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Title

Liposomes loaded with a STING pathway ligand, cyclic di-GMP, enhance cancer immunotherapy against metastatic melanoma

Author

Takashi Nakamura^{a†}, Hiroko Miyabe^{a†}, Mamoru Hyodo^b, Yusuke Sato^a, Yoshihiro Hayakawa^b, Hideyoshi Harashima^{a*}

^a*Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Japan;* ^b*Department of Applied Chemistry, Faculty of Engineering, Aichi Institute of Technology, Toyota, Japan*

[†]Takashi Nakamura and Hiroko Miyabe equally contributed to this study.

The authors disclose no potential conflicts of interest.

***Correspondence should be addressed to**

Hideyoshi Harashima, Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Hokkaido 060-0812, Japan.

Telephone: +81-11-706-3919, Fax: +81-11-706-4879

E-mail: harasima@pharm.hokudai.ac.jp

Abstract

Malignant melanomas escape immunosurveillance via the loss/down-regulation of MHC-I expression. Natural killer (NK) cells have the potential to function as essential effector cells for eliminating melanomas. Cyclic di-GMP (c-di-GMP), a ligand of the stimulator of interferon genes (STING) signal pathway, can be thought of as a new class of adjuvant against cancer. However, it is yet to be tested, because technologies for delivering c-di-GMP to the cytosol are required. Herein, we report that c-di-GMP efficiently activates NK cells and induces antitumor effects against malignant melanomas when loaded in YSK05 lipid containing liposomes, by assisting in the efficient delivery of c-di-GMP to the cytosol. The intravenous administration of c-di-GMP encapsulated within YSK05-liposomes (c-di-GMP/YSK05-Lip) into mice efficiently induced the production of type I interferon (IFN) as well as the activation of NK cells, resulting in a significant antitumor effect in a lung metastasis mouse model using B16-F10. This antitumor effect was dominated by NK cells.

The infiltration of NK cells was observed in the lungs with B16-F10 melanomas. These findings indicate that the c-di-GMP/YSK05-Lip induces MHC-I non-restricted antitumor immunity mediated by NK cells. Consequently, c-di-GMP/YSK05-Lip represents a potentially new adjuvant system for use in immunotherapy against malignant melanomas.

Keywords: STING; c-di-GMP; liposome; adjuvant; melanoma; cancer immunotherapy

1. Introduction

Our immune system basically has the ability to eliminate tumor cells from our bodies. Spontaneous immune responses against cancer are believed to contribute to the control of tumor growth in cancer patients, because the infiltration of preexisting T cells in tumor microenvironments appears to affect the prognostic benefit [1-3]. In addition, the infiltration of preexisting T cells into a tumor microenvironment can be also beneficial for patients who are undergoing cancer immunotherapy using monoclonal antibodies specific for the T-lymphocyte-associated protein 4 (CTLA-4) and the programmed cell death protein 1 (PD-1) [4,5]. Thus, it appears likely that the activation of natural immune responses governs the success of cancer immunotherapy. Although, the mechanism by which the host initiates the spontaneous immune sensing of tumors remains largely unknown, Woo et al. recently provided a possible answer to this question. They reported that the major mechanism for the innate immune sensing of cancer occurs via the host simulator of interferon genes (STING) pathway [6]. The STING pathway functions as a cytosolic sensor of cytosolic double stranded DNA and STING recognizes 2'3'-cGAMP, a cyclic dinucleotide, which is produced by the action of cyclic-GMP-AMP (cGAMP) synthase (cGAS) [7]. The recognition of cGAMP by STING triggers a signal of tank binding kinase 1 (TBK1)- interferon regulator factor 3 (IRF3) pathway, resulting in the activation of antigen presenting cells (APCs), the production of type I interferons (IFNs), and the priming of CD8⁺ T cells against tumor antigens. On the other hand, spontaneous CD8⁺ T cell priming was found not to be defective in *Myd88*^{-/-}, *Trif*^{-/-}, *Tlr4*^{-/-}, *Tlr9*^{-/-}, *P2rx7*^{-/-}, or *Mavs*^{-/-} mice, indicating that other innate immune activating pathways are not involved [6]. Therefore, an adjuvant for activating the STING pathway represents a potential strategy with major implications for cancer immunotherapy, which is different from existing agonists such as toll-like receptor (TLR) ligands.

Cyclic di-GMP (c-di-GMP) is a cyclic dinucleotide and forms a cyclic guanosine dimer that contains two (3'-5') phosphate linkages and functions as a co-factor of cellulose synthase in *Gluconabactor xylinum* [8,9]. Our group recently reported that c-di-GMP stimulates innate immunity by inducing the production of type I IFNs via the STING-TBK1-IRF3 pathway [10-12]. C-di-GMP binds to ATP dependent RNA helicase (DDX41) in the cytosol and forms a complex with STING, resulting in a signal transmitted via the STING-TBK1-IRF3 pathway. Thus, c-di-GMP represents a new class of adjuvant for activating the STING pathway. Using c-di-GMP is problematic due to the presence of its two phosphate

groups which hinder its transport across the cell membrane. Woo et al. also reported that the production of type I IFN mediated by the recognition of tumor derived DNA via the STING pathway was required for the success of delivery systems such as Lipofectamine2000 [6]. We recently succeeded in efficiently delivering c-di-GMP to the cytosol by encapsulating it into liposomes [13]. Liposomes containing the YSK05 lipid, a synthetic lipid that was developed in our laboratory, were used to efficiently deliver c-di-GMP to the cytosol. YSK05 is a pH-sensitive cationic lipid and possesses a high fusogenic activity which enhances endosomal escape [14]. C-di-GMP loaded YSK05-liposomes (c-di-GMP/YSK05-Lip) drastically enhanced the production of IFN- β via the STING-TBK1-IRF3 pathway, compared with the free form of c-di-GMP and c-di-GMP loaded Lipofectamine2000 [13]. We also verified an antitumor effect mediated by c-di-GMP/YSK05-Lip in a model tumor. Mice treated with c-di-GMP/YSK05-Lip and ovalbumin (OVA) as a model antigen showed significant OVA-specific cytotoxic T lymphocyte (CTL) activity and preventative antitumor effect against OVA-expressing lymphomas, E.G7-OVA tumors [13]. The CTL activity and antitumor effects mediated by c-di-GMP/YSK05-Lip appear to be stronger than that for liposomes loaded with the TLR3 ligand, polyinosine-polycytidylic acid [15]. These data suggest that c-di-GMP could be an advantageous adjuvant for inducing antigen-specific adaptive immunity against cancer compared with other adjuvants that are currently in use.

Although the findings should be considered preliminary at this time, it appears that the c-di-GMP/YSK05-Lip induces major histocompatibility complex class I (MHC-I) restricted antitumor immunity, because the E.G7-OVA cells expressed MHC-I (Suppl. Fig. S1). CTL is the main effector cell in adaptive immunity and shows antigen-specific cytotoxicity by recognizing the antigen peptide present on MHC-I. On the other hand, the loss/down-regulation of MHC-I expression in tumors is a well-known phenomenon and results in the escape of immunosurveillance by CTL [16]. The best known example of this is malignant melanoma. Malignant melanoma is a very aggressive tumor and frequently shows a tendency to spread, and rapidly progresses to a metastatic stage [17]. The poor efficiency of chemotherapy and radiotherapy has stimulated melanoma researchers to consider the use of immunotherapy [18]. Natural killer (NK) cells can be a major effector cell for the elimination of melanomas. Thus, the development of an adjuvant for the efficient activation of NK cells would be desirable. However, the stimulation of NK cell activity and the antitumor effect against malignant melanomas has not been investigated in the case of c-di-GMP, because of the lack of a delivery system for its delivery into cells. The development of

the c-di-GMP/YSK05-Lip now permits us to investigate whether c-di-GMP can induce NK cell mediated antitumor effects against malignant melanomas. Addressing this issue will provide us with new insights into the area, because the activation of NK cells via the STING pathway in cancer immunotherapy has not received much attention in the past. In addition, the potential of the c-di-GMP/YSK05-Lip for use as an adjuvant system for cancer immunotherapy will be advanced.

In this study, we report on an investigation of the activation of NK cells and antitumor effects against B16-F10 tumors, an MHC-I negative malignant melanoma (Fig. S1), when c-di-GMP/YSK05-Lip was intravenously administered into mice. The findings show that c-di-GMP/YSK05-Lip induced a significant activation of NK cells and therapeutic antitumor effects against the lung metastasis of B16-F10. The antitumor effect was dominated by NK cells and the infiltration of NK cells into tumor tissue was observed. This is the first study to demonstrate MHC-I non-restricted antitumor immunity via NK cell activation against malignant melanomas as the result of a c-di-GMP treatment. Furthermore, our findings clearly show that c-di-GMP/YSK05-Lip can efficiently activate MHC-I restricted and non-restricted antitumor activities and represents a promising adjuvant system for use in cancer immunotherapy.

2. Materials and Methods

2.1. Cell line, animal and reagents

B16-F10 murine melanoma cells stably expressing luciferase (GL4) (B16-F10-luc2; Caliper Life Sciences, Hopkinton, MA) were grown in 10% heat-inactivated fetal bovine serum (FBS) in RPMI-1640 (SIGMA-Aldrich, St. Louis, MO). C57BL/6 female mice (6 to 8 weeks old) were obtained from Japan SLC, Inc. (Shizuoka, Japan). Experiments using mice were approved by the Pharmaceutical Sciences Animal Committee of Hokkaido University. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE) was purchased from Avanti Polar Lipid Inc. (Alabaster, AL). Cholesterol was obtained from SIGMA-Aldrich (St. Louis, MO). 1,2-dimyristoyl-*sn*-glycerol, methoxypolyethylene glycol (DMG-PEG2000) was purchased from the NOF Corporation (Tokyo, Japan). YSK05 was synthesized by a previously reported method [14]. C-di-GMP was obtained from Yamasa Corporation (Chiba, Japan). Mouse IgG1 κ was purchased from SIGMA-Aldrich. FITC anti-mouse CD3, PE anti-mouse NK1.1, purified anti-mouse NK1.1, APC anti-mouse NKp46, PE/Cy7 anti-mouse CD69, APC anti-mouse natural killer group D (NKG2D) and each isotype control were

obtained from Biolegend (San Diego, CA). Cy5 anti-mouse IgG was purchased from Life Technologies (Carlsbad, CA). Anti-mouse asialo GM1 was obtained from Wako (Osaka, Japan).

2.2. Preparation of c-di-GMP/YSK05-Lip

The c-di-GMP/YSK05-Lip was prepared by a previously reported procedure [13] with minor modifications. Briefly, YSK05, POPE, cholesterol and DMG-PEG2000 were mixed in 300 μ L of 90% t-BuOH, resulting in a total lipid amount of 400 nmol (YSK05:POPE:cholesterol:DMG-PEG2000 = 40:25:35:1). 500 nmol of c-di-GMP was dissolved in 1 mM citric buffer (pH 4.5) up to 150 μ L. This c-di-GMP solution was added dropwise to the lipid solution with vortexing. The c-di-GMP/lipid mixture was added to 1.6 mL of 1 mM citric buffer (pH 4.5) using a 25G needle-syringe with vortexing and was diluted with 4 mL of PBS. The solution was concentrated with an Amicon Ultra (MWCO 100,000) and washed with 4 mL of PBS.

The diameter of the liposomes was determined by dynamic light scattering, and zeta potentials were determined by laser-Doppler velocimetry with a ZETASIZER Nano (ZEN3600, Malvern Instruments Ltd., Malvern, WR, UK). The diameters of c-di-GMP/YSK05-Lip and YSK05-Lip were 183.6 ± 3.7 nm and 136.7 ± 6.1 nm, respectively. The zeta-potentials of c-di-GMP/YSK05-Lip and YSK05-Lip were -5.7 ± 2.6 mV and 4.2 ± 0.5 mV, respectively. The values are mean $\pm$ SEM (n=9).

To quantify the amount of encapsulated c-di-GMP, c-di-GMP/YSK05-Lip was lysed with 12 μ L of 5% SDS and diluted with 600 μ L of water. The lysate was applied to a manual reverse phase chromatography column (Wakogel 50C18, Wako, Japan) and the fraction eluted with 20% methanol was collected. The collected fraction was concentrated and a UV-vis spectrum obtained. The amount of c-di-GMP recovered was estimated via the absorbance at 252 nm ($\epsilon=24,700$) [8]. The recovery ratio was $6.1 \pm 0.1\%$. The value is the mean $\pm$ SEM (n=11).

2.3. Quantification of cytokine concentration in serum

Mice were intravenously administered with YSK05-Lip or the c-di-GMP/YSK05-Lip (equivalent to 3 μ g of c-di-GMP). Blood samples were collected at 1, 2, 4, 8 and 24 h. After allowing the blood to clot, the samples were centrifuged and the supernatants were used as serum samples. The levels of IFN- β , tumor necrosis factor (TNF)- α , interleukin (IL)-6 and

IFN- γ in serum were measured by ELISA kit (R&D systems, Basel, Switzerland). These experiments were carried out following the manufacturer's instructions.

2.4. Quantification of NK cell activation

Mice were intravenously administered with YSK05-Lip or the c-di-GMP/YSK05-Lip (equivalent to 3 μ g of c-di-GMP). After 8 h, the spleen was collected and a single cell suspension of splenocytes was prepared. Mouse IgG1 κ was added to 1×10^6 splenocytes for blocking. After washing, the splenocytes were stained by FITC anti-mouse CD3, PE anti-mouse NK1.1, APC anti-mouse NKp46, PE/Cy7 anti-mouse CD69, APC anti-mouse NKG2D and each isotype control. The fluorescence of stained cells was measured by flow cytometer (SH800, Sony, Tokyo, Japan). NK cells were identified as CD3⁻/NK1.1⁺ cells. The values were calculated by subtracting the value of each isotype control from the values of individual samples.

2.5. Antitumor effect in lung metastatic tumor models

A mouse model of lung metastatic tumor was prepared as reported previously [19]. On day 0, 2×10^5 cells of B16-F10-luc2 in 200 μ L of PBS were injected into the tail vein of mice. On day 2, 4 and 8, PBS, YSK05-Lip or c-di-GMP/YSK05-Lip (equivalent to 3 μ g of c-di-GMP) in 200 μ L were intravenously administered into mice. For the depletion of NK cells, 100 μ L of 10 mg/mL anti-asialo GM1 antibody was intraperitoneally injected on days 1, 4, 7 and 10. The depletion of NK cells was confirmed 24 h after the anti-asialo GM1 antibody treatment. The spleen was collected and a single cell suspension of splenocytes was prepared. Mouse IgG1 κ was added to 1×10^6 splenocytes for blocking. After washing, the splenocytes were stained with FITC anti-mouse CD3, PE anti-mouse NK1.1 and each isotype control. The fluorescence of the stained cells was measured by flow cytometer (Gallios, Indianapolis, IN). NK cells were identified as CD3⁻/NK1.1⁺ cells. The values were calculated by subtracting the value of each isotype control from the values of each sample. On day 21, the lungs were collected. To quantify tumor growth, the collected lungs were completely homogenized in 1 mL of lysis buffer (100 mM Tris-HCl, 2 mM EDTA, 0.1% TritonX-100, pH 7.8). The luciferase activities were then measured using a luminometer (Luminescencer-PSN, ATTO, Japan). Luciferase activities are expressed as relative light unit (RLU) per whole lung.

2.6. Histological analysis of infiltrated NK cells in lung

On day 0, 2×10^5 cells of B16-F10-luc2 in 200 μ L of PBS were injected into the tail vein of mice. On day 2, 4 and 8, PBS, YSK05-Lip or c-di-GMP/YSK05-Lip (equivalent to 3 μ g of c-di-GMP) in 200 μ L were intravenously administered into mice. On day 9, the lungs were collected. The collected lungs were mounted with O.C.T. compound (Sakura Finetek, Torrance, CA, USA). The frozen tissue was then sectioned (5 μ m in thickness) on a microtome. The sections were washed with PBS and treated with PBS containing 1.5% bovine serum albumin and 0.1% Tween20 for blocking for 1 h at room temperature. After washing with PBS, the sections were stained with anti-mouse NK1.1 antibody overnight at 4°C. After washing with PBS, the sections were then stained with Cy5 anti-mouse IgG for 1 h at room temperature. After the section was counterstained with Hoechst 33342, the section was observed by confocal laser scanning microscopy (CLSM) (A1, Nikon, Tokyo, Japan).

2.7. Quantitative analysis of infiltrated NK cells in lung by RT-PCR

On day 0, 2×10^5 cells of B16-F10-luc2 in 200 μ L of PBS were injected into the tail vein of mice. On day 2, 4 and 8, PBS, YSK05-Lip or c-di-GMP/YSK05-Lip (equivalent to 3 μ g of c-di-GMP) in 200 μ L were intravenously administered into mice. On day 9, the lungs were collected and RNA was isolated using a RNeasy miniprep kit (QIAGEN, Hilden, Germany) according to the manufacturer's instruction. 10 μ L of 10 $\times$ DNase I buffer (TaKaRa, Otsu, Japan), 1 μ L of Ribonuclease inhibitor (TaKaRa) and 4 μ L of DNase I (TaKaRa) were added to 85 μ L of RNA solution and the mixture was incubated for 1 h at 37°C. 100 μ L of DEPC treated water and 200 μ L of phenol/chloroform (5:1) were added and mixed. After centrifugation, 200 μ L of chloroform was added to the supernatant. After centrifugation, the supernatant was mixed with 500 μ L of EtOH and 15 μ L of 5 M NaCl and the mixture was further incubated for 1 h at -80°C. After centrifugation, the pellet was washed with cold EtOH and dissolved in 25 μ L of DEPC treated water. Each sample was then reverse transcribed using a PrimeScript reverse transcription (RT) reagent Kit (TaKaRa) with an oligo-dT primer. 5-fold diluted cDNA was subjected to PCR with Taq DNA polymerase and primers specific for each. PCR was performed for a number of cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec, and extension at 72°C for 40 sec. 18s rRNA was used as an internal control. The amplified products were subjected to gel electrophoresis (2% agarose) and visualized by Gel Red (Wako). Band intensities were measured with Gel Doc EZ Imager

(Bio-Rad, Hercules, CA). Relative mRNA levels of each gene were represented as band intensities of each gene divided by that of 18s rRNA. The following primer pairs were used: NK1.1 (forward, 5'-GGACACAGCAAGTATCTACC-3'; reverse, 5'-AGACTCGCACTAAGACACTC-3'), NKp46 (forward, 5'-ACTACTCATCACAGGAGGTG-3'; reverse, 5'-CCTCTTCATGATCTTTCCTC-3'), 18s rRNA (forward, 5'-GGTAACCCGTTGAACCCCAT-3'; reverse, 5'-CAACGCAAGCTTATGACCCG-3'), synthesized by SIGMA-GENOSYS (St. Louis, MO).

2.8. Statistical analysis

Comparisons between the two treatments were performed by an unpaired t-test. Comparisons between multiple treatments were made using one-way or two-way ANOVA, followed by the Tukey-Kramer test. A P-value of <0.05 was considered to be significant.

3. Results

3.1. Cytokine profile after the c-di-GMP/YSK05-Lip treatment

The production of IFN- β caused by the c-di-GMP/YSK05-Lip was observed in RAW264.7 cells in a previous study [13], but it was not examined under in vivo condition. Thus, we first investigated cytokine production in mice after an intravenous administration of c-di-GMP/YSK05-Lip. Mice were intravenously administered the c-di-GMP/YSK05-Lip or the YSK05-Lip (without c-di-GMP) and the concentration of cytokines in serum was measured by ELISA. As a result, c-di-GMP/YSK05-Lip treatment induced a significantly higher production of IFN- β , TNF- α and IL-6 than YSK05-Lip (Fig. 1). Following the production of IFN- β , TNF- α and IL-6, a significant higher production of IFN- γ was observed in mice that had been treated with c-di-GMP/YSK05-Lip (Fig. 1). The activation of the STING pathway leads to the production of type I IFNs and proinflammatory cytokines [7,20]. The activated NK cells secrete TNF- α and IFN- γ [21]. Thus, these results indicate that NK cells are likely activated by the c-di-GMP/YSK05-Lip.

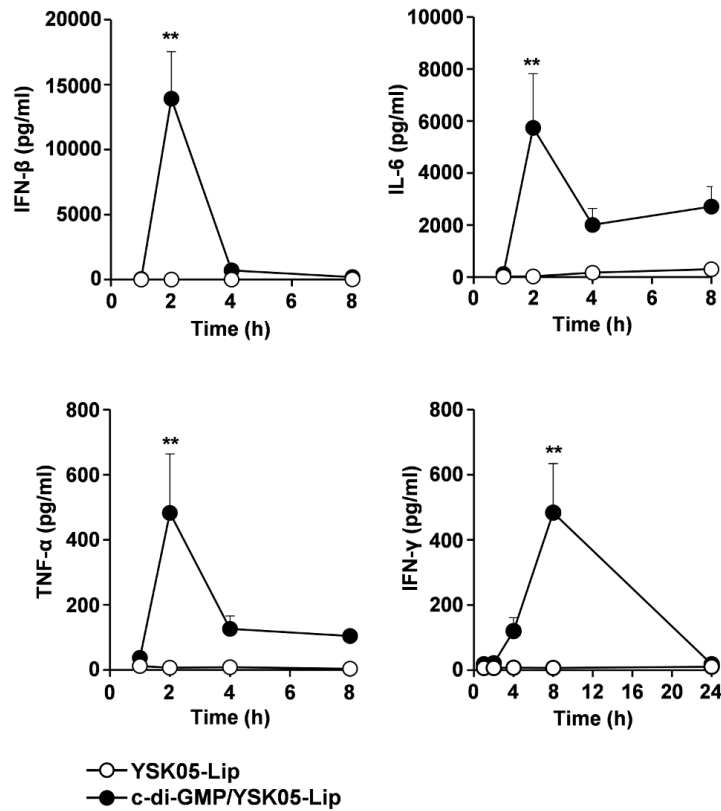


Fig. 1. Cytokine profiles in mice treated with the c-di-GMP/YSK05-Lip. Mice were intravenously administered with YSK05-Lip or c-di-GMP/YSK05-Lip (equivalent to 3 μ g of c-di-GMP). After 1, 2, 4,

8 and 24 h, blood sample were collected. The levels of IFN- β , TNF- α , IL-6 and IFN- γ in serum were measured by ELISA. Data are the mean $\pm$ SEM (n=3, **P<0.01). Statistical analyses were performed by two-way ANOVA, followed by the Tukey-Kramer test.

3.2. Activation of NK cells by c-di-GMP/YSK05-Lip treatment

We next examined the activation of NK cells in the spleens of mice treated with c-di-GMP/YSK05-Lip. Mice were intravenously injected with PBS, the YSK05-Lip or the c-di-GMP/YSK05-Lip and splenocytes were prepared at 8 h after the treatment. The splenocytes were then stained with an anti-CD3 antibody, an anti-NK1.1 antibody, an anti-NK1.1 antibody and an anti-CD69 antibody. The NK cell population was identified as CD3⁻/NK1.1⁺ cells (Fig. 2a). The CD3⁺ cell population was excluded and the remaining cells were analyzed on NK1.1/NK1.1 or NK1.1/CD69 plots (Fig. 2b). In the mouse, the spleen contains a large number of NK cells compared with other tissues and stimuli such as adjuvants, tumor and pathogens, induce the recruitment of NK cells, resulting in a decrease of the number of NK cells in the spleen [22]. As shown in Fig. 2c, the percentage of NK cells in the spleen were decreased. On the other hand, the expression of activation markers of NK cells, NKG2D and CD69, was significantly increased as the result of the c-di-GMP/YSK05-Lip treatment (Fig. 2d and e). These results indicate that the c-di-GMP/YSK05-Lip treatment efficiently induced the activation of NK cells.

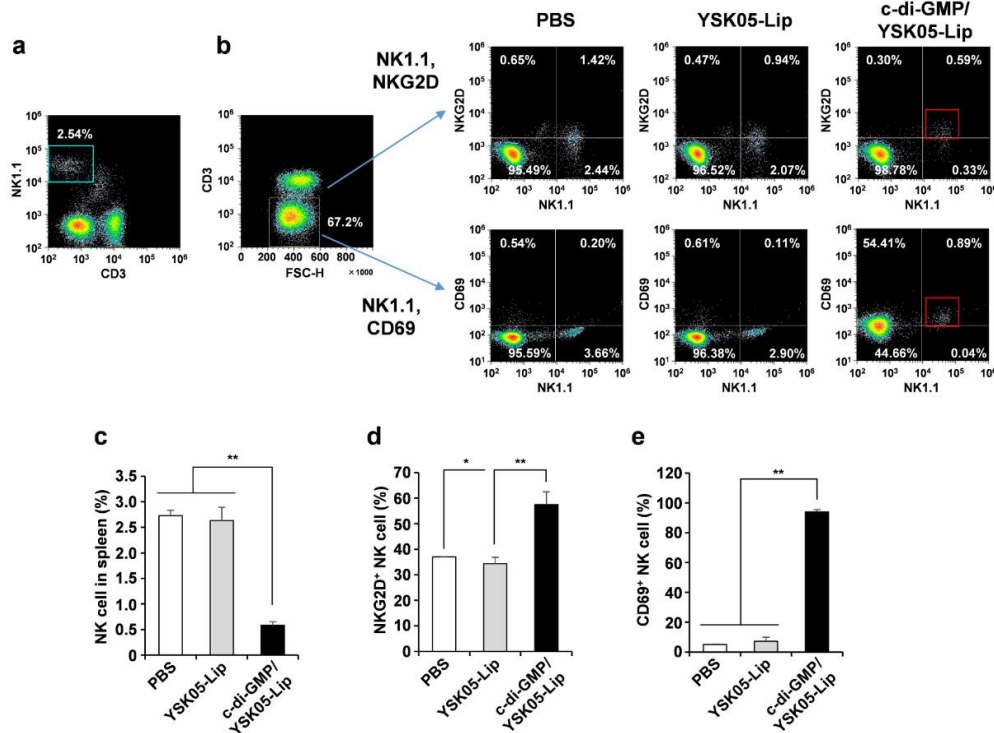


Fig. 2. Activation of NK cells in mice treated with the c-di-GMP/YSK05-Lip. Mice were intravenously administered with PBS, YSK05-Lip or c-di-GMP/YSK05-Lip (equivalent to 3 μ g of c-di-GMP). After 8 h, the spleen was collected and a single cell suspension of splenocyte was prepared. The splenocytes were stained with FITC anti-mouse CD3, PE anti-mouse NK1.1, APC anti-mouse NKG2D, PE/Cy7 anti-mouse CD69 and APC anti-mouse NK1.1 antibodies. The fluorescence of the stained cells was measured by flow cytometry. (a) NK cells were identified as CD3⁺/NK1.1⁺ cells. (b) The CD3⁺ cell population was excluded and the remaining cells were analyzed on NK1.1/NKG2D or NK1.1/CD69 plots. (c) The percentage of NK cells in spleen. Data are the mean $\pm$ SEM (n=3, **P<0.01). Statistical analysis was performed by one-way ANOVA, followed by the Tukey-Kramer test. (d) The percentage of NKG2D positive NK cells in spleen. Data are the mean $\pm$ SEM (n=3, *P<0.05, **P<0.01). Statistical analysis was performed by one-way ANOVA, followed by the Tukey-Kramer test. (e) The percentage of CD69 positive NK cells in spleen. Data are the mean $\pm$ SEM (n=3, **P<0.01). Statistical analysis was performed by one-way ANOVA, followed by the Tukey-Kramer test.

3.3. Therapeutic antitumor effect against lung metastatic melanoma by c-di-GMP/YSK05-Lip treatment

We next evaluated the therapeutic antitumor effect of the c-di-GMP/YSK05-Lip treatment in a lung metastatic mouse model with a B16-F10 melanoma. On day 0, mice were injected with B16-F10-luc2. On day 2, 4 and 8, PBS, YSK05-Lip or c-di-GMP/YSK05-Lip (equivalent to 3 μ g of c-di-GMP) were intravenously administered into mice, and the lungs were then collected on day 21. A striking decrease in tumor clusters was observed in mice that had been treated with c-di-GMP/YSK05-Lip, compared with the control groups (Fig. 3a).

The quantitative analysis based on luciferase activity also showed a significant inhibition of tumor growth in mice that had been treated with c-di-GMP/YSK05-Lip, compared with the control groups (Fig. 3b). These data suggest that the c-di-GMP/YSK05-Lip resulted in evoking an MHC-I non-restricted antitumor effect.

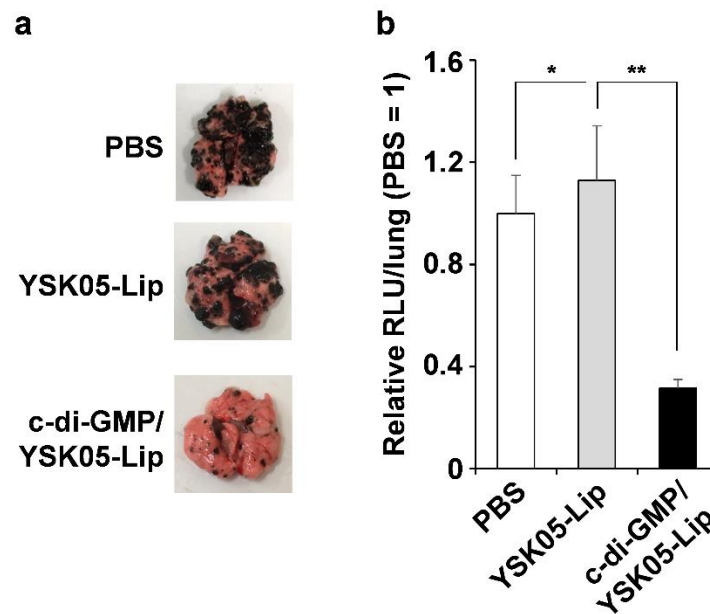


Fig. 3. Antitumor effect mediated by c-di-GMP/YSK05-Lip in a lung metastatic mouse model with B16-F10 melanoma. On day 0, 2×10^5 cells of B16-F10-luc2 were intravenously injected into mice. PBS, YSK05-Lip or c-di-GMP/YSK05-Lip (equivalent to 3 μ g of c-di-GMP) were intravenously administered into mice on day 2, 4 and 8. On day 21, the lungs were collected and the luciferase activity was measured. (a) Pictures of the lungs collected on day 21. (b) Quantitative analysis of lung metastasis by measuring luciferase activities. Luciferase activities are expressed as relative light units (RLU) per whole lung. The value of PBS treated group represent 1. Values are the mean $\pm$ SEM (n=7-9, *P<0.05, **P<0.05). Statistical analysis was performed by one-way ANOVA, followed by the Tukey-Kramer test.

3.4. NK cells are the major effector cells in the antitumor effect mediated by c-di-GMP/YSK05-Lip

Because the c-di-GMP/YSK05-Lip treatment induced an MHC-I non-restricted antitumor effect against a B16-F10 melanoma, we pursued the role of NK cells in antitumor activity. The injection of the anti-asialo GM1 antibody induces the depletion of NK cells [23]. Mice were intraperitoneally injected with an anti-asialo GM1 antibody and the population of NK cells was measured by flow cytometry 24 h after the antibody treatment. The population of NK cells in the mice treated with anti-asialo GM1 antibody was drastically decreased compared with the mice treated with saline (Fig. 4a). Subsequently, mice were intravenously

injected with B16-F10-luc2 cells on day 0, and with PBS or c-di-GMP/YSK05-Lip (equivalent to 3 μ g of c-di-GMP) on days 2, 4 and 8. For the depletion of NK cells, an anti-asialo GM1 antibody was intraperitoneally injected on days 1, 4, 7 and 10. Repeated injections with the antibody continued to result in the NK cells being depleted [23]. On day 21, the lungs were collected. A decrease in tumor clusters was observed in the mice that had been treated with c-di-GMP/YSK05-Lip in the absence of an anti-asialo GM1 antibody as well as the result of Fig. 3 (Fig. 4b). On the other hand, the anti-asialo GM1 antibody treatment clearly inhibited the effect of the c-di-GMP/YSK05-Lip and tumor growth was comparable to that of the PBS treated mice group (Fig. 4b). Similarly, a quantitative analysis based on luciferase activity measurements showed that the tumor growth between PBS treated mice and c-di-GMP/YSK05-Lip treated mice was comparable in the presence of the anti-asialo GM1 antibody (Fig. 4c). On the other hand, tumor growth was significantly inhibited in the c-di-GMP/YSK05-Lip treated mice that had not been treated with the anti-asialo GM1 antibody (Fig. 4c). These results indicate that NK cells are critical for the antitumor effect mediated by the c-di-GMP/YSK05-Lip against B16-F10 lung metastasis.

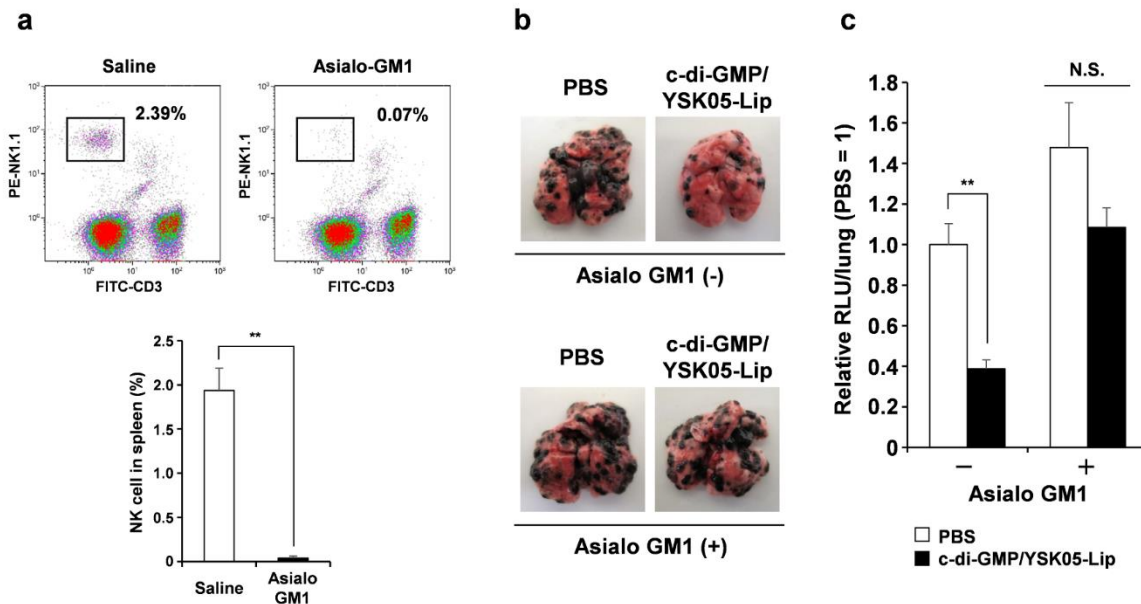


Fig. 4. The effect of NK cell depletion on the antitumor activity mediated by the c-di-GMP/YSK05-Lip in a lung metastatic mouse model with B16-F10 melanoma. (a) The depletion of NK cells by the injection of an anti-asialo GM1 antibody. Mice were intraperitoneally injected with anti-asialo GM1 antibody. After 24 h, the spleen was collected and a single cell suspension of splenocytes was prepared. The splenocytes were stained with FITC anti-mouse CD3 and PE anti-mouse NK1.1. The fluorescence of the stained cells was measured by flow cytometry. NK cells were identified as CD3⁺/NK1.1⁺ cells. Data are the mean $\pm$ SEM (n=3, **P<0.01). Statistical analysis was performed by the unpaired t-test. (b) and (c) On day 0, 2×10^5

cells of B16-F10-luc2 were intravenously injected into mice. PBS, YSK05-Lip or c-di-GMP/YSK05-Lip (equivalent to 3 μ g of c-di-GMP) were intravenously administered into mice on day 2, 4 and 8. For the depletion of NK cells, anti-asialo GM1 antibody was intraperitoneally injected on day 1, 4, 7 and 10. On day 21, the lungs were collected and luciferase activity was measured. (b) Pictures of the lungs collected on day 21. (c) Quantitative analysis of lung metastasis by measuring luciferase activities. Luciferase activities are expressed as relative light units (RLU) per whole lung. The value of PBS treated group represent 1. Values are the mean $\pm$ SEM (n=4, **P<0.05). N.S.: not significant. Statistical analysis was performed by the unpaired t-test.

The infiltration of NK cells in lungs with B16-F10 melanomas was also investigated. Mice were intravenously injected with B16-F10-luc2 cells on day 0, and with PBS or c-di-GMP/YSK05-Lip (equivalent to 3 μ g of c-di-GMP) on days 2, 4 and 8. On day 9, the lungs were collected, and a histological analysis and quantitative analysis by RT-PCR were carried out. As a result, numerous green dots corresponding to NK cells were clearly observed in the lung of mice with the B16-F10 melanoma that had been treated with c-di-GMP/YSK05-Lip (Fig. 5a). On the other hand, the number of green dots was clearly low in the lungs of mice with the B16-F10 melanoma that had been treated with PBS or YSK05-Lip (Fig. 5a). In addition to the histological analysis, a quantitative analysis of mRNA levels of NK cell markers by RT-PCR in each lung with B16-F10 melanoma was carried out and the results are shown in Fig. 5b, c and d. The mRNA levels of NK1.1 and NKp46 were significantly increased in the mice treated with c-di-GMP/YSK05-Lip compared with the control groups (Fig. 5b, c and d). These facts suggest that the c-di-GMP/YSK05-Lip treatment stimulates the infiltration of NK cells into lungs with the B16-F10 melanoma.

Consequently, these findings suggest that NK cells can be considered to be major effector cells in the antitumor effect mediated by c-di-GMP/YSK05-Lip in a lung metastatic mouse model with a B16-F10 melanoma.

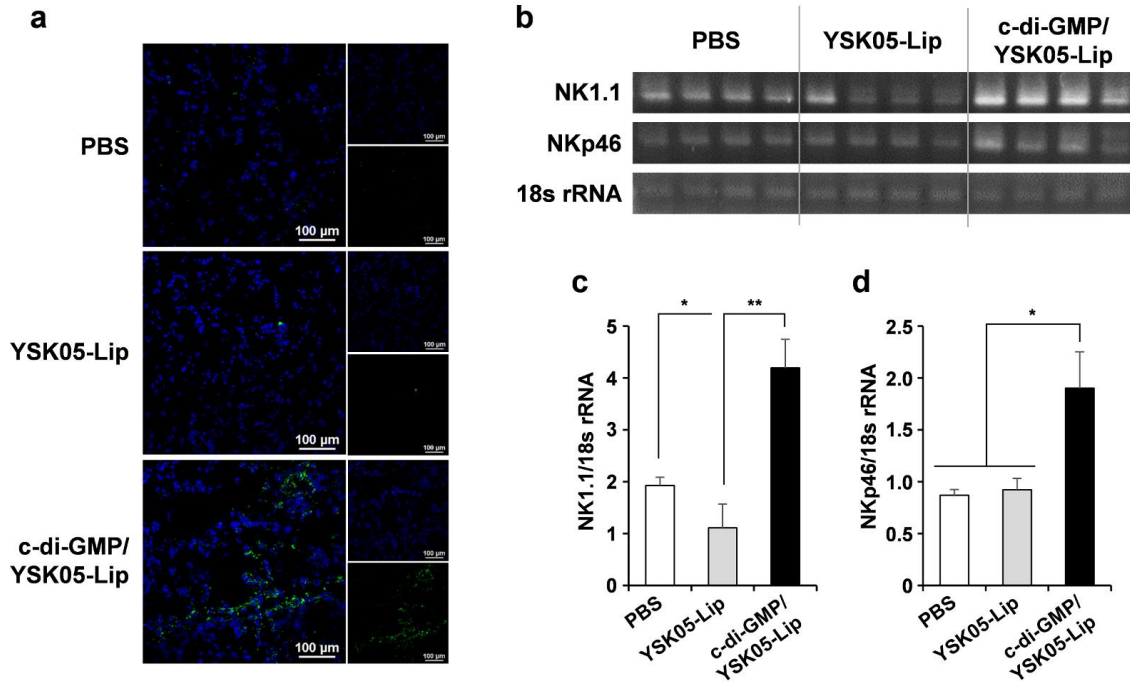


Fig. 5. The infiltration of NK cells into lungs by the c-di-GMP/YSK05-Lip in a lung metastatic mouse model with B16-F10 melanoma. On day 0, 2×10^5 cells of B16-F10-luc2 were intravenously injected into mice. PBS, YSK05-Lip or c-di-GMP/YSK05-Lip (equivalent to 3 μ g of c-di-GMP) were intravenously administered into mice on day 2, 4 and 8. On day 9, the lungs were collected. (a) CLSM images obtained by histological analysis of lung. Green fluorescence and blue fluorescence show NK cell (Cy5) and nuclei (Hoechst 33342), respectively. Bars=100 μ m. (b) Quantitative analysis of infiltrated NK cells in lung by RT-PCR. PCR products were subjected to 2% agarose gel electrophoresis and visualized by Gel Red. The results of four individual experiments are shown. (c) and (d) Band intensities were measured with Gel Doc EZ Imager. Relative mRNA levels of NK1.1 (c) and NKp46 (d) are represented as band intensities of each gene divided by that of 18s rRNA. Values are the mean $\pm$ SEM (n=4, *P<0.01, **P<0.05). Statistical analyses were performed by one-way ANOVA, followed by the Tukey-Kramer test.

4. Discussion

We previously established that the c-di-GMP/YSK05-Lip has some promise for use as an adjuvant system, both in vitro and in vivo [13]. The c-di-GMP/YSK05-Lip was found to efficiently activate APCs via the STING-TBK1-IRF3 pathway and to induce antigen-specific cellular immunity. Furthermore, a c-di-GMP/YSK05-Lip treatment evoked an MHC-I restricted antitumor effect against E.G7-OVA tumors in the preventative scheme. In addition to the preventative antitumor effect, we also confirmed a therapeutic antitumor effect

against E.G7-OVA tumors (Suppl. Fig. S2). Based on the findings reported herein, the c-di-GMP/YSK05-Lip efficiently activates NK cells, resulting in the infiltration of NK cells into the lung and the inhibition of tumor growth in a lung metastatic mouse model with a B16-F10 melanoma which is an MHC-I negative tumor. Therefore, these findings suggest that c-di-GMP represents a potentially potent adjuvant for producing antitumor immunity mediated by NK cells against a malignant melanoma, and that the c-di-GMP/YSK05-Lip efficiently activates both MHC-I restricted and non-restricted antitumor activities. It is quite likely that c-di-GMP/YSK05-Lip could be applied to several type of cancers, because the level of MHC-I is different in each cancer [16].

There are two currently accepted models for the *in vivo* activation of NK cells [24]. One is that NK cells directly recognize pathogens, cancers and adjuvants via pattern recognition receptors (PRRs), resulting in their activation, because NK cells usually circulate in a steady state. The other is that NK cells are activated by mature DCs in lymphoid tissues. DCs recognize pathogens, cancers and adjuvants via PRRs and become mature. The mature DCs provide direct activating signals for NK cells through cell-cell contact [24-26], and also produce proinflammatory cytokines and type I IFNs. Furthermore, recent studies have reported that the activation of IRF3 and IRF5 in DCs induces NK cell activation via cell-to-cell contact [24,27]. In this study, the drastic production of IFN- β and of proinflammatory cytokines (IL-6 and TNF- α) were observed as the result of the c-di-GMP/YSK05-Lip treatment (Fig. 1), and the activation of NK cells occurred in the spleen (Fig. 2). The efficient NK activation appears to be due to the stability of c-di-GMP/YSK05-Lip in the blood. The stability of c-di-GMP/YSK05-Lip was not high in mouse serum, because we used DMG-PEG as a PEG lipid in this study (Fig. S3). Nanoparticles containing DMG-PEG are rapidly cleared from the blood and rapidly accumulate in the spleen and liver [28]. Spleen contains high levels of APCs and NK cells. The intravenously-injected c-di-GMP/YSK05-Lip would rapidly accumulate in the spleen. Moreover, c-di-GMP/YSK05-Lip is taken up by APCs and facilitates IFN- β production and APC maturation [13]. Hence, it appears that the NK cell activation by c-di-GMP/YSK05-Lip can be induced by mature APCs (Fig. 6).

NK cells play an important role in host defense against tumors. In the experiment using NK cell deficient mice, NK cells were found to play a critical role in suppressing tumor metastasis and outgrowth [29]. In humans, NK cell infiltration in tumors may be associated with a better prognosis in squamous cell lung, gastric and colorectal carcinomas [30]. In addition, human tumor cells with a loss of or down regulation of MHC-I or that produce a

variety of altered self stress-inducible proteins are ideal targets of NK cells [31]. The effector function of NK cells is controlled by the expression of NK cell activating ligands and a negative ligand (MHC-I) on the cell surface [32]. NK cells spare normal cells that express self MHC-I and low amounts of NK cell activating ligands (inhibitory signal > activating signal). On the other hand, NK cells selectively kill abnormal cells that lack or down-regulate MHC-I or upregulate NK cell activating ligands (inhibitory signal < activating signal). The B16-F10 melanoma cells used in this study lack self MHC-I (Suppl. Fig. S1). Thus, they appeared to be the target of NK cells, resulting in the inhibition of tumor growth (Fig. 3 and 5). Moreover, the c-di-GMP/YSK05-Lip efficiently increased the expression of NKG2D, a lectin-like activating receptor, on the surface of NK cells (Fig. 2d). NKG2D clearly influences the NK cell mediated immune surveillance of experimentally induced tumors [33,34]. Thus, it is possible that the NK cells that are activated by the c-di-GMP/YSK05-Lip received a stronger activating signal, leading to a more efficient tumor elimination (Fig. 6). Given the importance of NK cell activation in clinical effects and the prognosis in human cancer patients, the c-di-GMP/YSK05-Lip has the potential for use as an adjuvant in cancer immunotherapy.

The STING pathway has a quite important role in cancer immunosurveillance and cancer immunotherapy. It was recently reported that a major mechanism for the innate immune sensing of cancer is via a cytosolic DNA-STING pathway [6]. This sensing was not associated with other innate signaling pathways such as TLR, the retinoic acid-inducible gene I (RIG-I) or the melanoma differentiation-associated protein 5 (MDA5) pathways. Tumor-derived DNA is a ligand for the STING pathway, which is taken up by host APCs, localized to the cytosol, and is associated with the *in vivo* TBK1-IRF3-STING dependent production of IFN. The activation of APCs via the STING pathway induces maximal spontaneous T cell priming against tumor antigens. As mentioned in the introduction section, the spontaneous T cell responses in a tumor environment appear to contribute to the control of tumor growth and to be associated with the clinical benefits associated with cancer immunotherapy. In fact, Woo et al. showed that the therapeutic effect of anti-CTLA4 and anti-PD-1 antibodies was absent in STING-deficient mice [6]. Moreover, it has been reported that STING-pathway-mediated cytosolic DNA sensing is required for radiation-mediated tumor immunity [35]. Therefore, the c-di-GMP/YSK05-Lip has the potential to enhance a wide range of cancer immunotherapies such as cancer vaccines, immune checkpoint inhibitors and radiotherapy, because the stimulation of the STING-pathway is critical for the induction and maximization

of spontaneous T cell responses.

This represents with first demonstration of the activation of NK cells, resulting in an antitumor effect against MHC-I negative tumors, mediated by c-di-GMP (Fig. 6). Based on our previous report and this study, the findings suggest that c-di-GMP/YSK05-Lip can efficiently induce MHC-I restricted and non-restricted antitumor activities. In addition, the mechanism responsible for the immune activation by the c-di-GMP/YSK05-Lip, which is associated with STING-TBK1-IRF3 pathway, may be advantageous and potent compared with other adjuvants. Therefore, c-di-GMP/YSK05-Lip promises to become a next generation adjuvant for cancer immunotherapy.

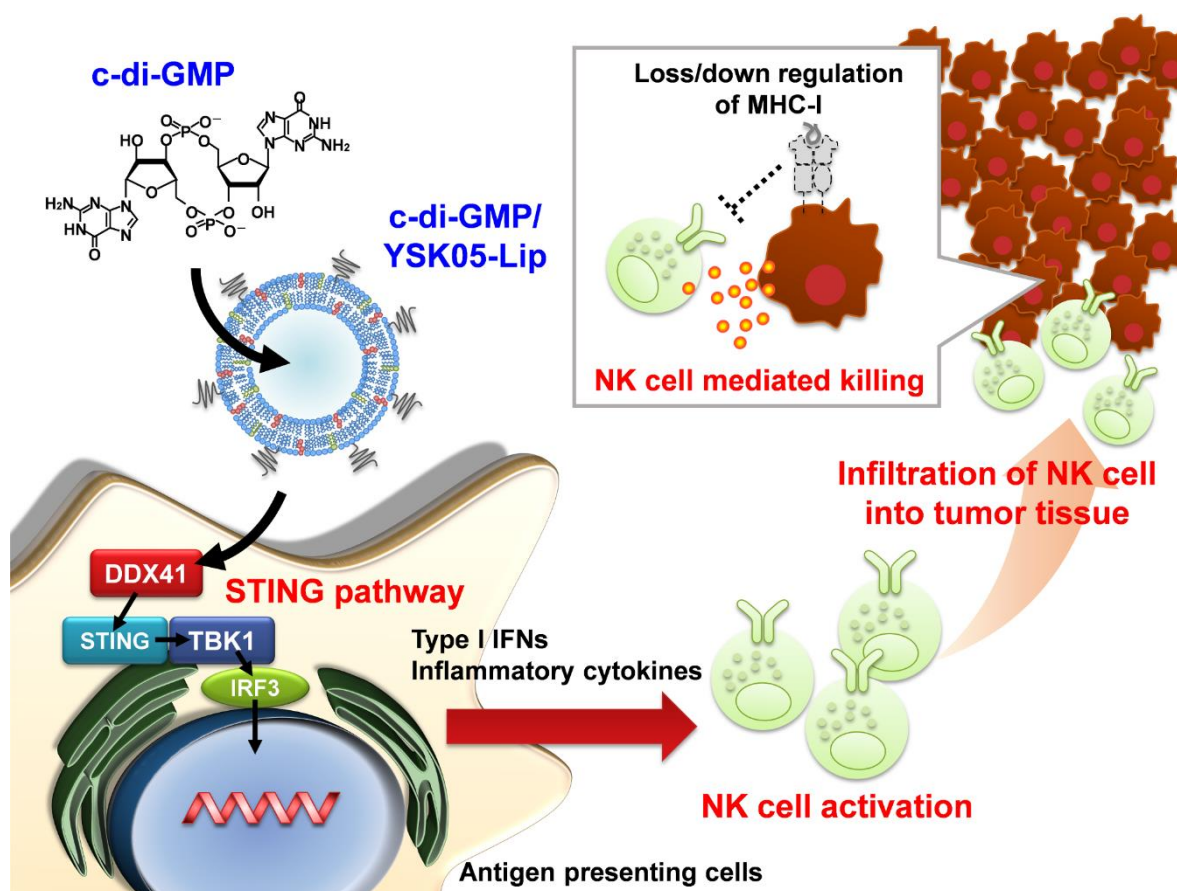


Fig. 6. Machinery associated with the antitumor effect against malignant melanomas by c-di-GMP/YSK05-Lip. The c-di-GMP loaded into the YSK05-Lip is delivered to the cytosol of antigen presenting cells. The c-di-GMP binds to DDX41, resulting in recognition by STING. The activation of STING signaling pathway leads to the production of type I IFNs and proinflammatory cytokines. They activate NK cells and the activated NK cells then infiltrate into a lung with a malignant melanoma (B16-F10). B16-F10 cells are killed by the activated NK cells.

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