

[Document Name] Description

[Title of Invention] METHOD FOR TREATING CANCER USING
ARTIFICIAL ADJUVANT CELL (aAVC)

[Technical Field]

[0001]

The present invention relates to an effective and safe method for treating or preventing a cancer using an artificial adjuvant cell (aAVC).

[Background Art]

[0002]

Natural killer T (NKT) cells are immune cells having the features of T cells and natural killer (NK) cells, and are activated by a CD1d ligand composed of glycolipid loaded on a major histocompatibility complex (MHC)-like molecule CD1d on antigen presenting cells (APC). The activated NKT cells are known to induce enhancement in direct cytotoxicity mediated by perforin/granzyme B, etc. as well as to produce cytokines such as interferon gamma (IFN- γ) or interleukin-4 (IL-4), thereby inducing the differentiation and proliferation of the NKT cells themselves and activating T cells, NK cells and B cells.

[0003]

CD1d ligands capable of activating NKT cells include, for example, α -galactosylceramide (α -GalCer),

α -C-galactosylceramide (α -C-GalCer), 7DW8-5, and isoglobotrihexosylceramide (iGb3). Known CD1d-expressing APCs include, for example, macrophages, dendritic cells (DC), B cells, and B cell lymphocytic leukemia (B-CLL) cells.

[0004]

NKT cells activated by DC pulsed with α -GalCer exhibit cytotoxicity to various cancer cells. It has been reported that DC pulsed with α -GalCer more strongly induces IFN- γ -producing NKT cells and exhibits a high antitumor effect, as compared with administration of free α -GalCer (Non Patent Literatures 1 and 2). Clinical trials have also been conducted targeting lung cancer patients (Non Patent Literatures 3 and 4).

[0005]

It has been reported that in mouse models, the administration of α -GalCer induces an antitumor effect by the activation of innate immunity via NKT cell activation, whereas adverse effects such as hepatocyte necrosis are manifested due to the production of inflammatory cytokines associated with the activation of NKT cells, etc. (Non Patent Literature 5). Furthermore, in clinical trials of α -GalCer, the administration of α -GalCer has induced immune response leading to an antitumor effect, and on the other hand, has been found to cause adverse events showing deterioration of general conditions, such as headache, vomiting, chills, and a

feeling of malaise, indicating the possibility of inducing various adverse reactions (Non Patent Literature 6).

[0006]

Fujii et al. have prepared aAVC by causing human-derived cells to exogenously express CD1d and a cancer antigen, and further pulsing the cells with α -GalCer (Patent Literatures 1 to 3). This aAVC activates NKT cells via its CD1d/ α -GalCer complex, and the activated NKT cells produce cytokines such as IFN- γ , which activates NK cells or the like. In mouse models, the administration of aAVC has been shown to have an NK cell-dependent antitumor effect (Patent Literatures 1 to 3 and Non Patent Literatures 7 and 8). aAVC administered to mice is immediately killed by activated NKT cells *in vivo*, and fragments of the aAVC are taken up into dendritic cells. The dendritic cells with the aAVC fragment thus taken up present the cancer antigen incorporated in MHC on the cell surface, and induce cancer antigen-specific T cells. In mouse models, the administration of aAVC has also been shown to have an antitumor effect via the induction of cancer antigen-specific T cells (Patent Literatures 1 to 3 and Non Patent Literatures 7 and 8). Thus, aAVC has been shown to be capable of strongly inducing two immune mechanisms, i.e., innate immunity activation shown by NK cell activation mediated by NKT cell activation and induction

of adaptive immunity shown by induction of antigen-specific T cells. However, no study has yet been conducted to determine a proper clinical dose of aAVC that exerts an antitumor effect by innate immunity activation and can be safely used.

[Citation List]

[Patent Literature]

[0007]

[Patent Literature 1] International Publication No. WO 2007/097370

[Patent Literature 2] International Publication No. WO 2010/061930

[Patent Literature 3] International Publication No. WO 2013/018778

[Non Patent Literature]

[0008]

[Non Patent Literature 1] "The Journal of Immunology", (USA), 1999; 163 (5): 2387-2391

[Non Patent Literature 2] "Nature Immunology", (USA), 2002; 3 (9): 867-874

[Non Patent Literature 3] "The Journal of Immunology", (USA), 2009; 182 (4): 2492-2501

[Non Patent Literature 4] "Clinical Cancer Research", (USA), 2005; 11 (5): 1910-1917

[Non Patent Literature 5] "Anticancer Research", 2016; 36 (7): 3667-3672

[Non Patent Literature 6] "Clinical Cancer Research",
(USA), 2002; 8 (12): 3702-3709

[Non Patent Literature 7] "Cancer Research", (USA), 2013;
73 (1): 62-73

[Non Patent Literature 8] "Cancer Research", (USA), 2016;
76 (13): 3756-3766

[Summary of Invention]

[Technical Problem]

[0009]

An object of the present invention is to provide an effective and safe method for treating or preventing a cancer using aAVC.

[Solution to Problem]

[0010]

The present inventors have conducted diligent studies on the clinical application of aAVC. The present inventors have found that the amount of α -GalCer loaded on aAVC in the course of aAVC manufacturing processes varies depending on the type of cells and conditions for pulsing cells with α -GalCer, etc. The need of standardizing the amount of α -GalCer loaded on aAVC has thereby been found in order to produce aAVC as medicaments. Accordingly, the present inventors have established a method for measuring the amount of α -GalCer loaded on cell surface in order to quantify the amount of

α -GalCer loaded on aAVC. The present inventors have further confirmed that adding various concentrations of α -GalCer into the medium when pulsing aAVC with α -GalCer results in variations in the amount of α -GalCer loaded on the cell (Example 2). The present inventors have further found that the dose of α -GalCer loaded on aAVC is related to the induction of an antitumor effect and adverse reactions attributable to innate immunity activation mediated by NKT cells (Examples 3 to 5). In this way, the present inventors have successfully found the dose of α -GalCer loaded on aAVC, and the amount of α -GalCer loaded on aAVC in a pharmaceutical composition comprising aAVC, which are preferred in terms of effectiveness and safety in the treatment and prevention of a cancer using aAVC. Specifically, the present invention provides an effective and safe method for treating or preventing a cancer using aAVC, aAVC for effective and safe treatment or prevention of a cancer, and a pharmaceutical composition comprising the same, etc.

[0011]

Specifically, the present invention may include the following aspects as a medically or industrially useful substance or method.

[0012]

[1] A method for treating or preventing a cancer, comprising the step of administering a human-derived cell to a human,

wherein the cell expresses exogenous CD1d, and has α -GalCer loaded on the cell surface,
and wherein the cell is administered to the human such that a single dose of α -GalCer loaded on the cell surface ranges from 1.7 ng to 275 ng per kg body weight of the human.

[2] The method according to [1], wherein the human-derived cell expresses an exogenous cancer antigen.

[3] The method according to [1] or [2], wherein the CD1d is human CD1d.

[4] The method according to any of [1] to [3], wherein the human-derived cell is a human embryonic kidney cell 293 (HEK293)-derived cell.

[5] The method according to any of [1] to [4], wherein the amount of α -GalCer loaded on the cell surface is in the range of 3.9 to 275 ng per 1×10^6 cells.

[6] A pharmaceutical composition for treating or preventing a cancer, comprising a human-derived cell, wherein the cell expresses exogenous CD1d and has α -GalCer loaded on the cell surface,
and wherein the composition is administered to a human such that a single dose of α -GalCer loaded on the cell surface ranges from 1.7 ng to 275 ng per kg body weight of the human.

[7] The pharmaceutical composition according to [6], wherein the human-derived cell expresses an exogenous cancer antigen.

[8] The pharmaceutical composition according to [6] or [7], wherein the CD1d is human CD1d.

[9] The pharmaceutical composition according to any of [6] to [8], wherein the human-derived cell is a human embryonic kidney cell 293 (HEK293)-derived cell.

[10] The pharmaceutical composition according to any of [6] to [9], wherein the amount of α -GalCer loaded on the cell surface is in the range of 3.9 to 275 ng per 1×10^6 cells.

[11] Use of a human-derived cell for producing a pharmaceutical composition for treating or preventing a cancer,

wherein the cell expresses exogenous CD1d and has α -GalCer loaded on the cell surface,

and wherein the composition is administered to a human such that a single dose of α -GalCer loaded on the cell surface ranges from 1.7 ng to 275 ng per kg body weight of the human.

[12] The use according to [11], wherein the human-derived cell expresses an exogenous cancer antigen.

[13] The use according to [11] or [12], wherein the CD1d is human CD1d.

[14] The use according to any of [11] to [13], wherein the human-derived cell is a human embryonic kidney cell 293 (HEK293)-derived cell.

[15] The use according to any of [11] to [14], wherein the amount of α -GalCer loaded on the cell surface is in the range of 3.9 to 275 ng per 1×10^6 cells.

[16] A human-derived cell for use in treating or preventing a cancer, wherein the cell expresses exogenous CD1d and has α -GalCer loaded on the cell surface, and wherein the cell is administered to a human such that a single dose of α -GalCer loaded on the cell surface ranges from 1.7 ng to 275 ng per kg body weight of the human.

[17] The human-derived cell according to [16], wherein the human-derived cell expresses an exogenous cancer antigen.

[18] The human-derived cell according to [16] or [17], wherein the CD1d is human CD1d.

[19] The human-derived cell according to any of [16] to [18], wherein the human-derived cell is a human embryonic kidney cell 293 (HEK293)-derived cell.

[20] The human-derived cell according to any of [16] to [19], wherein the amount of α -GalCer loaded on the cell surface is in the range of 3.9 to 275 ng per 1×10^6 cells.

[21] A method for producing a human-derived cell that expresses exogenous CD1d and has α -GalCer loaded on the cell surface, wherein the cell is administered to a human such that a single dose of α -GalCer loaded on the cell

surface ranges from 1.7 ng to 275 ng per kg body weight of the human, and wherein the method comprises the step of culturing human-derived cells expressing exogenous CD1d in a culture medium containing 56 ng/mL to 3000 ng/mL α -GalCer.

[22] The method according to [21], further comprising the step of measuring the amount of α -GalCer loaded on the cells obtained by the culture step, and selecting a cell wherein the amount of α -GalCer loaded on the cell surface is in the range of 3.9 to 275 ng per 1×10^6 cells.

[Advantageous Effects of Invention]

[0013]

The method of the present invention can be used for effectively and safely preventing or treating a cancer using aAVC.

[Brief Description of Drawings]

[0014]

[Figure 1] Figure 1 shows a change in survival rate after administration of aAVC(3T3)-WT1 in a mouse lung metastasis model in which B16 melanoma cells were intravenously injected. B16-F10 cells were administered to the tail vein of each 7-week-old C57BL/6J female mice at 2×10^4 cells, and 3 hours after administration, BICANATE or each dose of aAVC(3T3)-WT1 was administered to the tail vein of the mice (n = 16 in each group). The

survival of the mice was examined for 61 days after the administration. The survival time of the aAVC(3T3)-WT1 administration group was compared with the survival time of the BICANATE administration group by use of the log-rank test to determine a significance probability P-value. In the drawing, * and ** indicate that P-value is less than significance levels 0.05/3 and 0.01/3, respectively, corrected by the Bonferroni's method.

[Figure 2] Figure 2 shows the proportion (%) of NKT cells in spleen cells after administration of aAVC(3T3)-WT1 or each aAVC-WT1 described in Table 2. BICANATE or each dose of aAVC(3T3)-WT1 or aAVC-WT1 was administered to the tail vein of each 5-week-old C57BL/6J female mice (n = 3 in each group). Three days after administration, the spleen was collected, and NKT cells were detected by flow cytometry. The proportion of NKT cells was defined as the proportion of CD1d/Gal-dimer-positive and CD19-negative cells in lymphocyte-fraction cells of a forward scatter and side scatter cytogram, and indicated by mean $\pm$ standard error in the graph. The numeric values on the X-axis indicate the amount of α -GalCer loaded on aAVC (ng/ 10^6 cells).

[Figure 3] Figure 3 shows results of measuring the body weights of male mice or female mice in a toxicity study after single-dose intravenous administration of aAVC-WT1 in mice. BICANATE or each dose of aAVC-WT1 was administered once to the tail vein of each 8-week-old

female or male C57BL/6J mice (n = 5 in each group). The body weights were measured on the day before administration (day -1), the day of administration (day 0), the day following administration (day 1), 3 days after administration (day 3) and 7 days after administration (day 7), and shown in a graph.

[Figure 4] Figure 4 shows results of measuring the food consumptions of male mice or female mice in a toxicity study for single-dose intravenous administration in mice. BICANATE or each dose of aAVC-WT1 was administered once to the tail vein of each 8-week-old female or male C57BL/6J mice (n = 5 in each group). The food consumptions per day (g/day) were measured from 3 days before administration to the day before administration (days -3 to -1), from the day of administration to the day following administration (days 0 to 1), from the day following administration to 3 days after administration (days 1 to 3), and from 3 days after administration to 7 days after administration (days 3 to 7), and shown in a graph.

[Description of Embodiments]

[0015]

Hereinafter, the present invention will be described in detail. However, the present invention is not limited to the embodiments described below. Scientific terms and technical terms used in relation to the present invention

have meanings generally understood by those skilled in the art, unless otherwise specified herein.

[0016]

<Method for treating or preventing cancer according to the present invention>

The present invention provides the following method for treating or preventing a cancer:

a method for treating or preventing a cancer, comprising the step of administering a human-derived cell to a human, wherein the cell expresses exogenous or endogenous CD1d , and has α -GalCer loaded on the cell surface, and wherein the cell is administered to the human such that a single dose of α -GalCer loaded on the cell surface ranges from 1.7 ng to 275 ng per kg body weight of the human.

[0017]

1. aAVC

The method of the present invention can employ a human-derived cell that expresses exogenous or endogenous CD1d, and has α -GalCer loaded on the cell surface (also referred to as aAVC in the present specification). aAVC may further express one or more exogenous or endogenous cancer antigen(s). Although, aAVC without expressing cancer antigens(s) can activate NKT cells and shows a certain degree of antitumor effect, aAVC expressing a cancer antigen(s) has/have more advantage to induce

adaptive immunity against a cancer. aAVC can be easily prepared by those skilled in the art using a method known in the art (e.g., Non Patent Literatures 7 and 8 and Patent Literatures 1 to 3).

[0018]

The human-derived cell used in the present invention can be a cell derived from an arbitrary human tissue of, for example, stomach, small intestine, large intestine, lung, pancreas, kidney, liver, thymus gland, spleen, prostate, ovary, uterus, bone marrow, skin, muscle, or peripheral blood. The human-derived cell used in the present invention may have an ability to proliferate. In one embodiment, the human-derived cell used in the present invention is a non-hemocyte cell. The human-derived cell used in the present invention can be a cell derived from a particular type of cell in a human tissue (e.g., epithelial cells, endothelial cells, epidermal cells, stromal cells, fibroblasts, adipocytes, mammary gland cells, mesangial cells, pancreatic β cells, nerve cells, glial cells, exocrine epithelial cells, endocrine cells, skeletal muscle cells, smooth muscle cells, myocardial cells, osteoblasts, embryonic cells, and immune cells). The human-derived cell used in the present invention may be a normal cell or a cancer cell. In one embodiment, the human-derived cell used in the present invention is a normal cell. In one embodiment, the human-derived cell used in the present invention can

be a human embryonic kidney cell 293 (HEK293) cell (J. Gen. Virol.; 1977; 36: 59-74), a WI-38 cell, an SC-01MFP cell, and an MRC-5 cell, or a cell derived from any of these cells. In one embodiment, the human-derived cell used in the present invention is a HEK293-derived cell. In one embodiment, the human-derived cell used in the present invention is a FreeStyle™ 293-F cell.

[0019]

In one embodiment, the human-derived cell used in the present invention is an immortalized cell or a cell line derived from a human tissue. The immortalized cell and the cell line can be prepared by use of methods known to those skilled in the art.

[0020]

In one embodiment, the human-derived cell used in the present invention is a human-derived induced pluripotent stem cell (iPS cell) or an embryonic stem cell (ES cell). The iPS cell and the ES cell can be prepared by use of methods known to those skilled in the art. In one embodiment, the human-derived cell used in the present invention is a cell derived from a human-derived induced pluripotent stem cell (iPS cell) or an embryonic stem cell (ES cell).

[0021]

The CD1d used in the present invention can be naturally occurring CD1d or a modified form having its functions. CD1d may be CD1d which is endogenously

expressed in the human-derived cell used, or may be CD1d which is exogenously expressed in the human-derived cell used. In one embodiment, the expression means expression at any location of a cell. In one embodiment, the expression means expression on the cell surface. In one embodiment, aAVC expresses exogenous CD1d. In one embodiment, the CD1d used in the present invention is CD1d derived from a mammal (e.g., humans, monkeys, mice, rats, dogs, and chimpanzees). In one embodiment, the CD1d used in the present invention is human CD1d.

[0022]

In the present specification, the terms "exogenous" or "exogenously " are used interchangeably so as to refer to artificial transfer of a gene or a nucleic acid into a cell of interest by an operation such as genetic engineering or gene transfer, and the gene or the nucleic acid artificially introduced into the cell of interest, or a protein expressed therefrom. The exogenous gene may be operably linked to a promoter sequence which drives the expression of the gene.

[0023]

In the present specification, the term "endogenous" or "endogenously" means that a cell originally possesses a material, for example, gene, a nucleic acid or a protein.

[0024]

In the present specification, the term "derived" is used to indicate an animal species from which a cell has been obtained. For example, the human-derived cell means that the cell is a cell obtained from a human or a cell line obtained by subculturing the cell. For example, the human-derived cell means that the cell is a human cell.

[0025]

In the present specification, the term "identity" means a value of Identity obtained from the parameters provided as defaults using EMBOSS Needle (Nucleic Acids Res.; 2015; 43: W580-W584). The parameters described above are as follows:

Gap Open Penalty = 10

Gap Extend Penalty = 0.5

Matrix = EBLOSUM62

End Gap Penalty = false

[0026]

In one embodiment, the human CD1d is a protein consisting of the amino acid sequence represented by SEQ ID NO: 4. In one embodiment, the human CD1d is a protein that consists of an amino acid sequence derived from the amino acid sequence represented by SEQ ID NO: 4 with the deletion, substitution, insertion, and/or addition of 1 or several amino acids or in a certain embodiment, 1 to 10, 1 to 7, 1 to 5, 1 to 3, or 1 or 2 amino acids, and has a function of CD1d. In one embodiment, the human CD1d is a protein that consists of an amino acid sequence

having at least 90% or more, 95% or more, 96% or more, 97% or more, 98% or more, or 99% or more identity to the amino acid sequence represented by SEQ ID NO: 4, and has a function of CD1d. In one embodiment, the human CD1d is a protein consisting of an amino acid sequence encoded by the nucleotide sequence represented by SEQ ID NO: 3.

[0027]

Examples of the function of CD1d include the ability to bind to a CD1d ligand (e.g., α -GalCer). The ability of CD1d to bind to a CD1d ligand may be easily evaluated by those skilled in the art using a method known in the art. Alternatively, the function of CD1d may be evaluated by using the ability of aAVC to activate human NKT cells as an index. This ability to activate human NKT cells can be evaluated by a method described in Patent Literature 1 or Example 4 of the present application.

[0028]

In one embodiment, the aAVC used in the present invention expresses a cancer antigen. An arbitrary protein whose expression is found in cancer cells can be used as the cancer antigen used in the present invention. Examples of cancer antigens include Wilms tumor 1 (WT-1), human carbohydrate antigen 125 (CA-125), carcinoembryonic antigen (CEA), human telomerase reverse transcriptase (hTERT), mucin-1 (Muc-1), mucin-2 (Muc-2), cancer/testis antigen 1B (CTAG1B/NY-ESO-1), prostatic acid phosphatase

(PAP), prostate specific antigen (PSA), prostate specific membrane antigen (PSMA), survivin b, mutant ras, and mutant p53. In one embodiment, the cancer antigen used in the present invention is Wilms tumor 1 (WT-1). In one embodiment, the WT-1 is human WT-1. In one embodiment, the human WT-1 is a protein consisting of the amino acid sequence represented by SEQ ID NO: 2. In one embodiment, the human WT-1 is a protein consisting of an amino acid sequence derived from the amino acid sequence represented by SEQ ID NO: 2 with the deletion, substitution, insertion, and/or addition of 1 or several amino acids or in a certain embodiment, 1 to 10, 1 to 7, 1 to 5, 1 to 3, or 1 or 2 amino acids. In one embodiment, the human WT-1 is a protein consisting of an amino acid sequence having at least 90% or more, 95% or more, 96% or more, 97% or more, 98% or more, or 99% or more identity to the amino acid sequence represented by SEQ ID NO: 2. In one embodiment, aAVC expresses one cancer antigen. In one embodiment, aAVC expresses a plurality of cancer antigens. The cancer antigen may be a naturally occurring cancer antigen or a modified form thereof as long as aAVC expressing the cancer antigen exhibits an antitumor effect through an immunological effect against the cancer antigen. The cancer antigen may be an antigen endogenously expressed in the human-derived cell used or may be an antigen exogenously expressed in the human-derived cell used. In one embodiment, aAVC expresses an

exogenous cancer antigen. In one embodiment, aAVC expresses a plurality of exogenous cancer antigens.

[0029]

α -GalCer is one of the CD1d ligands and a substance represented by CAS RN: 158021-47-7 and a molecular formula: $C_{50}H_{99}NO_9$ (molecular weight: 858.34). α -GalCer may be synthesized according to a technique known in the art, or a commercially available product (e.g., α -Galactosylceramide (Funakoshi Co., Ltd., Cat. KRN7000)) may be used. The loading of α -GalCer onto cells may be performed by culturing cells expressing CD1d in a medium containing α -GalCer as described in the section "2. Method for preparing aAVC".

[0030]

2. Method for preparing aAVC

The present invention may comprise preparing cells at a stage before loading of α -GalCer (also referred to as aAVC precursor cells in the present specification), and loading α -GalCer onto the aAVC precursor cells. The present invention also provides a method for preparing a cell, wherein the cell expresses exogenous CD1d, and has α -GalCer loaded on the cell surface; wherein the cell is administered to the human such that a single dose of α -GalCer loaded on the cell surface ranges from 1.7 ng to 275 ng per kg body weight of the human; and wherein the method comprises the step of culturing human-derived cells expressing exogenous CD1d in a culture medium

containing 56 ng/mL to 3000 ng/mL α -GalCer. The present invention also provides a method for preparing a cell, wherein the cell expresses exogenous CD1d, and has α -GalCer loaded on the cell surface; wherein the cell is administered to the human such that a single dose of α -GalCer loaded on the cell surface ranges from 1.7 ng to 275 ng per kg body weight of the human; and wherein the method comprises the step of culturing human-derived cells expressing exogenous CD1d in a culture medium containing 56 ng/mL to 3000 ng/mL α -GalCer and the step of selecting a cell wherein the amount of α -GalCer loaded on the cell surface is in the range of 3.9 to 275 ng per 1×10^6 cells.

[0031]

2-1. Preparation of aAVC precursor cell

The aAVC precursor cells can be prepared by introducing either CD1d gene or a gene encoding a cancer antigen, or CD1d gene and a gene encoding a cancer antigen to human-derived cells. The CD1d gene and the gene encoding a cancer antigen can be designed and prepared by use of a standard molecular biological and/or chemical method by obtaining nucleotide sequences encoding amino acid sequences from NCBI RefSeq ID or GenBank Accession numbers. These genes can be synthesized, for example, by use of a phosphoramidite method based on their nucleotide sequences, or can be prepared by combining DNA fragments obtained through

polymerase chain reaction (PCR) from cDNA libraries. The transfer of the genes of interest to human-derived cells can be performed using an expression vector containing the gene to be introduced in a form of cDNA, mRNA or the like. Alternatively, the genes of interest may be introduced directly to human-derived cells by a method such as electroporation, lipofection or the like. Further, after introducing the gene of interest into human-derived cells to prepare aAVC progenitor cells, the cells may be cultured and proliferated.

[0032]

The expression vector used in the present invention is not particularly limited as long as the expression vector enable to express CD1d or the cancer antigen of interest, or CD1d and the cancer antigen of interest in human-derived cells. The expression vector may be, for example, a plasmid vector (e.g., pcDNA series (Thermo Fisher Scientific Inc.), pALTER®-MAX (Promega Corp.), and pHEK293 Ultra Expression Vector (Takara Bio Inc.)), or a viral vector (e.g., lentivirus, adenovirus, retrovirus, and adeno-associated virus). For example, pLVSIN-CMV/EF1 α vector (Takara Bio Inc.) or pLenti vector (Thermo Fisher Scientific Inc.) for use in the preparation of lentivirus can be used in the production of a viral vector. When CD1d and a cancer antigen are exogenously expressed in human-derived cells, CD1d and

the cancer antigen may be expressed by using either one vector or separate vectors.

[0033]

The expression vector may contain a promoter operably linked to the gene encoding CD1d or a cancer antigen. As a promoter, any of a promoter which constitutively promotes expression and a promoter that is driven by a drug (e.g., tetracycline or doxycycline) or the like can be used. Examples of the promoter constitutively promoting expression include promoters derived from viruses such as CMV (cytomegalovirus), RSV (respiratory syncytial virus), and SV40 (simian virus 40), actin promoter, and EF (elongation factor) 1 α promoter. Examples of the inducible promoter include tetracycline responsive factor (TRE3G promoter), cumate operator sequence, λ operator sequence (12 $\times$ λ Op), and heat shock promoter.

[0034]

The expression vector may contain a start codon and a stop codon. In this case, the expression vector may contain an enhancer sequence, an untranslated region, a splice junction, a polyadenylation site, or a replicable unit, etc. The expression vector may also contain a gene capable of serving as a marker for confirming the expression of the gene of interest (e.g., a drug resistance gene, a gene encoding a reporter enzyme, or a gene encoding a fluorescent protein).

[0035]

In one embodiment, the aAVC precursor cells are prepared by introducing CD1d gene to human-derived cells using a viral vector. In one embodiment, the aAVC precursor cells used in the present invention is a cell caused to exogenously express CD1d with a viral vector. In this embodiment, the aAVC precursor cells may contain a gene encoding CD1d, the gene being operably linked to a promoter. In one embodiment, the aAVC precursor cells are prepared by introducing CD1d gene and a gene encoding a cancer antigen to human-derived cells using a viral vector. In one embodiment, the aAVC precursor cells used in the present invention is a cell in which CD1d and a cancer antigen have been exogenously expressed using a viral vector. In this embodiment, the aAVC precursor cells may contain CD1d gene and a gene encoding a cancer antigen, the genes being each operably linked to a promoter.

[0036]

In one embodiment, the aAVC precursor cells are prepared by introducing CD1d gene to human-derived cells using a lentivirus vector. In one embodiment, the aAVC precursor cells used in the present invention is a cell in which CD1d has been exogenously expressed using a lentivirus vector. In one embodiment, the aAVC precursor cells are prepared by introducing CD1d gene and a gene encoding a cancer antigen to human-derived cells using a

lentivirus vector. In one embodiment, the aAVC precursor cells used in the present invention is a cell in which CD1d and a cancer antigen have been exogenously expressed using a lentivirus vector.

[0037]

The aAVC precursor cells are cultured by a method known in the art. For example, MEM medium (Science; 1952; 122: 501), DMEM medium (Virology; 1959; 8: 396-397), RPMI1640 medium (J. Am. Med. Assoc.; 1967; 199: 519-524), 199 medium (Proc. Soc. Exp. Biol. Med.; 1950; 73: 1-8), FreeStyle™ 293 Expression Medium (Thermo Fisher Scientific Inc., Cat. 12338022), CD 293 Medium (Thermo Fisher Scientific Inc., Cat. 11913019), or Expi293™ Expression Medium (Thermo Fisher Scientific Inc., Cat. A1435101) can be used as a basal medium. The culture medium can contain, for example, serum (e.g., fetal bovine serum), a serum replacement (e.g., KnockOut Serum Replacement: KSR), a fatty acid or a lipid, an amino acid, vitamin, a growth factor, a cytokine, an antioxidant, 2-mercaptoethanol, pyruvic acid, a buffer, an inorganic salt, or an antibiotic. The culture conditions (e.g., culture conditions such as culture time, temperature, medium pH, and CO₂ concentration) can be appropriately selected by those skilled in the art. The medium pH is preferably approximately 6 to 8. The culture temperature is not particularly limited and is, for example, approximately 30 to 40°C, preferably

approximately 37°C. The CO₂ concentration is approximately 1 to 10%, preferably approximately 5%. The culture time is not particularly limited, and the culture is performed for approximately 15 to 336 hours. If necessary, aeration or stirring may be performed. In the case of using a promoter that is driven by a drug such as tetracycline or doxycycline, the method may comprise the step of culturing the aAVC precursor cells in a medium supplemented with the drug to induce the expression of CD1d and the cancer antigen. This step can be performed in accordance with a gene induction method using a general gene induction system.

[0038]

2-2. Loading of α -GalCer onto cell

aAVC having α -GalCer loaded on the cell surface is prepared by pulsing the aAVC precursor cells with α -GalCer. In the present specification, the phrase "having α -GalCer loaded on the cell surface" refers to a state where α -GalCer is bound to the surface of an aAVC cell. The amount of α -GalCer bound can be measured by use of a method described in the section "2-3. Measurement of amount of α -GalCer loaded on aAVC". In the present specification, the term "pulsing" with α -GalCer refers to contacting aAVC precursor cells with α -GalCer to bind the α -GalCer to the cell surface of the aAVC precursor cells expressing CD1d. The pulsing can be performed in a cell culture medium.

[0039]

The conditions for pulsing the aAVC precursor cells (e.g., the timing of adding α -GalCer to a cell culture medium, the concentration of α -GalCer in the culture medium and culture time) can be appropriately adjusted by those skilled in the art in consideration of the aAVC precursor cells used and culture conditions. The concentration of α -GalCer to be added to the culture medium for the aAVC precursor cells is not particularly limited and can be appropriately selected within the range of, for example, 56 ng/mL to 3000 ng/mL.

[0040]

The preparation of the aAVC precursor cells and the pulsing of the cells with α -GalCer may be performed at the same time by the gene introduction of CD1d or a cancer antigen or CD1d and a cancer antigen to human-derived cells in the presence of α -GalCer.

[0041]

After the loading of α -GalCer onto the cell surface, α -GalCer that has not been loaded onto the cell surface (an excess of α -GalCer in the culture medium) can be removed.

[0042]

After pulsing the aAVC precursor cells with α -GalCer, the proliferation of aAVC may be arrested by using an artificial method. The method for arresting the proliferation of aAVC is not particularly limited. For

example, a method of arresting cell proliferation by exposure to radiation of particle rays such as radioactive rays (e.g., X-ray or γ -ray), or a method of adding a drug such as mitomycin C may be used.

[0043]

2-3. Measurement of the amount of α -GalCer loaded on aAVC

The present inventors have established a method for measuring the amount of α -GalCer loaded on aAVC cell surface. The measurement of the amount of α -GalCer loaded on aAVC cell surface can be performed by preparing cell extracts of aAVC and quantifying α -GalCer in the extracts by a method using mass spectrometry (MS) (e.g., liquid chromatography-tandem mass spectrometry (LC-MS/MS)).

[0044]

In a general quantitative analysis using LC-MS/MS, the quantitative analysis of α -GalCer in a sample is conducted by the following steps.

Step 1: Standard substances of α -GalCer prepared at several concentrations are analyzed.

Step 2: Temporal change in ionic strength (mass chromatogram) is obtained for m/z (mass/charge ratio) of ions derived from the standard substances, and peak areas of the mass chromatogram are calculated. Step 3: A calibration curve is prepared from the relationship between the peak area of each substance determined in the step 2 and the concentration of the substance.

Step 4: A sample to be quantified is analyzed to calculate the mass chromatogram peak area of α -GalCer in the sample.

Step 5: The concentration of α -GalCer in the sample corresponding to the peak area in the mass chromatogram of the step 4 is calculated on the basis of the calibration curve prepared in the step 3.

[0045]

The measurement of α -GalCer by LC-MS/MS can be performed by, for example, a method of ionizing α -GalCer into precursor ions and product ions by an electrospray ionization method. The column and the composition of the mobile phase for use in liquid chromatography in LC-MS/MS may be any combination of a column and a mobile phase as long as the combination used permits separation of α -GalCer from cell-derived components and permits discrimination of α -GalCer from cell-derived components by MS/MS.

[0046]

In one embodiment, the amount of α -GalCer loaded on the aAVC cell surface is in the range of 3.9 ng to 275 ng per 1×10^6 cells. In one embodiment, the amount of α -GalCer loaded on the aAVC cell surface can be in the range of 10 to 275 ng, 10 to 140 ng or 10 to 100 ng per 1×10^6 cells.

[0047]

3. Treatment or prevention of cancer with aAVC

The treatment or prevention method of the present invention can involve administration to a subject such that the single dose of α -GalCer loaded on the cell surface of aAVC to be administered to a human is a predetermined dose.

[0048]

In one embodiment, the treatment or prevention method of the present invention is characterized in that an aAVC is administered to a subject such that the single dose of α -GalCer loaded on the cell surface of aAVC to be administered to a human ranges from 1.7 ng to 275 ng per kg body weight of the human.

[0049]

In one embodiment, the single dose of α -GalCer loaded on the cell surface of aAVC to be administered to a human can be in a particular numeric range. The particular numeric range can be a numeric range included in the range of 1.7 ng to 275 ng (i.e., a numeric range from an upper limit value of 275 ng or less to a lower limit value of 1.7 ng or more) per kg body weight of the human. The numeric range has, for example, a numeric value of 275 ng or less, 270 ng or less, 260 ng or less, 250 ng or less, 240 ng or less, 238 ng or less, 230 ng or less, 220 ng or less, 210 ng or less, 200 ng or less, 190 ng or less, 180 ng or less, 170 ng or less, 160 ng or less, 150 ng or less, 140 ng or less, 130 ng or less, 120 ng or less, 110 ng or less, 100 ng or less, 90 ng or

less, 80 ng or less, 70 ng or less, 66 ng or less, 60 ng or less, 50 ng or less, 47 ng or less, 40 ng or less, 36 ng or less, 30 ng or less, 24 ng or less, 20 ng or less, 19 ng or less, 18 ng or less, 17 ng or less, 16 ng or less, 15 ng or less, 14 ng or less, 13 ng or less, 12 ng or less, 11 ng or less, 10 ng or less, 9 ng or less, 8 ng or less, 7 ng or less, 6.6 ng or less, 5 ng or less, 4.7 ng or less, 4 ng or less, 3.6 ng or less, 3 ng or less, or 2 ng or less as its upper limit value and a numeric value of 270 ng or more, 260 ng or more, 250 ng or more, 240 ng or more, 238 ng or more, 230 ng or more, 220 ng or more, 210 ng or more, 200 ng or more, 190 ng or more, 180 ng or more, 170 ng or more, 160 ng or more, 150 ng or more, 140 ng or more, 130 ng or more, 120 ng or more, 110 ng or more, 100 ng or more, 90 ng or more, 80 ng or more, 70 ng or more, 66 ng or more, 60 ng or more, 50 ng or more, 47 ng or more, 40 ng or more, 36 ng or more, 30 ng or more, 24 ng or more, 20 ng or more, 19 ng or more, 18 ng or more, 17 ng or more, 16 ng or more, 15 ng or more, 14 ng or more, 13 ng or more, 12 ng or more, 11 ng or more, 10 ng or more, 9 ng or more, 8 ng or more, 7 ng or more, 6.6 ng or more, 6 ng or more, 5 ng or more, 4.7 ng or more, 4 ng or more, 3.6 ng or more, 3 ng or more, 2.4 ng or more, 2 ng or more, or 1.7 ng or more as a lower limit value. The particular numeric range can be a numeric range included in one or more ranges selected from the group consisting of, for example, the range of

1.7 ng to 50 ng, the range of 50 ng to 100 ng, the range of 100 ng to 150 ng, the range of 150 ng to 200 ng, the range of 200 ng to 275 ng, the range of 1.7 ng to 238 ng, the range of 1.7 ng to 170 ng, the range of 6.6 ng to 238 ng, or the range of 6.6 ng to 170 ng.

[0050]

In one embodiment, the amount of α -GalCer loaded on the cell surface of aAVC to be administered to a human may be determined by using the proportion of NKT cells in spleen cells in the subject after administration as an index. An elevated proportion of NKT cells in spleen cells is considered to enhance the effect of administration.

[0051]

In one embodiment, the amount of α -GalCer loaded on the cell surface of aAVC to be administered to a human is in the range of 3.9 ng to 275 ng per 1×10^6 cells. In one embodiment, the amount of α -GalCer loaded on the cell surface of aAVC to be administered to a human can be in the range of 10 to 275 ng, 10 to 140 ng or 10 to 100 ng per 1×10^6 cells.

[0052]

aAVC can be administered to a subject in need of treatment or prevention of a cancer by use of a method known to those skilled in the art. In the case of administering aAVC to a subject, the aAVC can be administered to the subject in the form of a

pharmaceutical composition comprising aAVC and a pharmaceutically acceptable excipient. The pharmaceutical composition comprising aAVC can be prepared by a commonly used method using an excipient commonly used in the art, i.e., an pharmaceutical excipient, a pharmaceutical carrier, or the like. For the formulation of the pharmaceutical composition, an excipient, a carrier, an additive, or the like appropriate for its dosage form can be used within a pharmaceutically acceptable range. Examples of the dosage form of the pharmaceutical composition include parenteral agents such as injections and infusions.

[0053]

Examples of the cancer to be targeted by the treatment or prevention of the present invention include, but are not particularly limited to: hematologic cancers such as acute lymphoblastic leukemia (ALL), acute myeloid leukemia (AML), Hodgkin's lymphoma, non-Hodgkin's lymphoma, B cell lymphoma, multiple myeloma, and T cell lymphoma; solid cancers such as myelodysplastic syndrome, adenocarcinoma, squamous cell carcinoma, adenosquamous carcinoma, anaplastic cancer, large-cell cancer, non-small cell lung cancer, small-cell lung cancer, mesothelioma, skin cancer, breast cancer, prostate cancer, bladder cancer, vaginal cancer, neck cancer, head and neck cancer, uterine cancer, uterine cervical cancer, liver cancer, gallbladder cancer, bile duct cancer,

kidney cancer, pancreatic cancer, lung cancer, colon cancer, colorectal cancer, rectum cancer, small intestinal cancer, stomach cancer, esophageal cancer, testis cancer, ovary cancer, bladder cancer, and brain tumor; cancers of bone tissues, cartilage tissues, adipose tissues, muscle tissues, vascular tissues and hematopoietic tissues; sarcomas such as chondrosarcoma, Ewing's sarcoma, malignant hemangioendothelioma, malignant schwannoma, osteosarcoma, and soft tissue sarcoma; and blastomas such as hepatoblastoma, medulloblastoma, nephroblastoma, neuroblastoma, pancreatoblastoma, pleuropulmonary blastoma, and retinoblastoma.

[0054]

The dose of aAVC to a human and the number of doses thereof can be appropriately adjusted according to the type, position, and severity of the cancer, the age, body weight and condition of the subject to be treated, etc. The dose of aAVC can be set to an arbitrary amount within the range of, for example, 1×10^3 cells/kg to 1×10^8 cells/kg per dose to a subject. In one embodiment, the dose of aAVC is 1.7×10^3 cells/kg, 1.7×10^4 cells/kg, 1.7×10^5 cells/kg, or 1.7×10^6 cells/kg. In one embodiment, the dose of aAVC can be set to an arbitrary amount within the range of 6.2×10^3 cells/kg to 7.0×10^7 cells/kg. In this context, the lower limit value is the number of cells per kg body weight of the human when

cells having α -GalCer loaded in an amount of 275 ng/ 10^6 cells are administered such that the single dose of α -GalCer loaded on the cell surface is 1.7 ng/kg. The upper limit value is the number of cells per kg body weight of the human when cells having α -GalCer loaded in an amount of 3.9 ng/ 10^6 cells are administered such that the single dose of α -GalCer loaded on the cell surface is 275 ng/kg. In one embodiment, the dose of aAVC can be in a particular numeric range. The particular numeric range can be a numeric range included in the range of 6.2×10^3 cells/kg to 7.0×10^7 cells/kg (i.e., a numeric range from an upper limit value of 7.0×10^7 cells/kg or less to a lower limit value of 6.2×10^3 cells/kg or more). The numeric range has, for example, a numeric value of 7.0×10^7 cells/kg or less, 6.0×10^7 cells/kg or less, 5.0×10^7 cells/kg or less, 4.0×10^7 cells/kg or less, 3.0×10^7 cells/kg or less, 2.0×10^7 cells/kg or less, 1.7×10^7 cells/kg or less, 1.3×10^7 cells/kg or less, 1.0×10^7 cells/kg or less, 9.0×10^6 cells/kg or less, 8.0×10^6 cells/kg or less, 7.0×10^6 cells/kg or less, 6.0×10^6 cells/kg or less, 5.0×10^6 cells/kg or less, 4.3×10^6 cells/kg or less, 4.0×10^6 cells/kg or less, 3.0×10^6 cells/kg or less, 2.7×10^6 cells/kg or less, 2.0×10^6 cells/kg or less, 1.7×10^6 cells/kg or less, or 1.0×10^6 cells/kg or less as its upper limit value and a numeric value of 1.0×10^4 cells/kg or more, 1.7×10^4 cells/kg or more, 2.0×10^4 cells/kg or more, $3.0 \times$

10^4 cells/kg or more, 4.0×10^4 cells/kg or more, 4.4×10^4 cells/kg or more, 5.0×10^4 cells/kg or more, 6.0×10^4 cells/kg or more, 6.6×10^4 cells/kg or more, 7.0×10^4 cells/kg or more, 8.1×10^4 cells/kg or more, 9.0×10^4 cells/kg or more, 1.0×10^5 cells/kg or more, 1.7×10^5 cells/kg or more, 2.0×10^5 cells/kg or more, 3.0×10^5 cells/kg or more, 3.2×10^5 cells/kg or more, 4.0×10^5 cells/kg or more, 5.0×10^5 cells/kg or more, 6.0×10^5 cells/kg or more, 6.6×10^5 cells/kg or more, 7.0×10^5 cells/kg or more, 8.0×10^5 cells/kg or more, or 9.0×10^5 cells/kg or more as a lower limit value. The particular numeric range can be a numeric range included in one or more ranges selected from the group consisting of, for example, the ranges of 1.7×10^3 cells/kg to 1.7×10^6 cells/kg, 1.7×10^3 cells/kg to 1.7×10^5 cells/kg, 1.7×10^3 cells/kg to 1.7×10^4 cells/kg, 1.7×10^4 cells/kg to 1.7×10^6 cells/kg, 1.7×10^4 cells/kg to 1.7×10^5 cells/kg, 1.7×10^5 cells/kg to 1.7×10^6 cells/kg, 6.6×10^4 cells/kg to 1.7×10^7 cells/kg, 1.7×10^5 cells/kg to 2.7×10^7 cells/kg, 6.6×10^5 cells/kg to 1.7×10^7 cells/kg, 8.1×10^4 cells/kg to 1.3×10^7 cells/kg, 3.2×10^5 cells/kg to 8.0×10^6 cells/kg, 4.4×10^4 cells/kg to 7.0×10^6 cells/kg, 4.4×10^4 cells/kg to 7.0×10^6 cells/kg, 2.6×10^5 cells/kg to 4.3×10^6 cells/kg, 1.7×10^4 cells/kg to 2.7×10^6 cells/kg, and 6.6×10^4 cells/kg to 1.7×10^6 cells/kg.

[0055]

As for a method for administering aAVC to a human, the aAVC can be administered by, for example, intravenous, intratumoral, intradermal, subcutaneous, intramuscular, intraperitoneal, or intra-arterial injection or infusion.

[0056]

The treatment or prevention method of the present invention can be used in combination with an additional cancer treatment method. Examples of the additional cancer treatment method include surgery, radiotherapy, hematopoietic stem cell transplantation, and treatment with other anticancer agents.

[0057]

<Pharmaceutical composition, etc. of present invention>

The present invention also provides a pharmaceutical composition for the treating or preventing a cancer, comprising a human-derived cell. The pharmaceutical composition comprises a human-derived cell that expresses exogenous or endogenous CD1d and has α -GalCer loaded on the cell surface, and is administered to a human such that a single dose of α -GalCer loaded on the surface of the cell to be administered to the human ranges from 1.7 ng to 275 ng per kg body weight of the human. The human-derived cell may further express one or more exogenous or endogenous cancer antigen(s). The present invention also provides use of a human-derived cell for manufacturing a pharmaceutical composition for the treatment or

prevention of a cancer, wherein the cell expresses exogenous or endogenous CD1d and has α -GalCer loaded on the cell surface, and the composition is administered to a human such that a single dose of α -GalCer loaded on the surface of the cell to be administered to the human ranges from 1.7 ng to 275 ng per kg body weight of the human. The human-derived cell may further express one or more exogenous or endogenous cancer antigen(s). The present invention also provides a human-derived cell for the treatment or prevention of a cancer, wherein the cell expresses exogenous or endogenous CD1d and has α -GalCer loaded on the cell surface, and the cell is administered to a human such that a single dose of α -GalCer loaded on the surface of the cell to be administered to the human ranges from 1.7 ng to 275 ng per kg body weight of the human. The human-derived cell may further express one or more exogenous or endogenous cancer antigen(s).

[0058]

Embodiments regarding the cell used (aAVC) and a method for producing the same, and its use in the treatment or prevention of a cancer, etc. in the pharmaceutical composition, the use, and the cell of the present invention mentioned above are as described in the section "Method for treating or preventing cancer according to the present invention".

[0059]

<Method for producing pharmaceutical composition, etc.
according to the present invention>

The present invention also provides a method for producing a pharmaceutical composition for treating or preventing a cancer, comprising a human-derived cell. The method for producing a pharmaceutical composition according to the present invention can be a method comprising: providing a human-derived cell that expresses exogenous or endogenous CD1d and has α -GalCer loaded on the cell surface; and packaging the cell as a pharmaceutical composition that is administered to a human such that a single dose of α -GalCer loaded on the cell surface is in the range of 1.7 ng to 275 ng per kg body weight of the human. The method may further comprise the step of arresting the growth of the human-derived cell by a method such as exposure to radiation. In one embodiment, the method for producing a pharmaceutical composition according to the present invention can be a method comprising: providing a human-derived cell that expresses exogenous or endogenous CD1d and has α -GalCer loaded on the cell surface; selecting a cell or determining a cell number to be packaged as a pharmaceutical composition by using the amount of α -GalCer loaded on the cell surface as an index; and packaging the selected or determined cell to obtain a pharmaceutical composition. In this embodiment, the total amount of α -GalCer loaded on the surface of cells

contained in the pharmaceutical composition can be in a numeric range defined by multiplying an arbitrary numeric value from 1.7 ng to 275 ng by an average human body weight. The method for producing a pharmaceutical composition according to the present invention may further involve a method for measuring the amount of α -GalCer loaded on cell surface, described in the section <Method for measuring amount of α -GalCer loaded on cell>. The method for producing a pharmaceutical composition according to the present invention may further comprise: selecting a cell population wherein the amount of α -GalCer loaded per cell is in a defined numeric range; and packaging the selected cell population as a pharmaceutical composition. The amount of α -GalCer loaded per cell can be in the range of, for example, 3.9 ng to 275 ng, 10 to 275 ng, 10 to 140 ng or 10 to 100 ng per 1×10^6 cells. The produced pharmaceutical composition is administered at the dose described above to a human.

[0060]

<Method for measuring amount of α -GalCer loaded on cell>

The present invention also provides the following method for measuring the amount of α -GalCer loaded on cells:

a method for measuring the amount of α -GalCer loaded on cells, wherein the cells express CD1d and have α -

GalCer loaded on the cell surface, wherein the method comprises the steps of:
preparing cell extracts of the cells; and
subjecting the cell extracts to mass spectrometry (MS) to measure the amount of α -GalCer in the extracts.

[0061]

In one embodiment, the mass spectrometry (MS) is liquid chromatography-tandem mass spectrometry (LC-MS/MS).

[0062]

In one embodiment, the cells used in this method are human-derived cells that express exogenous or endogenous CD1d and have α -GalCer loaded on the cell surface (aAVC). In one embodiment, the cells used in this method are human-derived cells that express exogenous or endogenous CD1d and one or more exogenous or endogenous cancer antigen(s) and have α -GalCer loaded on the cell surface (aAVC). In one embodiment, the cells used in this method are human-derived cells that express exogenous CD1d and one or more exogenous cancer antigen(s) and have α -GalCer loaded on the cell surface (aAVC).

[0063]

Particular Examples to be referred to will be provided here for the further understanding of the present invention. However, these examples are given for illustrative purposes and do not limit the present invention.

[Examples]

[0064]

[Example 1-1: Preparation of aAVC-WT1]

Lentiviruses were prepared using plasmids carrying WT1, CD1d, and Tet3G (reverse tetracycline regulatory transactivator) genes, respectively. By introducing those genes into FreeStyle™ 293-F cells (Thermo Fisher Scientific, Cat.R79007) using said lentivirus carrying each of the genes, aAVC expressing CD1d and WT1 and loaded α -GalCer onto the cell surface was constructed.

[0065]

(1) Construction of plasmids for lentivirus preparation

A gene having a 5'-terminally added XhoI recognition sequence and a 3'-terminally added NotI recognition sequence in the WT1 gene (SEQ ID NO: 1) was inserted into the XhoI-NotI site of pLV SIN-CMV Pur plasmid (Takara Bio Inc., Cat. 6183) to construct pLV SIN-CMV-WT1 plasmid. The gene of SEQ ID NO: 1 was prepared by optimization for human codons through the artificial gene synthesis service of Thermo Fisher Scientific Inc. based on the amino acid sequence of Wilms tumor protein isoform D (NCBI Reference Sequence: NP_077744.3). The pLV SIN-CMV-WT1 plasmid was cleaved with restriction enzymes ClaI and EcoRI to remove CMV promoter. Then, the cut ends were blunted with T4 DNA Polymerase (Toyobo Co., Ltd., Cat. TPL-101). Ligation reaction was performed using DNA

Ligation Kit (Takara Bio Inc., Cat. 6023) to construct pLVSIN- Δ -WT1 plasmid. TRE3G promoter excised from pTRE3G (Takara Bio Inc., Cat. 631173) with restriction enzymes EcoRI and SalI was inserted to the EcoRI-XhoI site of the pLVSIN- Δ -WT1 plasmid to construct pLVSIN-TRE3G-WT1 plasmid. The pLVSIN-TRE3G-WT1 plasmid was cleaved with restriction enzymes BamHI and MluI to remove PGK promoter and puromycin resistance gene. Then, the cut ends of the plasmid were blunted with T4 DNA Polymerase. Ligation reaction was performed using DNA Ligation Kit to construct pLVSIN-TRE3G-WT1 Δ pur plasmid.

[0066]

pLVSIN-CMV Pur plasmid was cleaved with restriction enzymes XhoI and MluI to remove PGK promoter and puromycin resistance gene. The CD1d gene (SEQ ID NO: 3) was amplified by PCR using primers with sequences complementary to 15 nucleotides at both sides of the XhoI-MluI site of the plasmid, respectively. The amplified CD1d gene was inserted into the cleaved plasmid described above using In-Fusion® HD Cloning Kit (Takara Bio Inc., Cat. 639648) to construct pLVSIN-CMV-CD1d Δ pur plasmid. The gene of SEQ ID NO: 3 was prepared by optimization for human codons through the artificial gene synthesis service of Thermo Fisher Scientific Inc. based on the amino acid sequence (SEQ ID NO: 4) of antigen-presenting glycoprotein CD1d isoform 1 precursor (NCBI Reference Sequence: NP_001757.1).

[0067]

The Tet3G gene was amplified from pCMV-Tet-On 3G plasmid (Takara Bio Inc., Cat. 631335) by use of PCR. The amplified gene was inserted to the XhoI-MluI site of pLVSIN-CMV Pur plasmid using In-Fusion® HD Cloning Kit to construct pLVSIN-CMV-Tet3G Δ pur plasmid.

[0068]

(2) Lentivirus preparation

WT1-loaded lentivirus, CD1d-loaded lentivirus and Tet3G-loaded lentivirus were prepared using the pLVSIN-TRE3G-WT1 Δ pur plasmid, the pLVSIN-CMV-CD1d Δ pur plasmid, and the pLVSIN-CMV-Tet3G Δ pur plasmid prepared in (1).

[0069]

30 μ g of the pLVSIN-TRE3G-WT1 Δ pur plasmid and 30 μ L of ViraPower Lentiviral Packaging Mix (Thermo Fisher Scientific Inc., Cat. K497500) were mixed. Opti-MEM™ I Reduced Serum Medium (Thermo Fisher Scientific Inc., Cat. 31985-070) was added thereto for adjustment to 1 mL and mixed, and the mixture was left standing for 5 minutes (A). 60 μ L of PEIpro® in vitro DNA transfection reagent (Polyplus-transfection SE, Cat. 115-010) and 940 μ L of Opti-MEM™ I Reduced Serum Medium were mixed and left standing for 5 minutes (B). The (A) and (B) were mixed and left standing at room temperature for 15 minutes. Then, gene introduction was performed by adding the whole amount to 293T cells (ATCC Cat. CRL-3216) inoculated to Falcon® Vent Cap Type Flask 800 mL, Slant Neck (Corning

Inc., Cat. 353138). The inoculation of the 293T cells was performed on the day before gene introduction. The cells were cultured at 37°C under 5% CO₂ conditions in DMEM medium (Thermo Fisher Scientific Inc., Cat. 10569-010) containing 10% fetal bovine serum (SAFC Biosciences, Cat. 12007C (γ ray-irradiated product)) and 0.1% gentamycin (Thermo Fisher Scientific Inc., Cat. 15750-060). After the gene introduction, the cells were cultured for 2 days. A culture supernatant containing WT1 gene-loaded lentivirus was recovered by centrifuging the culture medium at 230 × g at room temperature for 5 minutes. The supernatant was filtered through a 0.22 μm filter (Merck KGaA, Cat. SLGV033RS). A 4 × PEG solution (32% Poly(ethylene glycol) BioUltra, 6000 (Merck KGaA, Cat. 81253-250G), 0.4 M NaCl (Kanto Chemical Co., Inc., Cat. 37144-02), and 0.04 M HEPES (Thermo Fisher Scientific Inc., Cat. 15630-080)) was added thereto in an amount of 1/3 of the amount of the supernatant, mixed, and left standing overnight at 4°C. The mixture was centrifuged at 2500 × g at 4°C for 30 minutes and the supernatant was removed. The supernatant was completely removed after an additional centrifugation at 2500 × g at 4°C for 30 minutes. The precipitates were suspended in an appropriate amount of Opti-MEM™ I Reduced Serum Medium. A recovered culture supernatant was concentrated 30-fold to obtain WT1-loaded lentivirus.

[0070]

The Tet3G-loaded lentivirus was prepared with almost the same procedure as above using the pLVSIN-CMV-Tet3G Δ pur plasmid. The culture period after gene introduction was set to 2 days or 3 days. The still standing after mixing with the 4 $\times$ PEG solution was carried out at 4°C overnight or for 4 days. FreeStyle 293 Expression Medium (Thermo Fisher Scientific Inc., Cat. 12338018) was used as the final suspension medium. [0071]

12 μ g of the pLVSIN-CMV-CD1d Δ pur plasmid and 36 μ L of ViraPower Lentiviral Packaging Mix were mixed. OptiPro™ SFM (Thermo Fisher Scientific Inc., Cat. 12309-019) was added thereto for adjustment to 6 mL and mixed, and the mixture was left standing for 5 minutes (A-2). 144 μ L of Lipofectamine 2000 CD Transfection Reagent (Thermo Fischer Scientific Inc., Cat. 12566-014) and 6 mL of OptiPro™ SFM were mixed and left standing for 5 minutes (B-2). The A-2 and B-2 were mixed and left standing at room temperature for 20 minutes. Then, gene introduction was performed by adding the whole amount to 293FT cells (Thermo Fisher Scientific Inc., Cat. R70007) inoculated to Falcon® Vent Cap Type Flask 800 mL, Slant Neck. The medium used for culturing the 293FT cells was prepared by adding G418 (Thermo Fisher Scientific Inc., Cat. 10131-027) and MEM Non-Essential Amino Acids Solution (100X) (Thermo Fisher Scientific Inc., Cat. 11140050) at final concentrations of 500 μ g/mL and 0.1

mM, respectively, to DMEM medium containing 10% fetal bovine serum (SAFC Biosciences, Cat. 12007C (γ ray-irradiated product)) and 0.1% gentamycin. After the gene introduction, the cells were cultured for 2 days. A culture supernatant containing CD1d gene-loaded lentivirus was recovered by centrifuging the culture medium at $540 \times g$ at 4°C for 10 minutes. The supernatant was filtered through a $0.44 \mu\text{m}$ filter (Merck KGaA, Cat. SLHV033RS). PEG-itTM Virus Precipitation Solution (5x) (System Biosciences, LLC, Cat. LV825A-1) was added thereto in an amount of 1/5 of the amount of the supernatant, mixed, and left standing overnight at 4°C . The mixture was centrifuged at $1500 \times g$ at 4°C for 30 minutes for removal of a supernatant, and centrifuged again at $1500 \times g$ at 4°C for 5 minutes to completely remove the supernatant. The pellets were suspended in an appropriate amount of FreeStyle 293 Expression Medium. A recovered culture supernatant was concentrated 75-fold to obtain CD1d-loaded lentivirus.

[0072]

(3) Preparation of FreeStyle 293F_WT1_CD1d_Tet3G cell

FreeStyleTM 293-F cells were infected sequentially with the 3 types of lentiviruses prepared in (2) to obtain FreeStyle 293F_WT1_CD1d_Tet3G cells. The FreeStyleTM 293-F cells were cultured in FreeStyle 293 Expression Medium containing 0.1% gentamycin. The FreeStyleTM 293-F cells at a concentration of 1×10^6

cells/mL were prepared and inoculated at a volume of 1 mL/well to Falcon® 12-Well Flat Bottom Multiwell Cell Culture Plate with Lid (Corning Inc., Cat. 353043; hereinafter, referred to as a 12-well plate). 50 µL of the WT1-loaded lentivirus and 100 µL of the Tet3G-loaded lentivirus prepared in (2) were added to each well. After centrifugation at 540 × g at room temperature for 30 minutes, the cells were gently suspended by pipetting, and shake-cultured. After 3 days, the cells were passaged from the 12-well plate to Corning® Polycarbonate 125 mL Erlenmeyer Flask with Vent Cap (Corning Inc., Cat. 431143; hereinafter, referred to as a 125 mL Erlenmeyer flask), further passaged at 3-day or 4-day intervals, and cultured for 11 days. These cells were inoculated again to a 12-well plate. 100 µL of the CD1d-loaded lentivirus prepared in (2) was added to each well. The second lentivirus infection was performed by the same procedures as in the first one. After 1 day, the cells were passaged from the 12-well plate to a 125 mL Erlenmeyer flask, further passaged at 3-day or 4-day intervals, and cultured for 10 days. The obtained cells were used as FreeStyle 293F_WT1_CD1d_Tet3G cells.

[0073]

(4) Cloning

Single-cell cloning was performed by the limiting dilution method from the FreeStyle 293F_WT1_CD1d_Tet3G cells prepared in (3). Clones stably expressing all the

genes were selected. The cells were inoculated at 1 cell/well to a 96-well plate and passaged as the cells proliferated. The expression levels of the WT1 protein and the CD1d protein in the proliferated cells were measured by ELISA for WT1 and by flow cytometry for CD1d to select clones stably expressing the WT1 and CD1d proteins. The expression of WT1 was induced via Tet-On System by the addition of doxycycline (Takara Bio Inc., Cat. 631311) with a final concentration of 100 ng/mL to the medium, and evaluated. The measurement by ELISA was performed in accordance with a general procedure of sandwich ELISA using Anti-Wilms' tumor Antibody, NT clone 6F-H2 (Merck KGaA, Cat. MAB4234-C) as an antibody for immobilization, Anti WT1 antibody (Wuxi AppTec Co., Ltd., Cat. AP11964c) as a primary antibody, and Rabbit IgG Horseradish Peroxidase-conjugated Antibody (R&D Systems, Inc., Cat. HAF008) as a secondary antibody. The measurement by flow cytometry was performed using APC Mouse Anti-Human CD1d antibody (BD Biosciences, Cat. 563505) and FACSVerse™ (BD Biosciences). The selected clones were used as aAVC-WT1 precursor cells.

[0074]

(5) Preparation of aAVC-WT1 differing in amount of α -GalCer loaded

By adding various concentrations of α -GalCer (whose synthesis was commissioned by Juzen Chemical Corp. under manufacturing agreement) to aAVC-WT1 precursor cells and

culturing the cells, aAVCs differing in the amount of α -GalCer loaded (also referred to as aAVC-WT1) are prepared.

[0075]

The aAVC-WT1 precursor cells were cultured, and doxycycline at a final concentration of 100 ng/mL and an α -GalCer solution at a final concentration of 56 ng/mL, 167 ng/mL, 500 ng/mL, 1500 ng/mL or 3000 ng/mL were added to the medium to pulse the cells with α -GalCer. The culture of the aAVC-WT1 precursor cells supplemented with the 1500 ng/mL α -GalCer solution was independently performed with three different culture volumes. The α -GalCer solution was prepared by a method conforming to Example 2(1) described later. 2 days after addition of α -GalCer, the cells were recovered by the centrifugation method, washed, concentrated, and irradiated with X-ray at a dose of 30, 40 or 100 Gy using X-ray irradiation apparatus MBR-1520R-3 or MBR-1520R-4 (Hitachi Power Solutions Co., Ltd.) to prepare aAVC-WT1.

[0076]

[Example 1-2: Preparation of mouse-type aAVC(3T3)-WT1]

Mouse-type aAVC (also referred to as aAVC(3T3)-WT1) was prepared by introducing mRNAs of the WT1 gene and the CD1d gene into mouse NIH/3T3 cells (ATCC, Cat. CRL-1658), and adding α -GalCer (synthesized by Juzen Chemical Corp. under manufacturing agreement) thereto.

[0077]

The WT1 gene excised from pcDNA3-ATG-WT1 described in WO2013/018778 with restriction enzymes HindIII and EcoRI was inserted into the HindIII-EcoRI site of pGEM-4Z plasmid (Promega Corp., Cat. P2161) to prepare pGEM-4Z-WT1 plasmid. Mouse CD1d gene consisting of the nucleotide sequence shown in SEQ ID NO: 5 (synthesized by optimization for mouse codons through the artificial gene synthesis service of Thermo Fisher Scientific Inc. based on the amino acid sequence (SEQ ID NO: 6) of Antigen-presenting glycoprotein CD1d1, UniProt: P11609-1 was inserted into the HindIII-BamHI site of the pGEM-4Z plasmid to construct pGEM-4Z-mCD1d plasmid. The pGEM-4Z-WT1 plasmid and the pGEM-4Z-mCD1d plasmid were cleaved and linearized with EcoRI and BamHI, respectively. WT1 mRNA and CD1d mRNA were prepared using the plasmids as templates using mMACHINE™ T7 ULTRA Transcription Kit (Thermo Fisher Scientific Inc., Cat. AMB13455). NIH/3T3 cells cultured for 2 days in Dulbecco's Modified Eagle medium (Thermo Fisher Scientific Inc., Cat. 10569) containing 10% fetal bovine serum supplemented with 500 ng/mL α -GalCer (synthesized by Juzen Chemical Corp. under manufacturing agreement) were recovered. WT1 mRNA and CD1d mRNA were added to the cell suspension and electroporated (poring pulse: voltage: 150 V, pulse width: 8 ms, pulse interval: 50 ms, the number of times: 2, decay rate: 40%, polarity: +, transfer pulse: voltage: 20 V, pulse width: 50 ms, pulse

interval: 50 ms, the number of times: ± 5 , decay rate: 10%, polarity: +/-) using NEPA21 electroporator (Nepa Gene Co., Ltd.). The electroporated cells were recovered and irradiated with X-ray at a dose of 30 Gy using X-ray irradiation system MBR-1520R-3 (Hitachi Power Solutions Co., Ltd.) or MBR-1520R-4 to prepare aAVC(3T3)-WT1.

[0078]

[Example 2: Measurement of concentration of α -GalCer loaded on aAVC-WT1 and aAVC(3T3)-WT1]

(1) Preparation of α -GalCer standard solution

28 g of sucrose (Merck KGaA, Cat. S7903) and 3.75 g of L-histidine (Merck KGaA, Cat. H8000) were added to 400 mL of water for injection (Thermo Fisher Scientific Inc., Cat. A12873-02) and dissolved by heating in a constant temperature water bath at 80°C. 100 mg of α -GalCer and 25 g of 10% polysorbate 20 (MP Biomedicals, LLC, Cat. 194724) were added to the solution, and the mixture was heated for over 1 hour in a constant temperature bath at 80°C. After confirmation that solid matter in the solution was dissolved, the amount was adjusted to 500 g by the addition of water for injection. This solution was used as a 200 μ g/mL α -GalCer solution. The 200 μ g/mL α -GalCer solution was diluted with a diluent solution (10% water and 90% mobile phase B (70% ethanol, 29.5% methanol, and 0.5% formic acid)) to prepare α -GalCer solutions having a concentration of 1000 ng/mL, 500

ng/mL, 100 ng/mL, 50 ng/mL, 10 ng/mL, 5 ng/mL, or 2.5 ng/mL, which were used as α -GalCer standard solutions.
[0079]

(2) Preparation of cell extract

1 mL or 0.25 mL of Milli-Q water was added to 1×10^6 cells of aAVCs prepared by pulsing with various concentration of α -GalCer in Example 1. The cells were homogenized for 20 seconds by using an ultrasonic homogenizer (SMT Co., Ltd., Cat. UH-50). 900 μ L of mobile phase B was added to 100 μ L of the cell homogenate, and the mixture was stirred for over 10 minutes. After centrifugation at $10000 \times g$ for 5 minutes, 250 μ L of supernatant was collected into a polypropylene screw vial (GL Sciences Inc., Cat. 1030-61024) to prepare cell extracts.

[0080]

(3) LC-MS/MS measurement

The α -GalCer standard solutions prepared in (1) and the cell extracts prepared in (2) were measured by multiple reaction monitoring of LC-MS/MS. The measurement was carried out with the number of measurements set to 1 for the α -GalCer standard solutions and to 6, 3, or 1 for the cell extracts as shown in Table 1 of (5). LC employed 1200 series (Agilent Technologies, Inc.) was used as LC, and 6410 Triple Quad LC-MS (Agilent Technologies, Inc., Cat. G6410B) was used as MS. Samples were separated by LC. 10 μ L each of the α -GalCer

standard solutions and the cell extracts was applied to Accucore-150-C4 (Thermo Fisher Scientific Inc., Cat. 16526-103030) column, and the samples were separated using mobile phase A (0.1% formic acid) and mobile phase B (70% ethanol, 29.5% methanol, and 0.5% formic acid) on a gradient from 60% to 100% of the mobile phase B. The flow rate to the column was 0.4 ml/min, and the column temperature was 40°C. The separated solutions were sequentially introduced to MS, and compounds in the solutions were ionized by the electrospray ionization method. 858.7 [m/z] was selected as a precursor ion of α -GalCer from the generated ions. This precursor ion was further decomposed to detect 696.7 [m/z] as a product ion of α -GalCer. Values determined in advance by analyzing the α -GalCer standard solutions by the LC-MS/MS were used for the precursor ion and the product ion.

[0081]

(4) Analysis

α -GalCer concentrations were quantified using analysis software (Agilent Technologies, Inc., Agilent MassHunter Quantitative Analysis). The retention time of α -GalCer was determined from results of measuring the α -GalCer standard solutions, and measurement samples were confirmed to exhibit a similar retention time, and the amount of α -GalCer in each of the measurement samples analyzed by the peak area method. A calibration curve was prepared from the results of analyzing the α -GalCer

standard solutions prepared in (1), and the samples of interest were quantified. For the preparation of the calibration curve, measurement results obtained from 6 points of 1000 ng/mL, 500 ng/mL, 100 ng/mL, 50 ng/mL, 10 ng/mL, and 5 ng/mL or from 4 or 5 points among 6 points of 500 ng/mL, 100 ng/mL, 50 ng/mL, 10 ng/mL, 5 ng/mL, and 2.5 ng/mL were used.

[0082]

(5) Results of measuring α -GalCer content

Results of measuring the amount of α -GalCer loaded on aAVC(3T3)-WT1 or aAVC-WT1 by LC-MS/MS are shown in the table below.

In Table 1, the three values shown at the α -GalCer addition concentration of 1500 ng / mL indicate the amount of α -GalCer loaded on the cells obtained in three independent experiments with different culture scales.

[Table 1]

Type	α -GalCer addition concentration (ng/mL)	Amount loaded (ng/10 ⁶ cells)	The number of measurements
aAVC(3T3)-WT1	500	2.4*	1
aAVC-WT1	56	3.9*	3
	167	10	3
	500	21	3
	1500	39	3
	1500	140	6
	1500	275	6
	3000	100	3

* represents "less than the lower limit value of quantification" when the number of the measured cells is 10⁶ cells.

[0083]

[Example 3: Determination of the dose of aAVC(3T3)-WT1 cells showing pharmacological efficacy]

The antitumor effect by activation of NKT cells mediated by α -GalCer of aAVC(3T3)-WT1 was evaluated in a mouse lung metastasis model in which B16 melanoma cells were intravenously injected. B16-F10 melanoma cells (ATCC, Cat. CRL-6475) suspended in PBS were administered at 2×10^4 cells to the tail vein of each 7-week-old C57BL/6J female mouse (Charles River Laboratories Japan, Inc.) (n =16 in each group) to prepare a mouse lung metastasis model in which B16 melanoma cells were intravenously injected. 3 hours after the administration, aAVC(3T3)-WT1 suspended in BICANATE Injection (Otsuka Pharmaceutical Co., Ltd.) where the loaded amount of α -GalCer is 2.4 ng/ 10^6 cells (the uppermost part of Table 1 in Example 2) was administered at 1.7×10^4 cells/kg, 1.7×10^5 cells/kg, or 1.7×10^6 cells/kg to the tail veins of the mice. 200 μ L of BICANATE Injection was administered to a control group. The results of examining survival over 61 days from the administration of B16-F10 showed that the median survival time of the control group was 29.5 days, whereas the median survival time of the aAVC(3T3)-WT1 administration group was 31.5 days for the 1.7×10^4 cells/kg administration group, 42.0 days for the 1.7×10^5 cells/kg administration group, and 37.0 days for the 1.7×10^6 cells/kg administration group. When the survival

times were compared by the log-rank test, the survival time of the groups given aAVC(3T3)-WT1 administered at 1.7×10^5 cells/kg or 1.7×10^6 cells/kg was significantly prolonged as compared with the control group (Figure 1). Accordingly, aAVC(3T3)-WT1 was found to be effective at 1.7×10^5 cells/kg for this model. It has been reported that the antitumor effect of cells having α -GalCer loaded thereon in this model is attenuated due to a deficiency of NKT cells in mice or depletion of NK cells (J. Immunolo.; 2007; 178: 2853-2861). Thus, the antitumor effect of aAVC(3T3)-WT1 shown in this model is thought to be mediated by the activation of NKT cells by α -GalCer loaded thereon.

[0084]

[Example 4: Amount of α -GalCer loaded and NKT cell activation]

In order to estimate the pharmacological effective dose of human-type aAVC-WT1 using mouse-type aAVC(3T3)-WT1, the ability to activate NKT cells in mice was measured for aAVC(3T3)-WT1 and each aAVC-WT1 described in Table 1 of Example 2, and compared. The dose of α -GalCer necessary for exerting a pharmacological effect mediated by NKT cell activation by the administration of aAVC-WT1 to a human was determined using the results obtained above. The proportion of NKT cells in spleen cells after administration of aAVC(3T3)-WT1 was measured as an index for the ability to activate NKT cells.

[0085]

aAVC(3T3)-WT1 or each aAVC-WT1 loaded with the amount of α -GalCer given in Table 1 of Example 2, and suspended in BICANATE Injection was administered at 1.7×10^4 cells/kg, 1.7×10^5 cells/kg, or 1.7×10^6 cells/kg to the tail vein of each 5-week-old C57BL/6J female mouse (Charles River Laboratories Japan, Inc.) (n = 3 in each group). 200 μ L of BICANATE Injection was administered to a control group. 3 days after administration, the mouse was sacrificed, and the spleen was excised. Spleen cells were prepared from the excised spleen according to a general method. NKT cells included in the spleen cells were stained with α -GalCer-bound recombinant soluble dimeric mouse CD1d:Ig fusion (CD1d/Gal-dimer, Becton, Dickinson and Company, Cat. 557599), APC-conjugated anti-mouse IgG1 (Becton, Dickinson and Company, Cat. 560089) and FITC-conjugated anti-mouse CD19 (BioLegend, Inc., Cat. 115506), and measured by flow cytometry. The proportion of NKT cells was calculated by analyzing the proportion of CD1d/Gal-dimer-positive and CD19-negative cells in lymphocyte-fraction cells of a forward scatter and side scatter cytogram using FlowJo ver. 10.2 software (FlowJo, LLC). As a result, a tendency was shown that the proportion of NKT cells in spleen cells was increased in accordance with the increase of the amount of α -GalCer loaded on aAVC-WT1 and the increase of the number of administered cells (Figure 2). These results suggest

that the amount of α -GalCer administered is markedly strongly correlated with NKT cell activation rather than the number of administered cells.

[0086]

The proportion of NKT cells in spleen cells was 1.1% (shaded bar of aAVC(3T3)-WT1 in Figure 2) when aAVC(3T3)-WT1 was administered at 1.7×10^5 cells/kg (dose that significantly prolonged survival time as compared with a control group in Example 3). In the aAVC-WT1 administered mouse, the minimum dose of α -GalCer exhibiting an NKT cell proportion of 1.1% or more was 1.7 ng/kg (Figure 2, shaded bar of aAVC-WT1 with α -GalCer 10 ng/ 10^6 cells; and Table 2, when 10 ng/ 10^6 cells of α -GalCer was administered at 1.7×10^5 cells/kg, 1.7 ng/kg was administered in term of a dose of the α -GalCer). This suggests that an α -GalCer dose of 1.7 ng/kg or more can significantly elevate the proportion of NKT cells in spleen cells.

Even the minimum amount of α -GalCer loaded on the aAVC cell surface, corresponding to a concentration of 3.9 ng/ 10^6 cells, showed an NKT cell proportion of 1.1% or more as long as the dose of α -GalCer was one exceeding 1.7 ng/kg (Figure 2, filled bar graph of aAVC-WT1 wherein the amount of the loaded α -GalCer was 3.9 ng/ 10^6 cells). This suggests that it is desirable to use cells loaded with α -GalCer at a concentration of 3.9 ng/ 10^6 cells or higher.

[0087]

Table 2 summarizes the types of the cells, the amount of the loaded α -GalCer, the numbers of administered cells and the doses of α -GalCer in administration at said numbers of administered cells to mice, used in Examples 3 and 4.

[Table 2]

Type	Amount of α -GalCer loaded (ng/ 10^6 cells)	The number of administered cells (cells/kg)	α -GalCer dose (ng/kg)
aAVC(3T3)-WT1	2.4*	1.7×10^4	0.041
		1.7×10^5	0.41
		1.7×10^6	4.1
aAVC-WT1	3.9*	1.7×10^4	0.066
		1.7×10^5	0.66
		1.7×10^6	6.6
	10	1.7×10^4	0.17
		1.7×10^5	1.7
		1.7×10^6	17
	21	1.7×10^4	0.36
		1.7×10^5	3.6
		1.7×10^6	36
	39	1.7×10^4	0.66
		1.7×10^5	6.6
		1.7×10^6	66
	100	1.7×10^4	1.7
		1.7×10^5	17
		1.7×10^6	170
	140	1.7×10^4	2.4
		1.7×10^5	24
		1.7×10^6	238
	275	1.7×10^4	4.7
		1.7×10^5	47
		1.7×10^6	468

* represents "less than the lower limit value of quantification" when the number of the measured cells is 10^6 cells.

[0088]

[Example 5: Toxicity study after single-dose intravenous administration in mice]

Toxicity of aAVC-WT1 (amount of α -GalCer loaded: 275 ng/ 10^6 cells) for after single-dose intravenous

administration to mice was evaluated. Eight-week old C57BL/6J female or male mice (Charles River Laboratories Japan, Inc.) were used in each group consisting of five mice. As the administration group, four groups: a vehicle control group, a low-dose group (1×10^6 cells/kg), a middle-dose group (1×10^7 cells/kg) and a high-dose group (1×10^8 cells/kg), were set. BICANATE Injection (Otsuka Pharmaceutical Co., Ltd.) was used as the vehicle to prepare the cell administration solution. aAVC-WT1 was suspended in BICANATE Injection to prepare 1×10^5 cells/mL administration solution for the low-dose group, 1×10^6 cells/mL administration solution for the middle-dose group, and 1×10^7 cells/mL administration solution for the high-dose group, respectively. The dose to the mice was set to 10 mL/kg. The amount of the dosing solution was calculated for each mouse base on the body weight measured on the day of administration, and the vehicle or each cell dosing solution was administered once into the tail vein of each mouse. The mice after the administration were subjected to examination of general conditions and body weight measurement over 7 days after the administration. Then the mice was sacrificed on the 7 days of post-administration and subjected to necropsy. No effects (death, moribundity, other decrease in locomotor activity, etc.) due to administration of vehicle or each cell dosing solution were observed during the observation period of general

conditions (observed 3 times in total before administration, 1 hour after administration, and 4 hours after administration on the day of administration, and once a day from the day after administration until necropsy). The body weight measurement was performed on the day before administration, on the day of administration, on the day after administration, 3 days and 7 days after administration. Decrease in body weight was found on 3 days after administration in females and males in the high-dose group (Figure 3). In food consumption measurement (the food consumptions per day were measured from 3 days before administration to the day before administration, from the day of administration to the day after administration, from the day after administration to 3 days after administration, and from 3 days after administration to 7 days after administration), decrease in food consumption was found from the day of administration to the day after administration and from the day after administration to 3 days after administration in females in the middle-dose group and females and males in the high-dose group (Figure 4). From the results of this study, no effects on body weight and food consumption was found in the low-dose group, therefore, it was considered that the dose of 1×10^6 cells/kg is the upper limit of the dose that does not affect body weight and food consumption

(equivalent to 275 ng/kg of α -GalCer dose calculated from the amount of α -GalCer loaded).

[Industrial Applicability]

[0089]

The present invention is expected to be useful in effective and safe treatment or prevention of a cancer using aAVC.

[Free Text of Sequence Listing]

[0090]

"Artificial Sequence" will be described in the numeric caption <223> of the sequence listing given below.

The nucleotide sequence set force in SEQ ID NO: 1 of the sequence listing is a nucleotide sequence encoding human WT1 protein, and the amino acid sequence set force in SEQ ID NO: 2 of the sequence listing is an amino acid sequence encoded by the sequence of SEQ ID NO: 1. The nucleotide sequence set force in SEQ ID NO: 3 of the sequence listing is a nucleotide sequence encoding human CD1d protein, and the amino acid sequence set force in SEQ ID NO: 4 of the sequence listing is an amino acid sequence encoded by the sequence of SEQ ID NO: 3. The nucleotide sequence set force in SEQ ID NO: 5 of the sequence listing is a nucleotide sequence encoding mouse CD1d protein, and the amino acid sequence set force in

SEQ ID NO: 6 of the sequence listing is an amino acid sequence encoded by the sequence of SEQ ID NO: 5.

[Sequence Listing]

[Document Name] Claims

[Claim 1]

A method for treating or preventing a cancer, comprising the step of administering a human-derived cell to a human, wherein the cell expresses exogenous CD1d, has α -galactosylceramide (α -GalCer) loaded on the cell surface, and wherein the cell is administered to the human such that a single dose of α -GalCer loaded on the cell surface ranges from 1.7 ng to 275 ng per kg body weight of the human.

[Claim 2]

The method according to claim 1, wherein the human-derived cell expresses an exogenous cancer antigen.

[Claim 3]

The method according to claim 1 or 2, wherein the CD1d is human CD1d.

[Claim 4]

The method according to any of claims 1 to 3, wherein the human-derived cell is a human embryonic kidney cell 293 (HEK293)-derived cell.

[Claim 5]

The method according to any of claims 1 to 4, wherein the amount of α -GalCer loaded on the cell surface is in the range of 3.9 to 275 ng per 1×10^6 cells.

[Claim 6]

A pharmaceutical composition for treating or preventing a cancer, comprising a human-derived cell, wherein the cell expresses exogenous CD1d and has α -GalCer loaded on the cell surface, and wherein the composition is administered to a human such that a single dose of α -GalCer loaded on the cell surface is 1.7 ng to 275 ng per kg body weight of the human.

[Claim 7]

The pharmaceutical composition according to claim 6, wherein the human-derived cell expresses an exogenous cancer antigen.

[Claim 8]

The pharmaceutical composition according to claim 6 or 7, wherein the CD1d is human CD1d.

[Claim 9]

The pharmaceutical composition according to any of claims 6 to 8, wherein the human-derived cell is a human embryonic kidney cell 293 (HEK293)-derived cell.

[Claim 10]

The pharmaceutical composition according to any of claims 6 to 9, wherein the amount of α -GalCer loaded on the cell surface is in the range of 3.9 to 275 ng per 1×10^6 cells.

[Claim 11]

Use of a human-derived cell for producing a pharmaceutical composition for treating or preventing a cancer,

wherein the cell expresses exogenous CD1d and has α -GalCer loaded on the cell surface,

and wherein the composition is administered to a human such that a single dose of α -GalCer loaded on the cell surface ranges from 1.7 ng to 275 ng per kg body weight of the human.

[Claim 12]

The use according to claim 11, wherein the human-derived cell expresses an exogenous cancer antigen.

[Claim 13]

The use according to claim 11 or 12, wherein the CD1d is human CD1d.

[Claim 14]

The use according to any of claims 11 to 13, wherein the human-derived cell is a human embryonic kidney cell 293 (HEK293)-derived cell.

[Claim 15]

The use according to any of claims 11 to 14, wherein the amount of α -GalCer loaded on the cell surface is in the range of 3.9 to 275 ng per 1×10^6 cells.

[Claim 16]

A human-derived cell for use in treating or preventing a cancer,

wherein the cell expresses exogenous CD1d and has α -GalCer loaded on the cell surface,
and wherein the cell is administered to a human such that a single dose of α -GalCer loaded on the cell surface ranges from 1.7 ng to 275 ng per kg body weight of the human.

[Claim 17]

The human-derived cell according to claim 16,
wherein the human-derived cell expresses an exogenous cancer antigen.

[Claim 18]

The human-derived cell according to claim 16 or 17,
wherein the CD1d is human CD1d.

[Claim 19]

The human-derived cell according to any of claims 16 to 18, wherein the human-derived cell is a human embryonic kidney cell 293 (HEK293)-derived cell.

[Claim 20]

The human-derived cell according to any of claims 16 to 19, wherein the amount of α -GalCer loaded on the cell surface is in the range of 3.9 to 275 ng per 1×10^6 cells.

[Claim 21]

A method for producing a human-derived cell that expresses exogenous CD1d and has α -GalCer loaded on the cell surface, wherein the cell is administered to a human such that a single dose of α -GalCer loaded on the cell

surface ranges from 1.7 ng to 275 ng per kg body weight of the human, and wherein the method comprises the step of culturing human-derived cells expressing exogenous CD1d in a culture medium containing 56 ng/mL to 3000 ng/mL α -GalCer.

[Claim 22]

The method according to claim 21, further comprising the step of measuring the amount of α -GalCer loaded on the cells obtained by the culture step, and selecting a cell wherein the amount of α -GalCer loaded on the cell surface is in the range of 3.9 to 275 ng per 1×10^6 cells.