

VACCINE COMPRISING NATURAL KILLER CELLS LOADED WITH LIGANDS OF NATURAL KILLER T CELLS AND CANCER ANTIGENS

BACKGROUND OF THE INVENTION

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1. Field of the Invention

The present invention relates to a vaccine comprising natural killer cells loaded with ligands of natural killer T cells and cancer antigens.

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2. Description of the Related Art

Natural killer cells are well known as important innate immune cells that prevent the development, growth, and metastasis of cancer by eliminating cancer
15 cells and virus-infected cells through degranulation of cytotoxic granules containing perforin and granzyme, death receptor-mediated apoptosis, and IFN- γ production (Vivier et al., *Science*, 331: 44-49, 2011). Although similar to cytotoxic CD8⁺ T cells in that they cause
20 cytotoxicity to target cells, natural killer cells, unlike cytotoxic T cells, have no antigen specificity, do not require sensitization or immunization, and can kill cancer cells with low MHC class I expression that cytotoxic T cells cannot attack, thus playing a unique
25 role in anticancer immunity (Uzhachenko and Shanker.,

Frontiers in Immunology, 10: 1906, 2019). Due to the unique characteristics of natural killer cells that are different from cytotoxic CD8⁺ T cells, active research is being conducted on anticancer immunotherapy focusing
5 on enhancing the cytotoxic capabilities of natural killer cells such as CAR-NKs (Chimeric Antigen Receptor-NKs) (Shimasaki et al., *Nature Reviews Drug Discovery*, 19: 200-218, 2020).

Invariant natural killer T cells (iNKTs) are innate
10 immune cells that express invariant T cell receptors and are known to play an important role in various immune diseases such as cancer, infectious diseases, and autoimmune diseases, mainly through cytokine secretion (Bendelac et al., *Annual Review of*
15 *Immunology*, 25: 297-336, 2007). Alpha-galactosylceramide (α -GC), a representative ligand of natural killer T cells, is a glycolipid extracted from the sponge *Agelasmauritianus*. It is presented by the CD1d molecule and binds to the V α 14-J α 18⁺ T cell
20 receptor of natural killer T cells, thereby activating invariant natural killer T cells (Kawano et al., *Science*, 278: 1626-1629, 1997). Natural killer T cells activated by α -GC rapidly produce various cytokines such as IL-4 and IFN- γ , which activate natural killer
25 cells and induce acquired immune responses, including

maturation of dendritic cells and activation and differentiation of T cells and B cells (Kaer et al., *Nature Reviews Immunology*, 5: 31-42, 2005).

It has been reported that cancer cells loaded with
5 α -GC can also function as antigen-presenting cells
capable of inducing natural killer T cell and natural
killer cell responses and cancer antigens-specific T
cell immune responses (Shimizu et al., *The Journal of*
Immunology, 178: 2853-2861, 2007 & Chung et al.,
10 *OncoImmunology*, 1:2, 141-151, 2012). It has also been
shown that α -GC-loaded B cells or T cells delivered
with antigens via peptide or viral vectors can also
acquire the characteristics of antigen-presenting cells
(Chung et al., *Cancer Research*, 66: 6843-6850, 2006 &
15 Chung et al., *OncoImmunology*, 1:2, 141-151, 2012). This
indicates that α -GC loading and delivery of cancer
antigens to cells expressing CD1d molecules can be
utilized as a cell vaccine for anticancer therapy.

Accordingly, the present inventors have added the
20 function of antigen-presenting cells by changing the
immunogenicity of natural killer cells through loading
natural killer T cell ligand, α -GC, on natural killer
cells having cytotoxic effects and delivering antigens,
and confirmed that the natural killer cells can induce

anticancer immune responses, thereby completing the present invention.

SUMMARY OF THE INVENTION

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It is an object of the present invention to provide an immunotherapeutic or preventive vaccine comprising natural killer cells loaded with ligands of natural killer T cells and cancer antigens.

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To achieve the above object, the present invention provides an immunotherapeutic or preventive vaccine comprising natural killer cells loaded with ligands of natural killer T cells and cancer antigens.

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ADVANTAGEOUS EFFECT

The present invention relates to an immunotherapeutic and preventive vaccine comprising
5 natural killer cells loaded with ligands of natural killer T cells and cancer antigens, and more particularly, to an immunotherapeutic or preventive vaccine comprising natural killer cells loaded with
10 alpha-galactosylceramide (hereinafter, α -GC), which is a natural killer T cell ligand and a type of glycolipid. The composition of the present invention can be used as an anticancer immunotherapeutic agent because natural killer cells are easier to obtain than dendritic cells, and immunization with natural killer cells loaded with
15 ligands of natural killer T cells and antigens induces significant levels of cytotoxic T lymphocyte responses as well as therapeutic effects on malignant tumors.

BRIEF DESCRIPTION OF THE DRAWINGS

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Figure 1a is a diagram showing the IFN- γ production capacity of natural killer T cells (NKT) and natural killer cells (NK) in the spleen induced by the vaccine comprising natural killer cells loaded with α -GC and/or
25 transected with adenovirus expressing antigens.

Figure 1b is a graph showing the IFN- γ production by the natural killer T cells (NKT) of Figure 1a.

Figure 1c is a graph showing the IFN- γ production by the natural killer cells (NK) of Figure 1a.

5 Figure 2a is a diagram showing the experimental method for confirming the activity of a cytotoxic T cell response by the vaccine comprising natural killer cells loaded with α -GC and/or transected with adenovirus expressing antigens.

10 Figure 2b is a set of histograms showing the results of measuring the in vivo cytotoxic T cell activity after administration of NK/ α -GC, NK/Adk35GM, or NK/ α -GC/Adk35GM and injection of target cells loaded with GP100₂₅₋₃₃ peptide labeled with CFSE.

15 Figure 2c is a graph showing the cytotoxic T cell activity of Figure 2b.

Figure 2d is a set of histograms showing the results of measuring the in vivo cytotoxic T cell activity after administration of NK/ α -GC/Ad-E6E7 and
20 injection of target cells loaded with HPV16 E6₄₉₋₅₇ and HPV16 E7₄₉₋₅₇ peptides labeled with CFSE.

Figure 2e is a graph showing the cytotoxic T cell activity of Figure 2d.

Figure 3a is a diagram showing the experimental
25 method for confirming the anticancer effect of the

vaccine comprising natural killer cells loaded with α -GC and transected with adenovirus expressing antigens.

Figure 3b is a graph showing the anticancer effect (change in tumor size) of the vaccine comprising natural
5 killer cells loaded with α -GC and transected with adenovirus expressing antigens (NK/ α -GC/Ad-E6E7).

Figure 3c is a graph showing the anticancer effect (mouse survival rate) of the vaccine comprising natural
killer cells loaded with α -GC and transected with
10 adenovirus expressing antigens (NK/ α -GC/Ad-E6E7).

Figure 4a is a diagram showing the experimental method for confirming the activation of antigen-specific cytotoxic T cells by the vaccine comprising natural killer cells co-loaded with α -GC and peptides.

15 Figure 4b is a set of histograms showing the in vivo cytotoxic T cell activity induced by the vaccine comprising natural killer cells loaded with α -GC (NK/ α -GC), the vaccine comprising natural killer cells loaded with ovalbumin₂₅₇₋₂₆₄ peptide (NK/OVA pep), or the vaccine
20 comprising natural killer cells co-loaded with α -GC and ovalbumin₂₅₇₋₂₆₄ peptide (NK/ α -GC/OVA pep).

Figure 4c is a graph showing the cytotoxic T cell activity of Figure 4b.

Figure 5a is a diagram showing the experimental
25 method for confirming the anticancer effect of the

vaccine comprising natural killer cells co-loaded with α -GC and peptides.

Figure 5b is a graph showing the anticancer effect (change in tumor size) of the vaccine comprising natural
5 killer cells co-loaded with α -GC and peptides (NK/ α -GC/OVA pep).

Figure 3c is a graph showing the anticancer effect (mouse survival rate) of the vaccine comprising natural
killer cells co-loaded with α -GC and peptides (NK/ α -
10 GC/OVA pep).

DESCRIPTION OF THE PREFERRED EMBODIMENTS

Hereinafter, the present invention is described
15 in detail.

It is well known that dendritic cells (DCs) loaded with alpha-galactosylceramide (α -GC) activate invariant natural killer T (NKT) cells (van der Vliet HJ, et al., J Immunol Methods., 1;247(1-2):61-72, 2001). The
20 present inventors confirmed the effect of inducing cytotoxic T lymphocyte responses in B cells, monocytes, and immature myeloid cells loaded with α -GC (Korean Patent Publication No. 10-2007-0105662 and Korean Patent Publication No. 10-2009-0051598), and confirmed

whether similar effects were observed in natural killer cells loaded with α -GC.

Accordingly, the present inventors produced a vaccine by altering the immunogenicity of natural
5 killer cells through α -GC loading and antigen delivery to natural killer cells with cytotoxic effects.

Antigen delivery using viral vectors can deliver cancer antigens to cells with high efficiency, making it suitable for large-scale production of cell
10 therapeutics targeting cancers expressing specific cancer antigens. When a virus capable of expressing the above antigen is used, the entire antigen can be introduced, so it is applicable to all people without being limited to a specific haplotype of the major
15 histocompatibility complex, and has the advantage of being able to induce not only a cellular immune response but also a humoral immune response. On the other hand, viral vectors are limited in their application to personalized cancer therapy.

20 The method of antigen delivery through peptide loading, when used clinically, is limited to the haplotype of an individual's major histocompatibility complex (MHC), so it cannot be used universally, and has the disadvantage of presenting only a single
25 epitope. However, neoepitopes, the peptide sequences

that are created by highly tumor-specific mutations that exist only in cancer cells, not normal cells, and can induce cancer cell-specific immune responses, are known as ideal targets for personalized anticancer immunotherapy for cancer patients. Anticancer immunotherapy using an immune cell vaccine that delivers neoepitopes discovered for personalized treatment for cancer patients is expected to induce the generation of more cancer cell-specific T cells than conventional immunotherapy, thereby minimizing the side effects of anticancer treatment by preventing damage to normal cells, while simultaneously inducing a strong anticancer treatment effect.

Therefore, natural killer cells were isolated from mice, loaded with α -GC, and a natural killer cell vaccine was constructed by delivering antigens in two ways (either by transferring the antigen gene into a viral vector expressing the antigen or by peptide loading) (see Example 1).

First, the present inventors constructed a natural killer cell vaccine loaded with α -GC and delivered with a viral vector expressing the antigen, and confirmed that administration of the vaccine could activate natural killer T cells and natural killer cells. As a result, it was confirmed that natural killer T cells

and natural killer cells were activated in mice that received α -GC-loaded natural killer cells and natural killer cells loaded with α -GC and adenovirus-delivered cancer antigens GP100 and MAGE-A3 (see Figures 1a to 5 1c). In addition, an in vivo CTL assay was performed to determine whether the vaccine could induce a cytotoxic immune response by activating antigen-specific cytotoxic T lymphocytes. As a result, it was confirmed that effective cytotoxic responses were induced by the 10 natural killer cell vaccine loaded with α -GC and delivered with cancer antigens (GP100 and MAGE-A3 or HPV16/18 E6E7) via adenovirus (see Figures 2a to 2e). In addition, the anticancer effect of the vaccine was confirmed using HPV16/18 E6E7. As a result, it was 15 confirmed that the vaccine exhibited significant anticancer effects by suppressing tumor size and increasing mouse survival rate (see Figures 3a to 3c).

Next, the present inventors constructed a natural killer cell vaccine loaded with α -GC and antigenic 20 peptides and determined whether administration of the vaccine induced cytotoxic T cell responses. As a result, it was confirmed that an effective cytotoxic response was induced by the natural killer cell vaccine loaded with α -GC and OVA₂₅₇₋₂₆₄ peptides (see Figures 4a to 4c). 25 In addition, the anticancer effect of the vaccine was

investigated, and it was confirmed that it exhibited a significant anticancer effect that suppressed the tumor size and increased the mouse survival rate (see Figures 5a to 5c). The natural killer cells of the present invention not only have a cytotoxic function and thus have an anticancer effect by themselves, but also acquire properties as antigen-presenting cells that activate antigen-specific cytotoxic T cell responses by loading ligands of natural killer T cells, especially α -GC, and antigen delivery, thereby improving the therapeutic effect on malignant tumors. Therefore, a vaccine composition containing the above cells can be used as a cancer treatment agent.

The ligands of the above natural killer T cells include alpha-galactosylceramide (α -GC), alpha-glucuronosylceramide, phosphatidylinositol tetramannoside, isoglobotrihexosylceramide, ganglioside GD3, phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, sulfitide, beta-galactosylceramide, lipophosphoglycan, glycoinositol phospholipids, beta-anomericgalactosylceramide and alpha-anomericgalactosylceramide, which are analogues of alpha-galactosylceramide, bacterial lipid antigens, and variants of alpha-galactosylceramide.

The above antigen may be any antigen that can be used as a vaccine and induce an immune response, and includes antigens derived from pathogens including pathogenic bacteria, viruses, and parasites, or cancer
5 antigens, and can be the full length or a fragment of the above antigen.

The antigens derived from the pathogenic bacteria include Bordetella pertussis antigens (pertussis toxin, filamentous haemagglutinin, and pertactin), tetanus
10 toxoid, diphtheria toxoid, Helicobacter pylori antigens (capsular polysaccharides of serogroups A, B, C, Y, and W-135), pneumococcal antigen (Streptococcus pneumoniae type 3 capsular polysaccharide), tuberculosis antigen, cholera antigen (cholera toxin B subunit),
15 staphylococcal antigen (staphylococcal enterotoxin B), shigella antigen (shigella polysaccharides), Borrelia sp. antigen, Candida albicans antigen, and Plasmodium antigen.

The antigens derived from the viruses include
20 influenza virus antigens (haemagglutinin and neuraminidase), human papilloma virus (HPV) antigen (glycoprotein), vesicular stomatitis virus antigen (vesicular stomatitis virus glycoprotein), cytomegalovirus (CMV) antigen, hepatitis virus antigens
25 (hepatitis A(HAV), B(HBV), C(HCV), D(HDV) and G(HGV))

antigens) (core antigen and surface antigen), respiratory syncytial virus (RSV) antigen, herpes simplex virus antigen, human immunodeficiency virus (HIV) antigens (GP-120, GP-160, p18, Tat, Gag, Pol, 5 Env) and combinations thereof.

The cancer antigens include gp100, melanoma antigen gene (MAGE), human papilloma virus (HPV) E6/E7, tyrosinase, tyrosinase-related protein-1 (TRP-1), tyrosinase-related protein-2 (TRP-2), murinoglobulin 1 10 (MUC-1), carcinoembryonic antigen (CEA), p53, α -fetoprotein, breast cancer protein expressed by Her-2/*neu*, proteinase 3, WT-1, PAP, PSA, PSMA, G250, BAGE, GAGE, NY-ESO- 1, MART-1, MCIR, Ig Idiotype, CDK4, caspase-8, β -catenin, CIA, BCR/ABL, EBV LMP2a, HCV, 15 HHV-8, 5T4, tumor-specific mutation-derived neoantigens and combinations thereof.

In addition, the antigen may be directly loaded into natural killer cells in the form of peptides, lipopolysaccharides, polysaccharides, glycoproteins, 20 or polynucleotides including DNA and RNA, and can be transfected into natural killer cells by recombinant viruses and expressed and loaded. Compared to the cell vaccine loaded with peptides, the cell vaccine introduced with the entire antigen via viruses are 25 applicable to all people, not limited to the haplotype

of the major histocompatibility complex, and can induce immune responses specific to multiple epitopes, and in particular, has the advantage of being able to induce humoral and cellular immune responses simultaneously.

5 The antigen can be expressed by introducing it through a recombinant virus. The virus introduced into natural killer cells for antigen expression as mentioned above includes adenovirus, retrovirus, vaccinia virus, Pox virus, and Sindbis virus, but not
10 always limited thereto. In addition to the method using viruses, the following methods can be applied for antigen gene delivery: 1) a method of transducing DNA by binding it to liposomes to protect the DNA from enzymatic degradation or to allow it to be taken up
15 into endosomes, 2) a method of increasing the efficiency of DNA delivery into cells by combining a molecular conjugate consisting of a protein or a synthetic ligand to the DNA [ex: asialoglycoprotein, transferrin, polymeric IgA], 3) A method for delivering antigen genes
20 by increasing the efficiency of DNA delivery into cells through a novel DNA delivery system using PTD (protein transduction domain) [ex: Mph-1], and 4) In addition to the above methods, peptides can be used or antigenic proteins can be applied to natural killer cells to cause
25 the natural killer cells to present the antigen.

The vaccine of the present invention can additionally include, in addition to the natural killer T cell ligands and natural killer cells, one or more effective ingredients having the same or similar effect
5 with them. The vaccine can also include, in addition to the above-mentioned effective ingredients, one or more pharmaceutically acceptable carriers for the administration. The pharmaceutically acceptable carrier can be selected or be prepared by mixing more than one
10 ingredients selected from a group consisting of saline, Ringer's solution, buffered saline, dextrose solution, maltodextrose solution, glycerol, and ethanol. Other general additives such as anti-oxidative agent, buffer solution, bacteriostatic agent, etc., can be added. In
15 order to prepare injectable solutions such as aqueous solution, suspension and emulsion, diluents, dispersing agents, surfactants, binders and lubricants can be additionally added. The vaccine of the present invention can further be prepared in suitable forms for
20 each disease or according to ingredients by following a method represented in Remington's Pharmaceutical Science (the newest edition), Mack Publishing Company, Easton PA.

The vaccine of the present invention can be
25 administered by parenterally and the parenteral

administration includes subcutaneous injection, intravenous injection, intramuscular injection and intrathoracic injection. To prepare the vaccine as a formulation for parenteral administration, the natural
5 killer cells loaded with ligands of natural killer T cells, the natural killer cells loaded with ligands of natural killer T cells and peptides, or the natural killer cells infected with viruses expressing cancer antigens of the present invention is mixed with a
10 stabilizer or a buffering agent to produce a solution or suspension, which is then formulated as ampoules or vials.

The vaccine of the present invention can be formulated in a variety of forms according to
15 administration pathways. For example, the vaccine of the present invention can be prepared in the form of sterilized solution or suspension for injection, or in the form of freeze-dried formula using freeze-drying technique. The freeze-dried vaccine of the present
20 invention is supposed to be maintained typically at about 4°C and can be restored in a stabilizing solution containing or not containing an adjuvant such as saline or/and HEPES.

To accomplish the present invention, the effective
25 dose of the vaccine for administration is determined by

considering administration method, administration frequency, specific disease under treatment, severity of disease, disease history, whether or not a patient is under co-treatment with other drugs, age, height, weight, health condition and other physical conditions of a patient, but not always limited thereto. In general, as the weight of a patient under treatment increases, the dose of this preparation is preferably increased.

10 The vaccine can be administered by effective dose to induce immune response in a patient. For example, the vaccine can be administered to human once or a few times a day by the dosage of $1 \times 10^3 \sim 1 \times 10^9$ cells/kg, and more preferably 1×10^4 cells/kg $\sim 1 \times 10^8$ cells/kg. To
15 prepare a natural killer cell vaccine loaded with alpha-galactosylceramide, a medium has to be supplemented with alpha-galactosylceramide at the concentration of $1 \sim 2 \mu\text{g}/\text{ml}$ per $1 \times 10^6 \sim 1 \times 10^7$ natural killer cells/ ml . To
20 prepare a natural killer cell vaccine loaded with alpha-galactosylceramide and peptides, a medium has to be supplemented with alpha-galactosylceramide at the concentration of $1 \sim 2 \mu\text{g}/\text{ml}$ per $1 \times 10^6 \sim 1 \times 10^7$ natural killer cells/ ml and peptide at the concentration of $1 \sim 10 \mu\text{g}/\text{ml}$ per $1 \times 10^6 \sim 1 \times 10^7$ natural killer cells/ ml .

Alpha-galactosylceramide does not appear to induce toxicity in rodents and monkeys (Nakata et al., Cancer Res 58:1202-1207, 1988). No adverse effects were reported even in mice injected with 2200 $\mu\text{g/kg}$ of αGalCer (Giaccone et al., Clin Cancer Res 8:3702, 2002). In ongoing clinical trials, some side effects, such as mild headache, have been reported with systemic administration of αGalCer (Mie Nieda et al., Blood 103:383-389, Giaccone et al., Clin Cancer Res 8:3702, 2002), but these could be prevented by administration of paracetamol, and mild systemic side effects do not necessarily occur in these subjects (Giaccone et al., Clin Cancer Res 8:3702, 2002).

Hereinafter, the present invention will be described in detail by the following examples.

However, the following examples are only for illustrating the present invention, and the contents of the present invention are not limited thereto.

20

Example 1: Construction of natural killer cell vaccine
<1-1> Construction of natural killer cell vaccine in
which antigens are delivered by viral vectors
expressing the antigens

To isolate natural killer cells from mice, the mouse spleen and bone marrow were collected and then homogenized. After lysing red blood cells using ACK lysing buffer (Gibco), T cells, B cells, and neutrophils
5 expressing CD3 ϵ , CD19, or Ly6G on the cell surface were removed using microbeads (Miltenyibiotec), and then CD49b⁺ cells were obtained using anti-CD49b microbeads (Miltenyibiotec), and pure natural killer cells were isolated using a BD FACS Aria III instrument. The natural
10 killer cells isolated and purified as described above were placed in a medium containing serum and IL-2 (5 ng/mL) along with α -GC (1 μ g/mL), solvent (DMSO) and/or adenovirus (100 MOI) for cancer antigen (GP100 and MAGE-A3 cancer antigens or E6 and E7 cancer antigens of human
15 papillomavirus HPV types 16 and 18) gene delivery. After centrifugation at 20°C, 2000 rpm for 90 minutes in cell culture plates, the cells were cultured in a CO₂ incubator for 14~15 hours (37°C, 80~95% relative humidity, and 5% CO₂ concentration) to prepare natural
20 killer cells loaded with α -GC, natural killer cells transformed with adenovirus to express cancer antigens, natural killer cells loaded with α -GC and transformed with adenovirus to express cancer antigens. The prepared natural killer cell vaccine was washed three
25 times with Dulbecco's phosphate buffered saline (DPBS,

Welgene), dissolved in DPBS, and administered into the tail vein of mice.

<1-2> Construction of natural killer cell vaccine
5 loaded with antigen peptides

Viral vectors are suitable gene delivery vehicles for the large-scale production of cell therapeutics targeting cancer cells expressing specific cancer antigens, as they deliver cancer antigens common to
10 cancer cells with high efficiency. However, viral vectors have limitations in applying personalized treatment for cancer patients using neoepitopes, so the present inventors have constructed a natural killer cell vaccine using antigen delivery via peptide
15 loading.

Specifically, the natural killer cells isolated from C57BL/6 mice by the method of Example <1-1> were placed in a medium containing serum and IL-2 (5 ng/mL) together with α -GC (1 μ g/mL), solvent (DMSO), and/or
20 Ovalbumin₂₅₇₋₂₆₄ (OVA₂₅₇₋₂₆₄) peptide (2 μ g/mL), and cultured in a CO₂ incubator for 15~16 hours (37°C, 80~95% relative humidity, and 5% CO₂ concentration) to prepare a natural killer cell vaccine loaded with α -GC, a natural killer cell vaccine loaded with OVA₂₅₇₋₂₆₄
25 peptide, or a natural killer cell vaccine loaded with

α -GC and OVA₂₅₇₋₂₆₄ peptide. The prepared natural killer cell vaccine was washed three times with DPBS, dissolved in DPBS, and administered into the tail vein of mice.

5 Experimental Example 1: Confirmation of effectiveness
of natural killer cell vaccine in which antigens are
delivered by viral vectors expressing antigens
<1-1> Confirmation of induction of activation of
natural killer T cells and natural killer cells

10 The present inventors confirmed whether natural killer T cells and natural killer cells in the body could be activated through the natural killer cell vaccine administration.

Specifically, natural killer cells obtained from
15 C57BL/6 mice were used to generate natural killer cells loaded with α -GC (NK/ α -GC), natural killer cells transduced with adenovirus Adk35GM for cancer antigen (human GP100 and MAGE-A3) delivery (NK/Adk35GM), and natural killer cells loaded with α -GC and antigen-
20 delivered with adenovirus Adk35GM (NK/ α -GC/Adk35GM). Then, 8.5×10^4 natural killer cells were administered intravenously. After 17 hours, the level of IFN- γ production in natural killer T cells and natural killer cells in the spleen was measured by flow cytometry.

As a result, as shown in Figures 1a to 1c, it was confirmed that the administration of α -GC-loaded natural killer cells (NK/ α -GC and NK/ α -GC/Adk35GM) to mice stimulated natural killer T cells in the body and consequently activated natural killer cells, unlike the administration of transduced cells (NK/Adk35GM).

It is thought that the activation of these natural killer T cells and natural killer cells may contribute to the anticancer effect along with the induction of cytotoxic T cell responses.

<1-2> Confirmation of induction of cytotoxic T cell response

<1-2-1> Evaluation of efficacy of natural killer cell vaccine containing GP100 and MAGE-A3

To determine whether the adenovirus-transduced natural killer cell vaccine could induce antigen-specific cytotoxic T cell immune responses, an *in vivo* CTL assay was performed.

Specifically, natural killer cells obtained from C57BL/6 mice were used to construct α -GC-loaded or adenovirus Adk35GM antigen-delivered natural killer cell vaccines, which were immunized into C57BL/6 mice and a cytotoxicity assay was performed 10 days later (Figure 2a). First, spleen cells from the same mouse

were divided into two groups with equal amounts. The target cells loaded with GP100₂₅₋₃₃ peptide were labeled with 3 μ M CFSE (carboxyfluorescein diacetate succinimidyl ester) (CFSE^{high}), and the control cells
5 without the peptide were labeled with 0.3 μ M CFSE (CFSE^{low}), and then injected into the vaccinated mice in equal amounts. One day later, lysis of the target cells was measured by calculating the CFSE^{high} : CFSE^{low} ratio in mouse spleen cells by flow cytometry (Figure 2a). A
10 lower percentage of the CFSE^{high} cells loaded with antigen peptides indicates a higher antigen-specific cytotoxic T cell immune response.

As a result, as shown in Figures 2b and 2c, when immunized with natural killer cells (NK/Adk35GM, NK/ α -
15 GC/Adk35GM) introduced with antigens via adenovirus vector, an effective cytotoxic T cell immune response was observed.

<1-2-2> Evaluation of efficacy of natural killer cell vaccine containing E6 and E7 antigens of human papillomavirus (HPV) types 16 and 18

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To determine whether the natural killer cell-mediated vaccination could be applied to other cancer antigens, the present inventors evaluated the efficacy
25 of a natural killer cell vaccine containing E6 and E7

cancer antigens of human papillomavirus (HPV) types 16 and 18.

Specifically, C57BL/6 mice were immunized with 6.5×10^5 natural killer cells loaded with α -GC and engineered to express HPV16/18 E6E7 via adenovirus Ad-E6E7, and then an *in vivo* cytotoxicity assay was performed 8 days later. The target cells loaded with HPV16 E6₄₉₋₅₇ and E7₄₉₋₅₇ peptides were labeled with CFSE^{high}, while the control cells were labeled with CFSE^{low} without peptide loading, and the equal amount of cells from both groups were mixed and injected into the immunized mice. One day later, single spleen cells were harvested and the ratio of CFSE^{high} to CFSE^{low} cell populations was measured by flow cytometry.

As a result, as shown in Figures 2d and 2e, cytotoxicity against HPV16 E6E7 was confirmed to be induced *in vivo* in a group of mice that received natural killer cells loaded with α -GC and engineered to express HPV16/18 E6E7.

Based on the results of Experimental Examples <1-1> and <1-2> above, it can be inferred that both innate immune responses, activation of natural killer T cells and natural killer cells, and acquired immune responses, cytotoxic T cell immune responses, can be induced by administration of a natural killer cell

vaccine loaded with α -GC and antigen-delivered by an adenoviral vector, and thus exhibit potent anticancer therapeutic effects.

5 <1-3> Evaluation of anticancer effect of natural
killer cell vaccine

The present inventors investigated whether the natural killer cell vaccine administration induces anticancer immunity.

10 To this end, C57BL/6 mice were immunized with 2.6×10^5 NK/ α -GCs or NK/ α -GC/Ad-E6E7 4 days after subcutaneous implantation of 2×10^5 TC-1 cancer cells into the flanks of C57BL/6 mice (Figure 3a). Then, tumor size changes and mouse survival rates were measured.
15 The tumor size was calculated using the following equation by measuring the width, length, and height of the tumor using a caliper.

$$\text{(Tumor Size)} = \text{(Width)} \times \text{(Length)} \times \text{(Height)} \times (\pi / 6)$$

20 As a result, as shown in Figures 3b and 3c, when the natural killer cell vaccine (NK/ α -GC/Ad-E6E7) was administered, cancer growth was suppressed and a high survival rate was observed. In contrast, in the mice administered with NK/ α -GC, tumor growth was hardly
25 inhibited and a low survival rate was observed.

Through the above results, it was confirmed that the anticancer therapeutic effect could not be induced by activating natural killer T cells and natural killer cells loaded with α -GC alone.

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Experimental Example 2: Confirmation of effectiveness of natural killer cell vaccine loaded with antigen peptides

<2-1> Confirmation of induction of cytotoxic T cell response

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The present inventors investigated whether the natural killer cell vaccine loaded with the antigen peptides of Example <1-2> could induce cytotoxic T cell responses.

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C57BL/6 mice were immunized by intravenous administration of natural killer cells loaded with α -GC (NK/ α -GC), natural killer cells loaded with OVA₂₅₇₋₂₆₄ peptide (NK/OVA pep), or natural killer cells loaded with α -GC and OVA₂₅₇₋₂₆₄ peptide together (NK/ α -GC/OVA pep), and an *in vivo* cytotoxicity assay was performed 8 days later (Figure 4a).

20

As a result, as shown in Figures 4b and 4c, only in the mouse group administered with NK/ α -GC/OVA pep, the CFSE^{high} target cells loaded with OVA₂₅₇₋₂₆₄ peptide were mostly lysed. This confirmed that cytotoxic T cells

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can also be activated by the natural killer cell vaccine loaded with α -GC and peptides.

However, unlike that cytotoxic T cell responses were induced in the group administered with natural
5 killer cells to which only the antigen was delivered via an adenovirus vector without α -GC in Experimental Example <1-2> (Figure 2c), no cytotoxic T cell responses were observed in the group administered with NK/OVA pep loaded with peptide alone (Figure 4c). This suggests
10 that delivering the entire antigen via adenovirus to induce diverse immune responses specific to multiple epitopes is superior to presenting only a single peptide in inducing cytotoxic T cell immune responses.

15 <2-2> Evaluation of anticancer effect of natural killer cell vaccine

The present inventors investigated whether the natural killer cell vaccine loaded with antigen peptides could induce anticancer therapeutic effects
20 (Figure 5a).

To this end, C57BL/6 mice were immunized with 2.5×10^5 NK/OVA pep or NK/ α -GC/OVA pep 2 days after subcutaneous implantation of 1×10^5 B16F10-OVA cancer cells into the mice.

As a result, as shown in Figures 5b and 5c, when the natural killer cell vaccine (NK/ α -GC/OVA pep) was administered, cancer growth was significantly suppressed and a high survival rate was observed. In contrast, in the mice administered with NK/OVA pep, tumor growth was hardly inhibited and a low survival rate was observed. Therefore, it was revealed that peptide loading alone cannot induce sufficient anticancer therapeutic effects.

When combined with the above results and the anticancer effect data of Examples <1-3> (Figures 3b and 3c), it was confirmed that the natural killer cell vaccine prepared by loading α -GC and delivering antigen showed a stronger anticancer effect than the cell vaccine loaded with α -GC alone or delivered with antigen alone, inhibiting cancer growth and improving survival rate.

WHAT IS CLAIMED IS:

1. An immunotherapeutic and preventive vaccine comprising natural killer cells loaded with ligands of natural killer T cells and cancer antigens,

wherein the ligand of natural killer T cell is selected from the group consisting of alpha-galactosylceramide, alpha-glucuronosylceramide, phosphatidylinositol tetramannoside, isoglobotrihexosylceramide, ganglioside GD3, phosphatidylcholine, beta-galactosylceramide, lipophosphoglycan, glycoinositol phospholipids, beta-anomericgalactosylceramide and alpha-anomericgalactosylceramide, which are analogues of alpha-galactosylceramide, bacterial lipid antigens, and derivatives of alpha-galactosylceramide;

wherein the cancer antigen is selected from the group consisting of gp100, melanoma antigen gene (MAGE), human papilloma virus (HPV) E6/E7, tyrosinase, tyrosinase-related protein-1 (TRP-1), tyrosinase-related protein-2 (TRP-2), murinoglobulin 1 (MUC-1), carcinoembryonic antigen (CEA), p53, α -fetoprotein, breast cancer protein expressed by Her-2/neu, proteinase 3, WT-1, PAP, PSA, PSMA, G250, BAGE, GAGE, NY-ESO-1, MART-1, MCIR, Ig Idiotype, CDK4, caspase-8, β -catenin, CIA, BCR/ABL, EBV LMP2a, HCV, HHV-8, 5T4, and tumor-specific mutation-derived neoantigens.

2. The immunotherapeutic and preventive vaccine according to claim 1,

wherein the cancer antigen is in the form of a peptide, lipopolysaccharide, polysaccharide, glycoprotein, or polynucleotide including DNA and RNA.

3. The immunotherapeutic and preventive vaccine according to claim 1,

wherein the antigen is introduced into the natural killer cells by a recombinant virus.

4. The immunotherapeutic and preventive vaccine according to claim 3,

wherein the recombinant virus is adenovirus, retrovirus, vaccinia virus, pox virus, or Sindbis virus into which a gene expressing the cancer antigen has been introduced.