proliferate natural killer cells. However, this approach has the problems of low proliferation rate and purity (Biossel L. *et al*., *Biology of Blood and Marrow Transplantation*, 14, 1031-1038, 2008). On the other hand, the approach of using hematopoietic progenitor cells as seed cells is advantageous in terms of proliferation rate and purity but requires a long culture period and the use of a mixture of various cytokines and growth factors, making it difficult to commercialize natural killer cells in terms of cost (Fias AM *et al*., *Experimental Hematology* 36(1):61-68, 2008).

Seed cells such as PBMC, CD3- cells, CD3-CD56+ cells, and CD56+ cells are used for *ex vivo* expansion culture of natural killer cells. Cytokines such as IL-2, IL-12, IL-15, and IL-21 and OKT-3 antibodies stimulating LPS (Goodier *et al*., *J. Immunol.* 165(1):139-147, 2000) and CD3 (Condiotti *et al*., *Experimental Hematol.* 29(1):104-113, 2001) are also used as proliferation factors for natural killer cells. The above-mentioned proliferation factors allow natural killer cells to proliferate about 3-10-fold. However, the proliferation rate alone is insufficient to commercialize natural killer cells for therapeutic applications.

Recent research efforts have focused on methods for mass proliferation of natural killer cells using various types of feeder cells. Peripheral blood mononuclear, EBV-LCL, and K562 cell lines are used as representative feeder cell lines. The K562 cell line is a cell line derived from leukemia lacking HLA. The K562 cell line is a typical target that can be easily attacked by natural killer cells. Most feeder cells used to culture natural killer cells are proliferated by known methods, for example, by expressing 4-1BBL and

membrane-bound IL-15 in the K562 cell line (Fujisaki *et al*., *Cancer Res*. 69(9):4010-4017, 2009), expressing MICA, 4-1BBL, and IL-15 (Gong *et al*., *Tissue Antigens*, 76(6):467-475, 2010), and expressing 4-1BBL and membrane-bound IL-21 (Cecele JD *et al*, *PloSONE*, 7(1):e30264, 2012).

Detailed Description of the Invention

Problems to be Solved by the Invention

Under such circumstances, the inventors of the present invention have succeeded in developing an efficient method for *ex vivo* proliferation of natural killer cells from cord blood by co-culturing the cord blood-derived natural killer cells with CD4+ T cells expressing a costimulatory factor and a growth factor capable of increasing the proliferation of the natural killer cells.

Specifically, the inventors of the present invention have produced and used transformed CD4(+) T cells as feeder cells to efficiently culture natural killer cells and have found that co-culture of the transformed CD4(+) T cells and cord blood-derived mononuclear cells leads to increases in the proliferation rate and cytotoxicity of natural killer cells. Based on this finding, the present invention has been accomplished.

Means for Solving the Problems

One aspect of the present invention provides a method for culturing natural killer cells including co-culturing transformed CD4+ T cells and seed cells.

A further aspect of the present invention provides natural killer cells produced by the method.

Effects of the Invention

The method of the present invention uses transformed T cells to culture natural killer cells and enables effective proliferation and production of natural killer cells from a small amount of cord blood-derived seed cells. In addition, natural killer cells produced by the method of the present invention have improved cytotoxicity. Therefore, the method

of the present invention is useful in commercializing natural killer cells for cell therapy. Furthermore, natural killer cells produced by the method of the present invention are useful for cell therapy.

Brief Description of the Drawings

Fig. 1a shows the expressions of different genes in the Hut78 cell line, which were determined by FACS.

Fig. 1b shows the expressions of single genes transduced into the Hut78 cell line, which were determined by FACS.

Fig. 1c shows the expressions of double genes mTNF-α/OX40L and mTNF-α/4-1BBL transduced into the Hut78 cell line, which were determined by FACS.

Fig. 1d shows the expressions of double genes mbIL-21/OX40L and mbIL-21/4-1BBL transduced into the Hut78 cell line, which were determined by FACS.

Fig. 1e shows the expressions of triple genes transduced into the Hut78 cell line, which were determined by FACS.

Fig. 1f shows the expressions of quadruple genes transduced into the Hut78 cell line, which were determined by FACS.

Fig. 2a shows the proliferation rates of natural killer cells produced by co-culturing the Hut78 cell line transduced with different genes and cord blood-derived CD3(-) mononuclear cells.

Fig. 2b shows the proliferation rates of natural killer cells produced by co-culturing the H9 cell line transduced with different genes and cord blood-derived CD3(-) mononuclear cells.

Fig. 2c shows the proliferation rates of natural killer cells produced by co-culturing the Jurkat cell line transduced with different genes and cord blood-derived CD3(-) mononuclear cells.

Fig. 2d shows the proliferation rates of natural killer cells produced by co-culturing the Peer cell line transduced with different genes and cord blood-derived CD3(-) mononuclear cells.

Fig. 2e shows the proliferation rates of natural killer cells produced by restimulation at intervals of 14 or 16 days during co-culture of the Hut78 cell line transduced with triple genes and cord blood-derived CD3(-) mononuclear cells.

Fig. 3a shows the viabilities of natural killer cells produced by co-culturing the Hut78 cell line transduced with different genes and cord blood-derived CD3(-) mononuclear cells.

Fig. 3b shows the viabilities of natural killer cells produced by co-culturing the H9 cell line transduced with different genes and cord blood-derived CD3(-) mononuclear cells.

Fig. 3c shows the viabilities of natural killer cells produced by co-culturing the Jurkat cell line transduced with different genes and cord blood-derived CD3(-) mononuclear cells.

Fig. 3d shows the viabilities of natural killer cells produced by co-culturing the Peer cell line transduced with different genes and cord blood-derived CD3(-) mononuclear cells.

Fig. 3e shows the viabilities of natural killer cells produced by restimulations at intervals of 14 or 16 days during co-culture of the Hut78 cell line transduced with triple genes and cord blood-derived CD3(-) mononuclear cells.

Fig. 4a shows the purities (CD3-CD56+) of natural killer cells produced by co-culturing the Hut78 cell line transduced with different genes and cord blood-derived CD3(-) mononuclear cells.

Fig. 4b shows the purities (CD3-CD56+) of natural killer cells produced by co-culturing the H9 cell line transduced with different genes and cord blood-derived CD3(-) mononuclear cells.

Fig. 4c shows the purities (CD3-CD56+) of natural killer cells produced by co-culturing the Jurkat cell line transduced with different genes and cord blood-derived CD3(-) mononuclear cells.

Fig. 4d shows the purities (CD3-CD56+) of natural killer cells produced by co-culturing the Peer cell line transduced with different genes and cord blood-derived CD3(-

) mononuclear cells.

Fig. 4e shows the purities (CD3-CD56+) of natural killer cells produced by restimulations at intervals of 14 or 16 days during co-culture of the Hut78 cell line transduced with triple genes and cord blood-derived CD3(-) mononuclear cells.

Fig. 5a shows the activities (CD16+CD56+) of natural killer cells produced by co-culturing the Hut78 cell line transduced with different genes and cord blood-derived CD3(-) mononuclear cells.

Fig. 5b shows the expression levels of NKG2D as a phenotypic marker of natural killer cells produced by co-culturing the Hut78 cell line transduced with different genes and cord blood-derived CD3(-) mononuclear cells.

Fig. 5c shows the expression levels of NKp30 as a phenotypic marker of natural killer cells produced by co-culturing the Hut78 cell line transduced with different genes and cord blood-derived CD3(-) mononuclear cells.

Fig. 5d shows the expression levels of NKp44 as a phenotypic marker of natural killer cells produced by co-culturing the Hut78 cell line transduced with different genes and cord blood-derived CD3(-) mononuclear cells.

Fig. 5e shows the expression levels of NKp46 as a phenotypic marker of natural killer cells produced by co-culturing the Hut78 cell line transduced with different genes and cord blood-derived CD3(-) mononuclear cells.

Fig. 5f shows the expression levels of DNAM-1 as a phenotypic marker of natural killer cells produced by co-culturing the Hut78 cell line transduced with different genes and cord blood-derived CD3(-) mononuclear cells.

Fig. 5g shows the expression levels of CXCR3 as a phenotypic marker of natural killer cells produced by co-culturing the Hut78 cell line transduced with different genes and cord blood-derived CD3(-) mononuclear cells.

Fig. 6a shows the activities (CD16+CD56+) of natural killer cells produced by co-culturing the Hut78 cell line transduced with different genes and cord blood-derived CD3(-) mononuclear cells.

Fig. 6b shows the expression levels of NKG2D as a phenotypic marker of natural

killer cells produced by co-culturing the H9 cell line transduced with different genes and cord blood-derived CD3(-) mononuclear cells.

Fig. 6c shows the expression levels of NKp30 as a phenotypic marker of natural killer cells produced by co-culturing the H9 cell line transduced with different genes and cord blood-derived CD3(-) mononuclear cells.

Fig. 6d shows the expression levels of NKp44 as a phenotypic marker of natural killer cells produced by co-culturing the H9 cell line transduced with different genes and cord blood-derived CD3(-) mononuclear cells.

Fig. 6e shows the expression levels of NKp46 as a phenotypic marker of natural killer cells produced by co-culturing the H9 cell line transduced with different genes and cord blood-derived CD3(-) mononuclear cells.

Fig. 6f shows the expression levels of DNAM-1 as a phenotypic marker of natural killer cells produced by co-culturing the H9 cell line transduced with different genes and cord blood-derived CD3(-) mononuclear cells.

Fig. 6g shows the expression levels of CXCR3 as a phenotypic marker of natural killer cells produced by co-culturing the H9 cell line transduced with different genes and cord blood-derived CD3(-) mononuclear cells.

Fig. 7a shows the activities (CD16+CD56+) of natural killer cells produced by restimulations at intervals of 14 or 16 days during co-culture of the Hut78 cell line transduced with triple genes and cord blood-derived CD3(-) mononuclear cells and the expression levels of NKG2D as a phenotypic marker of the natural killer cells.

Fig. 7b shows the expression levels of NKp30, NKp44, NKp46, DNAM-1, and CXCR3 as phenotypic markers of natural killer cells produced by restimulations at intervals of 14 or 16 days during co-culture of the Hut78 cell line transduced with triple genes and cord blood-derived CD3(-) mononuclear cells.

Fig. 8a shows the cytotoxicities of natural killer cells, which were produced by co-culturing the Hut78 cell line transduced with different genes and cord blood-derived CD3(-) mononuclear cells, to tumor cells.

Fig. 8b shows the cytotoxicities of natural killer cells, which were produced by

co-culturing the H9 cell line transduced with different genes and cord blood-derived CD3(-) mononuclear cells, to tumor cells.

Fig. 8c shows the cytotoxicities of natural killer cells, which were produced by co-culturing the Jurkat cell line transduced with different genes and cord blood-derived CD3(-) mononuclear cells, to tumor cells.

Fig. 8d shows the cytotoxicities of natural killer cells, which were produced by co-culturing the Peer cell line transduced with different genes and cord blood-derived CD3(-) mononuclear cells, to tumor cells.

Fig. 8e shows the cytotoxicities of natural killer cells, which were produced by restimulations at intervals of 14 or 16 days during co-culture of the Hut78 cell line transduced with triple genes and cord blood-derived CD3(-) mononuclear cells, to tumor cells.

Fig. 9a is a diagram showing an administration schedule for evaluating the efficacy of natural killer cells using Raji mouse models.

Fig. 9b shows the survival rates of Raji animal models measured to confirm the efficacies of NK cells, RTX, and co-administration thereof.

Fig. 10a is a diagram showing an administration schedule for evaluating the efficacy of natural killer cells using Ramos mouse models.

Fig. 10b shows the survival rates of Ramos animal models measured to confirm the efficacies of NK cells, RTX, and co-administration thereof.

Best Mode for Carrying out the Invention

The present invention will now be described in detail.

One aspect of the present invention provides a method for culturing natural killer cells including co-culturing transformed CD4+ T cells and seed cells.

The transformed CD4+ T cells may express at least one gene selected from the group consisting of the 4-1BBL, mbIL-21, OX40L, and mTNF-α genes.

Specifically, a single gene may be expressed in the transformed CD4+ T cells. In this case, the gene may be 4-1BBL, mbIL-21, OX40L or mTNF-α. Alternatively, two

genes may be expressed in the transformed CD4+ T cells. In this case, the gene combination may be mbIL-21/4-1BBL, 4-1BBL/OX40L, mTNF-α/4-1BBL, mbIL-21/OX40L, mbIL-21/mTNF-α or mTNF-α/OX40L. In the Examples section that follows, mbIL-21/4-1BBL, mTNF-α/OX40L, mTNF-α/4-1BBL, and mbIL-21/OX40L as gene combinations were transduced into T cells.

Alternatively, three genes may be expressed in the transformed CD4+ T cells. In this case, the gene combination may be 4-1BBL/mbIL-21/OX40L, mbIL-21/OX40L/mTNF-α, mTNF-α/mbIL-21/4-1BBL or 4-1BBL/OX40L/mTNF-α. In the Examples section that follows, mTNF-α/mbIL-21/4-1BBL was transduced into T cells.

Alternatively, four genes may be expressed in the transformed CD4+ T cells. In this case, the gene combination may be mTNF-α/mbIL-21/OX40L/4-1BBL. In the Examples section that follows, mTNF-α/mbIL-21/OX40L/4-1BBL was transduced into T cells.

The term "4-1BBL" as used herein refers to a ligand that forms a trimer and binds to 4-1BB as a receptor. 4-1BBL is a member belonging to the TNF superfamily (TNFSF) and is also called CD137L. The 4-1BBL gene may be derived from human.

Specifically, the 4-1BBL gene may be NCBI Reference Sequence: NM_003811 but is not limited thereto. The 4-1BBL gene may have a nucleotide sequence encoding the amino acid sequence set forth in SEQ ID NO: 1. The encoding nucleotide sequence may be set forth in SEQ ID NO: 2.

The term "mbIL-21" as used herein refers to IL-21 designed to bind to the cell membrane. mbIL-21 may be a fusion protein in which IL-21 and a transmembrane protein are fused together. The transmembrane protein may be CD8α, specifically a transmembrane domain of CD8α.

Specifically, the IL-21 gene may be NCBI Reference Sequence: NM_021803.3 but is not limited thereto. The CD8α gene may be NCBI Reference Sequence: NM_001768 but is not limited thereto. The mbIL-21 is expressed in the form of IL-21 that binds to the cell membrane. The mbIL-21 gene may have a nucleotide sequence encoding the amino acid sequence set forth in SEQ ID NO: 3. The encoding nucleotide

sequence may be set forth in SEQ ID NO: 4.

The term “OX40L” as used herein refers to a ligand that binds to OX40. OX40L is also called TNFSF4, gp34, TXGP1, CD252 or CD134L. Specifically, the OX40L gene may be NCBI Reference Sequence: NM_003326 but is not limited thereto. The OX40L gene may have a nucleotide sequence encoding the amino acid sequence set forth in SEQ ID NO: 5. The encoding nucleotide sequence may be set forth in SEQ ID NO: 6.

The term “mTNF-α” as used herein refers to a gene in which alanine-valine, a tumor necrosis factor-alpha-converting enzyme (TACE) recognition site in the amino acid sequence of tumor necrosis factor-alpha, is point mutated to proline-valine on DNA. The alanine mutated to proline is randomly selected.

Specifically, the mTNF-α gene may have a nucleotide sequence encoding the amino acid sequence set forth in SEQ ID NO: 8. The encoding nucleotide sequence may be set forth in represented by SEQ ID NO: 9.

The 4-1BBL, mbIL-21, OX40L or mTNF-α gene may be transduced through a recombinant lentivirus. The gene transduction vector is not limited to a recombinant lentivirus.

The gene(s) may be transfected into the cells by biochemical, physical or virus-mediated transfection. FuGene6 (Roche, USA), Lipofectamine^{TM} 2000 (Invitrogen, USA) or ExGen 500 (MBI Fermentas International Inc. CANADA) may be used for biochemical transfection. Lipid-mediated transfection using lipofectamine may also be used.

The term “vector” as used herein refers to a gene construct containing an essential regulatory element operably linked such that that a gene insert introduced into the vector is expressed. The vector may be an expression vector that can express a target gene in a cell into which the vector has been introduced.

The gene-containing expression vector may be any expression vector that can express the gene in the CD4+ cell line. In the Examples section that follows, a pCDH-CMV-MCS-EF1-Puro (SBI, CD510B-1) or pCDH-CMV-MCS-EF1-Neo (SBI, CD514B-1) lentiviral vector was used as the expression vector.

The lentivirus refers to a virus belonging to the family of retroviruses characterized by a long incubation period. The lentivirus can deliver genetic information to the DNA of the host cell. The use of the lentivirus is one of the most effective approaches to use gene delivery vectors that can replicate in non-dividing cells.

The CD4+ T cells may be *ex vivo* isolated CD4+ T cells, *ex vivo* expanded cultured CD4+ T cells or CD4+ cell line (T lymphoma cell line). The CD4+ T cells may be auxiliary T cells and may be hybridomas obtained by fusion with cancer cells. Specifically, the CD4+ T cells may be selected from the group consisting of Hut78, H9, Jurkat, Loucy, Molt-3, Molt-13, Peer, RPMI8402, and TALL-01 cells. Preferably, the CD4+ T cells are Hut78, H9, Jurkat or Peer cells.

The term "feeder cell" as used herein refers to a cell that does not proliferate but can produce various metabolites to help the proliferation of a target cell due to its metabolic activity. The feeder cells are also called culture auxiliary cells and may be transformed CD4+ T cells that express at least one gene selected from the group consisting of the 4-1BBL, mbIL-21, OX40L, and mTNF-α genes.

The T cells as feeder cells may be inactivated cells that are inhibited from division/proliferation or may be non-inactivated cells. Preferably, the T cells are inactivated to ensure safety. The inactivation may be accomplished by any suitable method known in the art, for example, by irradiation with gamma-rays. Most of the non-inactivated T cells are tumor cells that can be killed by activated natural killer cells during culture.

The term "seed cell" as used herein refers to a cell that can proliferate to natural killer cells through appropriate culture. Specifically, the seed cells may be cord blood-derived mononuclear cells or cord blood-derived natural killer cells but are not limited thereto. The seed cells are preferably CD3(-) cells from which CD3(+) cells are eliminated.

According to the method of the present invention, natural killer cells may be cultured by mixing the feeder cells with the seed cells in a ratio ≥0.1:1. The ratio of the feeder cells to the seed cells may be specifically 0.1:1 to 50:1, more specifically 0.5:1 to

40:1, even more specifically 1:1 to 30:1, most specifically 2:1 to 20:1. In one embodiment, the ratio of the feeder cells to the seed cells may be 2.5:1 but is not particularly limited thereto. The "ratio" is based on the numbers of the feeder cells and the seed cells.

According to the method of the present invention, natural killer cells may be cultured by mixing the seed cells with the feeder cells only once for 5 to 60 days or by mixing the seed cells with the feeder cells twice or more for at least 60 days. Preferably, the culture is performed by mixing the seed cells with the feeder cells only once for 14 to 21 days but is not limited to these conditions.

According to the method of the present invention, natural killer cells may be cultured with a T lymphoma cell line in a general animal cell culture medium such as AIM-V medium, RPMI1640 medium, CellGro SCGM, X-VIVO20 medium, IMDM or DMEM. Suitable substances may be added to the culture medium. The suitable substances may be an antibody that has low affinity for and stimulates T cells and an interleukin but are not limited thereto.

The term "antibody that has low affinity for and stimulates T cells" as used herein refers to a protein that specifically responds to CD3 antigens belonging to a group of molecules that associate with T cell receptors (TCRs) to form antigen recognition complexes. The CD3 molecules have longer intracellular regions than the TCRs and play a role in transmitting antigen recognition signals to cells.

The antibody that has low affinity for and stimulates T cells is preferably an anti-CD3 antibody. Specifically, the anti-CD3 antibody may be OKT-3, UCHT1 or HIT3a.

The term "interleukin (IL)" as used herein refers to a biologically active proteinaceous substance produced by immunocompetent cells such as lymphocytes, monocytes, and macrophages. The interleukin belongs to a group of cytokines and may be IL-2, IL-15, IL-12, IL-18 or IL-21.

In the Examples section that follows, OKT-3 and IL-2 were added for culture. The antibody may be added at a concentration of 0.1 ng/ml to 1,000 ng/ml, preferably 10 ng/μl. The interleukin may be added at a concentration of 10 U/ml to 2,000 U/ml, preferably 1,000 U/ml. The medium may be supplemented with serum or plasma and an

additional growth factor that supports the proliferation of lymphocytes. The serum or plasma is not particularly limited to a particular type and is selected from commercially available animal-derived serum and plasma products. The serum or plasma is preferably human-derived serum or plasma.

The term “culture” as used herein refers to cell growth under appropriately artificially controlled environmental conditions. The transformed CD4+ T cells may be cultured by a suitable method widely known in the art. Specifically, the culture may be performed in a batch or fed batch process or may be continuously performed in a repeated fed batch process.

Progenitor cells suitable for the culture medium may be used. The above-described raw materials may be added to the culture in a batch, fed-batch or continuous manner during culture by an appropriate method, but the manner of addition is not particularly limited. A basic compound such as sodium hydroxide, potassium hydroxide or ammonia or an acidic compound such as phosphoric acid or sulfuric acid may be used in an appropriate manner to adjust the pH of the culture.

The use of the T cells as feeder cells selectively induces the culture of natural killer cells from the seed cells. When compared to the use of PBMC feeder cells from a donor, the use of the T cells as feeder cells enables more stable culture of natural killer cells without any difference depending on who is the donor during proliferation. Further, the use of MNCs from a donor as feeder cells makes *ex vivo* culture of cord blood seed cells difficult. Therefore, the use of T cells as feeder cells in the method of the present invention can ensure a large amount of therapeutic natural killer cells in an efficient and stable manner.

A further aspect of the present invention provides natural killer cells produced by the method.

The natural killer cells can be frozen and do not lose their functions even when thawed. In addition, the natural killer cells have increased cytotoxicity against tumor cell lines and secrete a large amount of cytokines due to their ability to express a high level of activating receptors such as NKp46, and as a result, their outstanding anticancer

activity can be expected. Therefore, when activated, the clinically applicable natural killer cells can be used in a large amount to produce effective cell therapeutic agents for tumors.

The natural killer cells can be used as active ingredients of a pharmaceutical composition for preventing or treating an infectious disease. In this case, the natural killer cells are present in an amount of 10 to 95% by weight, based on the total weight of the composition. The composition may further include one or more active ingredients with the same or similar functions.

The pharmaceutical composition may be formulated with one or more pharmaceutically acceptable carriers for administration.

The dose of the pharmaceutical composition can be adjusted depending on various factors, including type and severity of the disease, kinds and amounts of the active ingredients and other ingredients, type of the formulation, patient's age, weight, general health, sex, and diet, time and route of administration, secretion rate of the composition, treatment period, and presence of a concomitant drug. For the desired effect, the natural killer cells are used in an amount of 0.01×10^7 cells/kg to 1.0×10^9 cells/kg or 0.5×10^7 cells/kg to 1.0×10^8 cells/kg. The composition can be administered in single or divided doses per day.

The pharmaceutical composition may be administered to subjects by various methods known in the art. The route of administration may be appropriately selected by those skilled in the art in consideration of the mode of administration, the volume of the body fluid, the viscosity of the composition, and other factors.

Another aspect of the present invention provides a composition for culturing natural killer cells including transformed CD4+ T cells as active ingredients. Since the CD4+ T cells and gene(s) transduced into the cells have already been described above and a description thereof is omitted to avoid excessive duplication.

Mode for Carrying out the Invention

The present invention will be explained in detail with reference to the following examples. However, these examples are provided for illustrative purposes only and are

not intended to limit the scope of the present invention.

Example 1. Production of recombinant lentiviruses

Example 1.1. Construction of recombinant lentiviral vectors

pCDH-CMV-MCS-EF1-Puro (SBI, CD510B-1) or pCDH-CMV-MCS-EF1-Neo (SBI, CD514B-1) was used as a lentiviral vector. 4-1BBL (TNF superfamily member 9, TNFSF9), mbIL-21 (membrane bound IL-21), OX40L (TNF superfamily member 4 (TNFSF4) transcript variant 1), and mTNF-α (membrane bound TNF alpha) were used as genes for transduction.

Specifically, a 4-1BBL gene expression vector (Origene, RC211160) was used for the 4-1BBL gene (SEQ ID NO: 2) and a pcDNA3.1 vector (Genscript, US) carrying a codon-optimized mbIL-21 gene sequence was used for the mbIL-21 gene (SEQ ID NO: 4). Synthesis of the OX40L gene (SEQ ID NO: 6) was requested to Bioneer.

For the mTNF-α gene (SEQ ID NO: 9), RNA was extracted from peripheral blood mononuclear cells (PBMCs) and then CDS was obtained by reverse transcriptase (RT)-PCR. TNF-α was cleaved with tumor necrosis factor-alpha-converting enzyme (TACE) for secretion. For this cleavage, alanine-valine, a TACE recognition site in the amino acid sequence of TNF-α, was point mutated to proline-valine on DNA, such that TNF-α remained attached to the cell membrane. The point mutation was performed by substituting the guanine and adenine residues at positions 226 and 228 in the human mTNF-α gene (SEQ ID NO: 7) with cytosine and guanine, respectively.

The coding sequence (CDS) of each gene was amplified by PCR using primers suitable for the gene (Table 1).

[Table 1]

Gene	Primers	Sequence (5'→3')	SEQ ID NO:
4-1BBL	4-1BBL Forward	TCTAGAGCTAGCGAATTCGCCACCATGGAATACGCCTCTGACGCTT	10
	4-1BBL Reverse	TTCGCGGCCGCGGATCCTTATTCCGACCTCGGTGAAGG	11
mbIL-21	mbIL-21 Forward	TAGAGCTAGCGAATTCGCCACCGCCACCATGGCTCTGCCC	12
	mbIL-21 Reverse	TCGCGGCCGCGGATCCTCAATACAGGGTGATGACC	13
OX40L	OX40L Forward	TAGAGCTAGCGAATTCGCCACCATGGAACGGGTGCAAC	14
	OX40L Reverse	TCGCGGCCGCGGATCCTCACAAGACACAGAACTCCCC	15
mTNF-α	mTNF-α Forward	TAGAGCTAGCGAATTCGCCACCGCCACCATGGCTCTGCCC	16
	mTNF-α Reverse	TCGCGGCCGCGGATCCTCACAGGGCAATGATCCC	17

The gene and the lentiviral vector were treated with EcoRI and BamHI as restriction enzymes, followed by ligation with an In-Fusion HD cloning kit (Clontech, 639649). The ligated lentiviral vector was transformed into DH5α competent cells, followed by culture. Plasmid DNA was isolated from the transformed DH5α competent cells using a plasmid mini-prep kit (MACHEREY-NAGEL/740422.50). All plasmid DNA samples were outsourced for sequencing to confirm matching of the DNA sequences. The desired genes were transduced into cLV-CMV-MCS-IRES-Puro (puromycin), cLV-CMV-MCS-IRES-Neo (neomycin), and cLV-CMV-MCS-IRES-Bsd (blasticidin) in the same manner as described above. This gene transduction was outsourced to an external manufacturer.

Example 1.2. Production of concentrated lentiviruses

In this example, recombinant lentiviruses were produced. First, the 1.5×10^6-2×10^6 cells of the 293T cell line were inoculated into a 75T flask (Nunc, 156499) 2 days before transfection. The cells were cultured in an incubator at 5% CO_2 and 37 °C. When the 293T cells were cultured to a confluency of ~80-90%, the medium was replaced with 6 ml of OPTI-MEM (Gibco, 31985-088). Cells were cultured at 37 °C and 5% CO_2 for 30 min. A DNA mixture and a lipofectamine mixture (lipofectamine 2000, Life technologies, 11668500) were prepared as shown in Table 2.

[Table 2]

Mixture	Components
DNA mixture	6 μg target DNA, 6 μg Gag, 6 μg REV, 3 μg VSVG, 1 ml OPTI-MEM
Lipofectamine mixture	36 μl lipofectamine 2000, 1 ml OPTI-MEM

The components of each mixture were sufficiently mixed in a vortexer and left standing at room temperature for 3 min. Thereafter, the two mixtures were mixed together and allowed to stand at room temperature for at least 20 min. The 293T cells cultured in 6 ml of OPTI-MEM medium were treated with 2 ml of the DNA/lipofectamine mixture. After 4 h, the medium was replaced with DMEM (Gibco, 11995073) supplemented with

10% (v/v) FBS. Cells were cultured at 37 °C and 5% CO_2 for 48 h. 8 ml of the culture solution of 293T cells was collected and passed through a 0.45 μm filter (Millipore, SLHP033RS). The filtrate was concentrated to ≤250 μl with an Amicon Ultra-15 Centrifugal Filter Unit with Ultracel-100 membrane (Merckmillipore, UFC910096). The concentrated virus was aliquoted in appropriate amounts and stored at -80 °C.

Example 2. Production of transgenic T cells

Example 2.1. Lentiviral infection

0.5×10^6 cells of the cultured cell line, 1 ml of OPTI-MEM, 50 μl of the thawed lentiviral solution, and 10 μg/ml polybrene (Santa Cruz, C2013) were mixed and plated in a 6-well plate (Nunc, 140675), followed by spinoculation at 1800×g and 32 °C for 90 min. Then, cells were cultured in an incubator at 5% CO_2 and 37 °C for 2 h. The medium was replaced with a new one, followed by culture for 48 h.

Hut78 cell line (ATCC, TIB-161™) was cultured in IMDM (ATCC, 30-2005) supplemented with 20% (v/v) FBS. During subculture, the cell density was maintained at 1.5×10^5-2.0×10^5 cells/ml. H9 cell line (ATCC, HTB-176™) and Jurkat cell line (ATCC, TIB-152™) were cultured in RPMI1640 (ATCC, 30-2001) medium supplemented with 10% (v/v) FBS and their cell densities were maintained at 1.0×10^5-1.5×10^5 cells/ml and 0.5×10^5-1.0×10^5 cells/ml during subculture, respectively. Peer cell line was cultured in RPMI1640 medium supplemented with 20% (v/v) FBS. During subculture, the cell density was maintained at 3.0×10^5-5.0×10^5 cells/ml. All cell lines were subcultured at intervals of 2-3 days. 75T flasks were used as culture vessels and the volumes of the media were maintained at 15-20 ml.

The cell lines infected with recombinant lentiviruses were screened with antibiotics (Table 3).

[Table 3]

	Combination of transduced genes	Vector used	Cell line	Concentration of antibiotic used
Single gene expression	mTNF-α-mbIL-21	pCDH (System Biosciences, SBI)	Hut78	0.5 μg/ml puromycin (Life technologies, A1113802)
	OX40L4-1BBL	pCDH (System Biosciences, SBI)	Hut78	1 mg/ml G148 (Sigma Aldrich, A1720-5G)
Double gene expression	mTNF-a/OX40L mbIL-21/OX40L mTNF-a/4-1BBL	pCDH (System Biosciences, SBI)	Hut78	0.5 μg/ml puromycin 1 mg/ml G418
	mbIL-21/4-1BBL	cLV (Sirion)	Hut78 H9 Jurkat Peer	6 μg/ml Blasticidin (Invitrogen, R210-01) 1 mg/ml G418
Triple gene expression	mTNF-a/mbIL-21/4-1BBL	cLV (Sirion)	Hut78 H9 Jurkat	0.5 μg/ml puromycin 6 μg/ml Blasticidin 1 mg/ml G418
Quadruple gene expression	mTNF-a/mbIL-21/OX40L/4-1BBL	mTNF-a/mbIL-21/4-1BBL: cLV Ox40L: pCDH	Hut78	0.5 μg/ml puromycin 6 μg/ml Blasticidin 1 mg/ml G418

The antibiotics used for the transgenic cell lines are shown in Table 3.

Example 2.2. Confirmation of expressions of the transduced genes

In this example, the expressions of the transduced genes were confirmed by flow cytometry. Each of the cell lines subcultured in Example 2.1 was collected and centrifuged at 1,200 rpm for 5 min. Then, the culture solution was removed by suction. 2% (v/v) FBS was added to PBS to prepare a FACS buffer. Cells were counted after dilution with 1 ml of the FACS buffer. Cells were diluted to a density of 5×10^6 cells/ml with the FACS buffer. The diluted cell solution was added to 5 ml FACS tubes (Falcon, 352052) (100 μl per tube). Cells were stained with anti-human TNF-α (membrane)-PE (R&D systems, FAB210P), anti-human OX40L-PE (BD, 558184), anti-human 4-1BBL-PE (BD, 559446), anti-human IL-21-PE (eBioscience, 12-7219-42), 7-AAD (Beckman coulter, IM3630c), PE mouse IgG1 κ isotype control (BD Pharmingen, 555749), and PerCP-Cy5.5 mouse IgG1 κ isotype control (BD, 550795) antibodies. The expression level of the gene was analyzed by FACS (Figs. 1a to 1f).

The expressions of the transduced genes were confirmed by real time (RT)-qPCR. To this end, each of the cell lines subcultured in Example 2.1 was collected and centrifuged at 1,200 rpm for 5 min. Then, the culture solution was removed by suction.

Cells were counted after dilution with PBS. RNA was extracted from 1×10^6 cells using an RNA prep kit and quantified. cDNA was synthesized using a cDNA synthesis kit. RT-qPCR was performed using the synthesized cDNA. Primers used for the RT-qPCR are shown in Table 4.

[Table 4]

	Primers	Sequence (5'→3')	SEQ ID NO:
4.1BBL	Forward primer	TCTGAGACAGGGCATGTTTG	18
	Reverse primer	CCACCAGTTCTTTGGTGTCC	19
mTNF-α	Forward primer	AACCTCCTCTCTGCCATCAA	20
	Reverse primer	ATAGTCGGGCCGATTGATCT	21
mbIL-21	Forward primer	TGGAAACAATGAGCGAATCA	22
	Reverse primer	AACCGCTCCAGGAACTCTTT	23
hTOP1	Forward primer	CCAGACGGAAGCTCGGAAAC	24
	Reverse primer	GTCCAGGAGGCTCTATCTTGAA	25

The expression levels of the transduced genes in the cell lines are shown in Table 5.

[Table 5]

Ct value	TOP1	mTNF-α	mbIL-21	4.1BBL
H9	20.3	21.5	n.d	n.d
H9-mbIL-21-4.1BBL	20.0	22.2	19.5	19.4
H9-mTNF-α-mbIL-21-4.1BBL	19.9	18.2	18.1	18.2
Jurkat	20.1	30.7	n.d	n.d
Jurkat-mbIL-21-4.1BBL	27.4	37.0	36.4	34.1
Jurkat-mTNF-α-mbIL-21-4.1BBL	20.4	19.8	19.2	19.8
Peer	21.4	26.2	34.2	34.9
Peer-mbIL-21-4.1BBL	26.8	33.8	29.0	25.6
* n.d: not detected				

As can be seen from the results in Table 5, high expression levels of the genes were induced when transduced into the cell lines.

Example 3. Co-culture of CD3(-) PBMCs and transgenic T cells

Example 3.1. Preparation of cord blood-derived CD3(-) PBMCs as seed cells

Cord blood donated for research purposes was placed in a 50 ml tube and

centrifuged at 1,500 rpm for 10 min. After removal of the supernatant plasma, phosphate buffered saline (PBS, LONZA, 17-516Q) was added in a 1:1 ratio. Thereafter, cord blood mononuclear cells (MNCs) were isolated by ficoll density gradient centrifugation (Ficoll-Paque Plus, GE Healthcare, 17-1440-03) and the cell number was measured using an ADAM cell counter system (Nano Entec).

Seed cells from which CD3(+) cells were eliminated were obtained by the following procedure. First, 5×10^7 cord blood mononuclear cells were transferred to a new 50 ml tube and centrifuged at 1,200 rpm and 4 °C for 5 min. 2% (v/v) FBS and 2 mM EDTA were mixed in PBS to prepare a MACS running buffer. After completion of the centrifugation, 400 μl of the MACS running buffer and 100 μl of CD3 magnetic beads (Miltenyi biotech, 130-050-101) were added to the pellets and incubation was allowed to proceed at 4 °C for 20 min. The culture was washed with 10 ml of the MACS running buffer, centrifuged at 13,500 rpm and 4 °C for 8 min, and suspended in 0.5 ml of the MACS running buffer.

Cells were isolated on VarioMACS (Miltenyi Biotech) equipped with a CS column (column, Miltenyi Biotech, 130-041-305). Cells were recovered by washing the column until the final volume reached 20 ml. The recovered cells were placed in a new 50 ml tube, centrifuged at 1,200 rpm and 4 °C for 5 min, and suspended in a freezing medium. The cells were counted using an ADAM cell counter system. 5×10^6 cells per vial were frozen in liquid nitrogen.

The frozen CD3(-) cord blood mononuclear cells in each vial were thawed in a thermostatic bath at 37 °C, transferred to a 50 ml tube, suspended in PBS containing 0.6% (v/v) citrate-dextrose solution (ACD, Sigma-Aldrich, C3821), 0.2% (v/v) fetal serum bovine (FBS), and 2 mM EDTA, and centrifuged at 1,500 rpm and 4 °C for 10 min. The CD3(-) cord blood mononuclear cells were suspended in CellGro medium (Cellgenix, 20802-0500) and the cell number was measured using an ADAM cell counter system. The CD3(-) cord blood mononuclear cells were suspended to a density of 1×10^6 cells/ml in CellGro medium.

Example 3.2. Co-culture of the CD3(-) cord blood mononuclear cells and the transgenic T cells

The transgenic T cells produced in Example 2 were recovered from the culture flask and centrifuged at 1,200 rpm and 4 °C for 5 min. Thereafter, the cells were suspended in CellGro medium and counted using an ADAM cell counter system. The transgenic T cells were suspended to a density of 2.5×10^6 cells/ml in CellGro medium and were then inactivated by irradiation with gamma-rays at 20,000 cGy.

Natural killer cells were cultured by the following procedure. First, 1,000 IU of IL-2 (Proleukin Injection, Novartis Korea) and 10 ng/ml OKT-3 (eBioscience, 16-0037-85) were plated in a plastic culture plate. On day 0 of culture, the CD3(-) cord blood mononuclear cells and the transgenic T cells (each 0.25 ml) were added in a ratio of 1:2.5 and 0.25 ml of CellGro medium supplemented with 2% (v/v) human plasma was added. Cells were cultured statically in an incubator at 37 °C for 4 days.

On day 4 of culture, the equal amount of CellGro medium supplemented with 1% (v/v) human plasma and 1,000 IU/ml IL-2 was added, followed by stationary culture. Thereafter, cells were counted at intervals of 2-3 days. CellGro medium supplemented with 1% (v/v) human plasma and 1,000 IU/ml IL-2 was added and suspension culture was performed until day 21 such that the cell density was 1×10^6 cells/ml. When cells of the Jurkat cell line or Peer cell line were used as feeder cells, suspension culture was continued until day 11. When cells of the transgenic H9 and Hut78 cell lines were as feeder cells, suspension culture was continued until day 21.

The proliferation rates of the cultured natural killer cells were compared based on the total nucleated cells (TNCs). As a result, the proliferation rate of natural killer cells increased as much as 93-fold when co-cultured with the non-transgenic Hut78 cell line. In contrast, the proliferation rate of natural killer cells increased significantly when co-cultured with the Hut78 cell line transduced with one or more genes (mTNF-α, mbIL-21, 4-1BBL). Particularly, the proliferation rate of natural killer cells increased as much as 957-fold when co-cultured with the Hut78 cell line transduced with the genes mbIL-21/4-1BBL and increased as much as 1,138-fold when co-cultured with the Hut78 cell line

transduced with the genes mTNF-α/mbIL-21/4-1BBL (Table 6, Fig. 2a).

[Table 6]

Hut78+transduced gene(s)	Average	STDEV
Hut78 parental	92.7	90.4
mTNF-α	112.3	67.2
mbIL-21	448.1	251.4
OX40L	50.5	30.7
4.1BBL	274.9	189.6
mTNF-α+OX40L	204.5	123.2
mTNF-α+4.1BBL	389.1	352.1
mbIL-21+OX40L	372.0	189.2
mbIL-21+4.1BBL	957.0	537.4
mTNF-α+mbIL21+4.1BBL	1138.5	192.0
mTNF-α+OX40L+mbIL21+4.1BBL	823.1	330.0

When co-cultured with the non-transgenic H9 cell line, the proliferation rate increased 13-fold. In contrast, when co-cultured with the H9 cell line transduced with mbIL-21/4-1BBL and mTNF-α/mbIL-21/4-1BBL, the proliferation rates increased as much as 367- and 979-fold, respectively (Table 7 and Fig. 2b).

[Table 7]

H9+transduced gene(s)	Average	STDEV
H9 parental	12.6	4.3
mbIL21+4.1BBL	367.4	80.1
mTNF-α+mbIL21+4.1BBL	978.8	287.7

When co-cultured with other cell lines (Jurkat cell line and Peer cell line), the culture could be continued until day 11 of culture. Relatively high proliferation rates were observed in the cell lines transduced with the genes mbIL-21/4.1BBL or the genes mTNF-α/mbIL-21/4-1BBL (Tables 8 and 9, Figs. 2c and 2b)

[Table 8]

Jurkat+transduced gene(s)	Average (after culture for 11 days)	STDEV
Jurkat Parental	0.9	0.7
mbIL21+4.1BBL	36.3	4.8
mTNF-α+mbIL21+4.1BBL	43.6	6.6

[Table 9]

Peer+transduced gene(s)	Average (after culture for 11 days)	STDEV
Peer Parental	1.6	0.7
mbIL21+4.1BBL	14.3	4.1

The above results reveal that natural killer cells could be cultured at a higher proliferation rate from the CD3(-) cells isolated from cord blood mononuclear cells for 21 days when the transgenic feeder cells were used than when non-transgenic feeder cells were used.

Example 3.3. Restimulation of culture of natural killer cells using the Hut78 cells transduced with the genes mTNF-α/mbIL-21/4-1BBL

The transgenic T cells prepared in Example 2 were recovered from the culture flask and centrifuged at 1,200 rpm and 4 °C for 5 min. Thereafter, the cells were suspended in CellGro medium and counted using an ADAM cell counter system. The transgenic T cells were suspended to a density of 2.5×10^6 cells/ml in CellGro medium and were then inactivated by irradiation with gamma-rays at 20,000 cGy.

Natural killer cells were cultured by the following procedure. First, 1,000 IU of IL-2 and 10 ng/ml OKT-3 were plated in a plastic culture plate. On day 0 of culture, the CD3(-) cord blood mononuclear cells and the transgenic T cells (each 0.25-1 ml) were added in a ratio of 1:2.5 and 0.25-1 ml of CellGro medium supplemented with 2% (v/v) human plasma was added. Cells were cultured statically in an incubator at 37 °C for 4 days.

On day 4 of culture, the equal amount of CellGro medium supplemented with 1% (v/v) human plasma and 1,000 IU/ml IL-2 was added, followed by stationary culture. Thereafter, cells were counted at intervals of 2-3 days. CellGro medium supplemented

with 1% (v/v) human plasma and 1,000 IU/ml IL-2 was added and culture was continued such that the cell density was 1×10^6 cells/ml.

For restimulation, Hut78 cells transduced with mTNF-α/mbIL-21/4-1BBL were used in the same ratio on day 0 of culture. On day 16 of culture, the first restimulation was performed. First, the cultured natural killer cells were counted using an ADAM cell counter system and diluted to 1.5×10^6 cells/ml with CellGro medium. 0.25 ml of the dilution was plated in a plastic culture plate. The Hut78 cells transduced with the genes mTNF-α/mbIL-21/4-1BBL were suspended to 2.5×10^6 cells/ml in CellGro medium and were then inactivated by irradiation with gamma-rays at 10,000 cGy.

0.25 ml of the Hut78 cells transduced with the inactivated genes mTNF-α/mbIL-21/4-1BBL were plated in a plastic culture plate. 1000 IU/ml IL-2, 10 ng/ml of OKT-3, and 1% (v/v) human plasma were added to the plastic culture plate, followed by stationary culture in an incubator at 37 °C for 3 days. Thereafter, the cell number was measured at intervals of 2-3 days. Culture was continued in CellGro medium supplemented with 1% (v/v) human plasma and 1000 IU/ml IL-2 until the cell number reached 1×10^6 cells/ml. After the first restimulation, restimulation was performed through the feeder cells at intervals of 14 days (on days 32, 46, and 60) in the same manner as described above. Culture was continued until day 70.

As a result, the proliferation rate of natural killer cells increased 6.9×10^4-fold on day 32 of culture after the first restimulation, 3.7×10^6-fold on day 46 of culture after the second restimulation, 2.3×10^8-fold on day 60 of culture after the third restimulation, and 5.9×10^9-fold on day 70 of culture after the fourth restimulation. That is, natural killer cells persistently proliferated at high rates (Table 10, Fig. 2e).

[Table 10]

Days of culture	Average	STDEV
32	6.9×10^4	3.2×10^3
46	3.7×10^6	3.1×10^5
60	2.3×10^8	1.4×10^8
70	5.9×10^9	1.1×10^8

The results in Table 10 reveal that the periodic restimulations with the Hut78 cell line transduced with the genes mTNF-α/mbIL-21/4-1BBL led to a continuous increase in proliferation factor, demonstrating that cells of the Hut78 cell line are very suitable as feeder cells.

Experimental Example 1. Confirmation of cell viability of natural killer cells depending on the transduced gene

The *in-vitro* cell viabilities were compared and evaluated using an ADAM cell counter system. The ADAM cell counter system is a cell counter that uses PI staining solution capable of binding to cell nuclei. The number of viable cells was calculated by subtracting the number of dead cells from the total number of counted cells. Cell viability was calculated using the following equation I.

[Equation I]

Cell viability (%) = (Number of viable cells/total number of counted cells) × 100

When co-cultured with the transgenic Hut78 cell line, the viabilities of natural killer cells were around 90% regardless of whether the cell line was transduced with genes (Table 11, Fig. 3a).

[Table 11]

Hut78+transduced gene(s)	Average	STDEV
Parental	91	2.6
mTNF-α	92.8	2.1
mbIL-21	92.8	1.5
OX40L	90.3	1.3
4.1BBL	91.3	1.3
mTNF-α+OX40L	93.5	1.7
mTNF-α+4.1BBL	92.5	1.7
mbIL-21+OX40L	89	2.4
mbIL-21+4.1BBL	89.8	2.6
mTNF-α+mbIL-21+4.1BBL	89	3.4
QD	88.5	3.4

When cultured in the H9, Jurkat, and Peer cell lines transduced with the gene mbIL-21/4-1BBL and the genes mTNF-α/mbIL-21/4-1BBL, the cell viabilities of natural killer cells were >90% on day 21 of culture (H9) and day 11 of culture (Jurkat, Peer) (Tables 12 to 14, Figs. 3b to 3d).

[Table 12]

H9+transduced gene(s)	Average	STDEV
Parental	86	6.1
mbIL21+4.1BBL	91	3.1
mTNF-α+mbIL21+4.1BBL	94	0.6

[Table 13]

Jurkat+transduced gene(s)	Average	STDEV
Parental	80	6.1
mbIL21+4.1BBL	91	0.6
mTNF-α+mbIL21+4.1BBL	92	2.0

[Table 14]

Peer+transduced gene(s)	Average	STDEV
Parental	83.5	6.1
mbIL21+4.1BBL	91	0.6

Natural killer cells were cultured in the Hut78 cell line transduced with the genes mTNF-α/mbIL-21/4-1BBL with increasing number of restimulations. As a result, the viabilities of natural killer cells were >~90% despite the increasing number of restimulations (Table 15, Fig. 3e).

[Table 15]

Days of culture	32	42	60	70
Average	96.0	93.5	97.5	91.5
STDEV	1.4	0.7	0.7	4.9

These results reveal that the viability of natural killer cells was maintained at a high level even during continued culture for a long period of time, offering the possibility of long-term expansion culture of natural killer cells.

Experimental Example 2. Confirmation of purity of natural killer cells

Natural killer cells cultured for 21 days or natural killer cells cultured by repeated restimulations were collected and centrifuged at 1,200 rpm for 5 min. The culture solution was removed by suction. Cells were counted after dilution with 1 ml of the FACS buffer. Cells were diluted to 5×10^6 cells/ml with the FACS buffer. The diluted cell solution was added to 5 ml FACS tubes (Falcon, 352052) (100 μl per tube). The phenotypes of the cells were analyzed with the following antibodies:

Tube 1: anti-human CD3-FITC (BD Pharmingen, 555332), anti-human CD16-PE (BD Pharmingen, 555407), anti-human CD56-BV421 (BD Pharmingen, 562751)

Tube 2: anti-human CD14-FITC (BD Pharmingen, 555397), anti-human CD19-PE (BD Pharmingen, 555413), anti-human CD3-BV421 (BD Pharmingen, 562438)

Tube 3: anti-human CD3-FITC, anti-human NKG2D-PE (R&D system, FAB139P), anti-human CD56-BV421

Tube 4: anti-human CD3-FITC, anti-human NKp30-PE (BD Pharmingen, 558407), anti-human CD56-BV421

Tube 5: anti-human CD3-FITC, anti-human NKp44-PE (BD Pharmingen, 558563), anti-human CD56-BV421

Tube 6: anti-human CD3-FITC, anti-human NKp46-PE (BD Pharmingen, 557991), anti-human CD56-BV421

Tube 7: anti-human CD3-FITC, anti-human DNAM-1-PE (BD Pharmingen, 559789), anti-human CD56-BV421

Tube 8: anti-human CD3-FITC, anti-human CXCR3-PE (BD Pharmingen, 557185), anti-human CD56-BV421

Tube 9: anti-human CD3-FITC, PE mouse IgG1 κ isotype control (BD Pharmingen, 555749), anti-human CD56-BV421

Tube 10: FITC mouse IgG1 κ isotype control (BD Pharmingen, 555748), PE mouse IgG1 κ isotype control, BV421 mouse IgG1 κ isotype control (BD Pharmingen, 562438)

The cells in each tube were stained with one of the three fluorescent antibodies. Specifically, the cells in Tube 1 were stained with the anti-human CD56 antibody, the cells in Tube 2 were stained with the anti-human CD3 antibody, the cells in Tubes 3-9 were stained with the anti-human CD56 antibody, and the cells in Tube 10 were stained with the isotype control antibody.

The cells in the tubes were stained at a refrigeration temperature for 30 min. Thereafter, 2 ml of the FACS buffer was added to the stained cells, followed by centrifugation at 1,500 rpm for 3 min. The supernatant was discarded, 2 ml FACS buffer was added, followed by centrifugation at 2,000 rpm for 3 min. After removal of the supernatant, cells were suspended in 200 μl of cytofix buffer (fixation buffer, BD, 554655) and were then identified and examined for purity and phenotype using FACS LSRII Fortessa (BD Biosciences).

After co-culture of the CD3(-) cells isolated from cord blood mononuclear cells with the transgenic Hut78 cell line for 21 days, natural killer cells were identified and analyzed for purity. As a result, the contents of natural killer cells (CD3-CD56+) were found to be >90% in all conditions irrespective of whether the cell line was transduced with genes (Table 16, Fig. 4a).

[Table 16]

Hut78+transduced gene(s)	**Average**	**STDEV**
Parental	92.4	9.6
mTNF-α	96.8	2.5
mbIL-21	98.6	0.9
OX40L	95.7	3.6
4.1BBL	98	1.8
mTNF-α+OX40L	97.2	2.5
mTNF-α+4.1BBL	98.6	1
mbIL-21+OX40L	98.4	1.3
mbIL-21+4.1BBL	98.5	0.9
mTNF-α+mbIL-21+4.1BBL	98.7	0.9
QD	99.3	0.5

After co-culture with the H9, Jurkat or Peer cell line transduced with the genes mbIL-21/4-1BBL or mTNF-α/mbIL-21/4-1BBL, the cultured natural killer cells were identified and measured for purity. As a result, the purities of the natural killer cells produced by co-culture with the transgenic cell lines were maintained at higher levels than those by co-culture with the non-transgenic cell lines (Tables 17 to 19, Figs. 4b to 4d).

[Table 17]

H9+transduced gene(s)	Average	STDEV
Parental	91.5	4.1
mbIL21+4.1BBL	98.5	0.7
mTNF-α+mbIL21+4.1BBL	99	0.3

[Table 18]

Jurkat+transduced gene(s)	Average	STDEV
Parental	88.6	6.9
mbIL21+4.1BBL	97.6	1.3
mTNF-α+mbIL21+4.1BBL	97.5	0.8

[Table 19]

Peer+transduced gene(s)	Average	STDEV
Parental	79.2	14.6
mbIL21+4.1BBL	94.9	2.1

Natural killer cells were cultured while increasing the number of restimulations with the cell lines transduced with the three genes mTNF-α/mbIL-21/4-1BBL. The contents of the natural killer cells (CD3-CD56+) were found to be high (>90%) until day 60 of culture (Table 20, Fig. 4e).

[Table 20]

Days of culture	32	60
Average	99.7	97.8
STDEV	0.1	0.8

Experimental Example 3. Analysis of activation markers of natural killer cells

After co-culture of the CD3(-) cells isolated from cord blood mononuclear cells with the transgenic feeder cells for 21 days, the expressions of representative receptors of natural killer cells were analyzed.

When co-cultured with the Hut78 cell line, all CD16 cells were expressed at high levels. Activation markers NKG2D, NKp30, NKp44, NKp46, and DNAM-1 were expressed at higher levels without variations between donors when the feeder cells were transduced with double genes than when the feeder cells were not transduced or transduced with single genes (Figs. 5a to 5g).

The expression levels of CD16, NKG2D, DNAM-1, and CXCR3 were found to be higher when co-cultured with the feeder cells of the non-transgenic H9 cell line than when co-cultured with the feeder cells transduced with the genes mbIL-21/4-1BBL or the triple genes mTNF-α/mbIL-21/4-1BBL. The expression levels of other activation markers NKp30, NKp44, and NKp46 were found to be high without variations between donors. In conclusion, the feeder cells transduced with double and triple genes are useful in increasing the activity and tumor targeting ability of NK cells (Figs. 6a to 6g).

Natural killer cells were produced by restimulating the co-culture using the Hut78 cell line transduced with the triple genes mTNF-α/mbIL-21/4-1BBL, and their phenotypes were identified. As a result, the expressions of activation markers, including NKG2D, NKp44, NKp46, DNAM-1, and CXCR3, showed a tendency to decrease when the restimulation was performed 4 times during culture compared to when the restimulation was performed only once. These results revealed that the increasing number of restimulations led to a long culture period, which affected the expression levels of some of the activation markers (Figs. 7a and 7b).

Experimental Example 4. Confirmation of cytotoxicities of natural killer cells depending on genes transduced into T cells and co-culture with T cells

1×10^6 cells of the K562 cancer cell were placed in a 15 ml tube and centrifuged.

The cell pellets were suspended in RPMI1640 medium supplemented with 1 ml of 10% (v/v) FBS. After addition of 30 μl of 1 mM Calcein-AM (Molecular probe, C34852), the suspension was protected from light with aluminum foil, followed by staining in an incubator at 37 °C for 1 h.

The Calcein-AM stained tumor cell line was washed in RPMI1640 medium supplemented with 10-15 ml of 10% (v/v) FBS and centrifuged. The pellets were suspended to a density of 1×10^5 cells/ml in RPMI1640 medium supplemented with 10 ml of 10% (v/v) FBS. 1×10^6 natural killer cells were placed in a 15 ml tube and centrifuged. The pellets in a desired ratio (1:1) to the K562 cancer cell line were suspended in RPMI1640 medium supplemented with 10% (v/v) FBS. A mixture of the K562 cancer cell line and the natural killer cell line (each 100 μl) were plated in a 96-well U-bottom plate (Nunc, 163320). Fluorescence was measured in triplicate for each well and the measured values were averaged.

The stained K562 cancer cell line was added to spontaneous release wells (100 μl/well) and RPMI1640 medium supplemented with 10% (v/v) FBS was added thereto (100 μl/well). The stained K562 cancer cell line was added to maximum release wells (100 μl/well) and triple-distilled water containing 2% (v/v) Triton-X 100 was added thereto (100 μl/well).

The auto-fluorescence values of the RPMI1640 medium supplemented with 10% (v/v) FBS and the RPMI1640 medium supplemented with 2% (v/v) Triton-X 100 were corrected by subtracting the fluorescence value of a mixture of 100 μl of RPMI1640 medium supplemented with 10% (v/v) FBS and 100 μl of RPMI1640 medium supplemented with 2% (v/v) Triton-X 100 from the fluorescence value of 200 μl of RPMI1640 medium supplemented with 10% (v/v) FBS and adding the difference **A** to the maximum release value.

Incubation was allowed to proceed in an incubator at 37 °C for 4 h in the dark and the plate was then centrifuged at 2,000 rpm for 3 min. The supernatant was plated in a 96-well black plate (Nunc, 237108) (100 μl per well). The fluorescence value ($OD_{480/535}$ nm) was measured using a fluorescent plate reader (Perkin Elmer, VICTOR X3) and the

cytotoxicity (% of killing) of natural killer cells to tumor cells was calculated using the following equation II:

[Equation II]

% of killing = (average fluorescence value of sample well - average fluorescence value of spon. well)/[(average fluorescence value of max. well + **A**) - average fluorescence value of spon. well] × 100

The direct cytotoxicities of natural killer cells cultured using various types of feeder cells to the K562 cancer cell line were measured. As a result, the cytotoxicities of natural killer cells cultured using feeder cells transduced with the genes mbIL-21/4-1BBL gene and feeder cells transduced with the genes mTNF-α/mbIL-21/4-1BBL were higher than those of natural killer cells cultured using non-transgenic feeder cells (Figs. 8a to 8d).

The cytotoxicity of natural killer cells was maintained high without a significant difference until day 60 of culture despite the increasing number of restimulations with the Hut78 cell line transduced with the genes mTNF-α/mbIL-21/4-1BBL (FIG. 8e).

These results concluded that the feeder cells transduced with the genes mbIL-21/4-1BBL or the genes mTNF-α/mbIL-21/4-1BBL are useful in *ex vivo* expansion culture of high-purity natural killer cells with high cytotoxicity and activity than the non-transgenic feeder cells.

Example 4. Animal experiments

Example 4.1. Culture of natural killer cells using transgenic T feeder cells

500 or 1,000 IU of IL-2 (Proleukin Injection, Novartis Korea) and 10 ng/ml OKT-3 (eBioscience, 16-0037-85) were plated in a plastic culture plate. On day 0 of culture, CD3(-) cord blood mononuclear cells or peripheral blood mononuclear cells and transgenic T cells were added in a ratio of 1:2.5 and 0.25 ml of CellGro medium supplemented with 2% (v/v) human plasma was added. Cells were cultured statically in an incubator at 37 °C for 4 days. The cord blood mononuclear cells were used at a

concentration of 1000 IU/mL and the peripheral blood mononuclear cells were used at a concentration of 500 IU/mL.

Cord blood-derived natural killer cells were cultured by the following procedure. On day 4 of culture, the equal amount of CellGro medium supplemented with 1% (v/v) human plasma and 1,000 IU/ml IL-2 was added, followed by stationary culture. Thereafter, cells were counted at intervals of 2-3 days. CellGro medium supplemented with 1% (v/v) human plasma and 1,000 IU/ml IL-2 was added and culture was continued until day 14 such that the cell density was 1×10^6 cells/ml. On day 14, culture of natural killer cells was restimulated with the transgenic T feeder cells in a ratio of 1:2.5. Natural killer cells were cultured in CellGro medium supplemented with 1% (v/v) human plasma, OKT3, and IL-2. Thereafter, cells were counted at intervals of 2-3 days and CellGro medium supplemented with 1% (v/v) human plasma and 1000 IU/mL IL-2 were added. Culture was continued for additional 14 days (for a total of 28 days) such that the cell number was 1×10^6 cells/mL.

Cord blood-derived natural killer cells were cultured by the following procedure. On day 4 of culture, the equal amount of CellGro medium supplemented with 1% (v/v) human plasma and 500 IU/ml IL-2 was added, followed by stationary culture. Thereafter, cells were counted at intervals of 2-3 days. CellGro medium supplemented with 1% (v/v) human plasma and 500 IU/ml IL-2 was added and culture was continued until day 11 such that the cell density was 1×10^6 cells/ml. On day 11, culture of natural killer cells was restimulated with transgenic T feeder cells in a ratio of 1:2.5. Natural killer cells were cultured in CellGro medium supplemented with 1% (v/v) human plasma, OKT3, and IL-2. Thereafter, cells were counted at intervals of 2-3 days and CellGro medium supplemented with 1% (v/v) human plasma and 1000 IU/mL IL-2 were added. Culture was continued for additional 8-10 days (for a total of 19-21 days) such that the cell number was 1×10^6 cells/mL.

The cultured cells were suspended to 1×10^6 cells/mL in a freezing medium, frozen in a temperature-controlled freezer, and stored in liquid nitrogen.

The proliferation rates of the cultured natural killer cells were compared. As a

result, the cord blood-derived natural killer cells (CB-enFeeder) proliferated approximately 80,000-fold and the peripheral blood-derived natural killer cells (PBMC-enFeeder) proliferated 55,000-fold. There was no significant difference in proliferation rate between CB-enFeeder and PBMC-enFeeder (Table 21).

[Table 21]

	Average	STDEV
CB-enFeeder	79288.3	37381.0
PBMC-enFeeder	53649.3	16827.9

Example 4.2. Efficacy evaluation using Raji animal models

Cancer cells of the Raji-luc cell line were collected on the last day of culture and the cell density was adjusted to 5×10^5 cells/mL with PBS. The cells were injected into mice via the tail vein (0.2 mL (1×10^5 cells)/mouse)). Natural killer cells were injected at a density of 2×10^7 cells/200 μL via the tail vein. Rituxan (RTX) (Mabthera injection, Roche Korea) was diluted to 0.01 μg/100 μL with PBS. 100 μL of the dilution was injected subcutaneously into a weakened region between the scapula and the thoracic wall of each mouse. On the next day after cancer cell transplantation, NK cells were administered via the tail vein a total of 6 times using a restrainer. and RTX was administered subcutaneously once (Table 22, Fig. 9a).

[Table 22]

Group	Dose(s)	Dosing route(s)	Volume(s) (μl)	Number of heads
1	Freezing medium+IgG 0.01 μg/head	i.v+s.c	200+100	10
2	RTX 0.01 μg/head	s.c	100	10
3	PBMC-enFeeder NK 2×10^7 cells/head	i.v	200	10
4	CB-eFeeder NK 2×10^7 cells/head	i.v	200	10
5	PB-eFeeder NK+RTX	i.v+s.c	200+100	10
6	CB-eFeeder NK+RTX	i.v+s.c	200+100	10

All animals, including general symptoms and dead animals, were observed twice daily. After an observation was made as to whether the animals were dead or alive, the median survival times of the freezing medium control group and the groups treated with

natural killer cells and RTX were calculated to evaluate the effect of natural killer cells to prolong the survival rate. Over 26-122 days after transplantation of the Raji-luc cell line, dead animals were observed in the groups administered natural killer cells and RTX alone or in combination. The median survival time of the freezing medium control group until the last day (day 122) was 30 days. In contrast, the median survival times of the group administered RTX, the group administered PBMC-enFeeder, and the group administered CB-enFeeder were 48.5 days, 43 days, and 47 days, respectively. The median survival times of the group administered both PBMC-enFeeder and RTX and the group administered both CB-enFeeder and RTX were 55 days and 75.5 days, respectively (Table 23, Fig. 9b).

[Table 23]

Raji-luc models	Freezing medium	RTX	PBMC-enFeeder	CB-enFeeder	RTX+PBMC-enFeeder	RTX+CB-enFeeder
Median survival time (days)	29.5	48.5	43	47	55	75.5

Example 4.3. efficacy evaluation using Ramos animal models

Cancer cells of the Ramos cell line were collected on the last day of culture and the cell density was adjusted to 5×10^6 cells/mL with PBS. The cells were injected into mice via the tail vein (0.2 mL (1×10^6 cells)/mouse)). Natural killer cells were injected at a density of 2×10^7 cells/200 μL via the tail vein. RTX was diluted to 0.3 μg/100 μL with PBS. 100 μL of the dilution was injected subcutaneously into a weakened region between the scapula and the thoracic wall of each mouse. Natural killer cells were administered via the tail vein a total of 6 times using a restrainer from the fourth day after cancer cell transplantation and RTX was administered via the tail vein a total of 6 times from the third day after cancer cell transplantation (Table 24, Fig. 10a).

[Table 24]

Group	Dose(s)	Dosing route(s)	Volume(s) (μl)	Number of heads
1	Freezing medium+IgG 0.3 μg/head	i.v+i.v	200+100	10
2	RTX 0.3 μg/head	i.v	100	10
3	PBMC-enFeeder NK 2×10^7 cells/head	i.v	200	10
4	CB-eFeeder NK 2×10^7 cells/head	i.v	200	10
5	PB-eFeeder NK+RTX	i.v+i.v	200+100	10
6	CB-eFeeder NK+RTX	i.v+i.v	200+100	10

All animals, including general symptoms and dead animals, were observed twice daily. After an observation was made as to whether the animals were dead or alive, the median survival times of the freezing medium control group and the groups treated with natural killer cells and RTX were calculated to evaluate the effect of natural killer cells to prolong the survival rate. Over 34-110 days after transplantation of the Ramos cell line, dead animals were observed in the groups administered natural killer cells and RTX alone or in combination. The median survival time of the freezing medium control group until the last day (day 124) was 31 days. In contrast, the median survival times of the group administered RTX, the group administered PBMC-enFeeder, and the group administered CB-enFeeder were 49.5 days, 42 days, and 42.5 days, respectively. The median survival times of the group administered both PBMC-enFeeder and RTX and the group administered both CB-enFeeder and RTX were 63.5 days and 87.5 days, respectively (Table 25, Fig. 10b).

[Table 25]

Ramos models	Freezing medium	RTX	PBMC-enFeeder	CB-enFeeder	RTX+PBMC-enFeeder	RTX+CB-enFeeder
Median survival time (days)	31	49.5	42	42.5	63.5	87.5

Although the particulars of the present invention have been described in detail, it will be obvious to those skilled in the art that such particulars are merely preferred embodiments and are not intended to limit the scope of the present invention. Therefore, the substantial scope of the present invention is defined by the appended claims and their equivalents.

CLAIMS

1. A method for culturing natural killer cells comprising co-culturing transformed CD4+ T cells and seed cells.

2. The method according to claim 1, wherein the transformed CD4+ T cells express at least one gene selected from the group consisting of the 4-1BBL, mbIL-21, OX40L, and mTNF-α genes.

3. The method according to claim 2, wherein the 4-1BBL gene has a nucleotide sequence encoding the amino acid sequence set forth in SEQ ID NO: 1.

4. The method according to claim 3, wherein the encoding nucleotide sequence is set forth in SEQ ID NO: 2.

5. The method according to claim 2, wherein the mbIL-21 gene has a nucleotide sequence encoding the amino acid sequence set forth in SEQ ID NO: 3.

6. The method according to claim 5, wherein the encoding nucleotide sequence is set forth in SEQ ID NO: 4.

7. The method according to claim 2, wherein the OX40L gene has a nucleotide sequence encoding the amino acid sequence set forth in SEQ ID NO: 5.

8. The method according to claim 7, wherein the encoding nucleotide sequence is set forth in SEQ ID NO: 6.

9. The method according to claim 2, wherein the mTNF-α gene has a nucleotide sequence encoding the amino acid sequence set forth in SEQ ID NO: 8.

10. The method according to claim 9, wherein the encoding nucleotide sequence is set forth in represented by SEQ ID NO: 9.

11. The method according to claim 2, wherein the gene is transduced through a recombinant lentivirus.

12. The method according to claim 1, wherein the CD4+ T cells express the 4-1BBL or mbIL-21 gene.

13. The method according to claim 1, wherein the CD4+ T cells express the 4-1BBL and mbIL-21 genes.

14. The method according to claim 1, wherein the CD4+ T cells express the 4-1BBL, mbIL-21, and mTNF-α genes.

15. The method according to claim 1, wherein the CD4+ T cells express the 4-1BBL, mbIL-21, OX40L, and mTNF-α genes.

16. The method according to claim 1, wherein the CD4+ T cells are selected from the group consisting of Hut78, H9, Jurkat, Loucy, Molt-3, Molt-13, Peer, RPMI8402, and TALL-01 cells.

17. The method according to claim 1, wherein the seed cells are cord blood-derived mononuclear cells.

18. The method according to claim 1, wherein the seed cells are cells from which CD3(+) cells are eliminated.

19. The method according to claim 1, wherein the transformed CD4+ T cells and the seed cells are mixed in a ratio of 0.1:1 to 50:1.

20. The method according to claim 1, wherein the seed cells are mixed with the feeder cells only once for 5 to 60 days or twice or more for at least 60 days.

21. The method according to claim 1, wherein natural killer cells are cultured in a medium supplemented with an anti-CD3 antibody and an interleukin protein.

22. The method according to claim 21, wherein the anti-CD3 antibody is selected from the group consisting of OKT-3, UCHT1, and HIT3a.

23. The method according to claim 21, wherein the interleukin protein is selected from the group consisting of IL-2, IL-12, IL-15, IL-18, and IL-21.

24. Natural killer cells produced by the method according to claim 1.

25. A composition for culturing natural killer cells comprising transformed CD4+ T cells as active ingredients.

26. The composition according to claim 25, wherein the transformed CD4+ T cells express at least one gene selected from the group consisting of the 4-1BBL, mbIL-21, OX40L, and mTNF-α genes.