DESCRIPTION

Title of Invention: CAR-EXPRESSING T CELLS AND CAR EXPRESSION VECTOR

Technical Field

[0001]

The present invention relates to immune cells expressing a chimeric antigen receptor (hereinafter referred to as "CAR") (hereinafter referred to as "CAR-expressing immune cells") that can be used for cancer immunotherapy, typically, T cells expressing a CAR (hereinafter referred to as "CAR-T cells"), and an expression vector for producing the CAR-expressing immune cells (such as CAR-T cells). More specifically, the present invention relates to CAR-expressing immune cells (such as CAR-T cells) expressing predetermined cytokines and chemokines, and an expression vector for producing the cells.

Background Art

[0002]

Cancer immunotherapy by administration of CAR-T cells has been shown to be effective in hematopoietic malignancies such as leukemia and lymphoma, but there are cancer types and

cases for which no therapeutic effect is observed due to problems such as low survival efficiency of CAR-T cells in vivo and inhibition of the activity of CAR-T cells in the microenvironment of cancers by the tumor immunoavoidance mechanism of cancer cells, especially, in solid cancers. Therefore, CAR-T cells exhibiting a therapeutic effect and having higher antitumor activity are required, especially, in solid cancers.

[0003]

Patent Literature 1 and Non Patent Literature 1 disclose CAR-T cells that express IL-7 and CCL19 and are more excellent in antitumor activity than conventional CAR-T cells. However, Patent Literature 1 and Non Patent Literature 1 do not include specific descriptions of other combinations of cytokines and chemokines.

[0004]

Non Patent Literature 2 discloses CAR-T cells that have improved persistence independent of CAR signaling and express membrane-bound chimeric IL-15 (mbIL-15). However, Non Patent Literature 2 does not include specific descriptions of combinations of mbIL-15 with chemokines such as CCL19.

[0005]

Non Patent Literature 3 discloses that use of a fusion protein comprising IL-15 linked to an IL-15Rαsushi domain via a flexible linker can provide more potent activity in proliferation of lymphocytes (such as NK cells, NK-T cells, and memory CD8-positive cells), activation of dendritic cells, and the like than that caused by conventional combined use of IL-15 and IL-15Rαsushi domain. However, Non Patent Literature 3 does not include specific descriptions of combinations of IL-15 and IL-15Rαsushi domain with chemokines such as CCL19.

[0006]

Non Patent Literature 4 discloses that transfection of a secretory fusion protein of mouse IL-15 and IL-15Rα improves the viability and proliferative capacity of CD8-positive T cells. However, Non Patent Literature 4 does not include specific descriptions of combinations of IL-15 with chemokines such as CCL19.

[0007]

Non Patent Literature 5 discloses single-chain IL-27 (p28 and EBI3 linked by a flexible linker) and an effect thereof on treatment of inflammatory bowel disease (IBD). However, Non Patent Literature 5 does not include specific descriptions of CAR-T cells and combinations of single-chain IL-27 with chemokines such as CCL19.

[0008]

Patent Literature 2 discloses T cells expressing recombinant IL-7, recombinant IL-15, or combinations of these and discloses that expression of such cytokines improves T cell survival. However, Patent Literature 2 does not include specific descriptions of CAR-T cells and combinations of the specific cytokines with chemokines such as CCL19.

[0009]

Patent Literature 3 discloses a modified natural killer T cell containing an expression construct encoding IL-2, IL-4, IL-7, IL-15 or combinations of these, and a CAR construct. However, Patent Literature 3 does not include specific descriptions of combinations of the specific cytokines with chemokines such as CCL19.

[0010]

Patent Literature 4 discloses CAR-T cells expressing membrane-bound cytokines such as IL-7, IL-15 (IL-15/IL-15Rα fusion protein), and IL-21. However, Patent Literature 4 does not include specific descriptions of combinations of the specific cytokines with chemokines such as CCL19.

[0011]

Patent Literature 5 discloses a CAR-T cell expressing IL-15 and targeting CD19. However, Patent Literature 5 does

not include specific descriptions of combinations of IL-15 with chemokines such as CCL19.

[0012]

Non Patent Literature 6 discloses a CAR-T cell expressing IL-15 and/or IL-21 and targeting GPC3. However, Non Patent Literature 6 does not include specific descriptions of combinations of the specific cytokines with chemokines such as CCL19.

Citation List

Patent Literature

[0013]

Patent Literature 1: WO 2016/056228

Patent Literature 2: WO 2007/037780

Patent Literature 3: WO 2013/040371

Patent Literature 4: WO 2014/186469

Patent Literature 5: US 2013/0071414

Non Patent Literature

[0014]

Non Patent Literature 1: Adachi et al., Nature Biotechnology, VOL 36, No 4, 346-353

Non Patent Literature 2: Hurton et al., Proc Natl Acad Sci., 113 (48), E7788-97, 2016

Non Patent Literature 3: Mortier et al., J Biol Chem. 281

(3), 1612-9, 2006

Non Patent Literature 4: Rowley et al., Eur J Immunol. 39 (2), 491-506, 2009

Non Patent Literature 5: Sasaoka et al., Am J Physiol Gastrointest Liver Physiol 300: G568-576

Non Patent Literature 6: Barta et al., Armored Glypican-3-Specific CAR T cells for the Immunotherapy of Hepatocellular Carcinoma, ASGCT 2018, May 2018, abstract

Summary of Invention

Technical Problem

[0015]

It is an object of the present invention to provide immune cells (such as CAR-T cells) having higher antitumor activity than immune cells (such as CAR-T cells) expressing CAR alone (not expressing cytokines and/or chemokines).

Solution to Problem

[0016]

There are at least several hundreds of molecules in vivo that can control the functions of immune cells such as T cells. The inventors have found that the antitumor activity is enhanced by CAR-T cells expressing at least one selected from the group consisting of interleukin-15 (IL-15),

interleukin-18 (IL-18), interleukin-21 (IL-21), and interleukin-27 (IL-27), which are cytokines, in combination with CC chemokine ligand 19 (CCL19), which is a chemokine, together with CAR, as compared with cells expressing CAR alone, and the persistence and proliferation of CAR-T cells are especially improved, thereby accomplishing the present invention.

[0017]

IL-15, IL-18, IL-27, and CCL19 are cytokines and a chemokine that are not expressed by natural T cells (in which no exogenous genes are introduced) in vivo. IL-21 is a cytokine expressed by natural T cells in vivo (such as NK T cells and CD4-positive T cells). In the present invention, expression of the predetermined cytokine and chemokine means expression of the predetermined cytokine and chemokine at higher levels than in natural T cells in vivo by introducing genes for expressing the predetermined exogenous cytokine and chemokine into T cells.

[0018]

Further, the embodiment of gene transfer and expression of the aforementioned predetermined interleukins and chemokine together with CAR in T cells can be extended to an embodiment of gene transfer and expression thereof in

immune cells other than T cells, such as NK cells, monocytes, macrophages, and dendritic cells.

[0019]

The present invention includes at least the following aspects.

[1] A T cell expressing:

(1) a chimeric antigen receptor (CAR);

(2) at least one selected from the group consisting of interleukin-15 (IL-15), interleukin-18 (IL-18), interleukin-21 (IL-21), and interleukin-27 (IL-27); and

(3) CC chemokine ligand 19 (CCL19).

[2] The T cell according to item 1, wherein the (2) is IL-15.

[3] The T cell according to item 2, wherein the IL-15 is linked to IL-15Rα to form a fusion protein.

[4] The T cell according to item 3, wherein the fusion protein is a fusion protein comprising IL-15LSP linked to IL-15 ($IL15_{LSP}$), a fusion protein comprising IL-15Rα extracellular domain linked to IL-15 ($_{s}IL15_{RA}$), a fusion protein comprising IL-15 linked to full-length IL-15Rα ($_{mb}IL15_{RA}$), or a fusion protein comprising IL-15Rα containing a signal peptide and a sushi domain linked to IL-15 ($_{sushi}IL15$).

[5] The T cell according to item 3, wherein the fusion

protein is a fusion protein comprising IL-15 linked to full-length IL-15Rα ($_{mb}$IL15$_{RA}$), or a fusion protein comprising IL-15Rα containing a signal peptide and a sushi domain linked to IL-15 ($_{sushi}$IL15).

[6] A medicament comprising the T cell according to item 1.

[7] The medicament according to item 6, wherein the medicament is a therapeutic agent for cancer.

[8] The medicament according to item 7, wherein the cancer is melanoma, Merkel cell cancer, colorectal cancer, kidney cancer, breast cancer, ovarian cancer, fallopian tube cancer, cervical cancer, liver cancer, lung cancer, non-small cell lung cancer, head and neck cancer, small intestine cancer, prostate cancer, bladder cancer, rectal cancer, pancreatic cancer, Ewing's sarcoma, rhabdomyosarcoma, nasopharyngeal cancer, esophageal cancer, biliary tract cancer, neuroblastoma, osteosarcoma, acute myeloid leukemia, multiple myeloma, lymphoma, or leukemia.

[9] An expression vector comprising:

(1) a nucleic acid encoding a CAR;

(2) a nucleic acid encoding at least one selected from the group consisting of IL-15, IL-18, IL-21, and IL-27; and

(3) a nucleic acid encoding CCL19.

[10] The expression vector according to item 9, wherein the

(2) is IL-15.

[11] The expression vector according to item 10, wherein the IL-15 is linked to IL-15Rα to form a fusion protein.

[12] The expression vector according to item 11, wherein the fusion protein is a fusion protein comprising IL-15LSP linked to IL-15 ($IL15_{LSP}$), a fusion protein comprising IL-15Rα extracellular domain linked to IL-15 ($_{s}IL15_{RA}$), a fusion protein comprising IL-15 linked to full-length IL-15Rα ($_{mb}IL15_{RA}$), or a fusion protein comprising IL-15Rα containing a signal peptide and a sushi domain linked to IL-15 ($_{sushi}IL15$).

[13] The expression vector according to item 11, wherein the fusion protein is a fusion protein comprising IL-15 linked to full-length IL-15Rα ($_{mb}IL15_{RA}$), or a fusion protein comprising IL-15Rα containing a signal peptide and a sushi domain linked to IL-15 ($_{sushi}IL15$).

[14] A method for producing a CAR-T cell, comprising a step of introducing the expression vector according to item 9 into a T cell.

[15] A method for treating cancer, comprising a step of administering the T cell according to item 1 to a subject in need of cancer treatment.

[16] The treatment method according to item 15, wherein the cancer is melanoma, Merkel cell cancer, colorectal cancer,

kidney cancer, breast cancer, ovarian cancer, fallopian tube cancer, cervical cancer, liver cancer, lung cancer, non-small cell lung cancer, head and neck cancer, small intestine cancer, prostate cancer, bladder cancer, rectal cancer, pancreatic cancer, Ewing's sarcoma, rhabdomyosarcoma, nasopharyngeal cancer, esophageal cancer, biliary tract cancer, neuroblastoma, osteosarcoma, acute myeloid leukemia, multiple myeloma, lymphoma, or leukemia.

[17] The T cell according to item 1, for use as an active component for cancer treatment.

[18] The T cell according to item 17, wherein the cancer is melanoma, Merkel cell cancer, colorectal cancer, kidney cancer, breast cancer, ovarian cancer, fallopian tube cancer, cervical cancer, liver cancer, lung cancer, non-small cell lung cancer, head and neck cancer, small intestine cancer, prostate cancer, bladder cancer, rectal cancer, pancreatic cancer, Ewing's sarcoma, rhabdomyosarcoma, nasopharyngeal cancer, esophageal cancer, biliary tract cancer, neuroblastoma, osteosarcoma, acute myeloid leukemia, multiple myeloma, lymphoma, or leukemia.

[0020]

Hereinafter, the T cell of [1] above is also referred to as "T cell of the present invention", the medicament of [6] above is also referred to as "medicament of the present

invention", the expression vector of [9] above is also referred to as "expression vector of the present invention", and the method for producing a CAR-T cell of [14] above is also referred to as "the production method of the present invention".

[0021]

Those skilled in the art would be able to appropriately convert the embodiments (categories) of the invention in consideration of the contents disclosed herein as a whole. For example, the medicament of the present invention according to [6] above can be converted into "a method for treating cancer, comprising a step of administering a T cell expressing: (1) a CAR; (2) at least one selected from the group consisting of IL-15, IL-18, IL-21, and IL-27; and (3) CCL19 to a subject in need of cancer treatment", " a T cell expressing: (1) a CAR; (2) at least one selected from the group consisting of IL-15, IL-18, IL-21, and IL-27; and (3) CCL19 for use as an active component for cancer treatment", and "use of a T cell expressing: (1) a CAR; (2) at least one selected from the group consisting of IL-15, IL-18, IL-21, and IL-27; and (3) CCL19 for the manufacture of a medicament for treating cancer.

[0022]

Those skilled in the art would be able to appropriately convert the embodiment of the T cell of the present invention to be obtained by allowing the T cell to express the predetermined cytokine and chemokine together with a CAR, and relevant embodiments thereto such as the medicament of the present invention, the expression vector of the present invention, and the production method of the present invention into an embodiment of immune cells other than T cells (such as NK cells, monocytes, macrophages, and dendritic cells) expressing the predetermined cytokine and chemokine together with a CAR and relevant embodiments thereto such as a medicament, an expression vector, and a production method, in consideration of the contents disclosed herein as a whole.

Advantageous Effects of Invention

[0023]

The persistence and proliferation of the T cell of the present invention are improved by the T cell expressing the predetermined cytokine (such as IL-15) and chemokine (CCL19). Therefore, the antitumor activity by the CAR can be enhanced (e.g., reduction in the number of residual tumor cells, improvement in amount of IFNγ to be produced, and improvement in migration and accumulation of host immune cells (such as T cells, dendritic cells, NK cells) in the tumor site).

Further, the therapeutic effect on cancer can be improved by a medicament containing the T cell of the present invention. In particular, the T cell of the present invention may be involved in NK cell activation and therefore may be useful for the treatment of carcinomas sensitive to NK cells (such as melanoma, Merkel cell cancer, colorectal cancer, kidney cancer, breast cancer, ovarian cancer, fallopian tube cancer, cervical cancer, liver cancer, lung cancer, non-small cell lung cancer, head and neck cancer, small intestine cancer, prostate cancer, bladder cancer, rectal cancer, pancreatic cancer, Ewing's sarcoma, rhabdomyosarcoma, nasopharyngeal cancer, esophageal cancer, biliary tract cancer, neuroblastoma, osteosarcoma, acute myeloid leukemia, multiple myeloma, lymphoma, and leukemia). Further, since the enhancement of the antitumor activity reduces the number of cells to be administered, effects such as reduction in side effects such as CRS (Cytokine Release Syndrome) and reduction in production cost are expected.

[0024]

The combination of the predetermined cytokine and chemokine of the present invention exerts action and effect that various CAR-expressing immune cells with high antitumor activity can be obtained even in the case where the genes are transferred and expressed together with a CAR gene in immune

cells in general, that is, not only in T cells as mentioned above but also in NK cells, monocytes, macrophages, and dendritic cells.

Brief Description of Drawings

[0025]

[Figure 1] Figure 1 shows the results of analysis of the expression level of the CAR (the horizontal axis) and CD8 (the vertical axis) in the following T cells of Example (Experimental Example 1) by flow cytometry. The numerical value (%) in the figure represents the percentage of the cell population in each compartment with respect to the total cells. "Transduction (-)" is a result for transduction (-) T cells of Comparative Example 1 (T cells without transduction), "conv. CAR-T" is a result for conv. CAR-T cells of Comparative Example 2 (anti-human CD20 CAR-T cells), "15_{LSP} x 19 CAR-T" is a result for $IL15_{LSP}$ × CCL19 CAR-T cells of Example 1-1 ($IL15_{LSP}$_CCL19_anti-human CD20 CAR-T cells), "$_{s}15_{RA}$ x 19 CAR-T" is a result for $_{s}IL15_{RA}$ × CCL19 CAR-T cells of Example 1-2 ($_{s}IL15_{RA}$_CCL19_anti-human CD20 CAR-T cells), "$_{mb}15_{RA}$ x 19 CAR-T" is a result for $_{mb}IL15_{RA}$ × CCL19 CAR-T cells of Example 1-3 ($_{mb}IL15_{RA}$_CCL19_anti-human CD20 CAR-T cells), "$_{sushi}$15 x 19 CAR-T" is a result for $_{sushi}$IL15 × CCL19 CAR-T cells of Example 1-4 ($_{sushi}$IL15_CCL19_anti-human CD20 CAR-T

cells), "18 x 19 CAR-T" is a result for IL-18 × CCL19 CAR-T cells of Example 2 (IL18_CCL19_anti-human CD20 CAR-T cells), "21 x 19 CAR-T" is a result for IL-21 × CCL19 CAR-T cells of Example 3 (IL21_CCL19_anti-human CD20 CAR-T cells), and "$_{sc}$27 x 19 CAR-T" is a result for $_{sc}$IL27 × CCL19 CAR-T cells of Example 4 ($_{sc}$IL27_CCL19_anti-human CD20 CAR-T cells). The same applies also to Figures 2 to 6 below.

[Figure 2] Figure 2 shows the results of measurement of amounts of (A) IL-15, (B) IL-18, (C) IL-21, (D) IL-27, and (E) CCL19 secreted into the culture supernatant in the T cells in Example (Experimental Example 1) by ELISA.

[Figure 3] Figure 3 shows the results of measurement of the number of living cells in the T cells 3 days, 5 days, and 7 days after stimulation of activation by co-culture with human CD20 expressing P815 mastocytoma (hCD20/P815) in Example (Experimental Example 2).

[Figure 4] Figure 4 shows the results of histogram analysis of the staining intensity with a CytoTell reagent in the T cells in Example (Experimental Example 2). The peak of the histogram shows the number of generations due to cell division, and the numerical value (%) in the donut graph shows the percentage of gated fractions in the Thy1.2-positive T cell population (1 represents the undivided first generation, 2 represents the second generation after one

division, 3 represents the third generation after two divisions, 4 represents the fourth generation after three divisions, > 5 represents the fifth and subsequent generations after four or more divisions).

[Figure 5] Figure 5 shows the results of measurement of the tumor cytotoxic activity of the T cells in (A) target tumor cells (hCD20/P815) and (B) control tumor cells (P815) in Example (Experimental Example 3) by flow cytometry.

[Figure 6] Figure 6 shows the results of measurement of the concentration of IFNγ in the culture supernatant of the T cells at the time of co-culture with (A) target tumor cells (hCD20/P815), and (B) control tumor cells (P815) in Example (Experimental Example 3).

[Figure 7] Figure 7 shows the arrangement of genes and the like contained in the nucleotide sequence of SEQ ID NO: 1 (anti-human CD20 CAR DNA fragment).

[Figure 8] Figure 8 shows the arrangement of genes and the like contained in the nucleotide sequence of SEQ ID NO: 3 (MCS DNA fragment).

[Figure 9] Figure 9 shows the arrangement of genes and the like contained in the nucleotide sequence of SEQ ID NO: 4 ($IL15_{LSP}$_F2A_CCL19 DNA fragment).

[Figure 10] Figure 10 shows the arrangement of genes and the like contained in the nucleotide sequence of SEQ ID NO: 6

($_{s}$IL15$_{RA}$_F2A_CCL19 DNA fragment).

[Figure 11] Figure 11 shows the arrangement of genes and the like contained in the nucleotide sequence of SEQ ID NO: 8 ($_{mb}$IL15$_{RA}$_F2A_CCL19 DNA fragment).

[Figure 12] Figure 12 shows the arrangement of genes and the like contained in the nucleotide sequence of SEQ ID NO: 10 ($_{sushi}$IL15_F2A_CCL19 DNA fragment).

[Figure 13] Figure 13 shows the arrangement of genes and the like contained in the nucleotide sequence of SEQ ID NO: 12 (IL-18_F2A_CCL19 DNA fragment).

[Figure 14] Figure 14 shows the arrangement of genes and the like contained in the nucleotide sequence of SEQ ID NO: 14 (IL-21_F2A_CCL19 DNA fragment).

[Figure 15] Figure 15 shows the arrangement of genes and the like contained in the nucleotide sequence of SEQ ID NO: 16 ($_{sc}$IL27_F2A_CCL19 DNA fragment).

[Figure 16] Figure 16 shows the results of measurement of the antitumor activity (change in tumor volume) for a mouse melanoma tumor transplant model in Example (Experimental Example 4).

[Figure 17] Figure 17 shows the results of measurement of the antitumor activity (change in tumor volume) for a mouse colorectal cancer tumor model in Example (Experimental Example 4).

Description of Embodiments

[0026]

As used herein, "transfection" means introduction of any substance into a cell. A cell includes at least a cytoplasm and a nucleus thereinside.

[0027]

"Culture" or "culturing" means maintaining, proliferating, and/or differentiating cells outside tissues or outside the body, e.g., in a dish, a Petri dish, a flask, or a culture tank.

[0028]

"Pluripotency" means the ability to differentiate into tissues and cells having various different morphologies and functions, and the ability to differentiate into cells of any lineage of the three germ layers. "Pluripotency" is distinguished from "totipotency" which means the ability to differentiate into any tissue in the body including blastocysts, in that the pluripotency does not mean the ability to differentiate into blastocysts and therefore lacks the ability to form individuals.

[0029]

"Multipotency" means the ability to differentiate into cells of a limited number of lineages. For example,

mesenchymal stem cells, hematopoietic stem cells, and neural stem cells are multipotent but not pluripotent.

[0030]

Examples of "stem cells" include pluripotent stem cells.

[0031]

The "pluripotent stem cells" that can be used in the present invention refer to stem cells that can differentiate into tissues and cells having various different morphologies and functions of the living body and have the ability to differentiate into cells of any lineage of the three germ layers (endoderm, mesoderm, and ectoderm). Examples thereof include, but not specifically limited to, embryonic stem cells (ESCs), embryonic stem cells derived from cloned embryos obtained by nuclear transplantation, sperm stem cells, embryonic germ cells, and induced pluripotent stem cells (hereinafter also referred to as "iPSCs").

[0032]

Further, "multipotent stem cells" that can be used in the present invention refer to stem cells having the ability to differentiate into cells of a limited number of lineages. Examples of "multipotent stem cells" that can be used in the present invention include somatic stem cells derived from dental pulp stem cells, oral mucosa-derived stem cells, hair

follicle stem cells, culture fibroblasts, and bone marrow stem cell. Preferred pluripotent stem cells are ESCs and iPSCs.

[0033]

"Induced pluripotent stem cells (iPSCs)" refer to cells obtained by introducing a specific factor (nuclear reprogramming factor) into mammalian somatic cells or undifferentiated stem cells and reprogramming the cells. Currently, there are various types of "induced pluripotent stem cells". Other than iPSCs established by introducing four factors of Oct3/4, Sox2, Klf4, and c-Myc into mouse fibroblasts by Yamanaka, et al. (Takahashi K, Yamanaka S., Cell, (2006) 126: 663-676), human cell-derived iPSCs established by introducing the same four factors into human fibroblasts (Takahashi K, Yamanaka S., et al. Cell, (2007) 131: 861-872.), Nanog-iPS cells established by introducing the four factors and then sorting the cells using expression of Nanog as an index (Okita, K., Ichisaka, T., and Yamanaka, S. (2007). Nature 448, 313-317.), iPS cells produced by a method excluding c-Myc (Nakagawa M, Yamanaka S., et al. Nature Biotechnology, (2008) 26, 101-106), and iPS cells established by introducing six factors by a virus-free method (Okita K et al. Nat. Methods 2011 May; 8(5): 409-12, Okita K et al. Stem Cells. 31(3): 458-66.) can also be used.

Further, induced pluripotent stem cells, produced by Thomson, et al., established by introducing four factors of OCT3/4, SOX2, NANOG, and LIN28 (Yu J., Thomson JA. et al., Science (2007) 318: 1917-1920.), induced pluripotent stem cells produced by Daley, et al. (Park IH, Daley GQ. et al., Nature (2007) 451: 141-146), induced pluripotent stem cells produced by Sakurada, et al. (JP 2008-307007), and the like can also be used. Other than above, any of the induced pluripotent stem cells known in the art described in all the published papers (such as Shi Y., Ding S., et al., Cell Stem Cell, (2008) Vol 3, Issue 5, 568-574; Kim JB., Scholer HR., et al., Nature, (2008) 454, 646-650; and Huangfu D., Melton, DA., et al., Nature Biotechnology, (2008) 26, No 7, 795-797) or patents (such as JP 2008-307007, JP 2008-283972, US 2008-2336610, US 2009-047263, WO 2007-069666, WO 2008-118220, WO 2008-124133, WO 2008-151058, WO 2009-006930, WO 2009-006997, and WO 2009-007852) can also be used.

[0034]

As "induced pluripotent stem cells", various iPSC lines established by NIH, Institute of Physical and Chemical Research (RIKEN), Kyoto University, etc., can be used. Examples of human iPSC lines include HiPS-RIKEN-1A line, HiPS-RIKEN-2A line, HiPS-RIKEN-12A line and Nips-B2 line by RIKEN, and 253G1 line, 201B7 line, 409B2 line, 454E2 line,

606A1 line, 610B1 line and 648A1 line by Kyoto University. Alternatively, clinical grade cell lines provided by Kyoto University, Cellular Dynamics International, or the like, and cell lines for research and clinical use produced using such cell lines may be used.

[0035]

As "embryonic stem cells (ESCs)", mouse ESCs such as various mouse ESC lines established by inGenious targeting laboratory, RIKEN (Institute of Physical and Chemical Research), and the like can be used, and human ESCs such as various human ESC lines established by NIH, Institute of Physical and Chemical Research, Kyoto University, and Cellartis can be used. For example, human ESC lines such as CHB-1 to CHB-12 lines, RUES1 line, RUES2 line and HUES1 to HUES28 lines by NIH, H1 line and H9 line by WisCell Research, and KhES-1 line, KhES-2 line, KhES-3 line, KhES-4 line, KhES-5 line, SSES1 line, SSES2 line and SSES3 line by RIKEN can be used. Alternatively, clinical grade cell lines, and cell lines for research and clinical use produced using such cell lines may be used.

[0036]

The term "comprise(s) or comprising" means to include the elements following the term, but be not limited to the elements. Accordingly, inclusion of the elements following

the term is suggested, but exclusion of any other elements is not suggested. The term "consist(s) of or consisting of" means to include all the elements following the term, and be limited to the elements. Accordingly, the term "consist(s) of or consisting of" indicates that the listed elements are required or indispensable, and other elements do not exist substantially. The term "consist(s) essentially of or consisting essentially of" means to include any element following the term, and other elements are limited to those which do not affect the activity or action of the element specified in the present disclosure. Accordingly, the term "consist(s) essentially of or consisting essentially of" indicates that the listed elements are required or indispensable, but other elements are optionally selected and may or may not exist depending on whether or not the other elements affect the activity or action of the listed elements.

[0037]

The combination of the predetermined cytokine and chemokine of the present invention can provide CAR-expressing immune cells with high antitumor activity by transferring the genes into immune cells, especially T cells responsible for cell-mediated immunity of acquired immunity, and NK cells, monocytes, macrophages, and dendritic cells, which are

responsible for natural immunity, together with a CAR gene and expressing the genes. In other words, gene transfer and expression of the combination of the predetermined cytokine and chemokine of the present invention can impart further technical characteristics to T cells (CAR-T cells), NK cells (CAR-NK cells), monocytes (CAR-monocytes), macrophages (CAR-macrophages), and dendritic cells (CAR-dendritic cells) in which a CAR gene is transferred and expressed.

[0038]

"Immune cells" are not specifically limited, as long as they are cells having the ability to damage target cells (pathogenic cells) such as cancer cells by some mechanism of action (so-called immunity effector cells). Examples thereof include T cells responsible for cell-mediated immunity of acquired immunity, and NK cells, monocytes, macrophages, and dendritic cells, which are responsible for natural immunity. In one preferred embodiment, immune cells can be T cells. In another preferred embodiment, immune cells can be cells responsible for natural immunity such as NK cells, macrophages, and dendritic cells. It is considered that, while T cells have a considerable risk of causing GVHD by allogeneic (allo) transplantation even in the case where the HLA type matches, allo NK cells do not cause GVHD. Accordingly, off-the-shelf use is enabled by preparing

various HLA types of allo-immune cells. CAR-NK cells are described, for example, in US 2016/0096892, Mol Ther. 25(8): 1769-1781 (2017), etc., and CAR-dendritic cells, CAR-macrophages, etc., are described, for example, in WO 2017/019848, eLIFE. 2018 e36688, etc.

[0039]

Hereinafter, the present invention will be described with reference to embodiments adopting T cells as a representative example of immune cells. However, the present invention is not limited only to the embodiments in which the immune cells are T cells, and the present invention also includes embodiments in which the immune cells are NK cells, monocytes, macrophages, dendritic cells, or the like. In the following description, "(CAR-) T cells" can be appropriately read as "(CAR-) NK cells", "(CAR-) monocytes", "(CAR-) macrophages", "(CAR-) dendritic cells", or the like.

[0040]

Hereinafter, the T cell of the present invention will be described in detail.

[0041]

The T cell of the present invention expresses: (1) a CAR; (2) at least one selected from the group consisting of IL-15, IL-18, IL-21, and IL-27; and (3) CCL19.

[0042]

The T cell of the present invention may be any T cells such as αβ T cells, γδ T cells, $CD8^+$ T cells, $CD4^+$ T cells, tumor infiltrating T cells, memory T cells, naive T cells, and NK T cells, like T cells expressing common or known CARs.

[0043]

The T cell may be isolated and purified from immune cells infiltrating into body fluids such as blood and bone marrow fluid, tissues such as spleen, thymus, lymph nodes, or cancer tissues such as primary tumors, metastatic tumors, and cancerous ascites. Further, the T cell may be obtained by culturing induced pluripotent stem cells (iPS cells), embryonic stem cells (ES cells), other stem cells, progenitor cells, or the like, under appropriate conditions and thereby inducing and differentiating such cells into T cells.

[0044]

The T cell may be derived from humans or may be derived from mammals other than humans (non-human mammals). Examples of non-human mammals include mice, rats, hamsters, guinea pigs, rabbits, dogs, cats, pigs, cows, horses, sheep, and monkeys.

[0045]

(1) CAR

The CAR expressed by the T cell of the present invention is basically constituted by peptides at the sites

of (i) a single-chain antibody that recognizes a cell surface antigen of cancer cells, (ii) a transmembrane domain, and (iii) a signaling domain that induces T cell activation, which are linked via spacers, as needed, like common or known CARs.

[0046]

The single-chain antibody (i) that recognizes a cell surface antigen of cancer cells is typically a single-chain variable fragment (scFv) composed of a light-chain variable region and a heavy-chain variable region derived from antigen-binding sites of a monoclonal antibody that specifically binds to the antigen and a linker peptide that links such regions.

[0047]

The "cell surface antigen of cancer cells" targeted by the CAR may be a biomolecule that is specifically expressed in cancer cells and their progenitor cells, a biomolecule that has been newly expressed by canceration of cells, or a biomolecule with an increased expression level in cancer cells as compared with normal cells. Such an antigen is generally referred to as "tumor-associated antigen" (TAA), and examples thereof can include BCMA, B7-H3, B7-H6, CD7, CD10, CD19, CD20, CD22, CD23, CD24, CD30, CD33, CD34, CD38, CD41, CD44, CD56, CD70, CD74, CD97, CD123, CD133, CD138,

CD171, CD248, CAIX, CEA, c-Met, CS1 (CD319), CSPG4, CLDN6, CLD18A2, CYP1B1, DNAM-1, GD2, GD3, GM2, GFRα4, GPC3, GPR20, GPRC5D, globoH, Gp100, GPR20, GPRC5D, EGFR, EGFRvariant, EpCAM, EGP2, EGP40, FAP, FITC, HER2, HER3, HPV E6, HPV E7, hTERT, IgG κ chain, IL-11Ra, IL-13Ra2, KIT, Lewis A, Lewis Y, Legumain, LMP1, LMP2, Ly6k, LICAM, MAD-CT-1, MAD-CT-2, MAGE-A1, Melanoma-associated antigen 1, MUC1, MUC16, NA-17, NY-BR-1, NY-ESO-1, O-acetyl-GD2, h5T4, PANX3, PDGRFb, PLAC1, Polysialic acid, PSCA, PSMA, RAGE1, ROR1, sLe, SSEA-4, TARP, TAG-72, TEM7R, Tn antigen, TRAIL receptor, TRP2, TSHR, α fetoprotein, mesothelin, folic acid receptor α (FRα), folic acid receptor β (FRβ), FBP, UPK2, VEGF-R2, and WT-1, but is not limited to these examples.

[0048]

The transmembrane domain (ii) is a polypeptide serving as a region for fixing the CAR to the cell membrane of T cells. Examples of the transmembrane domain can include BTLA, CD3ε, CD4, CD5, CD8, CD9, CD16, CD22, CD28, CD33, CD37, CD45, CD64, CD80, CD86, CD134, CD137, CD154, 4-1BB (CD137), CTLA-4, GITR, ICOS, LAG3, OX40, SLAMF4 (CD244, 2B4), or transmembrane domains derived from the α or β chain of a T cell receptor. Alternatively, mutant transmembrane domains having an amino acid sequence with a homology of 80% or more, 85% or more, 90% or more, 91% or more, 92% or more, 93% or

more, 94% or more, 95% or more, 96% or more, 97% or more, 98% or more, or 99% or more, to the natural amino acid sequence of the aforementioned transmembrane domain can also be used. In one embodiment of the present invention, the transmembrane domain of CD8 is preferable as such a transmembrane domain.

[0049]

To the transmembrane domain, a hinge region which is a peptide (oligopeptide or polypeptide), consisting of any amino acid sequence, having a length of 1 to 100 amino acids, preferably 10 to 70 amino acids may be linked. Examples of the hinge region can include hinge regions derived from CD3, CD8, KIR2DS2, or IgG4, IgD or other immunoglobulins. Alternatively, mutant hinge regions having an amino acid sequence with a homology of 80% or more, 85% or more, 90% or more, 91% or more, 92% or more, 93% or more, 94% or more, 95% or more, 96% or more, 97% or more, 98% or more, or 99% or more, to the natural amino acid sequences of the aforementioned hinge regions can also be used. In one embodiment of the present invention, the hinge region of CD8 is preferable as such a hinge region.

[0050]

The signaling domain (iii) that induces T cell activation is a polypeptide serving as a region for transmitting signals within T cells when the single-chain

antibody recognizes the cell surface antigen of cancer cells and binds thereto. Examples of the signaling domain can include one or more intracellular domains selected from the group consisting of MHC class I molecules, TNF receptor proteins, immunoglobulin-like proteins, cytokine receptors, integrins, activated NK cell receptors, Toll-like receptors, B7-H3, BAFFR, BTLA, BY55 (CD160), CD2, CD3ζ, CD4, CD7, CD8α, CD8β, CD11a, CD11b, CD11c, CD11d, CD18, CD19, CD19a, CD27, CD28, CD29, CD30, CD40, CD49a, CD49D, CD49f, CD69, CD84, CD96 (Tactile), CD103, 4-1BB (CD137), CDS, CEACAM1, CRTAM, CNAM1 (CD226), DAP10, Fc Receptor-associated γchain, GADS, GITR, HVEM (LIGHTR), IA4, ICAM-1, ICOS (CD278), IL2Rβ, IL2Rγ, IL7Rα, ITGA4, ITGA6, ITGAD, ITGAE, ITGAL, ITGAM, ITGAX, ITGB1, ITGB2, ITGB7, KIRDS2, Ly9 (CD229), LAT, LFA-1 (CD11a/CD18), LIGHT, LTBR, NKG2C, NKG2D, NKp30, NKp44, NKp46, NKp80 (KLRF1), OX40, PAG/Cbp, PSGL1, SELPLG (CD162), SEMA4D (CD100), SLAM (SLAMF1, CD150, IPO-3), SLAMF4 (CD244, 2B4), SLAMF6 (NTB-A, Ly108), SLAMF7, SLAMF8 (BLAME), SLP-76, TNFR2, TRANCE/RANKL, VLA1, and VLA-6. Alternatively, mutant signaling domains (intracellular domains) having an amino acid sequence with a homology of 80% or more, 85% or more, 90% or more, 91% or more, 92% or more, 93% or more, 94% or more, 95% or more, 96% or more, 97% or more, 98% or more, or 99% or more, to the natural amino acid sequence of the

aforementioned signaling domain (intracellular domain) can also be used. In one embodiment of the present invention, the signaling domain preferably contains three intracellular domains, CD28, 4-1BB, and CD3ζ.

[0051]

In the case where the signaling domain contains a plurality of intracellular domains, the intracellular domains may be linked to each other via linker peptides (oligopeptides or polypeptides) consisting of 2 to 10 amino acids. Examples of such linker peptides can include a peptide consisting of a glycine-serine contiguous sequence.

[0052]

Each between the single-chain antibody and the transmembrane domain, and between the transmembrane domain and the signaling domain, a spacer region which is a peptide (oligopeptide or polypeptide) consisting of any amino acid sequence, having a length of 1 to 100 amino acids, preferably 10 to 50 amino acids may be included. Examples of the spacer region can include a peptide consisting of a glycine-serine contiguous sequence.

[0053]

The amino acid sequence of the aforementioned CAR expressed by the T cell of the present invention may be appropriately set depending on the application of the T cell,

typically, its functions as an active component of a medicament for treating cancer. As the amino acid sequence of the single-chain antibody (i) against the cell surface antigen of cancer cells, various amino acid sequences are known, and information on such amino acid sequences can also be used in the present invention. Alternatively, a new antibody against the cell surface antigen of desired cancer cells may be produced according to a conventional method, and information obtained by determining the amino acid sequence of the new antibody (preferably heavy-chain and light-chain variable regions, especially CDRs) may be used in the present invention. Further, various amino acid sequences derived from humans and mammals other than humans are known as the amino acid sequences of the transmembrane domain (ii) and the T cell activation signaling domain (iii), and such amino acid sequences are also registered in databases such as NCBI (National Center for Biotechnology Information; http://www.ncbi.nlm.nih.gov/guide/), and UniProt (The Universal Protein Resource; https://www.uniprot.org). Therefore, such information can also be used in the present invention.

[0054]

(2) Cytokine

The cytokine expressed by the T cell of the present invention is at least one selected from the group consisting of IL-15, IL-18, IL-21, and IL-27. The T cell of the present invention may express any one of the aforementioned four cytokines or two or more of them.

[0055]

IL-15 is a cytokine having functions related to T cell survival and activation, differentiation into memory T cells, NK cell activation, and the like. The term "IL-15" in the present invention includes not only full-length natural IL-15 protein but also various embodiments as long as the functions of IL-15 in the action and effect of the present invention are retained. That is, "IL-15" in the present invention includes not only the entire protein (polypeptide) consisting of the amino acid sequence of the natural IL-15, but also a part thereof (functional partial polypeptide) and variants of the whole or a part of the natural IL-15 in which one or a plurality of amino acid sequences are deleted, replaced or added to the natural amino acid sequence (preferably, within the range having the later-described homology), and further the whole or a part of the natural IL-15 or variants thereof linked to other proteins to form a fusion protein (e.g., those linked to the whole or a part of IL-15Rα to form a fusion protein). In the present invention, the state that

the T cell "expresses IL-15" includes expressing IL-15 in various forms as described above (the whole or a part of a protein having the natural amino acid sequence or variants thereof which may or may not be in the form of fusion proteins), as long as the action and effect of the present invention are not lost.

[0056]

Examples of IL-15 include the following four embodiments. All of these are known (see Non Patent Literature 2, Non Patent Literature 3, Non Patent Literature 4, and Patent Literature 4 above). However, IL-15 that can be used in the present invention is not limited to these specific examples, and various IL-15 modified according to the presence or absence and types of signal peptides and linkers in sequences, the order of elements, and other viewpoints (hereinafter also referred to also as "modified" IL-15) can also be used.

[0057]

First embodiment of IL-15: IL-15LSP/IL-15 (hereinafter also referred to as "$IL15_{LSP}$")

The "IL-15LSP" is a long-chain signal peptide consisting of 29 amino acids and binds to the N-terminal side of IL-15 in the first embodiment. Expression of the IL-15 of the first embodiment enhances the antitumor activity of the

CAR-T cells. The term "IL-15LSP" also includes not only the entire polypeptide consisting of the amino acid sequence of the natural IL-15LSP, but also a part thereof (functional partial polypeptide) and variants of the whole or a part of the natural IL-15LSP in which one or a plurality of amino acid sequences are deleted, replaced or added to the natural amino acid sequence (preferably, within the range having the later-described homology). As an example of the modified type of the first embodiment, "IL-15LSP" replaced with "IL-2SP" (a signal peptide of IL-2, which may be the whole or a part of the natural protein or variants thereof, like IL-15LSP) can be mentioned.

[0058]

Second embodiment of IL-15: IL-15/IL-15Rα extracellular domain (hereinafter also referred to as "$_{s}IL15_{RA}$")

The "IL-15Rα extracellular domain" refers to a part of IL-15Rα (interleukin 15 receptor α chain) excluding the transmembrane domain and the intracellular domain. The IL-15Rα extracellular domain includes a sushi domain (motif common to various binding proteins). The term "IL-15Rα extracellular domain" also includes not only the entire polypeptide consisting of the amino acid sequence of the natural IL-15Rα extracellular domain, but also a part thereof

(functional partial polypeptide) and variants of the whole or a part of the natural IL-15Rα in which one or a plurality of amino acid sequences are deleted, replaced or added to the natural amino acid sequence (preferably, within the range having the later-described homology). In the second embodiment, the IL-15Rα extracellular domain normally binds to the C-terminal side of IL-15 via a linker (e.g., a glycine-serine linker consisting of 26 amino acids). In the second embodiment, IL-2SP (a signal peptide of IL-2, which may be the whole or a part of the natural protein or variants thereof) normally binds to the N-terminal side of IL-15 instead of IL-15LSP. The second embodiment is of a secretory type and is an agonist that strongly binds to IL-15Rβ (interleukin 15 receptor β chain shared by the interleukin 2 receptor) and γc (a common γ chain shared as a receptor of interleukin 2, 4, 7, 9, 15, 21, or the like).

[0059]

Third embodiment of IL-15: IL-15/full-length IL-15Rα (hereinafter also referred to as "$_{mb}IL15_{RA}$")

The "full-length IL-15Rα" includes all of the extracellular domain, the transmembrane domain, and the intracellular domain of IL-15Rα and normally binds to the C-terminal side of IL-15 via a linker (e.g., a glycine-serine linker consisting of 20 amino acids) in the third embodiment.

The term "full-length IL-15Rα" also includes not only the entire protein (polypeptide) consisting of the amino acid sequence of the full-length natural IL-15Rα, but also variants of the full-length natural IL-15Rα in which one or a plurality of amino acid sequences are deleted, replaced or added to the natural amino acid sequence (preferably, within the range having the later-described homology). Further, IL-2SP (which may be the whole or a part of the natural protein or variants thereof) normally binds to the N-terminal side of IL-15 instead of IL-15LSP also in the third embodiment. The third embodiment is of a membrane-bound type, and expression thereof enhances the antitumor activity of the CAR-T cells.

[0060]

Fourth embodiment of IL-15: IL-15Rαsushi/IL-15 (hereinafter also referred to as "$_{sushi}$IL15")

In the fourth embodiment, IL-15Rα containing a signal peptide and a sushi domain normally binds to the N-terminal side of IL-15 via a linker (e.g., a linker consisting of 20 amino acids). The term "IL-15Rαsushi" also includes not only the entire polypeptide consisting of the amino acid sequence of the natural IL-15Rαsushi, but also a part thereof (functional partial polypeptide) and variants of the whole or a part of the natural IL-15Rαsushi in which one or a plurality of amino acid sequences are deleted, replaced or

added to the natural amino acid sequence (preferably, within the range having the later-described homology). The fourth embodiment is of a secretory type.

[0061]

In the present invention, IL-15 is preferably IL-15 (the whole or a part of a protein having the natural amino acid sequence or variants thereof) in the form of a fusion protein with IL-15Rα (the whole or a part of a protein having the natural amino acid sequence or variants thereof) in view of the persistence and proliferation of cells as described in Examples (Experimental Examples) below. For example, the aforementioned second, third, or fourth embodiments or their modified types are preferable, and the aforementioned third and fourth embodiments or their modified types are more preferable.

[0062]

IL-18 is a cytokine having functions related to T cell activation, NK cell activation, dendritic cell activation, and the like. The term "IL-18" in the present invention includes not only the full-length natural IL-18 protein but also various embodiments as long as the functions of IL-18 in the action and effect of the present invention are retained. That is, "IL-18" in the present invention includes not only the full-length protein (polypeptide) consisting of the amino

acid sequence of the natural IL-18, but also a part thereof (functional partial polypeptide) and variants of the whole or a part of the natural IL-18 in which one or a plurality of amino acid sequences are deleted, replaced or added to the natural amino acid sequence (preferably, within the range having the later-described homology), and further the whole or a part of natural IL-18 linked to other proteins to form a fusion protein. In the present invention, the term "express IL-18" in the context of T cell includes expressing IL-18 in various forms as described above (the whole or a part of a protein having the natural amino acid sequence or variants thereof which may or may not be in the form of fusion proteins), as long as the action and effect of the present invention are not lost.

[0063]

IL-21 is a cytokine having functions related to the functions of $CD8^+$ T cells, differentiation of $CD8^+$ T cells into memory T cells, NK cell survival, and the like. The term "IL-21" in the present invention includes not only the full-length natural IL-21 protein but also various embodiments as long as the functions of IL-21 in the action and effect of the present invention are retained. That is, "IL-21" in the present invention includes not only the full-length protein (polypeptide) consisting of the amino acid

sequence of the natural IL-21, but also a part thereof (functional partial polypeptide) and variants of the whole or a part of the natural IL-21 in which one or a plurality of amino acid sequences are deleted, replaced or added to the natural amino acid sequence (preferably, within the range having the later-described homology), and further the whole or a part of natural IL-21 linked to other proteins to form a fusion protein. In the present invention, the term "express IL-21" in the context of T cell includes expressing various forms of IL-21 as described above (the whole or a part of a protein having the natural amino acid sequence or variants thereof which may or may not be in the form of fusion proteins), as long as the action and effect of the present invention are not lost.

[0064]

IL-27 is a cytokine having functions related to T cell survival, NK cell activation, inhibition of tumor proliferation and angiogenesis, and the like. The term "IL-27" in the present invention includes not only the full-length natural IL-27 protein but also various embodiments as long as the functions of IL-27 in the action and effect of the present invention are retained. That is, "IL-27" in the present invention includes not only the full-length protein (polypeptide) consisting of the amino acid sequence of the

natural IL-27, but also a part thereof (functional partial polypeptide) and variants of the whole or a part of the natural IL-27 in which one or a plurality of amino acid sequences are deleted, replaced or added to the natural amino acid sequence (preferably, within the range having the later-described homology), and further the whole or a part of natural IL-27 linked to other proteins to form a fusion protein. In the present invention, the term "express IL-27" in the context of T cell includes expressing IL-27 in various forms as described above (the whole or a part of a protein having the natural amino acid sequence or variants thereof which may or may not be in the form of fusion proteins), as long as the action and effect of the present invention are not lost.

[0065]

Examples of IL-27 include a fusion protein (single-chain protein) in which p28 and EBI3 (Epstein-Barr virus-induced gene 3), which are two sub-units constituting IL-27, are linked by a linker, e.g., a linker consisting of 20 to 30 amino acids such as $(G_4S)_3$ (see Non Patent Literature 5 above, for example) or a linker consisting of GSTSGSGKPGSGEGSTKG (J Immunol. 183, 6217-26 (2009)). The terms "p28" and "EBI3" each also include the whole or a part

of a polypeptide with the natural amino acid sequence or variants thereof.

[0066]

The amino acid sequences of the aforementioned cytokines expressed by the T cell of the present invention may be appropriately set depending on the application of the T cell (CAR-T cell) of the present invention, typically, its functions as an active component of a medicament for treating cancer in consideration of the effect of CAR-T cells on enhancement of antitumor activity including improvement in persistence and proliferation of the CAR-T cells. The amino acid sequences of natural IL-15, IL-18, IL-21, IL-27, and IL-15Rα derived from humans and mammals other than humans (e.g., mice) are known and also registered in databases such as NCBI and UniProt, and therefore information thereof can be used also in the present invention. For example, SEQ ID NO: 18 shows the amino acid sequence of natural human IL-15 (the full length including signal peptide and propeptide parts) registered as UniProtKB-P40933, and SEQ ID NO: 19 shows the amino acid sequence of natural human IL-15Rα (the full length including a signal peptide, the sushi domain, the extracellular domain, etc.) registered as UniProtKB-Q13261. In the later-described embodiment in which a natural mouse amino acid sequence is replaced with a natural human amino

acid sequence in the amino acid sequence of a predetermined sequence ID number, the amino acid sequences of corresponding parts included in SEQ ID NOs: 18 and 19 can be referred to. Further, various variants, fusion proteins with other proteins, and their modified types of IL-15, IL-18, IL-21, IL-27, and IL-15Rα are known, and information on their amino acid sequences can be used also in the present invention.

[0067]

Examples of variants of IL-15, IL-18, IL-21, and IL-27 include those having an amino acid sequence with a homology of 80% or more, 85% or more, 90% or more, 91% or more, 92% or more, 93% or more, 94% or more, 95% or more, 96% or more, 97% or more, 98% or more, or 99% or more, as a whole or a partial region (domain), to their natural amino acid sequences derived from humans or mammals other than humans, for example, in the case where the signal peptide is replaced as shown below, as the homology of the region excluding the signal peptide. As a variant having a homology within such a range to a natural amino acid sequence derived from one species, a natural amino acid sequence derived from another species may be applicable (substantially included). Further, the signal peptide in the sequence of each of IL-15, IL-18, IL-21, and IL-27 may be replaced with a known signal peptide. In one aspect of the present invention, the amino acid

sequence of natural mouse IL-15, IL-18, IL-21, or IL-27 can be replaced with the amino acid sequence of a variant having such a homology, the amino acid sequence of natural human IL-15, IL-18, IL-21, or IL-27 that is substantially applicable to such a variant, or a further variant thereof in the amino acid sequences of proteins (polypeptides) expressed by the nucleotide sequences of SEQ ID NO: 4: $IL15_{LSP}$_F2A_CCL19 DNA fragment (DNA fragment #3), SEQ ID NO: 6: $_{s}IL15_{RA}$_F2A_CCL19 DNA fragment (DNA fragment #4), SEQ ID NO: 8: $_{mb}IL15_{RA}$_F2A_CCL19 DNA fragment (DNA fragment #5), SEQ ID NO: 10: $_{sushi}$IL15_F2A_CCL19 DNA fragment (DNA fragment #6), SEQ ID NO: 12: IL-18_F2A_CCL19 DNA fragment (DNA fragment #7), SEQ ID NO: 14: IL-21_F2A_CCL19 DNA fragment (DNA fragment #8), and SEQ ID NO: 16: $_{sc}$IL27_F2A_CCL19 DNA fragment (DNA fragment #9), or in the amino acid sequences of a fusion protein containing SEQ ID NO: 5: $IL15_{LSP}$, a fusion protein containing SEQ ID NO: 7: $_{s}IL15_{RA}$, a fusion protein containing SEQ ID NO: 9: $_{mb}IL15_{RA}$, a fusion protein containing SEQ ID NO: 11: $_{sushi}$IL15, a fusion protein containing SEQ ID NO: 13: IL-18, a fusion protein containing SEQ ID NO: 15: IL-21, and a fusion protein containing SEQ ID NO: 17: $_{sc}$IL27.

[0068]

Examples of variants of the IL-15Rα extracellular domain, the full-length IL-15Rα, and the IL-15Rαsushi include

those having an amino acid sequence with a homology of 80% or more, 85% or more, 90% or more, 91% or more, 92% or more, 93% or more, 94% or more, 95% or more, 96% or more, 97% or more, 98% or more, or 99% or more, to their natural amino acid sequences derived from humans or mammals other than humans. In one aspect of the present invention, the amino acid sequence of the natural mouse IL-15Rα extracellular domain, full-length IL-15Rα, or IL-15Rαsushi can be replaced with the amino acid sequence of a variant having such a homology, the amino acid sequence of the natural human IL-15Rα extracellular domain, the full-length IL-15Rα or the IL-15Rαsushi that is substantially applicable to such a variant, or a further variant thereof in the amino acid sequences of proteins (polypeptides) expressed by the nucleotide sequences of SEQ ID NO: 4: $IL15_{LSP}$_F2A_CCL19 DNA fragment (DNA fragment #3), SEQ ID NO: 6: $_{s}IL15_{RA}$_F2A_CCL19 DNA fragment (DNA fragment #4), SEQ ID NO: 8: $_{mb}IL15_{RA}$_F2A_CCL19 DNA fragment (DNA fragment #5), SEQ ID NO: 10: $_{sushi}$IL15_F2A_CCL19 DNA fragment (DNA fragment #6), or in the amino acid sequences of a fusion protein containing SEQ ID NO: 5: $IL15_{LSP}$, a fusion protein containing SEQ ID NO: 7: $_{s}IL15_{RA}$, a fusion protein containing SEQ ID NO: 9: $_{mb}IL15_{RA}$, and a fusion protein containing SEQ ID NO: 11: $_{sushi}$IL15.

[0069]

IL-15, IL-18, IL-21, and IL-27 are preferably derived from humans or mammals other than humans (e.g., mice) of the same type as those from which the T cell (or IL-15, IL-18, IL-21, and IL-27 receptors of the T cell) used for producing the T cell of the present invention is derived. Parts other than IL-15, IL-18, IL-21, and IL-27 (e.g., IL-15Rα) contained in the fusion proteins of IL-15, IL-18, IL-21, and IL-27, respectively, are also preferably derived from humans or mammals other than humans of the same type as those from which the T cell used for producing the T cell of the present invention is derived. In one aspect of the present invention, the amino acid sequence of natural mouse IL-15, IL-18, IL-21, or IL-27 or the amino acid sequence of the IL-15Rα extracellular domain, the full-length IL-15Rα, or the IL-15Rαsushi can be replaced with the natural human amino acid sequence thereof in the amino acid sequences of proteins (polypeptides) expressed by the nucleotide sequences of SEQ ID NO: 4: $IL15_{LSP}$_F2A_CCL19 DNA fragment (DNA fragment #3), SEQ ID NO: 6: $_{s}IL15_{RA}$_F2A_CCL19 DNA fragment (DNA fragment #4), SEQ ID NO: 8: $_{mb}IL15_{RA}$_F2A_CCL19 DNA fragment (DNA fragment #5), SEQ ID NO: 10: $_{sushi}$IL15_F2A_CCL19 DNA fragment (DNA fragment #6), SEQ ID NO: 12: IL-18_F2A_CCL19 DNA fragment (DNA fragment #7), SEQ ID NO: 14: IL-21_F2A_CCL19 DNA fragment (DNA fragment #8), SEQ ID NO: 16:

$_{sc}$IL27_F2A_CCL19 DNA fragment (DNA fragment #9), or in the amino acid sequences of a fusion protein containing SEQ ID NO: 5: IL15$_{LSP}$, a fusion protein containing SEQ ID NO: 7: $_{s}$IL15$_{RA}$, a fusion protein containing SEQ ID NO: 9: $_{mb}$IL15$_{RA}$, a fusion protein containing SEQ ID NO: 11: $_{sushi}$IL15, a fusion protein containing SEQ ID NO: 13: IL-18, a fusion protein containing SEQ ID NO: 15: IL-21, and a fusion protein containing SEQ ID NO: 17: $_{sc}$IL27.

[0070]

(3) Chemokine

The chemokine expressed by the T cell of the present invention is CCL19. CCL19 is mainly produced from dendritic cells and macrophages of lymph nodes and has a function of inducing migration of T cells, B cells, and mature dendritic cells via their receptor, CCR7. That is, "CCL19" in the present invention includes not only the full-length protein (polypeptide) consisting of the amino acid sequence of the natural CCL19, but also a part thereof (functional partial polypeptide) and variants of the whole or a part of the natural CCL19 in which one or a plurality of amino acid sequences are deleted, replaced or added to the natural amino acid sequence (preferably, within the range having the later-described homology).

[0071]

The amino acid sequence of the aforementioned chemokine expressed by the T cell of the present invention may be appropriately set depending on the application of the T cell (CAR-T cell) of the present invention, typically, its functions as an active component of a medicament for treating cancer in consideration of the effect of the CAR-T cell on enhancement of antitumor activity including improvement in persistence and proliferation of the CAR-T cell. The amino acid sequences of natural CCL19 derived from humans and mammals other than humans (e.g., mice) are known and also registered in databases such as NCBI and UniProt, and therefore information thereof can be used also in the present invention. Further, information on the amino acid sequences of known variants of CCL19 can also be used in the present invention.

[0072]

Examples of the variants of CCL19 include those having an amino acid sequence with a homology of 80% or more, 85% or more, 90% or more, 91% or more, 92% or more, 93% or more, 94% or more, 95% or more, 96% or more, 97% or more, 98% or more, or 99% or more, as a whole or a partial region, to the amino acid sequences of the natural CCL19 derived from humans or mammals other than humans. In one aspect of the present invention, the amino acid sequence of natural mouse CCL19 can

be replaced with the amino acid sequence of a variant having such a homology, the amino acid sequence of natural human CCL19 that is substantially applicable to such a variant, or a further variant thereof in the amino acid sequences of proteins (polypeptides) expressed by the nucleotide sequences of SEQ ID NO: 4: $IL15_{LSP}$_F2A_CCL19 DNA fragment (DNA fragment #3), SEQ ID NO: 6: $_{s}IL15_{RA}$_F2A_CCL19 DNA fragment (DNA fragment #4), SEQ ID NO: 8: $_{mb}IL15_{RA}$_F2A_CCL19 DNA fragment (DNA fragment #5), SEQ ID NO: 10: $_{sushi}$IL15_F2A_CCL19 DNA fragment (DNA fragment #6), SEQ ID NO: 12: IL-18_F2A_CCL19 DNA fragment (DNA fragment #7), SEQ ID NO: 14: IL-21_F2A_CCL19 DNA fragment (DNA fragment #8), and SEQ ID NO: 16: $_{sc}$IL27_F2A_CCL19 DNA fragment (DNA fragment #9).

[0073]

CCL19 is preferably derived from humans or mammals other than humans (e.g., mice) of the same type as the T cell used for producing the T cell of the present invention (CCL19 receptor of the T cell) or variants thereof.

[0074]

Hereinafter, the medicament of the present invention will be described in detail.

[0075]

The medicament of the present invention contains the T cell of the present invention as described above and may

further contain other components, as required. Those skilled in the art would be able to appropriately prepare the medicament of the present invention using the T cell of the present invention in consideration of the application (treatment target) and dosage form.

[0076]

The medicament of the present invention is mainly a medicament (anticancer agent) to treat cancer corresponding to the cell surface antigen of cancer cells (cancer-specific antigen) targeted by the CAR expressed by the T cell of the present invention. Accordingly, the type of cancer targeted by the medicament of the present invention is not specifically limited, as long as cancer cells expressing the antigen targeted by the CAR are contained in the cancer tissue, and a certain level of therapeutic effect is observed by the T cell of the present invention. Examples of the cancer that can be targeted by the medicament of the present invention can include cancers such as adenocarcinoma, squamous cell carcinoma, adenosquamous carcinoma, undifferentiated cancer, large cell cancer, small cell cancer, skin cancer (e.g., melanoma and Merkel cell cancer), breast cancer, prostate cancer, bladder cancer, vaginal cancer, neck cancer, head and neck cancer, uterine cancer, cervical cancer, liver cancer, kidney cancer, pancreatic

cancer, spleen cancer, lung cancer, non-small cell lung cancer, tracheal cancer, bronchial cancer, colon cancer, rectal cancer, small intestine cancer, colorectal cancer, stomach cancer, esophageal cancer, gallbladder cancer, testicular cancer, ovarian cancer, fallopian tube cancer, and nasopharyngeal cancer; cancers of bone tissue, cartilage tissue, adipose tissue, muscle tissue, vascular tissue, and hematopoietic tissue; sarcomas such as chondrosarcoma, Ewing's sarcoma, rhabdomyosarcoma, malignant hemangioendothelioma, malignant tumor, osteosarcoma, and soft tissue sarcoma; blastomas such as hepatoblastoma, medulloblastoma, nephroblastoma, neuroblastoma, pancreatoblastoma, pleuropulmonary blastoma, and retinoblastoma; embryonic cell tumors; lymphoma; leukemia, acute myeloid leukemia, and multiple myeloma. Preferably, examples thereof include carcinomas sensitive to NK cells (such as melanoma, Merkel cell cancer, colorectal cancer, kidney cancer, breast cancer, ovarian cancer, fallopian tube cancer, cervical cancer, liver cancer, lung cancer, non-small cell lung cancer, head and neck cancer, small intestine cancer, prostate cancer, bladder cancer, rectal cancer, pancreatic cancer, Ewing's sarcoma, rhabdomyosarcoma, nasopharyngeal cancer, esophageal cancer, biliary tract cancer, neuroblastoma, osteosarcoma, acute myeloid leukemia,

multiple myeloma, lymphoma, and leukemia). More preferably, examples thereof include melanoma, colorectal cancer, kidney cancer, multiple myeloma, lymphoma, and leukemia.

[0077]

Examples of components that can be contained in the medicament of the present invention other than the T cell include pharmaceutically acceptable additives, more specifically, saline, buffered saline, cell culture media, dextrose, water for injection, glycerol, ethanol, stabilizers, solubilizers, surfactants, buffers, preservatives, isotonic agents, fillers, and lubricants.

[0078]

The medicament of the present invention can be used by being administered to a subject in need of cancer treatment (such as cancer patients and cancer-bearing animals) by the same method as for known CAR-expressing T cells. Examples of the administration method include intratumor, intravenous, intraarterial, intramuscular, subcutaneous, and intraperitoneal injections.

[0079]

The amount of the T cell of the present invention to be contained in the medicament of the present invention can be appropriately adjusted, for example, depending on the application, dosage form, intended therapeutic effect, and

the like, in consideration of the type, location, and severity of cancer, the age, body weight, and condition of the subject to be treated, and the like. For example, the medicament of the present invention can be formulated so that the T cell of the present invention is administered in an amount of normally 1×10^4 to 1×10^{10}, preferably 1×10^5 to 1×10^9, more preferably 5×10^6 to 5×10^8, in a single dose.

[0080]

The administration interval of the medicament of the present invention is not specifically limited and can be appropriately adjusted in consideration of the amount of the T cell of the present invention to be administered at one time, and the like. The medicament of the present invention can be independently administered, for example, 4 times, 3 times, twice, or once a day, every other day, every 3 days, every 4 days, every 5 days, every 6 days, once a week, every 8 days, every 9 days, every 10 days, twice a week, once a month, or twice a month.

[0081]

The medicament of the present invention (anticancer agent) can be used in combination with known anticancer agents. Examples of the anticancer agents can include alkylating drugs such as cyclophosphamide, bendamustine, iosfamide, and dacarbazine; antimetabolites such as

pentostatin, fludarabine, cladribine, methotrexate, 5-fluorouracil, 6-mercaptopurine, and enocitabin; molecular targeted drugs such as rituximab, cetuximab, and trastuzumab; kinase inhibitors such as imatinib, gefitinib, erlotinib, afatinib, dasatinib, sunitinib, and trametinib; proteasome inhibitors such as bortezomib; calcineurin inhibitors such as cyclosporine and tacrolimus; antitumor antibiotics such as idarubicin, doxorubicin and mitomycin C; plant alkaloids such as irinotecan and etoposide; platinum preparations such as cisplatin, oxaliplatin, and carboplatin; hormone therapeutic drugs such as tamoxifen and bicalutamide; and immunoregulatory drugs such as interferon, nivolumab, and pembrolizumab.

[0082]

Use of the medicament of the present invention in combination with additional anticancer agent may be in any form of (a) using the additional anticancer agent and then the medicament of the present invention, (b) using the medicament of the present invention and the additional anticancer agent at the same time, or (c) using the medicament of the present invention and then the additional anticancer agent. When the medicament of the present invention is used in combination with the additional anticancer agent, it is expected that the therapeutic effect

on cancer is more improved, and the side effects due to the anticancer agents can be reduced by reducing the number of administrations or the doses of the medicament of the present invention and/or the other anticancer agent.

[0083]

Hereinafter, the expression vector of the present invention will be described in detail.

[0084]

The expression vector of the present invention contains: (1) a nucleic acid encoding a CAR; (2) a nucleic acid encoding at least one selected from the group consisting of IL-15, IL-18, IL-21, and IL-27; and (3) a nucleic acid encoding CCL19.

[0085]

In the present invention, the term "contain a nucleic acid encoding IL-15" in the context of expression vector includes comprising a nucleic acid encoding not only the full-length natural IL-15 protein but also IL-15 in various forms as described above. The same applies also to the term "contain a nucleic acid encoding IL-18", "contain a nucleic acid encoding IL-21", "contain a nucleic acid encoding IL-27", or "contain a nucleic acid encoding CCL19" in the context of expression vector.

[0086]

The "nucleic acid" may be any molecules obtained by polymerization of nucleotides and molecules having functions equivalent to the nucleotides. Examples thereof can include RNA, which is a polymer of ribonucleotides, DNA, which is a polymer of deoxyribonucleotides, a polymer mixing ribonucleotides and deoxyribonucleotides, and a nucleotide polymer containing nucleotide analogs. Further, the nucleic acid may be a nucleotide polymer containing nucleic acid derivatives. Further, the nucleic acid may be a single-stranded nucleic acid or a double-stranded nucleic acid. Further, the double-stranded nucleic acid also includes a double-stranded nucleic acid in which one strand is hybridized with the other strand under stringent conditions.

[0087]

The nucleotide analogs may be any molecules with modification of a ribonucleotide, a deoxyribonucleotide, RNA, or DNA, for improving the nuclease resistance, stabilizing, increasing the affinity with complementary strand nucleic acids, increasing the cell permeability, or visualizing cells, as compared with RNA or DNA. The nucleotide analogs may be naturally occurring molecules or non-natural molecules. Examples thereof include sugar-modified nucleotide analogs and phosphodiester bond-modified nucleotide analogs.

[0088]

The sugar-modified nucleotide analogs may be any analog obtained by adding any chemical structural material to or replacing with any chemical structural material part or the whole of the chemical structure of the sugar of the nucleotide. Specific examples thereof can include nucleotide analogs replaced with 2'-O-methyl ribose, nucleotide analogs replaced with 2'-O-propyl ribose, nucleotide analogs replaced with 2'-methoxyethoxy ribose, nucleotide analogs replaced with 2'-O-methoxyethyl ribose, nucleotide analogs replaced with 2'-O-[2-(guanidinium) ethyl] ribose, nucleotide analogs replaced with 2'-fluororibose, crosslinked artificial nucleic acids having two cyclic structures by introducing crosslinked structures into the sugar part (Bridged Nucleic Acids) (BNAs), more specifically, locked artificial nucleic acids in which the oxygen atom at the 2' position and the carbon atom at the 4' position are crosslinked via methylene (Locked Nucleic Acids) (LNAs), and ethylene crosslinked artificial nucleic acids (Ethylene bridged nucleic acids) (ENAs) [Nucleic Acid Research, 32, e175 (2004)], and further include peptide nucleic acids (PNAs) [Acc. Chem. Res., 32, 624 (1999)], oxypeptide nucleic acids (OPNAs) [J. Am. Chem. Soc., 123, 4653 (2001)], and peptide ribonucleic acids (PRNAs) [J. Am. Chem. Soc., 122, 6900 (2000)].

[0089]

The phosphodiester bond-modified nucleotide analogs may be any analog obtained by adding any chemical substance to or replacing with any chemical substance part or the whole of the chemical structure of the phosphodiester bond of the nucleotide. Specific examples thereof can include nucleotide analogs replaced with phosphorothioate bonds, and nucleotide analogs replaced with N3'-P5' phosphoramidite bonds [Cell technology, 16, 1463-1473 (1997)] [RNAi method and antisense method, Kodansha Ltd. (2005)].

[0090]

The nucleic acid derivatives may be any molecules obtained by adding another chemical substance to the nucleic acids, for improving the nuclease resistance, stabilizing, increasing the affinity with complementary strand nucleic acids, increasing the cell permeability, or visualizing cells, as compared with the nucleic acids. Specific examples thereof can include 5'-polyamine-added derivatives, cholesterol-added derivatives, steroid-added derivatives, bile acid-added derivatives, vitamin-added derivatives, Cy5-added derivatives, Cy3-added derivatives, 6-FAM-added derivatives, and biotin-added derivatives.

[0091]

The expression vector of the present invention needs only to be capable of producing the T cell of the present invention by contacting a T cell or its precursor, being introduced into the cell, and expressing a predetermined protein (polypeptide) encoded therein in the T cell. The embodiment thereof is not specifically limited. Those skilled in the art would be able to design and produce an expression vector capable of expressing a desired protein (polypeptide) in the T cell.

[0092]

The expression vector of the present invention may be linear or cyclic and may be a non-viral vector such as a plasmid, a viral vector, or a transposon vector.

[0093]

Examples of the viral vector can include a retroviral vector, a lentiviral vector, an adenoviral vector, and an adeno-associated viral vector. Preferable examples of the retroviral vector can include a pMSGV vector (Tamada k et al., ClinCancer Res 18:6436-6445 (2002)) and a pMSCV vector (available from Takara Bio Inc). In the case of using the retroviral vector, the genes contained in the vector are incorporated into the genome of a host cell (the T cell in the present invention), and therefore the genes can be stably expressed for a long period of time.

[0094]

In one embodiment of the present invention, one expression vector contains all elements of (1) a nucleic acid encoding a CAR, (2) a nucleic acid encoding at least one selected from the group consisting of IL-15, IL-18, IL-21, and IL-27, and (3) a nucleic acid encoding CCL19. In another embodiment of the present invention, a first expression vector contains one or two of the aforementioned elements (1) to (3), and a second expression vector contains the remaining element. In another embodiment of the present invention, a first expression vector contains the aforementioned element (1), a second expression vector contains the aforementioned element (2), and a third expression vector contains the aforementioned element (3). Accordingly, the expression vector of the present invention may mean a combination (set) of a plurality of expression vectors, that is, a combination of the aforementioned first and second expression vectors or a combination of the aforementioned first, second, and third expression vectors, depending on the embodiment. In the case where one expression vector contains a plurality of elements, the order in which those elements are arranged from the upstream side to the downstream side is not particularly limited.

[0095]

The nucleotide sequences of the predetermined three elements (CAR, cytokine, and chemokine) contained in the expression vector of the present invention may be designed so as to encode the amino acid sequences of proteins (polypeptides), respectively, corresponding to the proteins (polypeptides) selected as the elements. The nucleic acids (oligonucleotides) contained in the expression vector may be produced by chemically synthesizing oligo DNA or may be produced (cloned) as cDNA.

[0096]

The expression vector of the present invention may contain (in the case of the aforementioned combinations of a plurality of expression vectors, the expression vectors each independently may contain) the sequences of promoters, terminators, enhancers, start codons, stop codons, polyadenylation signals, nuclear localization signals (NLS), multicloning sites (MCS), and the like, that are involved in the expression of each gene, as required, in addition to the sequences (genes) encoding the aforementioned predetermined elements.

[0097]

In one embodiment of the present invention, in the case where one expression vector contains two or more of the aforementioned three elements, a gene encoding a self-

cleaving peptide (2A peptide) or IRES (Internal Ribozyme Entry Site) may be inserted between the elements.

[0098]

The 2A peptide is a self-cleaving peptide derived from a virus and has a characteristic that a predetermined position (position of one residue from the C-terminus) in the amino acid sequence is cleaved in the endoplasmic reticulum (Szymczak et al., Expert Opin. Biol. Ther. 5 (5): 627-638 (2005)). Therefore, the genes integrated before and after the 2A peptide will be expressed independently of each other in the cell. Examples of the 2A peptide include those derived from picornavirus, rotavirus, insect virus, aphthovirus, or trypanosoma virus.

[0099]

The expression vector of the present invention may further contain nucleic acids (nucleotide sequences) encoding "functional genes" such as a reporter gene (typically, a gene encoding a fluorescent protein), a drug selection gene, and a suicide gene.

[0100]

In the case of using a "drug resistance gene" as a functional gene, cells into which the expression vector of the present invention containing the drug resistance gene is introduced can be selected by adding a predetermined drug to

the medium in the method for producing a CAR-T cell of the present invention. Examples of the drug resistance gene include a kanamycin resistance gene, an ampicillin resistance gene, and a puromycin resistance gene. One of these genes may be used, or two or more of them may be used.

[0101]

In the case of using a "gene encoding a fluorescent protein" as a functional gene, T cells with the expression vector of the present invention introduced can be observed using a fluorescence microscope, or cells with the expression vector of the present invention introduced can be sorted using a flow cytometer (cell sorter), in the method for producing a CAR-T cell of the present invention. A typical example of the reporter gene is a gene encoding a fluorescent protein. Examples of the "gene encoding a fluorescent protein" include blue fluorescent proteins such as Sirius, BFP, and EBFP; cyan fluorescent proteins such as mTurquoise, TagCFP, AmCyan, mTFP1, MidoriishiCyan, and CFP; green fluorescent proteins such as TurboGFP, AcGFP, TagGFP, Azami-Green (e.g., hmAG1), ZsGreen, EmGFP, EGFP, GFP2, and HyPer; yellow fluorescent proteins such as TagYFP, EYFP, Venus, YFP, PhiYFP, PhiYFP-m, TurboYFP, ZsYellow, and mBanana; orange fluorescent proteins such as KusabiraOrange (e.g., hmKO2) and mOrange; red fluorescent proteins such as TurboRFP, DsRed-

Express, DsRed2, TagRFP, DsRed-Monomer, AsRed2, and mStrawberry; and near-infrared fluorescent proteins such as TurboFP602, mRFP1, JRed, KillerRed, mCherry, HcRed, KeimaRed (e.g., hdKeimaRed), mRasberry, and mPlum. Any one of these genes may be used, or two or more of them may be used. In the case of using two or more genes encoding fluorescent proteins, the fluorescent proteins preferably have different emission wavelengths so as not to interfere with the visibility of each other.

[0102]

In the case of using a "suicide gene" as a functional gene, the number of the T cells of the present invention in vivo can be controlled by administering a drug that activates the function of the suicide gene, depending on the course of cancer treatment, for example, at the stage when the tumor has disappeared. Examples of the suicide gene include a gene encoding diphtheria toxin A, herpes simplex thymidine kinase (HSV-TK), carboxypeptidase G2 (CPG2), carboxyl esterase (CA), cytosine deaminase (CD), cytochrome P450 (cyt-450), deoxycytidine kinase (dCK), nitroreductase (NR), purine nucleoside phosphorylase (PNP), thymidine phosphorylase (TP), varicella-zoster virus thymidine kinase (VZV-TK), xanthine-guanine phosphoribosyl transferase (XGPRT), or inducible caspase 9. Any one of these genes may be used, or two or

more of them may be used. Drugs that activate the function of each suicide gene are known, and examples thereof include ganciclovir for herpes simplex thymidine kinase (HSV-TK), and AP1903 that is a dimer-inducing compound for inducible caspase 9.

[0103]

Hereinafter, the method for producing a CAR-T cell of the present invention will be described in detail. The method for producing a CAR-T cell of the present invention contains a step of introducing the expression vector of the present invention into a T cell as described above (which will be hereinafter referred to as "expression vector-introducing step").

[0104]

The method for introducing the expression vector into a T cell can be made suitable for the embodiment of the expression vector. For example, the expression vector can be introduced into the T cell by known methods such as a virus infection method, a calcium phosphoric acid method, a lipofection method, a microinjection method, and an electroporation method. The expression vector of the present invention can be prepared in a form suitable for use in each method by known means and by using commercially available kits as appropriate (according to the instructions thereof).

In the expression vector-introducing step, T cells may be cultured by bringing such a preparation into contact with T cells, generally, in a medium to which a preparation containing the expression vector.

[0105]

In a preferred embodiment of the present invention, the expression vector of the present invention is introduced into T cells by a virus infection method. For example, a method in which the vector of the present invention and the packaging vector (plasmid) of each virus are transfected into a host cell, using a commercially available kit corresponding to each viral vector such as a retroviral vector, a lentiviral vector, an adenoviral vector, and an adeno-associated viral vector, to produce a recombinant virus, and thereafter T cells are infected with the recombinant virus obtained can be mentioned. Examples of the commercially available kit for viral vectors include Retrovirus packaging Kit Eco (available from Takara Bio Inc). Examples of the host cell include GP2-293 cell (available from Takara Bio Inc.), Plat-GP cell (available from Cosmo Bio Co., Ltd.), PG13 cell (ATCC CRL-10686), and PA317 cell (ATCC CRL-9078).

[0106]

The method for producing a CAR-T cell of the present invention may further contain other steps before and after

the expression vector-introducing step. Examples of the other steps include a step of culturing T cells, and a step including a process to cause a functional gene contained in the expression vector to function.

[0107]

The T cells into which the expression vector of the present invention is introduced can be normally pre-cultured in vitro by a known method. For example, T cells may be isolated and purified according to a conventional method from body fluids such as blood and bone marrow fluid; tissues such as spleen, thymus, and lymph nodes; or cancer tissues such as primary tumors, metastatic tumors, and cancerous ascites, which are collected from humans or nonhuman animals, and then cultured in an appropriate medium, and (a preparation containing) the expression vector of the present invention may be added to the medium. Alternatively, T cells (or precursors thereof) may be prepared in advance from iPS cells, ES cells, and other pluripotent stem cells through an appropriate differentiation-inducing step, and (a preparation containing) the expression vector of the present invention may be added to the medium.

[0108]

Further, in the case where the expression vector of the present invention contains a drug resistance gene as a

functional gene, a step of culturing T cells while adding a drug corresponding to the drug resistance gene used to the medium can be performed after the expression vector-introducing step, in order to sort T cells with the expression vector introduced. Those skilled in the art would be able to appropriately adjust the conditions for using the drug corresponding to the drug resistance gene such as the concentration in the medium and the culture period.

[0109]

In the case where the expression vector of the present invention contains a gene encoding a fluorescent protein (reporter gene) as a functional gene, a step of observing T cells with the expression vector introduced using a fluorescence microscope, a step of sorting T cells with the expression vector introduced using a cell sorter, and the like can be performed after the expression vector-introducing step. Those skilled in the art would be able to appropriately adjust the conditions for observation using a fluorescence microscope and selection using a cell sorter, such as irradiation with light having an excitation wavelength and detection of light having an emission wavelength corresponding to the fluorescent protein.

Examples

[0110]

The CAR in the following examples is a CAR targeting human CD20, specifically, an anti-human CD20 CAR consisting of anti-human CD20 scFv, mouse CD8 transmembrane domain, and mouse CD28_4-1BB_CD3ζ intracellular signal motif. Further, the cytokine in the following examples is any of mouse IL-15 ("$IL15_{LSP}$", "$_{s}$IL15RA", "$_{mb}IL15_{RA}$", or "$_{sushi}$IL15"); mouse IL-18; mouse IL-21; or a single-chain protein "$_{sc}$IL27" containing mouse IL-27 (p28 and EBI3). The chemokine in the following examples is mouse CCL19. Mouse-derived T cells expressing the CAR, the cytokine, and the chemokine were produced as follows and tested.

[0111]

[Table 1]

Example / Comparative Example	CAR	Cytokine	Chemokine
Example 1-1	Anti-human CD20scFv	$IL15_{LSP}$ (Mouse)	CCL19 (Mouse)
Example 1-2	Anti-human CD20scFv	$_{s}IL15_{RA}$ (Mouse)	CCL19 (Mouse)
Example 1-3	Anti-human CD20scFv	$_{mb}IL15_{RA}$ (Mouse)	CCL19 (Mouse)
Example 1-4	Anti-human CD20scFv	$_{sushi}$IL15 (Mouse)	CCL19 (Mouse)
Example 2	Anti-human CD20scFv	IL-18 (Mouse	CCL19 (Mouse)
Example 3	Anti-human CD20scFv	IL-21 (Mouse)	CCL19 (Mouse)
Example 4	Anti-human CD20scFv	$_{sc}$IL27 (Mouse)	CCL19 (Mouse)
Comparative Example 1	-	-	-
Comparative Example 2	Anti-human CD20scFv	-	-

[0112]

[Example 1-1] $IL15_{LSP}$ × CCL19 CAR-T cells

In a vector-producing step, three types of DNA fragments (DNA fragments 1 to 3) were first artificially synthesized in step 1, and then one expression vector containing all genes encoding the predetermined CAR, cytokine, and chemokine were produced using the three types of DNA fragments in step 2. Subsequently, in a CAR-T cell-producing step, a retroviral vector was first prepared using the expression vector in step 1, and then the predetermined genes were transduced into mouse T cells using the retroviral vector preparation in step 2.

[0113]

[A: Vector-producing step] Production of $IL15_{LSP}$_CCL19_anti-human CD20 CAR expression vector

Step 1: Synthesis of DNA fragments

DNA fragment #1: DNA fragment containing anti-human CD20 CAR

A DNA fragment (DNA fragment #1) containing a nucleotide sequence encoding anti-human CD20 CAR consisting of anti-human CD20 scFv, mouse CD8 transmembrane domain, and mouse CD28_4-1BB_CD3ζ intracellular signal motif was artificially synthesized. SEQ ID NO: 1 (Figure 7) shows the nucleotide sequence of the entire DNA fragment #1. In SEQ ID NO: 1, the bases at positions 3 to 785 form a sequence encoding the anti-human CD20 scFv, the bases at positions 795

to 1040 form a sequence encoding the mouse CD8 transmembrane domain, the bases at positions 1041 to 1637 form a sequence encoding the mouse CD28_4-1BB_CD3ζ intracellular signal motif (among them, the bases at positions 1041 to 1163 encode the intracellular domain of mouse CD28, the bases at positions 1164 to 1298 encode the intracellular domain of mouse 4-1BB, and the bases at positions 1299 to 1637 encode the polypeptide in the intracellular domain of mouse CD3ζ). SEQ ID NO: 2 shows the amino acid sequence of the fusion protein expressed by the nucleotide sequence at positions 3 to 1637 of DNA fragment #1.

[0114]

DNA fragment #2: DNA fragment containing stop codons and multicloning sites

A DNA fragment (DNA fragment #2) containing the nucleotide sequence of stop codons and the nucleotide sequence of multicloning sites (MCS) was artificially synthesized. SEQ ID NO: 3 (Figure 8) shows the nucleotide sequence of the entire DNA fragment #2.

[0115]

DNA fragment #3: DNA fragment for $IL15_{LSP}$ and CCL19

A DNA fragment (DNA fragment #3) containing a nucleotide sequence encoding 2A peptide (F2A) that is a self-cleaving peptide, mouse IL-2 signal peptide (IL-2SP), mouse

IL-15 construct "$IL15_{LSP}$" (fusion peptide consisting of IL-15LSP and IL-15), F2A, and mouse CCL19 was artificially synthesized. SEQ ID NO: 4 (Figure 9) shows the nucleotide sequence of the entire DNA fragment #3. In SEQ ID NO: 4, the bases at positions 7 to 81 form a sequence encoding the first F2A, the bases at positions 82 to 168 form a sequence encoding mouse $IL\text{-}15_{LSP}$, the bases at positions 169 to 567 form a sequence encoding mouse IL-15 (excluding stop codons), the bases at positions 568 to 642 form a sequence encoding the second F2A, and the bases at positions 646 to 969 form a sequence encoding mouse CCL19. SEQ ID NO: 5 shows the amino acid sequence of the entire fusion protein that is expressed by the nucleotide sequence at positions 7 to 969 of DNA fragment #3, self-cleaves at two F2A sites, and contains $IL15_{LSP}$.

[0116]

Step 2: Production of retrovirus expression vector

DNA fragment #1 and DNA fragment #2 were linked to produce a construct (anti-human CD20 CAR_MCS). The construct obtained was incorporated into a pMSGV retrovirus expression vector (Tamada k et al., Clin Cancer Res 18: 6436-6445 (2002)) by restriction enzyme (Nco I and Sal I) treatment, to produce an anti-human CD20 CAR_MCS-containing pMSGV retrovirus expression vector.

[0117]

Then, the nucleotide sequence at positions 1337 to 1637 of DNA fragment #1 and DNA fragment #3 were linked to produce a construct. The construct obtained was incorporated into an anti-human CD20 CAR_MCS-containing pMSGV retroviral vector by restriction enzyme (Sbf I and Sal I) treatment, to produce an anti-human CD20 CAR_F2A_IL15$_{LSP}$_F2A_CCL19-containing pMSGV retrovirus expression vector (IL15$_{LSP}$_CCL19_anti-human CD20 CAR expression vector).

[0118]

[B: CAR-T cell-producing step] Production of IL15$_{LSP}$_CCL19_anti-human CD20 CAR-T cells

Step 1: Preparation of retrovirus

Using Lipofectamine 3000 (available from Thermo Fisher SCIENTIFIC K.K.), the IL15$_{LSP}$_CCL19_anti-human CD20 CAR expression vector obtained by the vector-producing step was transfected into GP2-293 packaging cell line (Takara Bio Inc.) together with pCL-Eco plasmid (Novus Biologicals, LLC). DMEM with 10% deactivated FBS and antibiotics added was used as a culture solution for the GP2-293 packaging cell line. 48 hours after the transfection, the supernatant of the culture solution of the GP2-293 packaging cell line was collected, to form a preparation of the IL15$_{LSP}$_CCL19_anti-human CD20 CAR expression vector.

[0119]

Step 2: Transduction of mouse T cells

3×10^6 mouse T cells derived from mouse spleen and lymph nodes and purified were activated for 48 hours in the presence of an immobilized anti-mouse CD3 monoclonal antibody (3 μg/mL), an anti-mouse CD28 monoclonal antibody (1 μg/mL), and IL-2. Subsequently, the $IL15_{LSP}$_CCL19_anti-human CD20 CAR expression vector-containing preparation (GP2-293 cell culture supernatant) obtained in step 1 and mouse T cells activated as described above were mixed within the wells of a plate in which 25 μg/mL of retronectin is immobilized (registered trademark: Takara Bio Inc.), followed by centrifugation at 1500 rpm for 2 hours, and the mixture was cultured for 6 hours in the presence of IL-2. In order to remove retrovirus from the culture solution, mouse T cells were collected, transferred to a new complete RPMI medium containing IL-2, and further cultured for 42 hours, to obtain mouse T cells ($IL15_{LSP}$_CCL19_anti-human CD20 CAR-T cells, hereinafter referred to as "$IL15_{LSP}$ × CCL19 CAR-T cells") with an $IL15_{LSP}$_CCL19_anti-human CD20 CAR expression vector introduced, that is, expressing an anti-human CD20 CAR, $IL15_{LSP}$, and CCL19.

[0120]

The "complete RPMI medium" used for culturing the T cells was an RPMI-1640 medium to which deactivated 10% FBS, antibiotics, 50-mM 2-mercapto ethanol, and 25-mM HEPES buffer were added. A complete RPMI medium to which components separately described were further added, as required, was used below as a culture solution for the T cells.

[0121]

[Example 1-2] $_{s}IL15_{RA}$ (secretory IL-15/IL-15RA fusion protein) × CCL19 CAR-T cells

[A: Vector-producing step] Production of $_{s}IL15_{RA}$_CCL19_anti-human CD20 CAR expression vector

Using the same DNA fragment #1 and DNA fragment #2 as in step 1 of Example 1-1 and DNA fragment #4 as described below, instead of DNA fragment #3, a construct was produced in the same manner as in step 2 of Example 1-1, to obtain an anti-human CD20 CAR_F2A_$_{s}IL15_{RA}$_F2A_CCL19-containing pMSGV retrovirus expression vector ($_{s}IL15_{RA}$_CCL19_anti-human CD20 CAR expression vector).

[0122]

DNA fragment #4: DNA fragment for $_{s}IL15_{RA}$ and CCL19

A DNA fragment (DNA fragment #4) containing a nucleotide sequence encoding F2A, mouse IL-2SP, a mouse IL-15 construct "$_{s}IL15_{RA}$" (fusion peptide consisting of IL-15, a linker, and the IL-15Rα extracellular domain), F2A, and mouse

CCL19 was synthesized. SEQ ID NO: 6 (Figure 10) shows the nucleotide sequence of the entire DNA fragment #4. In SEQ ID NO: 6, the bases at positions 7 to 81 form a sequence encoding the first F2A, the bases at positions 82 to 141 form a sequence encoding mouse IL-2SP, the bases at positions 142 to 1137 form a sequence encoding $_{s}IL15_{RA}$ (among them, the bases at positions 142 to 540 encode mouse IL-15 (excluding signal sequences and stop codons), the bases at positions 541 to 618 serve as a linker, and the bases at positions 619 to 1137 encode the mouse IL-15Rα extracellular domain), the bases at positions 1138 to 1212 form a sequence encoding the second F2A, and the bases at positions 1216 to 1539 form a sequence encoding mouse CCL19. SEQ ID NO: 7 shows the amino acid sequence of the entire fusion protein that is expressed by the nucleotide sequence at positions 7 to 1539 of DNA fragment #4, self-cleaves at two F2A sites, and contains $_{s}IL15_{RA}$.

[0123]

[B: CAR-T cell-producing step] Production of $_{s}IL15_{RA}$_CCL19_anti-human CD20 CAR-T cells

A retrovirus-containing preparation was obtained in step 1 in the same manner as in the CAR-T cell-producing step of Example 1-1 except that the $_{s}IL15_{RA}$_CCL19_anti-human CD20 CAR expression vector obtained by the aforementioned vector-

producing step was used as a retrovirus expression vector, instead of the $IL15_{LSP}$_CCL19_anti-human CD20 CAR expression vector, to obtain mouse T cells expressing the genes of anti-human CD20 CAR, $_{s}IL15_{RA}$, and CCL19 contained in the retrovirus ($_{s}IL15_{RA}$_CCL19_anti-human CD20 CAR-T cells, hereinafter referred to as $_{s}IL15_{RA}$ × CCL19 CAR-T cells) in step 2.

[0124]

[Example 1-3] $_{mb}IL15_{RA}$ (membrane-bound IL-15/IL-15RA fusion protein) × CCL19 CAR-T cells

[A: Vector-producing step] Production of $_{mb}IL15_{RA}$_CCL19_anti-human CD20 CAR expression vector

Using the same DNA fragment #1 and DNA fragment #2 as in step 1 of Example 1-1 and DNA fragment #5 as described below, instead of DNA fragment #3, a construct was produced in the same manner as in step 2 of Example 1-1, to obtain an anti-human CD20 CAR_F2A_$_{mb}IL15_{RA}$_F2A_CCL19-containing pMSGV retrovirus expression vector ($_{mb}IL15_{RA}$_CCL19_anti-human CD20 CAR expression vector).

[0125]

DNA fragment #5: DNA fragment for $_{mb}IL15_{RA}$ and CCL19

A DNA fragment containing a nucleotide sequence encoding F2A, mouse IL-2SP, a mouse IL-15 construct "$_{mb}IL15_{RA}$" (fusion peptide consisting of IL-15, a linker, and full-length IL-15Rα), F2A, and mouse CCL19 was synthesized. SEQ

ID NO: 8 (Figure 11) shows the nucleotide sequence of the entire DNA fragment #5. In SEQ ID NO: 8, the bases at positions 7 to 81 form a sequence encoding the first F2A, the bases at positions 82 to 141 form a sequence encoding mouse IL-2SP, the bases at positions 142 to 1311 form a sequence encoding $_{mb}IL15_{RA}$ (among them, the bases at positions 142 to 540 encode mouse IL-15 (excluding signal sequences and stop codons), the bases at positions 541 to 618 serve as a linker, and the bases at positions 619 to 1311 encode mouse IL-15Rα (full-length)), the bases at positions 1312 to 1386 form a sequence encoding the second F2A, and the bases at positions 1390 to 1713 form a sequence encoding mouse CCL19. SEQ ID NO: 9 shows the amino acid sequence of the entire fusion protein that is expressed by the nucleotide sequence at positions 7 to 1713 of DNA fragment #5, self-cleaves at two F2A sites, and contains $_{mb}IL15_{RA}$.

[0126]

[B: CAR-T cell-producing step] Production of $_{mb}IL15_{RA}$_CCL19_anti-human CD20 CAR-T cells

A retrovirus-containing preparation was obtained in step 1 in the same manner as in the CAR-T cell-producing step of Example 1-1 except that the $_{mb}IL15_{RA}$_CCL19_anti-human CD20 CAR expression vector obtained by the aforementioned vector-producing step was used as a retrovirus expression vector,

instead of the $IL15_{LSP}$_CCL19_anti-human CD20 CAR expression vector, to produce mouse T cells expressing the genes of anti-human CD20 CAR, $_{mb}IL15_{RA}$, and CCL19 contained in the retrovirus ($_{mb}IL15_{RA}$_CCL19_anti-human CD20 CAR-T cells, hereinafter referred to as "$_{mb}$IL15 × CCL19 CAR-T cells") in step 2.

[0127]

[Example 1-4] $_{sushi}$IL15 (secretory IL-15RA/IL-15 fusion protein) × CCL19 CAR-T cells

[A: Vector-producing step] Production of $_{sushi}$IL15_CCL19_anti-human CD20 CAR expression vector

Using the same DNA fragment 1 and DNA fragment 2 as in step 1 of Example 1-1 and DNA fragment #6 as described below, instead of DNA fragment #3, a construct was produced in the same manner as in step 2 of Example 1-1, to obtain an anti-human CD20 CAR_F2A_$_{sushi}$IL15_F2A_CCL19-containing pMSGV retrovirus expression vector ($_{sushi}$IL15_CCL19_anti-human CD20 CAR expression vector).

[0128]

DNA fragment #6: DNA fragment for $_{sushi}$IL15 and CCL19

A DNA fragment containing a nucleotide sequence encoding F2A, a mouse IL-15 construct "$_{sushi}$IL15" (fusion peptide consisting of IL-15Rα sushi domain, a linker, and IL-15), F2A, and mouse CCL19 was synthesized. SEQ ID NO: 10

(Figure 12) shows the nucleotide sequence of the entire DNA fragment #6. In SEQ ID NO: 10, the bases at positions 7 to 81 form a sequence encoding the first F2A, the bases at positions 82 to 777 form a sequence encoding $_{sushi}$IL15 (among them, the bases at positions 82 to 375 encode the SP and the sushi domain of mouse IL-15Rα, the bases at positions 376 to 435 serve as a linker, and the bases at positions 436 to 777 encode mouse IL-15 (excluding signal sequences and propeptides)), the bases at positions 778 to 852 form a sequence encoding the second F2A, and the bases at positions 856 to 1179 form a sequence encoding mouse CCL19. SEQ ID NO: 11 shows the amino acid sequence of the entire fusion protein that is expressed by the nucleotide sequence at positions 7 to 1179 of DNA fragment #6, self-cleaves at two F2A sites, and contains $_{sushi}$IL15.

[0129]

[B: CAR-T cell-producing step] Production of $_{sushi}$IL15_CCL19_anti-human CD20 CAR-T cells

A retrovirus-containing preparation was obtained in step 1 in the same manner as in the CAR-T cell-producing step of Example 1-1 except that the $_{sushi}$IL15_CCL19_anti-human CD20 CAR expression vector obtained by the aforementioned vector-producing step was used as a retrovirus expression vector, instead of the IL15$_{LSP}$_CCL19_anti-human CD20 CAR expression

vector, to obtain mouse T cells expressing the genes of anti-human CD20 CAR, sushiIL15, and CCL19 contained in the retrovirus (sushiIL15_CCL19_anti-human CD20 CAR-T cells, hereinafter referred to as "sushiIL15 × CCL19 CAR-T cells") in step 2.

[0130]

[Example 2] IL-18 × CCL19 CAR-T cells

[A: Vector-producing step] Production of IL18_CCL19_anti-human CD20 CAR expression vector

Using the same DNA fragment 1 and DNA fragment 2 as in step 1 of Example 1-1 and DNA fragment #7 as described below, instead of DNA fragment #3, a construct was produced in the same manner as in step 2 of Example 1-1, to obtain an anti-human CD20 CAR_F2A_IL-18_F2A_CCL19-containing pMSGV retrovirus expression vector (IL18_CCL19_anti-human CD20 CAR expression vector).

[0131]

DNA fragment #7: DNA fragment for IL-18 and CCL19

A DNA fragment containing a nucleotide sequence encoding the first F2A, mouse IL-18, the second F2A, and mouse CCL19 was artificially synthesized. SEQ ID NO: 12 (Figure 13) shows the nucleotide sequence of the entire DNA fragment #7. In SEQ ID NO: 12, the bases at positions 7 to 81 form a sequence encoding the first F2A, the bases at

positions 82 to 657 form a sequence encoding mouse IL-18, the bases at positions 658 to 732 form a sequence encoding the second F2A, and the bases at positions 736 to 1059 form a sequence encoding mouse CCL19. SEQ ID NO: 13 shows the amino acid sequence of the entire fusion protein that is expressed by the nucleotide sequence at positions 7 to 1059 of DNA fragment #7 and self-cleaves at two F2A sites.

[0132]

[B: CAR-T cell-producing step] Production of IL18_CCL19_anti-human CD20 CAR-T cells

A retrovirus-containing preparation was obtained in step 1 in the same manner as in the CAR-T cell-producing step of Example 1-1 except that the IL18_CCL19_anti-human CD20 CAR expression vector obtained by the aforementioned vector-producing step was used as a retrovirus expression vector, instead of the $IL15_{LSP}$_CCL19_anti-human CD20 CAR expression vector, to obtain mouse T cells expressing the genes of anti-human CD20 CAR, IL-18, and CCL19 contained in the retrovirus (IL18_CCL19_anti-human CD20 CAR-T cells, hereinafter referred to as "IL18 × CCL19 CAR-T cells") in step 2.

[0133]

[Example 3] IL-21 × CCL19 CAR-T cells

[A: Vector-producing step] Production of IL21_CCL19_anti-human CD20 CAR expression vector

Using the same DNA fragment 1 and DNA fragment 2 as in step 1 of Example 1-1 and DNA fragment #8 as described below, instead of DNA fragment #3, a construct was produced in the same manner as in step 2 of Example 1-1, to obtain an anti-human CD20 CAR_F2A_IL-21_F2A_CCL19-containing pMSGV retrovirus expression vector (IL21_CCL19_anti-human CD20 CAR expression vector).

[0134]

DNA fragment #8: DNA fragment for IL-21 and CCL19

A DNA fragment containing a nucleotide sequence encoding the first F2A, mouse IL-21, the second F2A, and mouse CCL19 was artificially synthesized. SEQ ID NO: 14 (Figure 14) shows the nucleotide sequence of the entire DNA fragment #8. In SEQ ID NO: 14, the bases at positions 7 to 81 form a sequence encoding the first F2A, the bases at positions 82 to 567 form a sequence encoding mouse IL-21, the bases at positions 568 to 642 form a sequence encoding the second F2A, and the bases at positions 646 to 969 form a sequence encoding mouse CCL19. SEQ ID NO: 15 shows the amino acid sequence of the entire fusion protein that is expressed by the nucleotide sequence at positions 7 to 969 of DNA fragment #8 and self-cleaves at two F2A sites.

[0135]

[B: CAR-T cell-producing step] Production of

IL21_CCL19_anti-human CD20 CAR-T cells

A retrovirus-containing preparation was obtained in step 1 in the same manner as in the CAR-T cell-producing step of Example 1-1 except that the IL21_CCL19_anti-human CD20 CAR expression vector obtained by the aforementioned vector-producing step was used as a retrovirus expression vector, instead of the $IL15_{LSP}$_CCL19_anti-human CD20 CAR expression vector, to obtain mouse T cells expressing the genes of anti-human CD20 CAR, IL-21, and CCL19 contained in the retrovirus (IL21_CCL19_anti-human CD20 CAR-T cells, hereinafter referred to as "IL21 × CCL19 CAR-T cells") in step 2.

[0136]

[Example 4] $_{sc}$IL27 × CCL19 CAR-T cells

[A: Vector-producing step] Production of $_{sc}$IL27_CCL19_anti-human CD20 CAR expression vector

Using the same DNA fragment 1 and DNA fragment 2 as in step 1 of Example 1-1 and DNA fragment #9 as described below, instead of DNA fragment #3, a construct was produced in the same manner as in step 2 of Example 1-1, to obtain an anti-human CD20 CAR_F2A_scIL-27_F2A_CCL19-containing pMSGV retrovirus expression vector ($_{sc}$IL27_CCL19_anti-human CD20 CAR expression vector).

[0137]

DNA fragment #9: DNA fragment for $_{sc}$IL27 and CCL19

A DNA fragment containing a nucleotide sequence encoding the first F2A, a mouse IL-27 construct "scIL27" (fusion peptide consisting of p28, a linker, and EBI3), the second F2A, and CCL19 was artificially synthesized. SEQ ID NO: 16 (Figure 15) shows the nucleotide sequence of the entire DNA fragment #9. In SEQ ID NO: 16, the bases at positions 7 to 81 form a sequence encoding the first F2A, the bases at positions 82 to 1437 form a sequence encoding $_{sc}$IL27 (among them, the bases at positions 82 to 765 encode mouse EBI3, the bases at positions 766 to 819 serve as a linker, and the bases at positions 820 to 1437 encode mouse p28), the bases at positions 1438 to 1512 form a sequence encoding the second F2A, and the bases at positions 1516 to 1839 form a sequence encoding mouse CCL19. SEQ ID NO: 17 shows the amino acid sequence of the entire fusion protein that is expressed by the nucleotide sequence at positions 7 to 1839 of DNA fragment #9, self-cleaves at two F2A sites, and contains $_{sc}$IL27.

[0138]

[B: CAR-T cell-producing step] $_{sc}$IL27_CCL19_anti-human CD20 CAR-T cell-producing step

A retrovirus-containing preparation was obtained in step 1 in the same manner as in the CAR-T cell-producing step of Example 1-1 except that the $_{sc}$IL27_CCL19_anti-human CD20

CAR expression vector obtained by the aforementioned vector-producing step was used as a retrovirus expression vector, instead of the $IL15_{LSP}$_CCL19_anti-human CD20 CAR expression vector, to obtain mouse T cells expressing the genes of anti-human CD20 CAR, scIL27, and CCL19 contained in the retrovirus ($_{sc}$IL27_CCL19_anti-human CD20 CAR-T cells, hereinafter referred to as "$_{sc}$IL27 × CCL19 CAR-T cells") in step 2.

[0139]

[Comparative Example 1] Transduction (-) T cells

Mouse T cells that were only activated (hereinafter referred to as "transduction (-) T cells") were produced by the same procedure except that the production of the retroviral vector and the transfection into T cells using the retroviral vector were not performed, and an equivalent amount of a DMEM medium used for GP2-293 cell culture was added, instead of the $IL15_{LSP}$_CCL19_anti-human CD20 CAR expression vector preparation, in step 2 of step B of Example 1-1.

[0140]

[Comparative Example 2] Conv. CAR-T cells

[A: Vector-producing step] Production of anti-human CD20 CAR expression vector

An anti-human CD20 CAR_MCS-containing pMSGV retrovirus expression vector was produced using DNA fragment 1, DNA

fragment 2, and the pMSGV retroviral vector by the same procedure as in the middle of step 2 of step A of Example 1-1. This was used as a control retrovirus expression vector (anti-human CD20 CAR expression vector) that does not co-express cytokines and chemokines.

[0141]

[B: CAR-T cell-producing step] Production of anti-human CD20 CAR-T cells

A retrovirus-containing preparation was obtained in step 1 in the same manner as in the CAR-T cell-producing step of Example 1-1 except that an anti-human CD20 CAR expression vector was used as a retrovirus expression vector, instead of the $IL15_{LSP}$_CCL19_anti-human CD20 CAR expression vector, to obtain mouse T cells expressing only the anti-human CD20 CAR contained in the retrovirus (not expressing cytokines and chemokines) (anti-human CD20 CAR-T cells, hereinafter referred to as "conv. CAR-T cells") in step 2.

[0142]

[Experimental Example 1] Confirmation of expression level of CAR, cytokine, and chemokine introduced

[A: Measurement of CAR expression by flow cytometry]

Using the T cells produced in Examples and Comparative Examples above, the expression level of the anti-human CD20

CAR on the cell surface was analyzed by flow cytometry, as described below.

[0143]

A flow cytometer "BD FACSCanto™ II" (BD Biosciences) was used for flow cytometry, and a FlowJo software (BD Biosciences) was used for data analysis.

[0144]

The T cells were treated using a biotin-labeled protein L (which specifically binds to the κ light chain of anti-human CD20 scFv) and Brilliant Violet™ 421 (BV421)-bound streptavidin, to detect CAR expression. At the same time, the CD8-positive rate in each T cell population was measured using an allophycocyanin (APC)-bound anti-mouse CD8 monoclonal antibody (BioLegend). Since CD8-positive T cells are generally considered to have direct cytotoxic activity, the presence (positive rate) of CD8-positive T cells can support the potency.

[0145]

Figure 1 shows the results. It was confirmed that 60% or more of the cell population of the CAR-T cells in all Examples (and Comparative Example 2) expressed (i.e., were positive for) the anti-human CD20 CAR.

[0146]

[B: Measurement of amounts of cytokine and chemokine

secreted]

The culture supernatants of the T cells were collected 42 hours after the transduction, the concentrations of IL-15, IL-18, IL-21, IL-27, and CCL19 were measured using a commercially available ELISA kit (available from MBL only for IL-18, and R&D systems for others).

[0147]

Figure 2 shows the results. For IL-15 (Figure 2A), IL-15 was detected at a concentration of 250 pg/mL from the culture supernatant of the CAR-T cells of Example 1-1 (15LSP x 19 CAR-T). Meanwhile, the concentrations of IL-15 in the culture supernatants of the CAR-T cells of Example 1-2 (s15RA x 19 CAR-T), Example 1-3 (mb15RA x 19 CAR-T), and Example 1-4 (sushi15 x 19 CAR-T) were similar to those of non-transduced activated T cells (transduction (-): Comparative Example 1) and anti-human CD20 CAR-expressing T cells (conv. CAR-T: Comparative Example 2) as a control, and no secretion of IL-15 was observed by the ELISA kit used in Experimental Example 1. However, since Example 1-2, Example 1-3, and Example 1-4 exhibited a remarkable cell proliferation effect in Experimental Example 2, which will be described below, it is understood that IL-15 was expressed and secreted in these examples as well as in Example 1-1.

[0148]

For IL-18 (Figure 2B), IL-18 was detected at a concentration of 150 pg/mL or more from the culture supernatant of the CAR-T cells of Example 2 (18 x 19 CAR-T). Meanwhile, the amount secreted from the anti-human CD20 CAR-expressing T cells (conv. CAR-T: Comparative Example 2) as a control into the culture supernatant was as small as from the non-transduced activated T cells (transduction (-): Comparative Example 1).

[0149]

For IL-21 (Figure 2C), IL-18 was detected at a concentration of 400 pg/mL or more from the culture supernatant of the CAR-T cells of Example 3 (21 x 19 CAR-T). Meanwhile, the amount secreted from the anti-human CD20 CAR-expressing T cells (conv. CAR-T: Comparative Example 2) as a control was below the detection limit (Not Detected) as well as from the non-transduced activated T cells (transduction (-): Comparative Example 1).

[0150]

For IL-27 (Figure 2D), p28 that is a sub-unit of IL-27 was detected at a concentration of 250 pg/mL or more from the culture supernatant of the CAR-T cells of Example 4 (sc27 x 19 CAR-T). Meanwhile, the amount secreted from the anti-human CD20 CAR-T cells (conv. CAR-T: Comparative Example 2) as a control was similar to that from the non-transduced

activated T cells (transduction (-): Comparative Example 1) (at a concentration of about 50 pg/mL in the culture supernatant).

[0151]

For CCL19 (Figure 2E), while the concentrations in the culture supernatants of the anti-human CD20 CAR-T cells (conv. CAR-T: Comparative Example 2) as a control and the non-transduced activated T cells (transduction (-): Comparative Example 1) were below the detection limit, the concentrations in the culture supernatant of the CAR-T cells of Examples were 150 to 500 pg/mL.

[0152]

[Experimental Example 2] Evaluation of in vitro proliferative capacity of CAR-T cells

Using the T cells produced in Examples and Comparative Examples above, whether or not each cytokine produced from T cells (IL-15, IL-18, IL-21, and IL-27) exerted biological functions and exhibited immunoinducing effects was determined by measuring the number and proliferative capacity of the CAR-T cells, as follows.

[0153]

The T cells were stained with CytoTell™ UltraGreen (AAT Bioquest) and then co-cultured in the same well as that of P815 mastocytoma (hCD20/P815) treated with mitomycin C and

genetically transformed to express human CD20, followed by stimulation. After culturing for 3 days, 5 days, or 7 days from the start of stimulation, the cells were collected and analyzed by flow cytometry. The cells were stained with an APC-bound anti-Thy1.2 monoclonal antibody (eBioscience), and the number of Thy1.2-positive cells in the T cell (lymphocyte) population was counted as surviving T cells.

[0154]

Figure 3 shows the results. The transduction (-) T cells (Comparative Example 1) did not undergo activation stimulation even when co-cultured with hCD20/P815 cells, and the number of living cells was 1/10 or less on day 3 of culture. Meanwhile, it was confirmed on day 3 of culture that proliferation in the number of living cells equivalent to or greater than the conv. CAR-T cells (Comparative Example 2) as a control was maintained in the $IL15_{LSP}$ × CCL19 CAR-T cells (Example 1-1), the $_{s}IL15_{RA}$ × CCL19 CAR-T cells (Example 1-2), the $_{mb}IL15_{RA}$ × CCL19 CAR-T cells (Example 1-3), the $_{sushi}$IL15 × CCL19 CAR-T cells (Example 1-4), the IL-18 × CCL19 CAR-T cells (Example 2), the IL-21 × CCL19 CAR-T cells (Example 3), and the scIL27 × CCL19 CAR-T cells (Example 4), which were co-cultured with hCD20/P815 cells. Further, when the culture was continued until day 7, the $_{s}IL15_{RA}$ × CCL19 CAR-T cells (Example 1-2), the $_{mb}IL15_{RA}$ × CCL19 CAR-T cells

(Example 1-3), and the $_{sushi}$IL15 × CCL19 CAR-T cells (Example 1-4) especially exhibited remarkable cell proliferation. The $IL15_{LSP}$ × CCL19 CAR-T cells (Example 1-1), the IL-18 × CCL19 CAR-T cells (Example 2), the IL-21 × CCL19 CAR-T cells (Example 3), and the scIL27 × CCL19 CAR-T cells (Example 4) maintained the number of cells equivalent to the conv. CAR-T cells (Comparative Example 2) as a control from day 3 to day 7 of culture.

[0155]

In addition, histogram analysis of the staining intensity with a CytoTell reagent in the Thy1.2-positive cell population was performed. Figure 4 shows the results for each cell population 5 days after the start of stimulation. In the $IL15_{LSP}$ × CCL19 CAR-T cells (Example 1-1), the $_{s}IL15_{RA}$ × CCL19 CAR-T cells (Example 1-2), the $_{mb}IL15_{RA}$ × CCL19 CAR-T cells (Example 1-3), the $_{sushi}$IL15 × CCL19 CAR-T cells (Example 1-4), the IL-18 × CCL19 CAR-T cells (Example 2), and the IL-21 × CCL19 CAR-T cells (Example 3), the proportion of the cell population of the second generation (after one division) especially decreased, and the proportion of the cell population after the fifth generation (after four or more divisions) increased, as compared with the conv. CAR-T cells (Comparative Example 2) as a control.

[0156]

From the results shown in Figure 3 and Figure 4, it was revealed that the combinations of IL-15/IL-15Rα fusion protein and CCL-19 produced in the $_{s}IL15_{RA}$ × CCL19 CAR-T cells (Example 1-2), the $_{mb}IL15_{RA}$ × CCL19 CAR-T cells (Example 1-3), and the $_{sushi}$IL15 × CCL19 CAR-T cells (Example 1-4) were especially preferable for promoting survival and proliferation of CAR-T cells and exerting biological functions.

[0157]

[Experimental Example 3] Evaluation of tumor cytotoxic activity of CAR-T cells

Using the T cells produced in Examples and Comparative Examples above, the target antigen-specific tumor cytotoxic activity was examined.

[0158]

[A: Measurement of tumor cytotoxic activity by flow cytometry]

P815 mastocytomas (hCD20/P815) genetically transformed to express human CD20 were used as target tumor cells, and P815 mastocytomas (P815) that were not genetically transformed as above were used as control tumor cells.

[0159]

After the target tumor cells or control tumor cells were collected and seeded on a culture plate, the CAR-T cells

of Examples (the $IL15_{LSP}$ × CCL19 CAR-T cells (Example 1-1), the $_{s}IL15_{RA}$ × CCL19 CAR-T cells (Example 1-2), the $_{mb}IL15_{RA}$ × CCL19 CAR-T cells (Example 1-3), the $_{sushi}$IL15 × CCL19 CAR-T cells (Example 1-4), the IL-18 × CCL19 CAR-T cells (Example 2), the IL-21 × CCL19 CAR-T cells (Example 3), and the scIL27 × CCL19 CAR-T cells (Example 4)), or the CAR-T cells of Comparative Example 2 (conv. CAR-T cells) were added thereto as effector T cells, and the cells were co-cultured for 72 hours. The effector T cells were added so that the number of CAR-positive cells was 1/3 of the target tumor cells (E:T ratio = 1:3), and the total number of T cells seeded was set to be the same in each group by adding, according to the number of T cells seeded with the lowest CAR expression rate, non-transduced T cells to the other T cell groups. After co-culturing for 72 hours, all cells were collected, analyzed by flow cytometry, and measured for the number of living cells. The cells were stained with an APC-bound anti-Thy1.2 monoclonal antibody (eBioscience) and a Zombie Green Fixable Viability Kit (BioLegend), and the proportions of T cells and tumor cells in the living cell population were calculated from the Thy1.2-positive rate. The number of living cells was measured using NucleoCounter NC-200 (chemometec).

[0160]

Figure 5 shows the results. As shown in Figure 5A, the number of hCD20/P815 tumor cells after co-culturing for 72 hours was calculated from the Thy1.2-negative rate in the living cells. As a result, the number of hCD20/P815 tumor cells co-cultured with each of the $IL15_{LSP}$ × CCL19 CAR-T cells (Example 1-1), the $_{s}IL15_{RA}$ × CCL19 CAR-T cells (Example 1-2), the $_{mb}IL15_{RA}$ × CCL19 CAR-T cells (Example 1-3), the $_{sushi}$IL15 × CCL19 CAR-T cells (Example 1-4), the IL-18 × CCL19 CAR-T cells (Example 2), the IL-21 × CCL19 CAR-T cells (Example 3), or the scIL27 × CCL19 CAR-T cells (Example 4) significantly decreased as compared with the transduction (-) T cells (Comparative Example 1) and also further decreased as compared with the conv. CAR-T cells (Comparative Example 2), and the tumor cells survived was 1% or less. Meanwhile, no decrease in tumor cells, P815 cells not expressing human CD20, was observed in any group, as shown in Figure 5B, and no cytotoxicity non-specific to tumor cells not expressing human CD20 was confirmed.

[0161]

[B: IFNγ production measurement by ELISA assay]

As an index of activation of the target antigen-specific cytotoxic ability, interferon γ (IFNγ) produced from CAR-T cells was measured using a commercially available ELISA assay kit (R&D). The CAR-T cells of Examples (the $IL15_{LSP}$ ×

CCL19 CAR-T cells (Example 1-1), the $_{s}IL15_{RA}$ × CCL19 CAR-T cells (Example 1-2), the $_{mb}IL15_{RA}$ × CCL19 CAR-T cells (Example 1-3), the $_{sushi}$IL15 × CCL19 CAR-T cells (Example 1-4), the IL-18 × CCL19 CAR-T cells (Example 2), the IL-21 × CCL19 CAR-T cells (Example 3), and the scIL27 × CCL19 CAR-T cells (Example 4)), or the CAR-T cells of Comparative Example 2 (conv. CAR-T cells) were co-cultured with the P815 cells (control tumor cells) or hCD20-P815 cells (target tumor cells) to measure the amount of IFNγ in the culture supernatant 4 days after the co-culture.

[0162]

Figure 6 shows the results. As shown in Figure 6A, the level of IFNγ was higher than in the conv. CAR-T cells (Comparative Example 2), and about 15 ng/mL or more of IFNγ was detected in the culture supernatant of any of the $IL15_{LSP}$ × CCL19 CAR-T cells (Example 1-1), the $_{s}IL15_{RA}$ × CCL19 CAR-T cells (Example 1-2), the $_{mb}IL15_{RA}$ × CCL19 CAR-T cells (Example 1-3), the $_{sushi}$IL15 × CCL19 CAR-T cells (Example 1-4), the IL-18 × CCL19 CAR-T cells (Example 2), the IL-21 × CCL19 CAR-T cells (Example 3), or the scIL27 × CCL19 CAR-T cells (Example 4), which were co-cultured with the hCD20-P815 cells. The amount of IFNγ produced in the groups co-cultured with the transduction (-) T cells (Comparative Example 1) was below the detection limit. Meanwhile, the concentration of IFNγ in

the culture supernatant of the CAR-expressing cells co-cultured with the P815 cells was 10 pg/mL or less, as shown in Figure 6B.

[0163]

From the above results of Experimental Example 3 (Figure 5 and Figure 6), it was confirmed that all the T cells of Examples and Comparative Example 2 exhibited a human CD20-expressing cell, that is, target antigen-specific cytotoxic activity.

[0164]

[Experimental Example 4] Therapeutic effect on mouse tumor models

The therapeutic effect of the T cells produced in Example 1-3, Example 1-4, and Comparative Example 2 on a mouse melanoma tumor transplant model or a mouse colorectal cancer tumor model was examined using the following cancer-bearing mice.

[0165]

(Therapeutic effect on mouse melanoma tumor transplant model)

5×10^5 of mouse melanoma B16F10 genetically transformed to express human CD20 (B16F10-hCD20) was subcutaneously inoculated into C57BL/6N mice. An anticancer agent, cyclophosphamide (CPA, 50 mg/kg), was intraperitoneally administered on day 7 after the

inoculation, and 1 × 10^6 of the CAR-T cells of Example 1-3 ($_{mb}$IL15$_{RA}$xCCL19 CAR-T), the CAR-T cells of Example 1-4 ($_{sushi}$IL15xCCL19 CAR-T), or the cells of Comparative Example 2 (Conv. CAR-T) were intravenously administered on day 10. As control groups, a CAR-T untreated group with only CPA administration and a group inoculated with B16F10-hCD20 mouse melanoma and thereafter untreated were set. The tumor volume in mice was measured twice a week.

[0166]

Figure 16 shows the results of the tumor volume of the mouse melanoma tumor transplant model. The horizontal axis indicates the number of days after the inoculation with the day on which B16F10-hCD20 was subcutaneously inoculated into the mice taken as day 0, and the vertical axis indicates the tumor volume (tumor major diameter × (tumor minor diameter)^2/2 (mm^3)). The standard deviation was calculated in each experimental group. "No treatment" means an untreated group, "CPA" means a group with only CPA administration, "CPA+Conv." means a group with CPA administration and subsequent administration of Comparative Example 2 (Conv. CAR-T), "CPA+$_{mb}$IL15$_{RA}$x19" means a group with CPA administration and subsequent administration of the CAR-T cells of Example 1-3 ($_{mb}$IL15$_{RA}$xCCL19 CAR-T), and "CPA+$_{sushi}$IL15$_{RA}$x19" means a group with CPA administration and

subsequent administration of the CAR-T cells of Example 1-4 ($_{sushi}$IL15xCCL19 CAR-T).

[0167]

As shown in Figure 16, the effect of reducing the tumor volume was confirmed in the groups with administration of the CAR-T cells of Example 1-3 ($_{mb}$IL15$_{RA}$xCCL19 CAR-T) and the CAR-T cells of Example 1-4 ($_{sushi}$IL15$_{RA}$xCCL19 CAR-T), as compared with the groups with administration of Comparative Example 2 (Conv. CAR-T), the untreated groups (no treatment), and the groups with only CPA administration. Accordingly, it was revealed that the CAR-T cells of Example 1-3 ($_{mb}$IL15$_{RA}$xCCL19 CAR-T) and the CAR-T cells of Example 1-4 ($_{sushi}$IL15xCCL19 CAR-T) had excellent antitumor activity on the mouse melanoma tumor model.

[0168]

(Therapeutic effect on mouse colorectal cancer tumor transplant model)

5×10^5 of mouse colorectal cancer MC38 (MC38-hCD20) genetically transformed to express human CD20 was subcutaneously inoculated into C57BL/6N mice. An anticancer agent, cyclophosphamide (CPA, 50 mg/kg), was intraperitoneally administered on day 7 after the inoculation, and 1×10^6 of the CAR-T cells of Example 1-3 ($_{mb}$IL15$_{RA}$xCCL19 CAR-T), the CAR-T cells of Example 1-4

($_{sushi}$IL15xCCL19 CAR-T), or the cells of Comparative Example 2 (Conv. CAR-T) were intravenously administered on day 10. As control groups, a CAR-T untreated group with only CPA administration and a group inoculated with MC38-hCD20 mouse colorectal cancer and thereafter untreated were set. The tumor volume in mice was measured twice a week.

[0169]

Figure 17 shows the results of the tumor volume of the mouse colorectal cancer tumor transplant model. The horizontal axis indicates the number of days after the inoculation with the day on which MC38-hCD20 was subcutaneously inoculated into the mice taken as day 0, and the vertical axis indicates the tumor volume (tumor major diameter × (tumor minor diameter)^2/2 (mm^3)). The standard deviation was calculated in each experimental group. "No treatment" means an untreated group, "CPA" means a group with only CPA administration, "CPA+Conv." means a group with CPA administration and subsequent administration of Comparative Example 2 (Conv. CAR-T), "CPA+$_{mb}$IL15$_{RA}$x19" means a group with CPA administration and subsequent administration of the CAR-T cells of Example 1-3 ($_{mb}$IL15$_{RA}$xCCL19 CAR-T), and "CPA+$_{sushi}$IL15$_{RA}$x19" means a group with CPA administration and subsequent administration of the CAR-T cells of Example 1-4 ($_{sushi}$IL15xCCL19 CAR-T).

[0170]

As shown in Figure 17, the effect of reducing the tumor volume was confirmed in the groups with administration of the CAR-T cells of Example 1-3 ($_{mb}$IL15$_{RA}$xCCL19 CAR-T) and the CAR-T cells of Example 1-4 ($_{sushi}$IL15$_{RA}$xCCL19 CAR-T), as compared with the groups with administration of Comparative Example 2 (Conv. CAR-T), the untreated groups (no treatment), and the groups with only CPA administration. Accordingly, it was revealed that the CAR-T cells of Example 1-3 ($_{mb}$IL15$_{RA}$xCCL19 CAR-T) and the CAR-T cells of Example 1-4 ($_{sushi}$IL15xCCL19 CAR-T) had excellent antitumor activity also on the mouse colorectal cancer model.

[0171]

In Examples and Experimental Examples above, mouse-derived T cells, a cytokine (fusion protein with IL-15 linked to IL-15Rα or the like) and a chemokine (CCL19) each containing the natural mouse amino acid sequence, were used, and in vivo tests in mice were conducted. However, those skilled in the art would be able to understand that, also in embodiments related to mammals other than mice, preferably humans, that is, in the case of using human-derived T cells, a cytokine (fusion protein with IL-15 linked to IL-15Rα or the like), and a chemokine (CCL19) each containing the natural human amino acid sequence, or in vivo tests in humans

were conducted, the present invention could be carried out in the same manner, and the action and effect of the present invention would be exerted.

[0172]

For example, the fusion protein containing $IL15_{LSP}$ which was carried out (produced and used) using the natural mouse amino acid sequence shown in SEQ ID NO: 5 can be carried out using the amino acid sequences at positions 1 to 29 (signal peptide portion) and 49 to 162 (IL-15 portion) in the natural human amino acid sequence for IL-15 shown in SEQ ID NO: 18 or using nucleic acids having the nucleotide sequence encoding such amino acid sequences.

[0173]

The fusion protein containing $_{s}IL15_{RA}$ which was carried out using the natural mouse amino acid sequence shown in SEQ ID NO: 7 can be carried out using the amino acid sequence at positions 49 to 162 (IL-15 portion) in the natural human amino acid sequence for IL-15 shown in SEQ ID NO: 18 and the amino acid sequence at positions 31 to 205 (IL-15Rα extracellular domain) in the natural human amino acid sequence for IL-15Rα shown in SEQ ID NO: 19 or using nucleic acids having the nucleotide sequence encoding such amino acid sequences.

[0174]

The fusion protein containing $_{mb}IL15_{RA}$ which was carried out using the natural mouse amino acid sequence shown in SEQ ID NO: 9 can be carried out using the amino acid sequence at positions 49 to 162 (IL-15 portion) in the natural human amino acid sequence for IL-15 shown in SEQ ID NO: 18 and the amino acid sequence at positions 1 to 267 (full-length IL-15Rα) in the natural human amino acid sequence for IL-15Rα shown in SEQ ID NO: 19 or using nucleic acids having the nucleotide sequence encoding such amino acid sequences.

[0175]

The fusion protein containing $_{sushi}$IL15 which was carried out using the natural mouse amino acid sequence shown in SEQ ID NO: 11 can be carried out using the amino acid sequence at positions 49 to 162 (IL-15 portion) in the natural human amino acid sequence for IL-15 shown in SEQ ID NO: 18 and the amino acid sequence at positions 31 to 95 (sushi domain) in the natural human amino acid sequence for IL-15Rα shown in SEQ ID NO: 19 or using nucleic acids having the nucleotide sequence encoding such amino acid sequences.

Industrial Applicability

[0176]

The CAR-T cells according to the present invention that are excellent in persistence and proliferation are

suitably used for producing a medicament for treating cancer or the like. Further, the expression vector according to the present invention is suitably used for producing the CAR-T cells.

Sequence listing free text

[0177]

SEQ ID NO: 1: The nucleotide sequence of DNA fragment containing an anti-human CD20 CAR (DNA fragment #1)

SEQ ID NO: 2: The amino acid sequence of a fusion protein expressed by the nucleotide sequence at positions 3 to 1637 of DNA fragment #1

SEQ ID NO: 3: The nucleotide sequence of a MCS DNA fragment encoding stop codons and restriction enzyme sites (DNA fragment #2)

SEQ ID NO: 4: The nucleotide sequence of $IL15_{LSP}$_F2A_CCL19 DNA fragment (DNA fragment #3)

SEQ ID NO: 5: The amino acid sequence of the entire fusion protein that is expressed by the nucleotide sequence at positions 7 to 969 of DNA fragment #3, self-cleaves at two F2A sites, and contains $IL15_{LSP}$

SEQ ID NO: 6: The amino acid sequence of $_{s}IL15_{RA}$_F2A_CCL19 DNA fragment (DNA fragment #4)

SEQ ID NO: 7: The amino acid sequence of the entire fusion protein that is expressed by the nucleotide sequence at positions 7 to 1539 of DNA fragment #4, self-cleaves at two F2A sites, and contains $_{s}IL15_{RA}$

SEQ ID NO: 8: The nucleotide sequence of $_{mb}IL15_{RA}$_F2A_CCL19 DNA fragment (DNA fragment #5)

SEQ ID NO: 9: The amino acid sequence of the entire fusion protein that is expressed by the nucleotide sequence at positions 7 to 1713 of DNA fragment #5, self-cleaves at two F2A sites, and contains $_{mb}IL15_{RA}$

SEQ ID NO: 10: The nucleotide sequence of $_{sushi}$IL15_F2A_CCL19 DNA fragment (DNA fragment #6)

SEQ ID NO: 11: The amino acid sequence of the entire fusion protein that is expressed by the nucleotide sequence at positions 7 to 1179 of DNA fragment #6, self-cleaves at two F2A sites, and contains $_{sushi}$IL15

SEQ ID NO: 12: The nucleotide sequence of IL-18_F2A_CCL19 DNA fragment (DNA fragment #7)

SEQ ID NO: 13: The amino acid sequence of the entire fusion protein that is expressed by the nucleotide sequence at positions 7 to 1059 of DNA fragment #7 and self-cleaves at two F2A sites

SEQ ID NO: 14: The nucleotide sequence of IL-21_F2A_CCL19 DNA fragment (DNA fragment #8)

SEQ ID NO: 15: The amino acid sequence of the entire fusion protein that is expressed by the nucleotide sequence at positions 7 to 969 of DNA fragment #8 and self-cleaves at two F2A sites

SEQ ID NO: 16: $_{sc}$IL27_F2A_CCL19 DNA fragment (DNA fragment #9)

SEQ ID NO: 17: The amino acid sequence of the entire fusion protein that is expressed by the nucleotide sequence at positions 7 to 1839 of DNA fragment #9, self-cleaves at two F2A sites, and contains $_{sc}$IL27

SEQ ID NO: 18: The amino acid sequence of natural human IL-15 (full length including signal peptide and propeptide parts) registered as UniProtKB-P40933

SEQ ID NO: 19: The amino acid sequence of natural human IL-15Rα (full length including signal peptide, sushi domain, extracellular domain, etc.) registered as UniProtKB-Q13261

CLAIMS

1. A T cell expressing:

(1) a chimeric antigen receptor (CAR);

(2) interleukin-15 (IL-15); and

(3) CC chemokine ligand 19 (CCL19),

wherein the IL-15 is linked to IL-15Rα to form a fusion protein, and wherein the fusion protein is

(i) a fusion protein comprising IL-15 linked to full-length IL-15Rα ($_{mb}IL15_{RA}$), or

(ii) a fusion protein comprising IL-15Rα containing a signal peptide and a sushi domain linked to IL-15 ($_{sushi}IL15$).

2. A pharmaceutical composition comprising the T cell according to claim 1.

3. An expression vector comprising:

(1) a nucleic acid encoding a CAR;

(2) a nucleic acid encoding IL-15; and

(3) a nucleic acid encoding CCL19,

wherein the IL-15 is linked to IL-15Rα to form a fusion protein, and wherein the fusion protein is

(i) a fusion protein comprising IL-15 linked to full-length IL-15Rα ($_{mb}IL15_{RA}$), or

(ii) a fusion protein comprising IL-15Rα containing a

signal peptide and a sushi domain linked to IL-15 ($_{sushi}$IL15).

4. A method for producing a CAR-T cell, comprising a step of introducing the expression vector according to claim 3 into a T cell.

5. Use of the T cell according to claim 1 for the manufacture of a pharmaceutical composition for treating cancer.

6. The use according to claim 5, wherein the cancer is melanoma, Merkel cell cancer, colorectal cancer, kidney cancer, breast cancer, ovarian cancer, fallopian tube cancer, cervical cancer, liver cancer, lung cancer, non-small cell lung cancer, head and neck cancer, small intestine cancer, prostate cancer, bladder cancer, rectal cancer, pancreatic cancer, Ewing's sarcoma, rhabdomyosarcoma, nasopharyngeal cancer, esophageal cancer, biliary tract cancer, neuroblastoma, osteosarcoma, acute myeloid leukemia, multiple myeloma, lymphoma, or leukemia.