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## **Doctoral Dissertation**



# **In vivo memory-like NK cell induction as CD11b<sup>high</sup>CD27<sup>low</sup> with STING-LNP and CpG-ODN protects against melanoma lung metastasis**

STING 搭載脂質ナノ粒子と CpG-ODN の併用による  
メモリー様 NK 細胞の誘導と メラノーマ肺転移予防への展開

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## ***Abstract***

### **1. Introduction**

Natural killer (NK) cells are effective in attacking tumor cells that escape from T cell attack. Recent discoveries have led to a paradigm shift in the characterization of NK cells. Historically, it was assumed that NK cells are short-lived cells associated with innate immune responses and lack any memory function towards any prior stimuli. Paradigm shifting experiments performed over the last decade have clearly identified that NK cells remember their prior experience with an enhanced response upon restimulation. Although numerous issues remain to be addressed, NK cells with memory functions can be roughly divided into two types: memory NK cells and memory-like NK cells. Memory NK cells that were found in viral infected models, are specific to viral antigens. While memory-like NK cells, which are non-specific for certain antigens, like cytokine-induced memory-like (CIML) NK cells, have long lasting effects, even after the discontinuation of a stimulus. Researchers are currently attempting to identify the phenotype of these CIML NK cells. Phenotypical characterization of memory NK cells differs depending on the nature of the induction and whether they are human or murine derived cells. The surface density of CD27 and CD11b divides the murine NK cells into 4 subsets. Starting from  $CD11b^{low}CD27^{low}$ , the most immature phenotype, then increasing the maturation as  $CD11b^{low}CD27^{high}$  then  $CD11b^{high}CD27^{high}$  till reaching the most mature stage of NK cells characterized as  $CD11b^{high}CD27^{low}$ . A brief stimulation with interleukin (IL)-12, IL-15 and IL-18 which is reported to result in CIML-NK cells, can function potently in cancer immunotherapy following their adoptive transfer and displayed a mature phenotype as  $CD11b^{high}CD27^{low}$ .

NK cells. However, the available knowledge on the memory-like NK cells induction, identification, and potential in vivo is limited. Here in we succeeded in developing and identifying memory-like NK cells in vivo by a combination of a stimulator of interferon genes (STING) agonist loaded in a lipid nanoparticle (STING-LNP) and oligodeoxynucleotides containing CpG motifs (CpG-ODN) that tended to enhance the production of IL-12, IL-18, and exerted a therapeutic effect against B16-F10 lung metastasis. This combination drastically increased CD11b<sup>high</sup>CD27<sup>low</sup> NK cells, the most potent population of NK cells, and induced a surprisingly strong preventative effect, indicating that CD11b<sup>high</sup>CD27<sup>low</sup> can be a phenotype of memory-like NK cells. These findings are the first to provide novel insights regarding the in vivo induction and the phenotypical characterization of CIML NK cells by using a combination of immunotherapeutic drugs which could not be achieved without the aid of the delivery system.

## **2. Results and discussion**

### **2.1. NK cells play a significant role in the enhancement of the antitumor effect of STING-LNP and CpG-ODN**

First, we examined the ability of this combination therapy to induce therapeutic efficacy against a B16-F10 lung metastasis model. The combination therapy significantly controlled the tumor burden. We subsequently examined the contribution of NK cells and CD8<sup>+</sup> T cells on the antitumor effect by the combination therapy. An anti-NK1.1 antibody treatment abrogated the antitumor effect by combination therapy, whereas following the anti-CD8a antibody treatment the antitumor effect was maintained. This data can confirm

that the antitumor effect caused by the combination therapy is largely dependent on NK cells and not T cells.

## **2.2. Combining the STING-LNP with CpG-ODN enhances the production of cytokines resulting in the induction of the CIML NK cells**

Following the intravenous administration of STING-LNP and/or CpG-ODN, the levels of IL-12, IL-18, IL-1 $\beta$  cytokines, essentially required for the development of memory-like NK cells, were checked. The combination therapy tended to enhance the production of the cytokines better than each monotherapy. These results indicate the importance of combining CpG-ODN with the STING-LNP. We next examined our hypothesis that the combination therapy induces, not only the activation of NK cells but also their differentiation into memory-like NK cells, the most potent phenotype of NK cells that are characterized as CD11b<sup>high</sup>CD27<sup>low</sup> NK cells. The combination therapy increased the expression of CD11b and killer cell lectin-like receptor G1 (KLRG1), differentiation marker, and decreased the expression of CD27 which indicates that STING-LNP and CpG-ODN enhance the maturation, activation of the NK cells. We also checked the induction of memory-like NK cells based on the simultaneous expression of CD11b and CD27, as we speculated that producing NK cells with the most mature and potent phenotype would lead to the generation of memory-like NK cells. The percentage of the CD11b<sup>+</sup> SP, single positive: CD11b<sup>high</sup>CD27<sup>low</sup>, population was exclusively and drastically increased following the administration of STING-LNP and CpG-ODN in comparison to all other groups. Which is related to the phenotype of memory NK cells induced by a viral infection with KLRG1<sup>high</sup> and CD27<sup>low</sup> expression.

### **2.3. Protection against the development of melanoma lung metastasis**

To evaluate the development of memory-like NK cells, a prophylactic protocol was initiated; only the combination therapy induced a surprisingly strong preventative effect; however, monotherapies failed. This preventative effect suggests that CD11b<sup>high</sup>CD27<sup>low</sup> might be the phenotype of memory-like NK cells.

### **2.4. Sustained existence of the CD11b<sup>high</sup>CD27<sup>low</sup> NK cells induced by STING-LNP and CpG-ODN**

To track the memory-like NK cells, CD11b<sup>high</sup>CD27<sup>low</sup> NK cells, induction and their prolonged existence following the combination therapy. A prophylactic protocol was initiated in which two cycles of the PBS or a combination of STING-LNP and CpG-ODN were intravenously administered on days 0 and 4. Followed by checking the phenotype of the NK cells by flow cytometry in the spleen on days 7, 14, 21 and 28. SP CD11b<sup>+</sup> NK cells was induced following the vaccination starting from day 7 and significantly in comparison to the PBS group on day 14 until reaching its peak on day 21 which persisted for at least one month. Moreover, the SP CD11b<sup>+</sup> KLRG1<sup>+</sup> expression level was drastically elevated on day 7 just following the two-cycle vaccination when compared to the other days and the PBS group. The sustained existence of the CD11b<sup>high</sup>CD27<sup>low</sup> NK cells at least for one month can explain the reason behind the prolonged protection against the development of the melanoma lung metastasis.

### **3. Conclusion**

This represents the first report of the in vivo induction, identification, and a confirmation of the memory function of the memory-like NK cells through a prophylactic effect via the use of an immunotherapeutic drugs. Our findings provide novel insights into the in vivo induction of CD11b<sup>high</sup>CD27<sup>low</sup> memory-like NK cells, thus paving the way for the development of efficient immunotherapies. Two cycles of a combination of STING-LNP and CpG-ODN induced sufficient levels of cytokines that are essential for the activation and differentiation of NK cells to memory-like CD11b<sup>+</sup> SP NK cells which could have extended lifetimes. This resulted in an enhanced antitumor effect and protection against the development of the melanoma lung metastasis model.

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## List of Abbreviation

**STING:** Stimulator of interferon genes

**LNP:** Lipid nanoparticle

**IL:** interleukin

**MHC-I:** Major histocompatibility complex-I

**CIML:** Cytokine induced memory-like

**BCG:** Bacillus Calmette Guerin

**PD-1:** Programmed cell death-1

**PD-L1:** Programmed cell death ligand-1

**DMG-PEG<sub>2000</sub>:** 1,2-dimirystoyl-sn-glycerol methoxyethyleneglycol

**C-di-GMP:** Cyclic di-GMP

**PBS:** Phosphate Buffered Saline

**CTL:** Cytotoxic T lymphocyte

**RLU:** Relative light units

**DN:** Double negative

**SP:** Single positive

**DP:** Double positive

**KIR:** Killer immunoglobulin receptor

**TGF- $\beta$ :** Transforming growth factor- $\beta$

**MCMV:** Murine cytomegalovirus

**IFN:** Interferon

**TNF:** Tumor necrosis factor

**TBK1:** TANK-binding kinase 1

**IKK:** I $\kappa$ B kinase

**H3K4me3:** Histone H3 lysine 4 tri-methylation

**H3K27ac:** Histone H3 lysine 27 acetylation

**GVDH:** Graft-versus-host disease

**ALL:** Acute lymphocytic leukemia

**AML:** Acute myeloid leukemia

**BSA:** Bovine serum albumin

**DDW:** Deionized distilled water

**DNA:** Deoxyribonucleic acid

**FACS:** Fluorescence activated cell sorting

**FCM:** Flow cytometry

**FBS:** Fetal bovine serum albumin

**PEG:** Polyethylene glycol

**PDI:** Poly dispersity index

**KLRG1:** killer cell lectin-like receptor G1

**ODN:** Oligodeoxynucleotides

**DNA:** Deoxyribonucleic acid

**IRF3:** Interferon regulatory factor 3

**NF- $\kappa$ B:** Nuclear factor- $\kappa$ B

**cGAS:** Cyclic GMP-AMP synthase

***cGAMP:*** cyclic GMP-AMP

***TME:*** Tumor microenvironment

***CCL3-5:*** Chemokine ligand 3-5

***SCID:*** Severe combined immunodeficient

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# *Chapter 1*

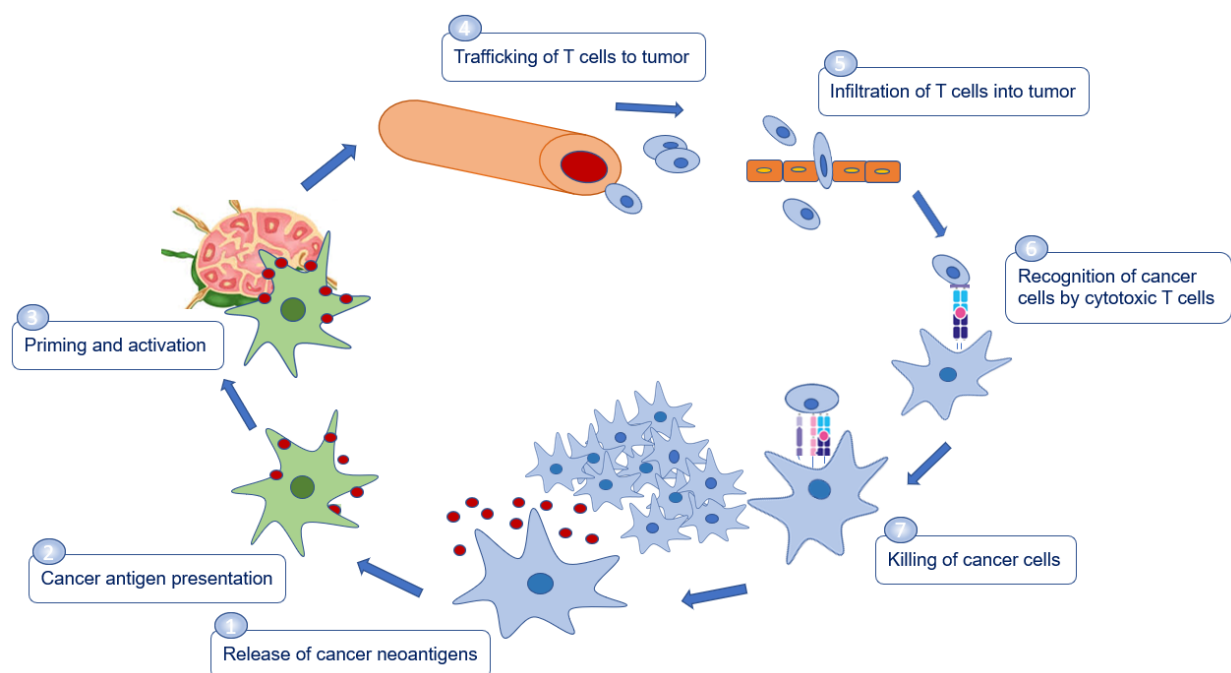
## ***General Introduction***

## ***General introduction***

Malignant melanoma is a virulent type of cancer with a high potential for metastasis. Considered as one of the most common causes of cancer-related mortalities globally. Worldwide, approximately 300,000 cases are diagnosed each year, and almost 60,000 melanoma-related deaths occur annually, considered as the deadliest type of skin cancer (1). The spread of melanoma can be cutaneous, to distant lymph nodes or to the visceral organs such as the lung (18-36%), liver (14-20%), brain (12-20%), and bones (11-17%) (2). Among these multiple sites, the lung is the one that most frequently involved in melanoma metastasis. The process of melanoma metastasis to the lungs involves complex interactions between melanoma cells and the host microenvironment. Melanoma cells undergo several steps including local invasion, intravasation, survival in the circulation, extravasation, and colonization of the lung tissue. After pulmonary metastasis the average survival is about 7.3 months (3). Most cases with pulmonary malignant melanoma undergo surgery interventions, for example, lobectomy or pneumonectomy followed by administration of combinational treatments. Especially, chemotherapy and immunotherapy such as interferon (IFN) or interleukin-2 (IL-2) and immune check point inhibitors (ICIs) (4, 5). Despite the availability of these therapeutic modalities, the therapeutic efficacy is quite limited (6, 7). Therefore, new advancements in the field of cancer immunotherapy to control the burden and the incidence of melanoma lung metastasis are essential.

The immune system is viewed as a network of biological processes that safeguard us from diseases. For an anticancer immune response to effectively destroy tumor cells, a series of steps, known as the Cancer-Immunity Cycle,

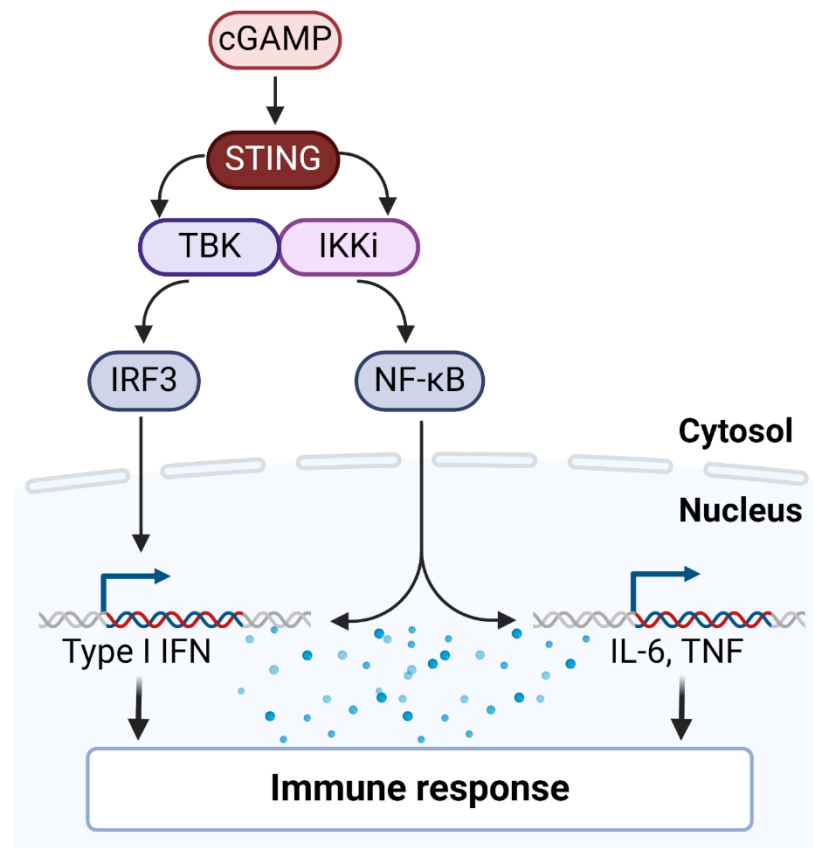
must be initiated and followed in sequence. (Fig. 1) (8). In summary, neoantigens released during oncogenesis are captured by dendritic cells, leading to T cell activation. These activated T cells then travel through the bloodstream and infiltrate the tumor tissue, where they kill tumor cells and release new neoantigens, repeating the cycle. However, in cancer patients, this process is often suboptimal. Consequently, numerous therapeutic strategies have recently been developed to repair or enhance the body's immune response.



**Fig. 1. The Cancer-Immunity Cycle, the schema is modified from (8).** The tumor-immunity cycle consists of a cascade of steps: **(1)** neoantigen release through oncogenesis, **(2)** neoantigen presentation by dendritic cells, **(3)** T-cell priming and activation, **(4)** trafficking of T lymphocytes through blood stream to tumor tissue, **(5)** infiltration of T lymphocytes into the tumor parenchyma

from the vasculature or tumor periphery, **(6)** recognition of tumor cells, and **(7)** cytotoxic T lymphocyte (CTL) destruction of tumor cells.

A key regulator of innate immune defense stimulation is the stimulator of interferon genes (STING). Recently, activating STING pathway has demonstrated significant potential in treating both primary and metastatic cancers, either as a standalone therapy or in combination with other treatments (9-11). STING is a signaling molecule located in the endoplasmic reticulum within the cytosol. When stimulated, it leads to the production of type-I IFN and other proinflammatory cytokines and chemokines necessary for priming CD8<sup>+</sup> T cells and natural killer (NK) cells. Briefly, cytosolic deoxyribonucleic acid (DNA) from tumors or pathogens is recognized by the cyclic GMP–AMP synthase (cGAS) enzyme, which generates cyclic GMP–AMP (cGAMP). This cGAMP then binds to the STING molecule, triggering its activation. STING subsequently activates the transcription factors interferon regulatory factor 3 (IRF3) and nuclear factor- $\kappa$ B (NF- $\kappa$ B) through the kinases TANK-binding kinase 1 (TBK1) and I $\kappa$ B kinase (IKK), respectively. IRF3 and NF- $\kappa$ B then move into the nucleus to induce the secretion of IFNs and other inflammatory cytokines, as illustrated in figure 2 (12).



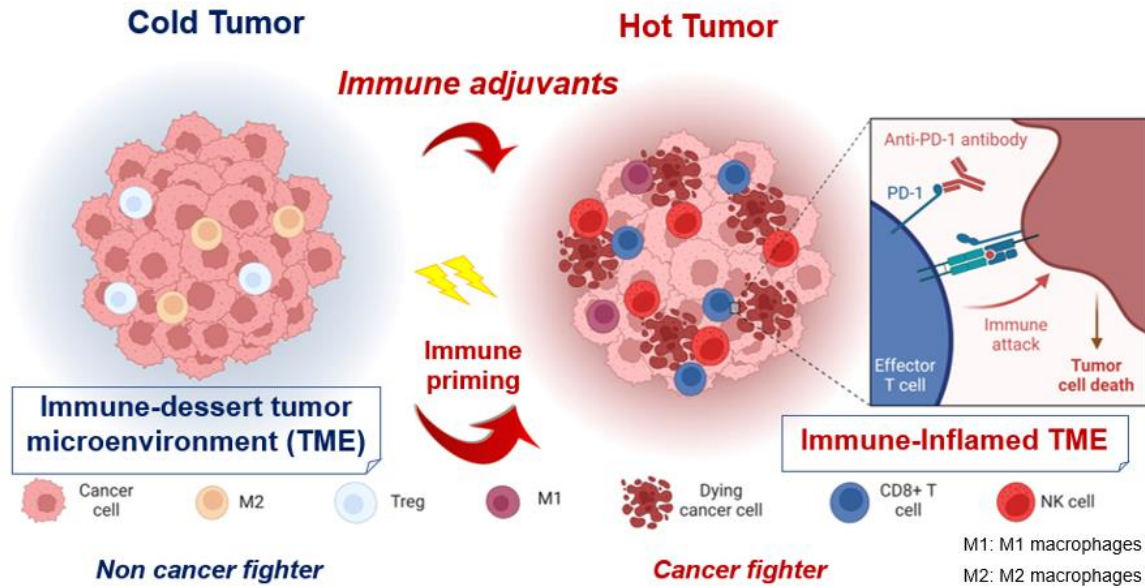
**Fig. 2. cGAS–cGAMP–STING signaling pathway.** cGAMP binding to STING, activates the downstream kinases TBK1 and IKK. TBK1 then activates the transcription factor IRF3, while IKK activates the transcription factor NF- $\kappa$ B. These activated transcription factors will be internalized into the nucleus for the expression of type I IFNs and cytokines.

Cyclic di-GMP (c-di-GMP), a STING agonist, is a cyclic dinucleotide that serves as a bacterial secondary messenger and is well-documented for its immunostimulatory properties (13). C-di-GMP is hydrophilic and contains two

phosphate groups, giving it a negative charge. This anionic nature impedes its ability to penetrate the cellular membrane and reach the cytosol, where the STING signaling molecule is located. Thus, there is a critical need for developing a drug delivery system that ensures its efficient cytosolic delivery. Over the past two decades, research has significantly advanced the use of nanotechnology for therapeutic applications, particularly in oncology (14-16). We have successfully encapsulated c-di-GMP into liposomes, enabling its efficient delivery to the cytosol (11). YSK05 lipid, our originally synthetic pH sensitive cationic lipid recognized for its high fusogenicity and superior ability to escape endosomes, was utilized for the efficient cytosolic delivery of small interfering RNA (11, 17). We previously showed that c-di-GMP/YSK05 liposome resulted in a seven-fold increase in the IFN- $\beta$  release when compared to another conventional liposome (11). Furthermore, we demonstrated that the c-di-GMP/YSK05-Liposome induces antitumor immunity, which is not reliant on the T-cells but rather mediated by NK cells (18).

A profound comprehension of the tumor microenvironment (TME) is crucial for determining the optimal immunotherapy approach. ICIs are ineffective in "cold tumors," referred to as immune desert, which are defined by a scarcity of effector immune cells and an abundance of immunosuppressive cells. Hence, igniting the TME and transitioning it into a "hot tumor," characterized by inflammation and infiltration of effector immune cells, is crucial (Fig. 3). We hypothesize that this can be accomplished by activating the STING signaling pathway. Additionally, besides priming immune cells, IFN signaling leads to the upregulation of Programmed cell death-1 (PD-1) and ligand-1 (PD-L1) on immune cells and tumor cells, respectively (19). However,

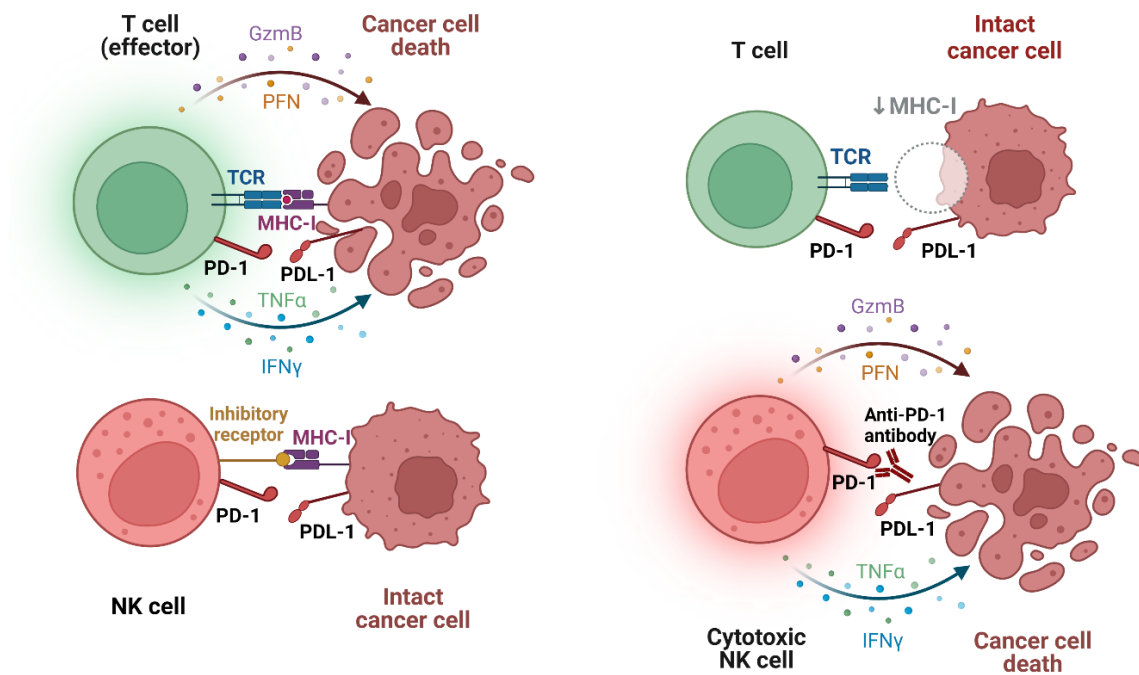
the interaction between PD-1 and PD-L1 leads to the suppression of NK cell effector function and subsequent exhaustion, thus its blockade by PD-1 antibody is essential. Therefore, the importance of combining STING agonist with a PD-1 antibody can be validated.



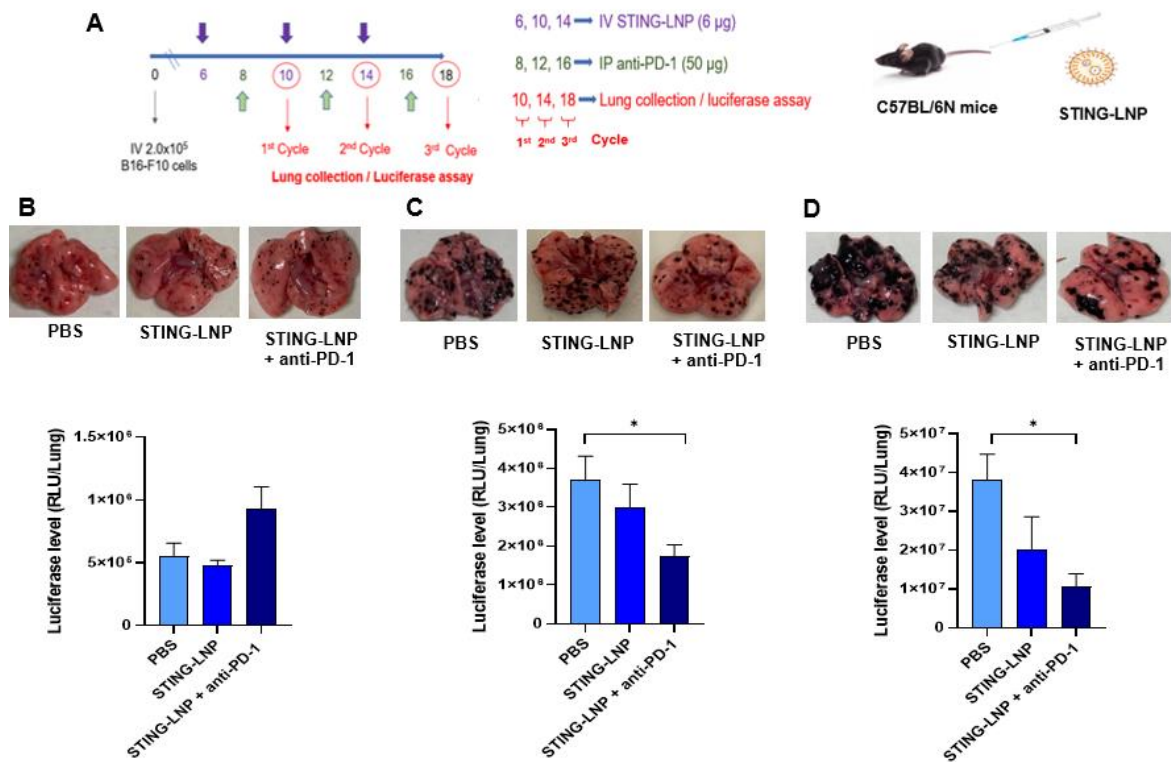
**Fig. 3. Cold tumor versus hot tumor.** Based on the spatial distribution of immune cells in the TME, a gradient of two immunophenotypes is observed: the immune-desert, and immune-inflamed phenotypes. In the immune-desert phenotype, the TME is overwhelmed with regulatory immune cells and inactivated ones while the effector ones are deficient. In the immune-inflamed phenotype, TME is overwhelmed with the activated immune cells such as T cells and NK cells. Notably, the immune-desert phenotype is non-responsive to ICIs, however, the inflamed one is responsive to ICIs.

The current focus is directed towards the priming of T cells following STING signaling stimulation (20-22). Here, we give more attention towards priming of NK cells by STING agonist aiming for the best cytotoxic effect in major histocompatibility complex-I (MHC-I) deficient tumors, where CD8<sup>+</sup> T cells are defective (Fig. 4). NK cells are lymphocytes of the innate immune system with cytotoxic potential against cancer cells. NK cells primarily function through two ways: (1) orchestrate the immune response via the cytokines and chemokines production (2) direct cell killing. NK cells secrete proinflammatory cytokines such as IFN- $\gamma$  and tumor necrosis factor (TNF) and chemokines such as chemokine ligand 3-5 (CCL3-5). NK cells also mediate direct killing of infected/tumor cells through releasing cytotoxic granules containing perforin or granzymes, as well as via death receptors ligands (23). We previously reported about overcoming the resistance towards PD-1 antibody by combining a STING-LNP to PD-1 antibody through NK cell mediated tumor attack (19, 24). The mechanism behind this revealed that NK cell activation by stimulating the STING pathway resulted in IFN- $\gamma$  secretion which induced PD-L1 expression on tumor cells. Which results in the establishment of PD-1/PD-L1 interaction between the activated NK cells and the tumor cells. The combination of PD-1 antibody can invigorate the NK cells resulting in the synergistic antitumor effect (19, 24). In addition, we previously reported about the importance of maneuvering the protocol setting to induce the best synergistic antitumor effect against melanoma lung metastasis (24). We confirmed that two cycles with two-day interval protocol was indispensable for induction of the best synergism following STING-LNP and PD-1 antibody (Fig. 5) (24). Mechanistic studies on the gene and cell level in the tumor, lung, and spleen

revealed the reason behind this two-day interval cycle dependency. The lack of synergism after one cycle and its significant existence following two cycles appears to be correlated to the changes in the TME (Fig.5 and Fig 6) (24). Two cycles of STING-LNP resulted in a drastic increase in NK1.1, NKG2D and IFN- $\gamma$  around 9-, 16- and 10-folds increase in comparison to the phosphate buffered saline (PBS) treatment, respectively, in the tumor colonies (Fig. 6) (24). In addition, flow cytometry (FCM) analysis showed a drastic enhancement in the population of the activated NK cells in the lung where the tumor metastasizes to (Fig. 7) (24). Collectively, one cycle of STING-LNP was insufficient to fire up the immune-desert TME, even though NK cell activation was slightly induced, but it was not capable of secreting IFN- $\gamma$  with no PD-1 or PD-L1 induction. However, upon administration of two cycles of STING-LNP, TME immune status was significantly activated, transforming into hot tumor, inflamed and filled with the activated NK cells, IFN- $\gamma$ , PD-1 and PD-L1 (Fig. 8) (24). Accordingly, anti-PD-1 resistance was overcome only after two cycles resulting in a substantial synergistic antitumor effect in a B16-F10 melanoma lung metastasis model. Therefore, the second dose of STING-LNP can be considered as a turning point for induction of the best synergism when PD-1 antibody is added (24). From these data that showed the importance of the second cycle and of the restimulation for induction of the best innate immune response. Our horizon was broadened not only towards the activation of the NK cells, but mainly towards their differentiation to memory-like NK cells following restimulation which will provide more enhanced responses that might have memory function (25).

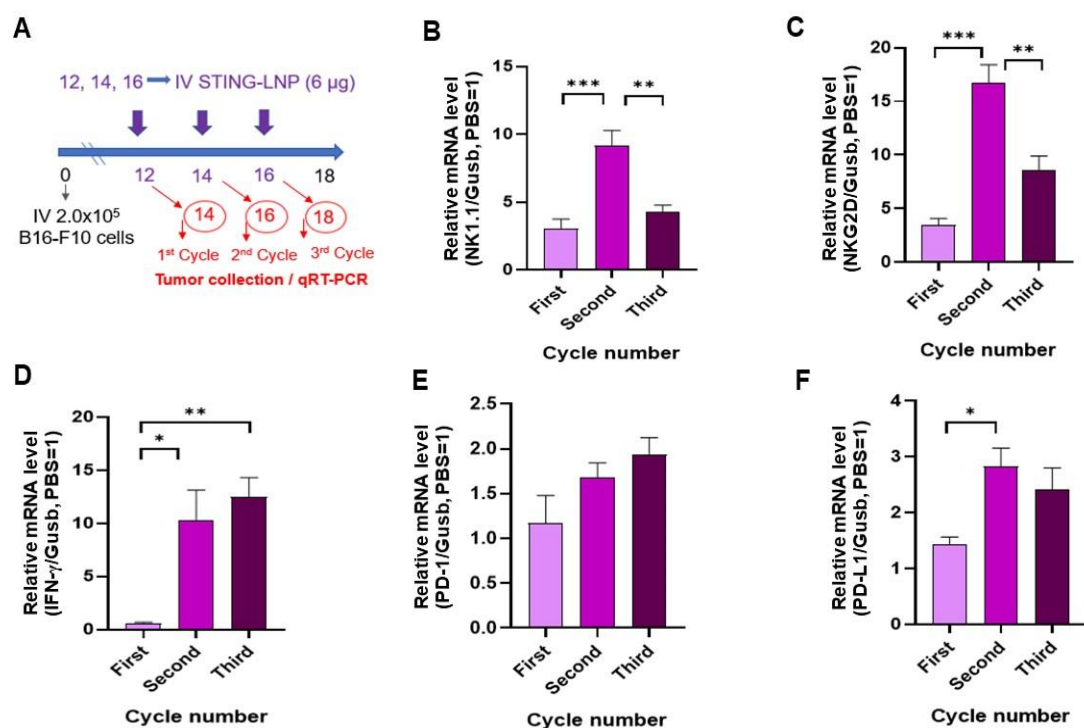


**Fig. 4. NK cell mediated cytotoxicity in MHC-I deficient tumors following innate immune priming.** MHC-I expression on the tumor cells is essential for the effector function of the T cells, but it inhibits the NK cell activity. In cases of tumors that loses the expression of the MHC-I, the antitumor effect is mainly dependent on the cytotoxic NK cells as T cells loses its effector function.

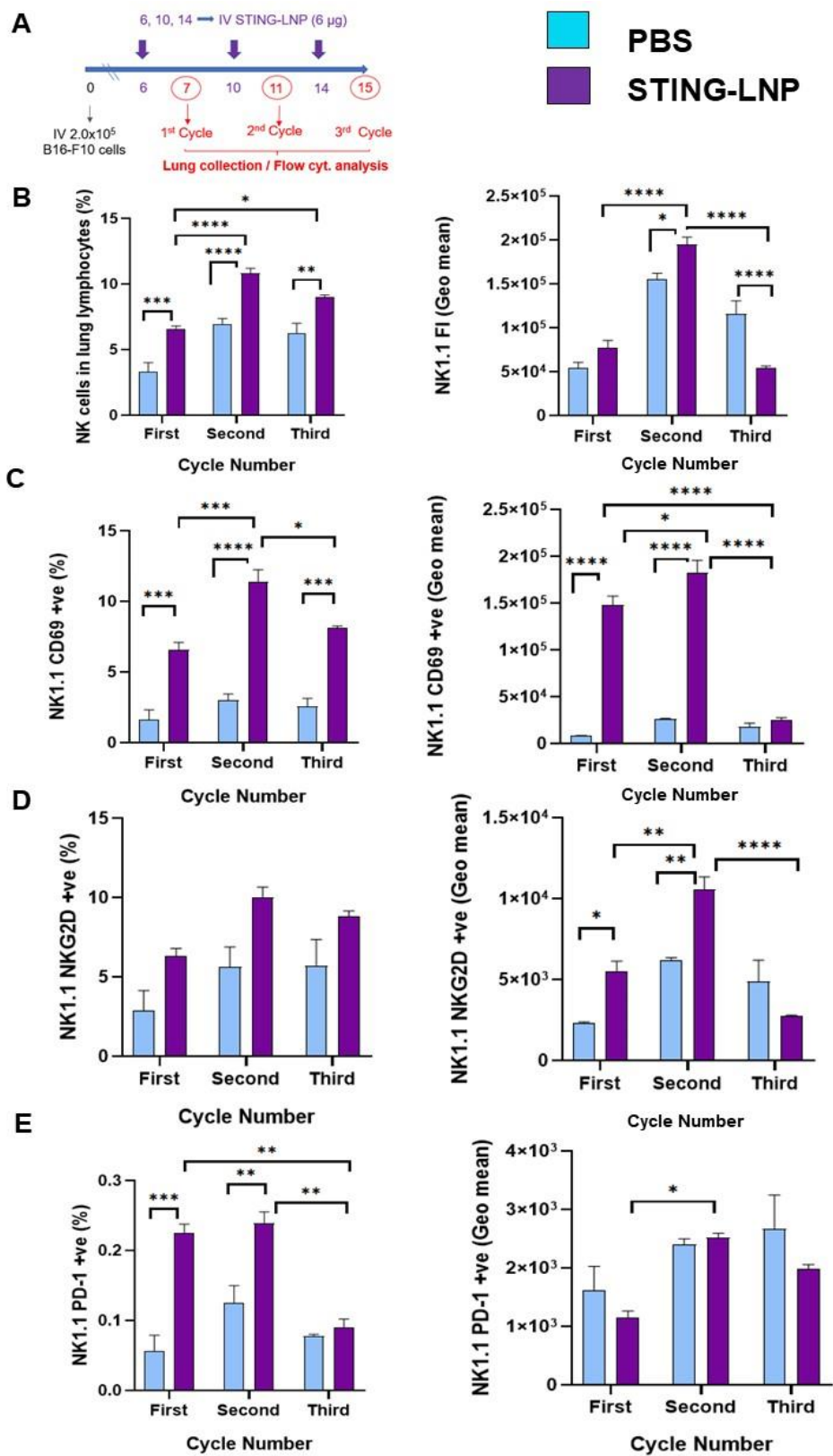


**Fig. 5. Cycle-dependent synergistic antitumor effect of STING-LNP and PD-1 antibody against melanoma lung metastasis. (A)** Treatment schedule for evaluating the impact of cycle number. Mice were intravenously injected with  $2 \times 10^5$  B16-F10-Luc2 cells. PBS or the STING-LNP (6  $\mu$ g/mouse of c-di-GMP) were intravenously injected. The PD-1 antibody (50  $\mu$ g/mouse) was intraperitoneally injected. **(B)** Antitumor effect by one-cycle treatment. Representative photos of the lungs of different treatment groups and quantitative analysis of lung metastasis based on measurement of luciferase activities. The values represent the mean  $\pm$  SEM (n = 4-6). **(C)** Antitumor effect by two-cycle treatment. Representative photos of the lungs of different treatment groups and quantitative analysis of lung metastasis based on

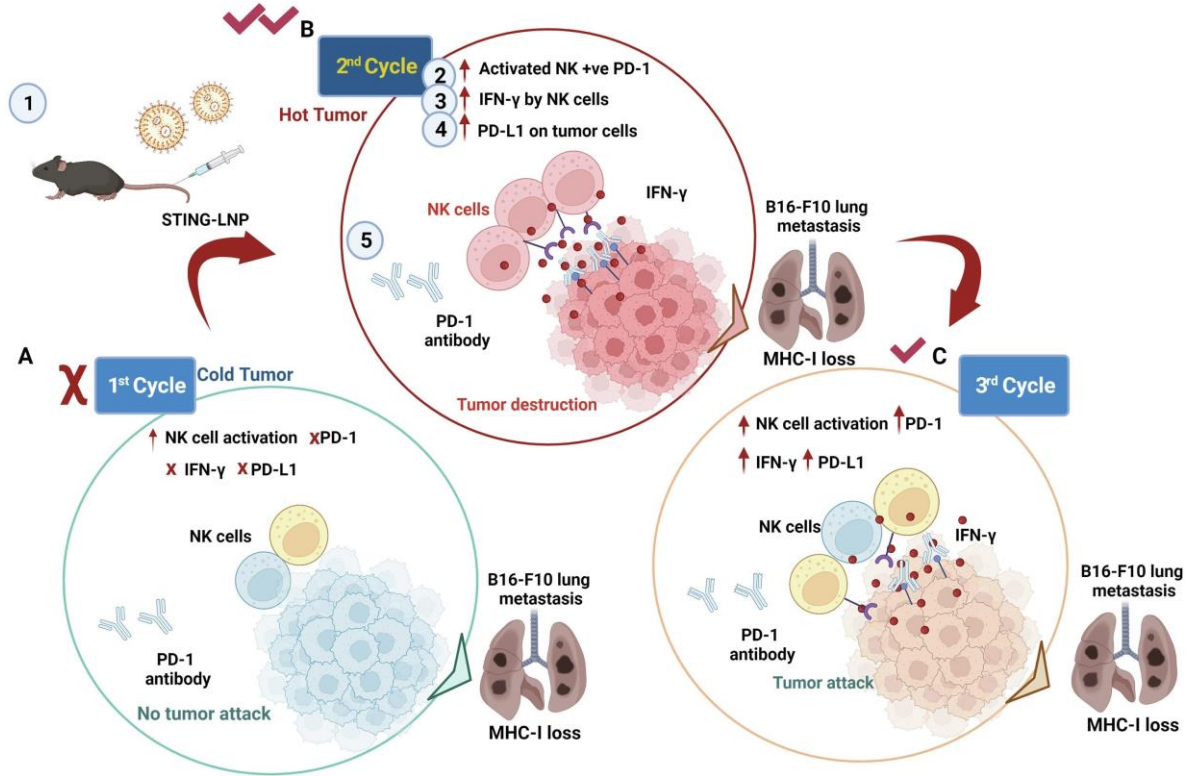
measurement of luciferase activities. The values represent the mean  $\pm$  SEM (n = 4-6, \*P<0.05). **(D)** Antitumor effect by three-cycle treatment. Representative photos of the lungs of different treatment groups and quantitative analysis of lung metastasis based on measurement of luciferase activities. The values represent the mean  $\pm$  SEM (n = 4-6, \*P<0.05).



**Fig. 6. Treatment with STING-LNP modulates the TME gene expression in a cycle-dependent manner.** (A) Treatment schedule for evaluating the gene expression. Mice with B16-F10 lung metastasis were intravenously administered with PBS or STING-LNP (6 µg of c-di-GMP LNP) at one cycle (day 12), two cycles (days 12 and 14) or three cycles (days 12, 14 and 16). The tumor colonies were collected 2 days after the final treatment. The mRNA levels were measured by qRT-PCR. (B) Fold change of NK1.1 gene expression. (C) Fold change of NKG2D gene expression. (D) Fold change of IFN-γ gene expression. (E) Fold change of PD-1 gene expression. (F) Fold change of PD-L1 gene expression. The value of PBS treated group represents 1. The values represent the mean ± SEM (n = 4–5, \*\*\*p<0.001; \*\*p<0.01; \*p<0.05).



**Fig. 7. Impact of cycle number on the NK cell activation in the lung. (A)** Treatment schedule for evaluating the NK cell activation. Mice with B16-F10 lung metastasis were intravenously administered with PBS or STING-LNP (6 µg of c-di-GMP) at one cycle (day 6), two cycles (days 6 and 10) or three cycles (days 6, 10 and 14). The lungs were collected 1 day after the final treatment. The lymphocyte fraction was analyzed by FCM. **(B)** The percentage of NK cell population and the FI (geo mean) of NK1.1 expression. **(C)** The percentage of CD69<sup>+</sup> NK cell in living cells and the FI (geo mean) of CD69 expression in NK cells. **(D)** The percentage of NKG2D<sup>+</sup> NK cell in living cells and the FI (geo mean) of NKG2D expression in NK cells. **(E)** The percentage of PD-1<sup>+</sup> NK cell in living cells and the FI (geo mean) of PD-1 expression in NK cells. The percentage of marker<sup>+</sup> NK cells on y-axis indicates the ones present in the living cells (lymphocytes), calculated as following (NK cell % × (marker<sup>+</sup> NK cell %/100)). Statistical significance among PBS cycles or between PBS and STING-LNP from different cycles were not shown (meaningless). The values represent the mean ± SEM (n = 3-4, \*\*\*\*p<0.0001; \*\*\*p<0.001; \*\*p<0.01; \*p<0.05).



**Fig. 8. Summary for the mechanism behind the cycle-dependent synergistic antitumor activity of STING-LNP and PD-1 antibody in the lung metastasis model. (A)** Following one cycle administration of this combination, NK cells appeared to be slightly activated lacking PD-1 expression which was not sufficient for any IFN- $\gamma$  release and consequently, no PD-L1 expression on tumor cells. **(B)** Upon administration of the second cycle, the TME can be fired up and overwhelmed with the activated PD-1<sup>+</sup> NK cells with high level of IFN- $\gamma$ . Followed by induction of PD-L1 expression on tumor cells. Immunosuppression by the PD-1/PD-L1 interaction can be overcome by PD-1 antibody. Resulting in tumor destruction and the best synergism. **(C)** After the third cycle administration, NK cell activation was reduced but similar levels of PD-1, IFN- $\gamma$  and PD-L1 were expressed.

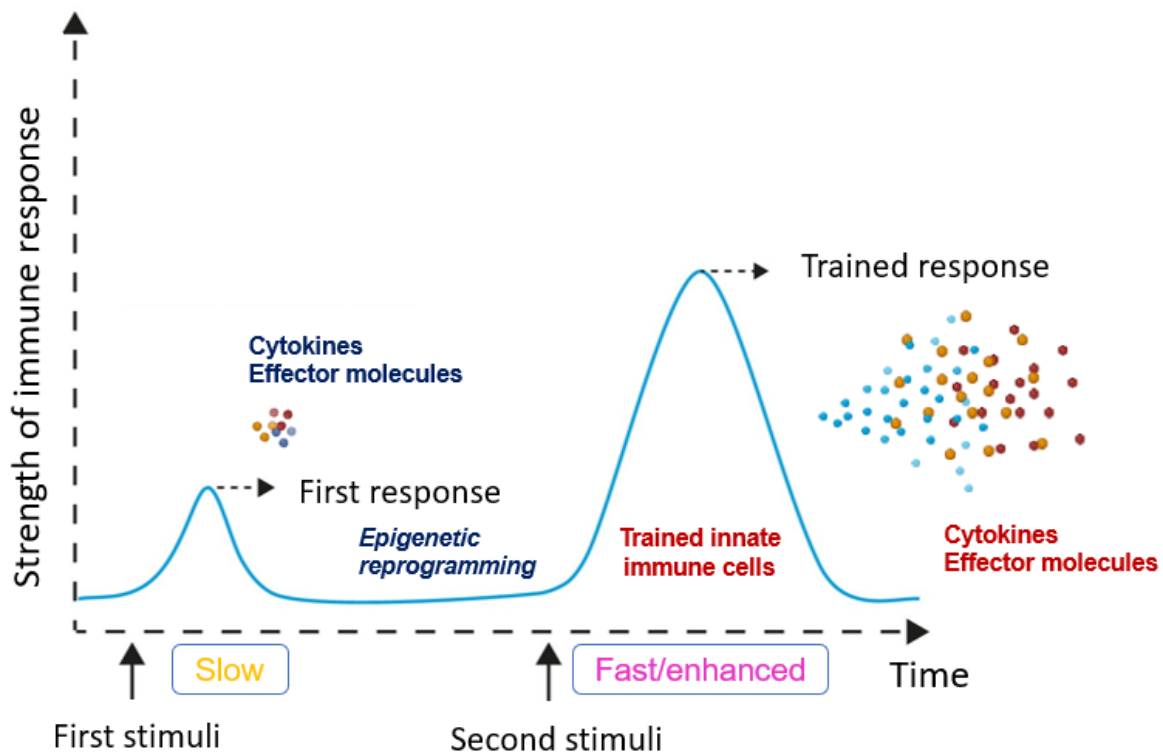
Immunological memory is an important evolutionary characteristic that improves host survival upon reinfection. Classically host immunity is classified into innate and adaptive responses. Adaptive immune responses require specialized cells such as T and B lymphocytes that can identify a specific pathogen and can provide memory responses upon the second exposure (26). While the innate immune responses are nonspecific and known to lack immunological memory. However, many reports have shown that plants and non-vertebrates that lack T and B lymphocytes had immunological memory and could prevent reinfection (26, 27) as described in table 1. In addition, over the last decade paradigm shifting experiments have shown that innate immune cells such as NK cells can remember a prior stimulus with enhanced responses following the restimulation (28). This can imply that the innate immune system has a memory function. Moreover, many vaccination reports have shown an evidence of memory function based on the innate immunity, such as in severe combined immunodeficient (SCID) mice, which lack T/B lymphocytes, have demonstrated that Bacille Calmette-Guerin (BCG) could still protect against disseminated candidiasis (29), this suggest that some vaccinations and even infections can confer broad protection against other pathogens through the underlying mechanisms of trained immunity as shown in table 1. Trained immunity an emerging concept, first introduced in 2011, in immunology that refers to the long-term functional reprogramming of innate immune cells following an initial challenge, resulting in an enhanced response and memory function to subsequent infections (30) (Fig. 9). Trained immunity is primarily driven by epigenetic reprogramming and metabolic changes in innate immune cells influenced by the first stimulus and the signaling pathways that are

subsequently activated. Key mechanisms include epigenetic and metabolic reprogramming (31). Epigenetic modifications such as histone methylation, acetylation, and DNA methylation alter the transcriptional landscape of innate immune cells, leading to a heightened state of readiness (31). Key histone modifications involved in trained immunity include histone H3 lysine 4 trimethylation (H3K4me3) and histone H3 lysine 27 acetylation (H3K27ac), which are associated with active transcription (31). On the other hand, metabolic shifts including increased glycolysis (the Warburg effect), enhanced oxidative phosphorylation, and changes in the mevalonate pathway, support the energetic and biosynthetic needs of trained immune cells (31). These metabolic and epigenetic changes are crucial for the sustained activation and trained property of these cells. It was reported previously that immunological memory granted by trained immunity lasts at least for some months (29), one year (32) and can last up to 5 years (33).

**Table 1: Evidence of the innate immunological memory.**

| Living organism          | Experimental model         | Efficacy                               | Citations |
|--------------------------|----------------------------|----------------------------------------|-----------|
| Plants                   |                            |                                        |           |
| Various plants           | Bacteria, viruses or fungi | Protection against reinfection.        | (34, 35)  |
| Non-vertebrates          |                            |                                        |           |
| <i>Anopheles gambiae</i> | <i>Plasmodium</i>          | Protection against <i>Plasmodium</i> . | (36)      |

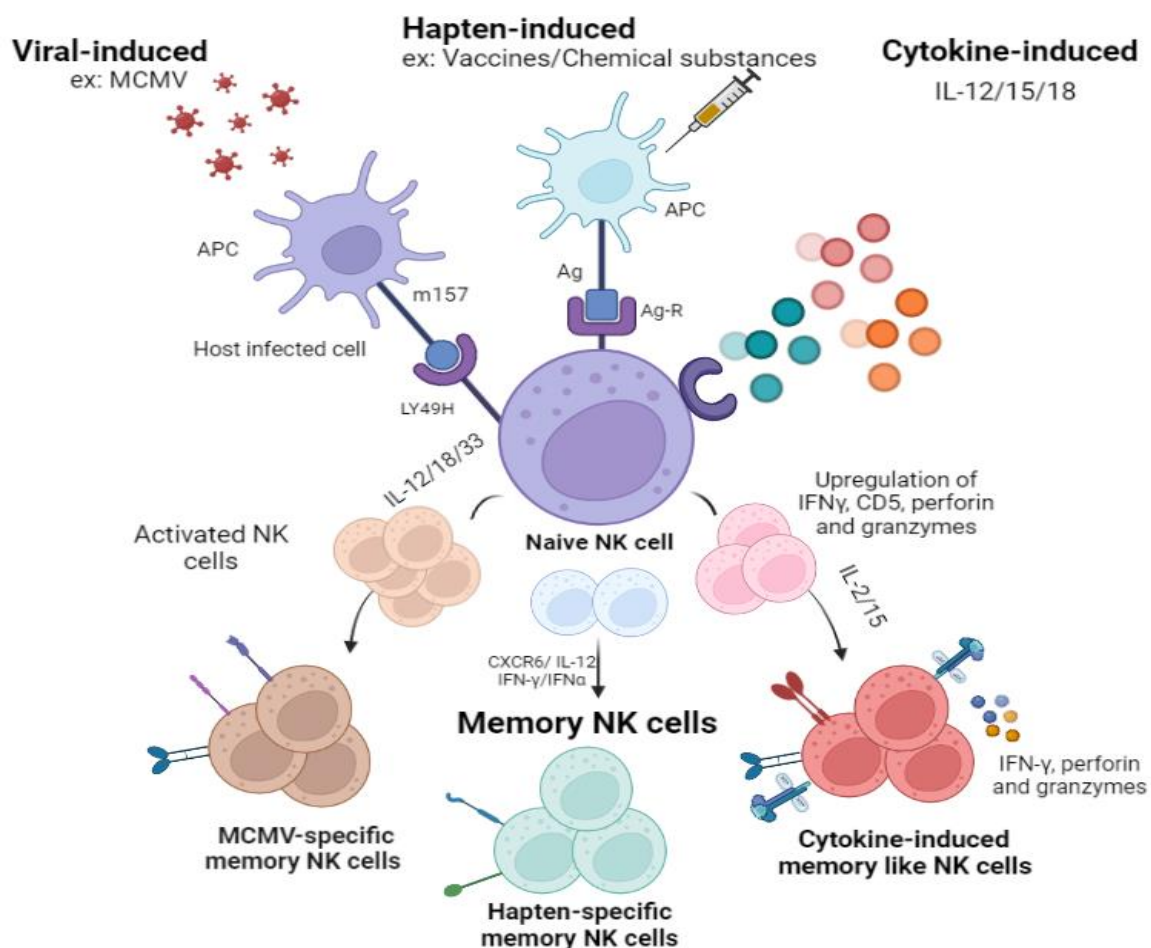
|                     |                                 |                                           |      |
|---------------------|---------------------------------|-------------------------------------------|------|
| <i>Drosophila</i>   | <i>Streptococcus pneumoniae</i> | Protection against <i>S. pneumoniae</i> . | (37) |
| Scleractinian coral | Tissue transplantation          | Rejection                                 | (38) |
| Vertebrates         |                                 |                                           |      |
| SCID mice           | <i>Candida albicans</i>         | Protection against Candidiasis.           | (39) |
| Mice                | BCG                             | Protection against Candidiasis.           | (29) |
| Mice                | Murine cytomegalovirus (MCMV)   | Protection against MCMV.                  | (40) |
| Human               | BCG                             | Protection against secondary infection.   | (41) |



**Fig. 9. The concept behind trained immunity.** Initial training starts with exposure to a primary stimulus, which triggers metabolic and epigenetic reprogramming in innate immune cells, including myeloid and innate lymphoid cells. Once these activated cells return to their baseline state, some epigenetic changes persist. Upon restimulation, these residual epigenetic modifications enable a more robust proinflammatory response from the innate immune system. This heightened response upon restimulation is referred to as trained immunity.

NK cells with memory responses have been defined and classified based on the type of stimuli (28). Following the exposure of the NK cells to a specific hapten, viral infection, or a combination of cytokine mixture of IL-12, IL-18

and IL-15, memory or memory-like NK cells will be induced, respectively (40, 42, 43) (Fig. 10). Memory NK cells that were found in viral infected models, are specific to viral antigens (40). While memory-like NK cells, which are non-specific for certain antigens, like cytokine-induced memory-like (CIML) NK cells, have long lasting effects, even after the discontinuation of a stimulus. These types of memory or memory-like NK cell are distinct, variably specific to the activation stimulus, and have unique functional and molecular biology (41). Thus, it is important to note in mind the different types of NK cell memory, and it is essential to consider each type (Fig. 10).

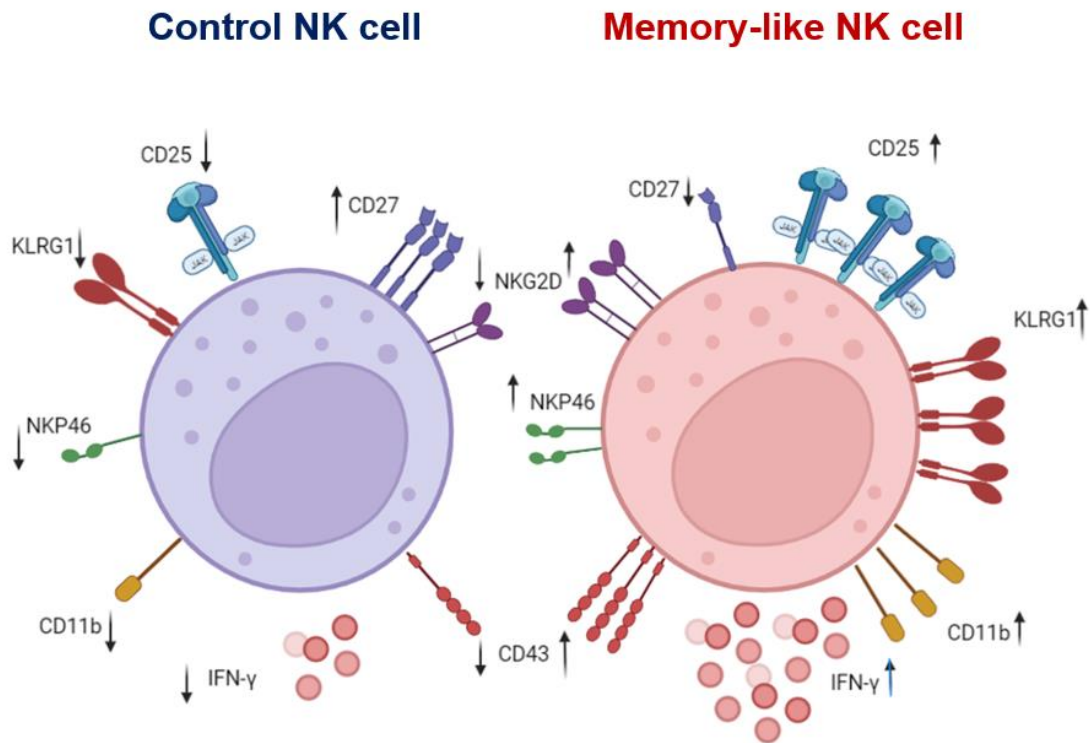


**Fig. 10. Summary of different ways of memory and memory-like NK cell induction.** Innate memory or memory-like responses are generally classified based on the type of stimulus the innate immune cells are exposed to as follows. Exposure of NK cells to, viral infection, specific haptens, or combination of IL-12, IL-15, and IL-18 cytokines results in memory or memory-like NK cells, respectively. Each type has a distinct phenotypical character and biological effect.

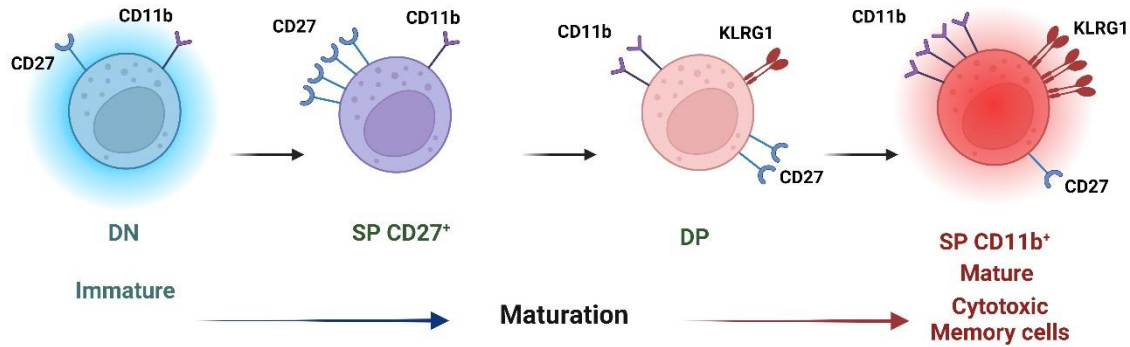
Recently, it was reported that CIML NK cells, developed in vitro with mixture of IL-12, IL-18 and IL-15 cytokines, are effective against established tumors as summarized in table 2. Currently, researchers are trying to identify the phenotype of these CIML NK cells. Phenotypical characterization of memory NK cells differs depending on the nature of the induction and whether they are human or murine derived cells. For instance, the phenotype of memory NK cells induced by a viral infection was characterized by high expression of killer cell lectin-like receptor G1 (KLRG1) and low expression of CD27 (40). In addition, human CIML NK cells showed an increase in CD94, NKG2D, NKG2A, NKp46, NKp44 and CD25 (44). While murine CIML NK cells showed an increase in CD25 and CD11b (45), collectively illustrated in figure 11. NK cell maturation can be dissected into four different stages based on the expression of CD27, a member of the TNF, and CD11b, as described in figure 12. Starting from  $CD11b^{low}CD27^{low}$ , the most immature phenotype, then increasing the maturation as  $CD11b^{low}CD27^{high}$  then  $CD11b^{high}CD27^{high}$  till reaching the most mature stage of NK cells characterized as  $CD11b^{high}CD27^{low}$  (46). A brief stimulation with IL-12, IL-15 and IL-18 which is reported to result

in CIML-NK cells, can function potently in cancer immunotherapy following their adoptive transfer and displayed a mature phenotype as CD11b<sup>high</sup>CD27<sup>low</sup> NK cells (44, 47, 48). However, the available knowledge on the memory-like NK cells induction, identification, and potential in vivo is limited. Therefore, here in we focused on the phenotypical characterization, in vivo induction, therapeutic and prophylactic effect of the memory-like NK cells that we speculate their induction following a combination of immune adjuvants (25).

In this study we successfully developed and identified memory-like NK cells in vivo via combining STING agonist loaded in a lipid nanoparticle STING-LNP (19, 24, 25) and oligodeoxynucleotides containing CpG motifs (CpG-ODN). This combination promoted the production of IL-12 and IL-18, exerting a therapeutic effect against B16-F10 lung metastasis. Notably, it significantly increased the population of CD11b<sup>high</sup>CD27<sup>low</sup> NK cells, which are considered the most potent subset of NK cells, leading to a remarkably strong preventative effect. These results suggest that CD11b<sup>high</sup>CD27<sup>low</sup> may represent a phenotype of memory-like NK cells. These findings provide new insights into the in vivo induction and phenotypical characterization of CIML NK cells using a combination of immunotherapeutic drugs, which could not be achieved without the support of the delivery system.



**Fig. 11. Collective description of the phenotypal character of the CIML NK cell in comparison to the control NK cells.** Cytokine, IL-12 + IL-18 + IL-15, pre-activated NK cells are characterized by the high expression of NKP46, CD11b, CD25, NKG2D, CD43, KLRG1 and low expression of CD27. In addition to the high levels of IFN- $\gamma$  secretion and other effector molecules in comparison to the control NK cells.



**Fig. 12. Maturation stages of NK cells.** The simultaneous distribution of CD11b and CD27 determines the maturation stage of NK cells. Their distribution subdivides NK cells into four subsets: starting from the  $CD11b^{low}CD27^{low}$  -->  $CD11b^{low}CD27^{high}$  -->  $CD11b^{high}CD27^{high}$  -->  $CD11b^{high}CD27^{low}$ . These developmental stages are linked to the gradual acquisition of NK-cell effector functions. DN: double negative, SP: single positive, DP: double positive.

**Table 2: Efficacy of the CIML NK cells against established tumors/diseases.**

| Therapy                                                                              | Cancer/disease type                                                       | Efficacy                                                                                                                                                                                                    | Citation |
|--------------------------------------------------------------------------------------|---------------------------------------------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|----------|
| Adoptively transferred murine IL-12, IL-15, and IL-18 activated NK cells.            | MHC class I-deficient RMA-S lymphoma or B16-Rae1 $\epsilon$ melanoma.     | Enhanced effector functions against the established tumors.                                                                                                                                                 | (49)     |
| Adoptive transfer of murine memory-like, IL-12, IL-15, IL-18 preactivated, NK cells. | Graft-versus-host disease (GVHD).                                         | Strongly suppressed acute GVHD and improved overall survival.                                                                                                                                               | (50)     |
| Preactivated human NK cells with IL-12, IL-15, and IL-18.                            | Acute myeloid leukemia (AML): K562 leukemia cells and primary AML blasts. | <i>In vitro</i> : enhanced IFN- $\gamma$ production and cell surface expression of CD107a, TNF. Superior effector cytokine production and enhanced cytotoxicity, compared to control conventional NK cells. | (44)     |

|                                                                        |                                                                 |                                                                                                                                            |          |
|------------------------------------------------------------------------|-----------------------------------------------------------------|--------------------------------------------------------------------------------------------------------------------------------------------|----------|
|                                                                        |                                                                 | <i>In vivo</i> : robust control of leukemia burden and prolonged the survival of leukemia-engrafted hosts. Complete remission in patients. |          |
| Adoptively transferred IL-12, IL-15, and IL-18 pre-activated NK cells. | Resistant T cell acute lymphoblastic leukemia (ALL).            | Delayed leukemia development <i>in vivo</i> .                                                                                              | (51)     |
| Human PBMCs were preactivated with IL-12, IL-15, and IL-18.            | Ovarian cancers: MA148, A1847, OVCAR5, and SKOV3.               | <i>In vitro</i> : enhanced IFN- $\gamma$ and TNF production.<br><i>In vivo</i> : tumor burden decrease.                                    | (52)     |
| IL12/15/18-activated human NK cells.                                   | Melanoma: K562, DM6, M14 cells, or autologous melanoma targets. | Drastic control of melanoma <i>in vitro</i> and <i>in vivo</i> .                                                                           | (53, 54) |

# Chapter 2

***In vivo memory-like NK cell induction via the combination of STING-LNP and CpG-ODN protects against melanoma lung metastasis***

# *Introduction*

## ***Introduction***

Significant efforts have recently been focused on developing tumor immune-based therapies that reduce tumor burden and extend patient survival and in some cases, inducing complete remission (55). Most of those T-cell-based immunotherapies have proven effectiveness against many types of cancers (56-58). However, because tumor cells for instance, melanoma cells can evade T-cell immune surveillance, this is considered to be a limitation to their effectiveness (59, 60). Therefore, utilizing innate immune cells and empowering them to fight against the cancer is critical, especially in tumors in which the MHC-I expression is lacking, which is considered to be the main cause of T-cell resistance (19, 24). MHC-I plays a crucial role in antigen presentation, essential for the effector function of T cells, but it acts as an inhibitory receptor for NK cells. (61, 62). Therefore, tumor cells that loses the expression of MHC-I would be expected to allow NK cells to attack and kill the tumor cells. Thus, NK cell targeting is a potent strategy in the case of MHC-I deficient tumors.

Recent discoveries have resulted in a paradigm shift in our understanding of the characterization of NK cells. Historically, it was assumed that NK cells have short life span associated with innate immune responses and lack any memory function towards any prior stimulus. Paradigm-shifting experiments over the past decade have clearly demonstrated that NK cells can remember their previous experiences, showing an enhanced response upon restimulation after an initial activation event. Thus, the discovery of NK cell subsets with memory functions has been revealed. Although many questions remain, NK cells with memory functions can be broadly categorized into two types: memory

NK cells and memory-like NK cells (63). Memory NK cells, discovered in viral infection models, are specific to viral antigens.(40). In contrast, memory-like NK cells, such as CIML NK cells, are not specific to certain antigens but have long-lasting effects even after the stimulus is absent or discontinued (64). However, the potential use of CIML NK cells in cancer therapy in vivo remains unclear, and information about their phenotype is still limited.

Research over the past decades has underscored the significant role of innate immune memory in host defense and in vaccinology (65, 66). It has been reported that memory-like NK cells can be induced by BCG, which has been found to confer heterologous protection against various infections and numerous solid cancers (32, 65, 67). Subsequent studies have demonstrated that memory-like features in natural killer cells can be generated using a combination of cytokines, resulting in enhanced functionality against many types of cancers (43, 45). Numerous preclinical and early-stage clinical trials have confirmed the safety and promising functional activity of these cytokine-induced memory-like (CIML) NK cells following adoptive transfer (44, 47, 68). Romee et al. observed that CIML NK cells could reduce tumor burden and improve survival in tumor-bearing mice (54). Additionally, complete remissions were noted in patients with refractory acute myeloid leukemia who received CIML NK cell infusions (44).

Researchers are currently working to identify the phenotype of CIML NK cells. This memory phenotypical character of NK cells varies based on the method of induction and whether the cells are derived from mice or humans (28). Human CIML NK cells exhibit increased levels of CD94, NKG2D, NKG2A, NKp46, NKp44, and CD25 (44). Murine CIML NK cells also show

increased levels of CD25 and CD11b (45). Additionally, adoptively transferred cytokine-preactivated murine NK cells display a mature phenotype characterized by CD11b<sup>high</sup>CD27<sup>low</sup>, KLRG1<sup>high</sup>, and CD43<sup>high</sup>, along with potent effector functions. However, this phenotype has not yet been confirmed to be associated with memory responses. (48).

To effectively induce antitumor responses mediated by NK cells in vivo, we developed a lipid nanoparticle loaded with a STING agonist (STING-LNP) (11, 18, 19, 24, 69). The STING pathway, a cytosolic sensor for DNA and cyclic dinucleotides, is a promising candidate for cancer immunotherapy (19, 24). Treatment with STING-LNP induced the production of systemic type I interferons (IFNs), leading to NK cell activation (18, 19, 70). However, STING agonists do not efficiently induce the production of IL-12 and IL-18, which are crucial for memory-like NK cell induction (71). On the other hand, CpG-ODN, an agonist of toll-like receptor 9 (TLR9), is a potent adjuvant for inducing Th1 cytokine responses, including IL-12 and IL-18 (72). Based on this information, we hypothesized that combining STING-LNP with CpG-ODN could efficiently induce the production of CIML NK cells.

In this study, we aimed to address the challenge of in vivo induction of memory-like NK cells by leveraging the advantages of a delivery system. We explored the potential of combining STING-LNP with CpG-ODN to induce the most mature and potent phenotype of NK cells in vivo, which we hypothesized to be CD11b<sup>high</sup>CD27<sup>low</sup> NK cells. The combination therapy tended to enhance the production of IL-12 and IL-18, significantly increasing the antitumor effect against B16-F10 lung metastasis. Flow cytometry analyses revealed that the combination therapy elevated the levels of the most mature phenotype of NK

cells, identified as CD11b<sup>high</sup>CD27<sup>low</sup> NK cells. Two treatment cycles were necessary for inducing these CD11b<sup>high</sup>CD27<sup>low</sup> NK cells. Furthermore, while monotherapies of STING-LNP or CpG-ODN failed to show a preventative antitumor effect, the combination therapy induced a robust and long-term preventative antitumor effect due to the sustained presence of CD11b<sup>high</sup>CD27<sup>low</sup> NK cells. This is the first report demonstrating that the CD11b<sup>high</sup>CD27<sup>low</sup> NK cells induced by STING-LNP and CpG-ODN combination therapy exhibit the phenotypical characteristics of CIML NK cells. These findings provide initial insights into the in vivo induction and phenotypical characterization of CIML NK cells using a combination of immunotherapeutic drugs, a feat that would not have been possible without the delivery system.

# *Materials and Methods*

## ***Materials***

### **Reagents, cell lines and mice**

YSK12-C4, (6Z, 9Z, 28Z, 31Z)-19-(4-(dimethylamino) butyl) heptatriaconta-6,9,28,31-tetraen-19-ol, was synthesized as previously reported (73-77). Cholesterol was obtained from Avanti Polar Lipids Inc. (Alabaster, AL). 1,2-dimirystoyl-sn-glycerol methoxyethyleneglycol 2000 ether (DMG-PEG<sub>2000</sub>) was purchased from the NOF Corporation (Tokyo, Japan). Cyclic di-GMP (c-di-GMP) was purchased from Cayman Chemical (Ann Arbor, MI). CpG-ODN (5'-ggGGTCAACGTTGAgggggg-3', with bases in lower case being phosphonothioate) was synthesized by Hokkaido System Science (Sapporo, Japan). FITC anti-mouse CD3, PE anti-mouse NK1.1, PE/Cyanine7 anti-mouse KLRG1, APC anti-mouse CD27, Alexa700 anti-mouse CD11b, APC/Cy7 anti-mouse CD43 antibodies and their isotype controls as well as the purified anti-mouse CD16/32 antibody and 7-AAD viability staining solution were purchased from Biolegend (San Diego, CA). The VersaComp Antibody capture bead kit was obtained from Beckman Coulter (Indianapolis, IN).

The B16-F10-luc2 cell line was purchased from the Caliper Life Science (Hopkinton, MA). B16-F10-luc2 cells were cultured in RPMI-1640 (high glucose) supplemented with 10% fetal bovine serum (FBS), 1 mM sodium pyruvate and 10 mM HEPES. C57BL/6N female mice (6-7 weeks old) were purchased from Japan SLC Inc. (Shizuoka, Japan). All animal experiments were approved by the Ethics of Pharmaceutical Science Animal Care Committee of Hokkaido University (approval number: 20-01226).

**Equipments:**

For cell culture:

- Clean bench NS-13B (Bio-Labo)
- Microscope BH-2 (Olympus)
- Microscope IX70 (Olympus)
- Centrifuge 3740 (Kubota)
- CO<sub>2</sub> incubator (Napco)

For STING-LNP preparation:

- Low speed refrigerated centrifuge (TOMY)
- Life Science UV/Vis Spectrophotometer (BECKMAN COULTER)
- Zetasizer Nano series (Malvern)

Others:

- Vortex (GENIE2 Scientific Industries)
- CytoFlex Flow Cytometer (Beckman Coulter)

**Reagents preparation:****1 mM Citrate Buffer (pH 4.5)**

10 mg of citric acid monohydrate dissolved in 40 ml deionized distilled water (DDW), pH was adjusted to 4.5 with 1 M NaOH. The solution was sterilized by 0.22 µm membrane filter, volume was adjusted to 50 ml by DDW and stored at 4 °C.

**10 mM HEPES Buffer (pH 7.4)**

119.2 mg of HEPES was dissolved in 40 ml DDW, pH was adjusted to 7.4 with NaOH. The solution was sterilized by 0.22  $\mu$ M membrane filter, volume was adjusted to 50 ml by DDW and stored at 4 °C.

**0.5% Trypsin 5 mM EDTA**

0.19 g EDTA.2Na.2H<sub>2</sub>O, 0.63 g NaCl and 0.19 g NaH<sub>2</sub>PO<sub>4</sub>.2H<sub>2</sub>O were dissolved in DDW. The pH of this solution was adjusted to 7.4 by 10 M NaOH, the final volume was adjusted to 80 ml by DDW. This solution was autoclaved under high pressure for its sterilization. Finally, 20 ml of 2.5% Trypsin was added to the sterilized solution in the clean bench and stored at 4 °C.

**Lysis Buffer (100 mM Tris-HCl, 1 mM EDTA, 0.1% TritonX-100, pH 7.8)**

6.06 g of Tris and 372 mg EDTA.2Na.2H<sub>2</sub>O were dissolved in DDW. The pH was adjusted to 7.8 by 1 M HCl, and the final volume was adjusted to 500 ml by DDW. The solution was sterilized by autoclaving, after that 500  $\mu$ l of TritonX-100 was added and stored at 4 °C.

**FACS Buffer (1% BSA, 0.1 NaN<sub>3</sub> in PBS)**

1 g sodium azide and 2.5 g of bovine serum albumin (BSA) were dissolved in 500 ml of D-PBS (-), mixed well and stored at 4 °C.

## ***Methods***

### **Preparation of the STING-LNP and its complexation with CpG-ODN**

The STING-LNPs were prepared as previously described (19). Typically, 150  $\mu$ L of 1 mM citrate buffer (pH 4.5) containing 500 nmol of c-di-GMP was added to 300  $\mu$ L of a lipid solution (YSK12-C4: cholesterol:DMG-PEG<sub>2000</sub>) = (85:15:1, mol ratio) in 90% t-butanol under vortexing. The resultant mixture was then added to 1.6 mL of 1 mM citrate buffer (pH 4.5) with continued vortexing followed by the addition of 4 mL PBS. Followed by filtering this solution with an Amicon Ultra (MWCO 100,000) and replacing it with 4 mL of PBS. The particle size and zeta-potential of the resulting STING-LNP were assessed with a Zetasizer Nano ZS ZEN3600 (Malvern Instruments, UK). To determine the amount of encapsulated c-di-GMP, the STING-LNP was lysed with 10% sodium deoxycholate and the UV absorbance at 252 nm ( $\epsilon = 24700$ ) was measured using 264 UV/Vis spectroscopy. The required amount of both the STING-LNP and CpG-ODN were mixed instantly prior to the injection. Similarly, the resulting complex was characterized.

### **Checking the levels of cytokines in serum through ELISA**

Mice were injected intravenously with either monotherapy of STING-LNP (4  $\mu$ g), CpG-ODN (150  $\mu$ g), CpG-ODN (250  $\mu$ g) or a combination therapy (a mixture of STING-LNP and CpG-ODN), 2 hours after, blood was collected, and the sera obtained was stored -80 °C. The concentration of IFN- $\beta$ , IL-12, IL-18 and IL-1 $\beta$  in the serum were quantified by ELISA kit (R&D systems, USA) following the manufacturer's instructions.

### **Evaluating antitumor and prophylactic effect by a luciferase assay**

For evaluating the antitumor effect, mice were inoculated by B16-F10-Luc2 cells ( $2 \times 10^5$ ) on day 0 to establish a melanoma lung metastasis model. Following the administration of PBS, STING-LNP (4  $\mu$ g), CpG-ODN (250  $\mu$ g) or combined therapies intravenously for two cycles on days 6 and 10, on day 14 the lungs were collected. For the depletion experiment, anti-NK1.1 or its isotype control clone C1.18.4 (200  $\mu$ g) or anti-CD8a or its isotype control clone LTF-2 (200  $\mu$ g) was injected intraperitoneally on days 5 and 9 which was followed by the intravenous administration of PBS or the STING-LNP and CpG-ODN on days 6 and 10. Lungs were collected on day 14 for luciferase activity measurements. For the prophylactic experiment, mice were intravenously administered with PBS as a control group, the STING-LNP (4  $\mu$ g), CpG-ODN (250  $\mu$ g) or combination therapy on days 0 and 4 for two cycles and the mice were challenged with the tumor cells either on day 5 or day 14 or day 24 or day 90, which was followed by lung collection on day 15 or day 28 or day 38 or day 104, respectively. Tumor metastasis towards the lungs was assessed as previously reported (19, 24). The lung sample was thoroughly homogenized in lysis buffer, and luciferase activity was measured using a luminometer (Luminescencer-PSN, ATTO, Japan). For the immune cell depletion experiment, the relative light units (RLU) per whole lung for the PBS-treated mice (PBS group) were normalized to 1.

### **FCM analysis for evaluating NK cell markers in the spleen**

To assess NK cell markers in the spleen using FCM, mice were injected with B16-F10-Luc2 cells ( $2 \times 10^5$ ) on day 0 to create a melanoma lung metastasis model. This was followed by the intravenous administration of PBS, STING-LNP (4  $\mu$ g), CpG-ODN (250  $\mu$ g) or the combination therapy on days 6 and 10 for two cycles. A single cell suspension from the spleen was prepared for the experiment of FCM on day 14. For the prophylactic protocol, mice received intravenous injections of PBS as a control, the STING-LNP (4  $\mu$ g), CpG-ODN (250  $\mu$ g) or the combination of each on days 0 and 4 for two cycles and tumor cells were inoculated either on day 5 or day 14 or day 24 followed by collecting the spleens on day 15 or day 28 or day 38, respectively. To track the induction and functional lifespan of memory-like NK cells, mice were intravenously administered with PBS as a control or the combination of STING-LNP (4  $\mu$ g) and CpG-ODN (250  $\mu$ g) on days 0 and 4 for two cycles followed by the spleen collection on days 7, 14, 21 and 28. To assess the effect of tumor cell inoculation on the level of memory-like NK cell induction, mice were intravenously administered with PBS as a control or the combination of STING-LNP (4  $\mu$ g) and CpG-ODN (250  $\mu$ g) on days 0 and 4 for two cycles and tumor cells were inoculated on day 24 in one group of mice followed by spleen collection on day 28. For FCM, the purified anti-mouse CD16/32 antibody was first added to the splenocytes for blocking. Followed by staining the splenocytes with the FITC anti-mouse CD3 Antibody, the PE anti-mouse NK1.1 Antibody, the PE/Cyanine7 anti-mouse KLRG1 antibody, the APC anti-mouse CD27 antibody, the Alexa700 anti-mouse CD11b antibody, the APC/Cy7 anti-mouse CD43 antibody and the corresponding isotype controls. 7-AAD was used to

stain the splenocytes and then the cells were analyzed using a CytoFlex (Beckman Coulter, Indianapolis, IN). Data analysis was performed using the FlowJo software.

### **Statistical analysis:**

All statistical analyses were performed with GraphPad Prism software. Comparisons between the two treatments were performed by an unpaired t-test. Comparison between multiple treatments were performed utilizing the one-way analysis of variance (ANOVA) followed by Tukey test. Statistical significance was designated as follows: \*\*\*\* $p < 0.0001$ ; \*\*\* $p < 0.001$ ; \*\* $p < 0.01$ ; \* $p < 0.05$ .

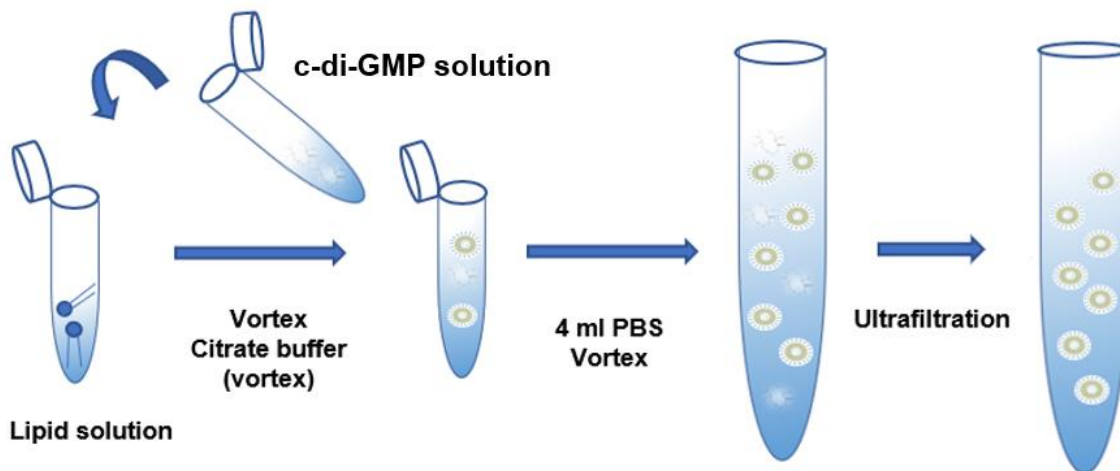
# *Results*

## **Results**

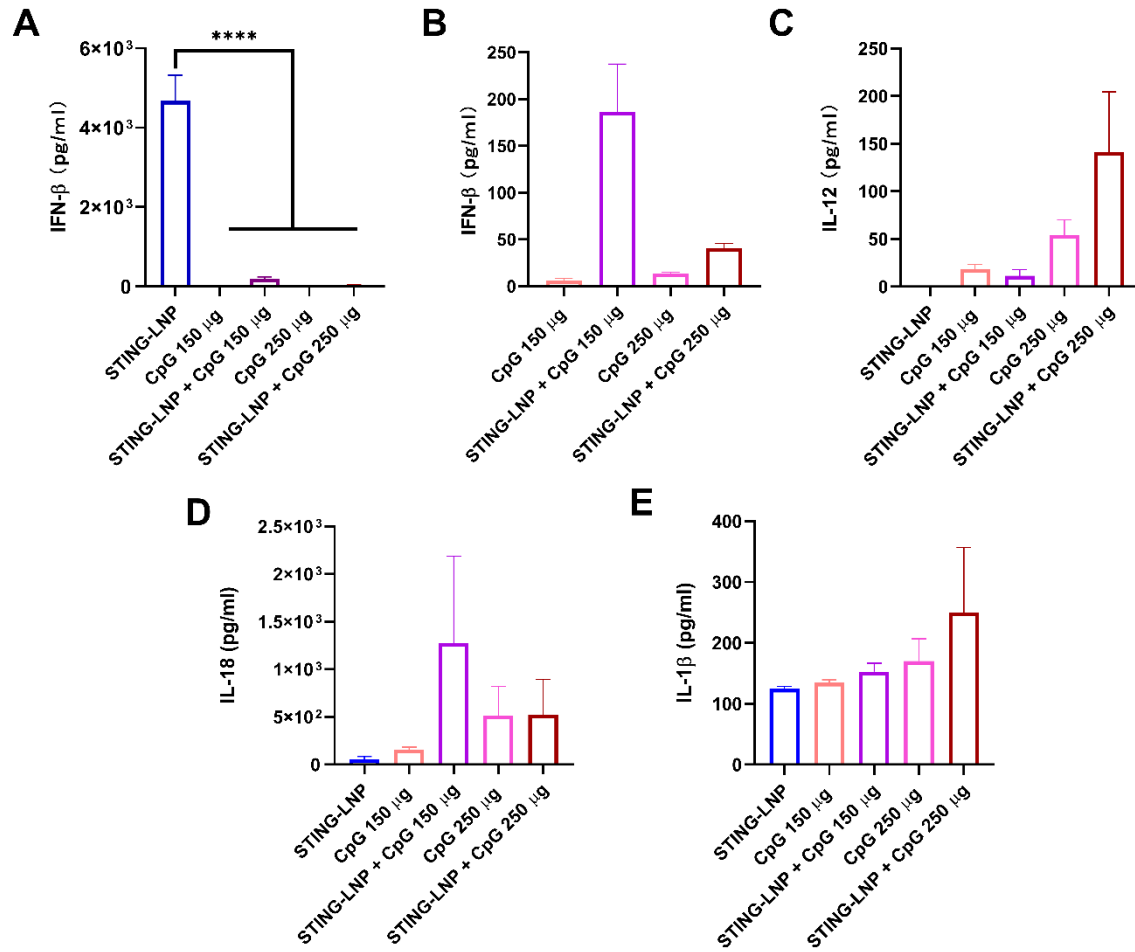
### **Combining the STING-LNP with CpG-ODN enhances the production of cytokines required for the induction of CIML NK cells**

The STING-LNP was prepared using YSK12-C4, an ionizable lipid known for its high affinity for immune cells and efficient cytosolic delivery due to its fusogenic activity (19, 24, 73, 74, 78). The lipid composition of the STING-LNP (YSK12-C4:cholesterol:1,2-dimyristoyl-rac-glycerol, methoxypolyethylene glycol 2000) at a molar ratio of 85:15:1 was prepared using the alcohol dilution method (Fig. 13) (19). The resulting LNPs had a diameter of  $170 \pm 15$  nm, a polydispersity index (PDI) of  $0.048 \pm 0.023$ , a zeta potential of  $7.1 \pm 0.4$  mV, and a c-di-GMP recovery rate of  $13 \pm 2\%$  (mean  $\pm$  SD, n=4). Previous studies have shown that STING-LNP remains stable for 270 days at 4 °C. We have previously showed that the physical characteristics of STING-LNP are stable for 270 days at 4 °C after preparation (19). The required amount of STING-LNP and CpG-ODN were mixed and characterized directly before injection as a combination therapy. The diameter, PDI and the zeta potential of the complex was  $195 \pm 10$  nm,  $0.082 \pm 0.021$  and  $-7.2 \pm 0.4$  mV, respectively (mean  $\pm$  SD, n=3). Two in vivo doses of CpG-ODN were screened for inducing the cytokines essential for NK cell activation and differentiation into CIML NK cells: IFN- $\beta$ , IL-12, IL-18, and IL-1 $\beta$  (45, 79). Mice were intravenously injected with the STING-LNP (4  $\mu$ g), CpG-ODN (150 or 250  $\mu$ g) or their combination. STING-LNP monotherapy significantly induced IFN- $\beta$ , whereas CpG-ODN alone induced very low levels. The combination therapy, however, induced much higher levels of IFN- $\beta$  compared to CpG-ODN monotherapy (Fig. 14A, 14B). STING-LNP monotherapy did not induce IL-12

and induced very low levels of IL-18. In addition, 150  $\mu\text{g}$  of CpG-ODN could induce very low levels of IL-12 and IL-18 (Fig. 14B and 14C). However, combining STING-LNP with 250  $\mu\text{g}$  of CpG-ODN induced high levels of both IL-12 and IL-18 (Fig. 14C and 14D). Furthermore, IL-1 $\beta$  levels were similar across all groups (Fig. 14E). These results highlight the importance of combining CpG-ODN with STING-LNP to induce IL-12 and IL-18. Suggesting that 250  $\mu\text{g}$  of CpG-ODN combined with the STING-LNP, which resulted in the highest level of IL-12, indispensable for memory-like NK cell development, can be considered as a good candidate for CIML NK cell induction. Thus, the dose of 250  $\mu\text{g}$  of CpG-ODN was selected to be combined with 4  $\mu\text{g}$  STING-LNP for the following experiments.



**Fig. 13. STING-LNP preparation method.** C-di-GMP solution and the lipid solution were mixed together, while vortexing using buffer system, for the purpose of STING-LNP preparation. The solution was ultrafiltered for the removal of the non encapsulated c-di-GMP.

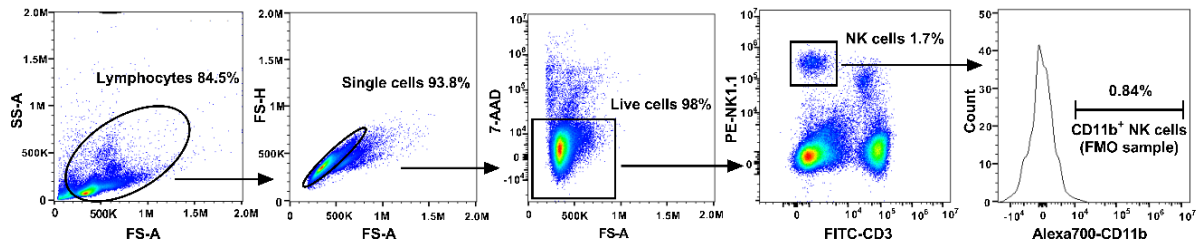


**Fig. 14. Combining STING-LNP with CpG-ODN enhances the production of cytokines.** (A) IFN-β concentration in serum (all groups). (B) IFN-β concentration in serum (without STING-LNP data). (C) IL-12 concentration in the serum. (D) IL-18 concentration in the serum (E) IL-1β concentration in the serum. Mice were intravenously injected with the STING-LNP (4 μg/mouse of c-di-GMP), CpG-ODN (150 or 250 μg) or combination of both. Blood samples were collected after 2 hours. Each cytokine concentration in the sera was measured by ELISA. The values represent the mean ± SEM (n = 3, \*\*\*\*p<0.0001).

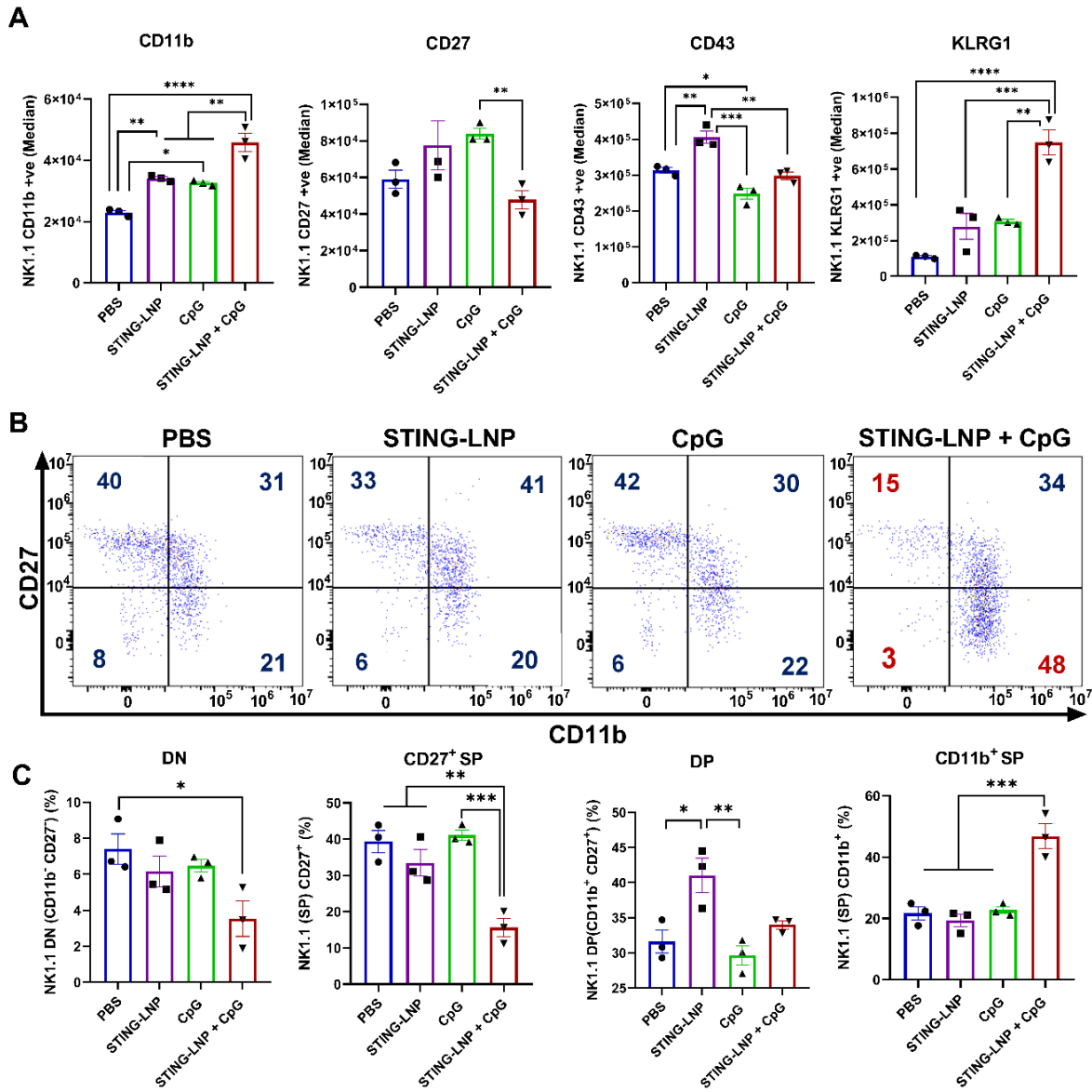
### **Combination of the STING-LNP and CpG-ODN stimulates the induction of memory-like NK cells**

We hypothesized that the combination therapy of STING-LNP and CpG-ODN induces not only the activation of NK cells but also promotes their differentiation into memory-like NK cells, the most mature phenotype characterized as CD11b<sup>high</sup> CD27<sup>low</sup> NK cells. To test this, mice with lung metastasis were intravenously administered with PBS, the STING-LNP, CpG-ODN or their combination for two cycles on days 6 and 10. The spleens were then collected and analyzed for NK cell maturation and activation and for identifying the presence of memory-like NK cells using FCM. First, gating the NK cell population (NK1.1<sup>+</sup>CD3<sup>-</sup>) was performed, then the expression of CD11b, CD27, CD43, and KLRG1 was checked (Fig. 15). The combination therapy of STING-LNP and CpG-ODN resulted in a significant increase in the CD11b expression and the percentage of CD11b<sup>+</sup> NK cells compared to the other groups (Fig. 16A and 17). Conversely, the level of CD27 expression and the percentage of CD27<sup>+</sup> NK cells were drastically reduced following the combination therapy when compared to the other groups (Fig. 16A and 17). Additionally, KLRG1 expression and the percentage of KLRG1<sup>+</sup> NK cells were significantly increased following the combination therapy when compared to the other groups (Fig. 16A and 17). These data indicate that combining STING-LNP and CpG-ODN can induce both the maturation and activation of NK cells. We further examined the induction of memory-like NK cells based on the simultaneous expression of CD11b and CD27, as we hypothesized that inducing the most mature phenotype of NK cells would result in memory-like NK cells generation. The surface density of CD11b and CD27 divides the

murine NK cells into four populations as follows; CD11b<sup>-</sup> CD27<sup>-</sup> cells (double negative: DN), CD11b<sup>-</sup> CD27<sup>+</sup> cells (single positive: CD27<sup>+</sup> SP) , CD11b<sup>+</sup> CD27<sup>+</sup> cells (double positive: DP) and CD11b<sup>+</sup> CD27<sup>-</sup> cells (CD11b<sup>+</sup> SP). These four subsets represent the developmental stages of the NK cells from the most immature phenotype, capable of cytokine secretion, to the most mature and potent NK cells with the highest cytolytic function (80). The dot plots in Fig. 16B show that the NK cell population changed significantly only after the combination therapy. The percentage of the DN population was comparable among all groups except for the combination group that was drastically reduced (Fig. 16C). Similarly, CD27<sup>+</sup> SP population percentage was comparable across all groups, but it was drastically decreased following the combination therapy (Fig. 16C). In addition, the DP population percentage was similar among all groups except for the monotherapy of STING-LNP which showed a slight increase but it was comparable to that of the combination therapy (Fig. 16C). Moreover, the CD11b<sup>+</sup> SP population percentage was exclusively and significantly increased following the administration of the combination therapy of STING-LNP and CpG-ODN when compared to the other groups (Fig. 16C). Despite these drastic changes in NK cell subsets following the four treatment groups; there was no significant change in the percentage of NK cells (Fig. 18). These results confirm that the combination therapy causes an increase in the numbers of the most mature and functional subset of NK cells as CD11b<sup>high</sup> CD27<sup>low</sup> with a high KLRG1 expression. This phenotype is similar to the phenotype of the memory NK cells induced by a viral infection with KLRG1<sup>high</sup> and CD27<sup>low</sup> expression (40).

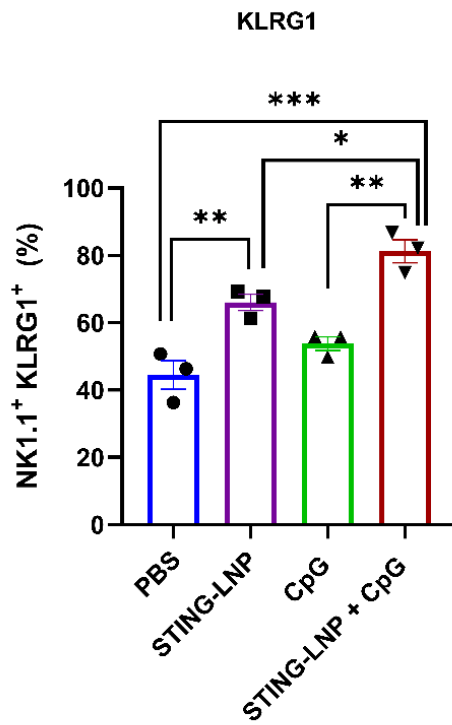
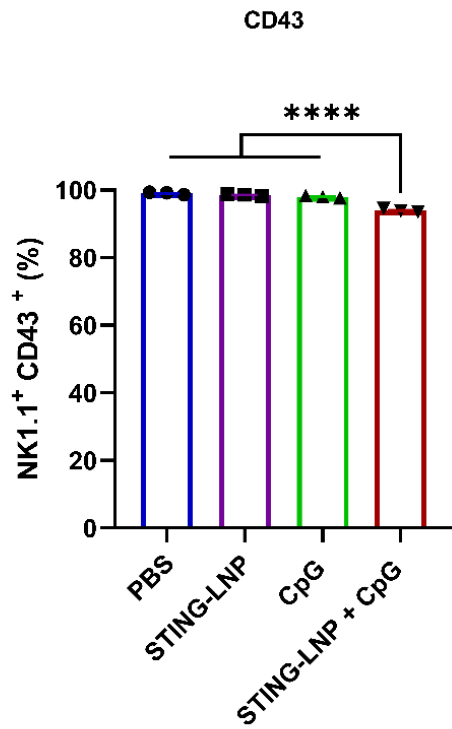
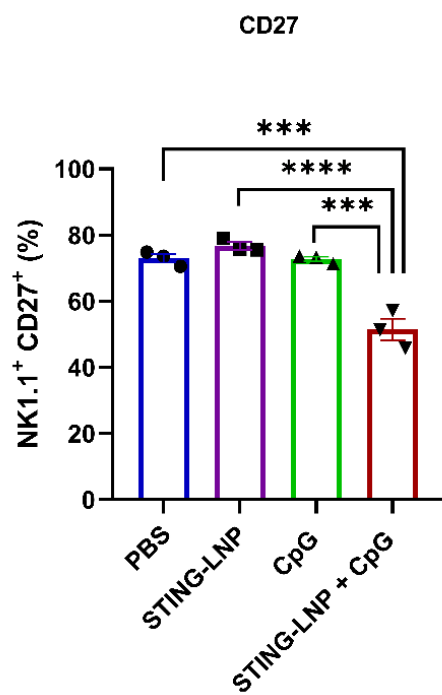
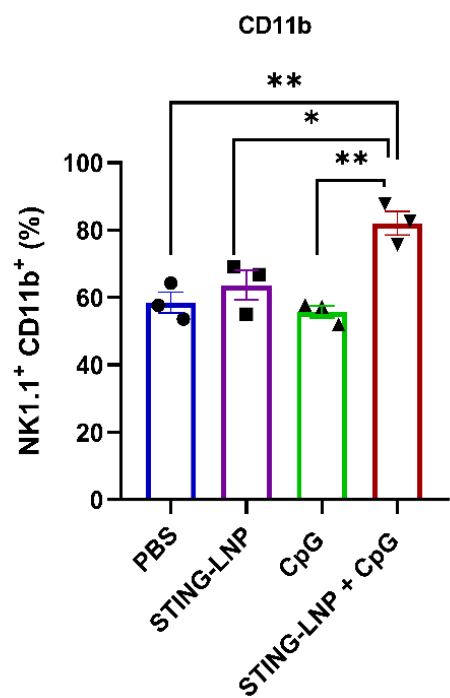


**Fig. 15. Gating strategy for FCM analysis in the spleen.** After gating live cells and lymphocytes, NK cells were identified as a CD3<sup>-</sup>NK1.1<sup>+</sup> cells. The CD11b<sup>+</sup> gate, the CD27<sup>+</sup> gate, CD43<sup>+</sup> gate and the KLRG1<sup>+</sup> gate were set by using an isotype control.

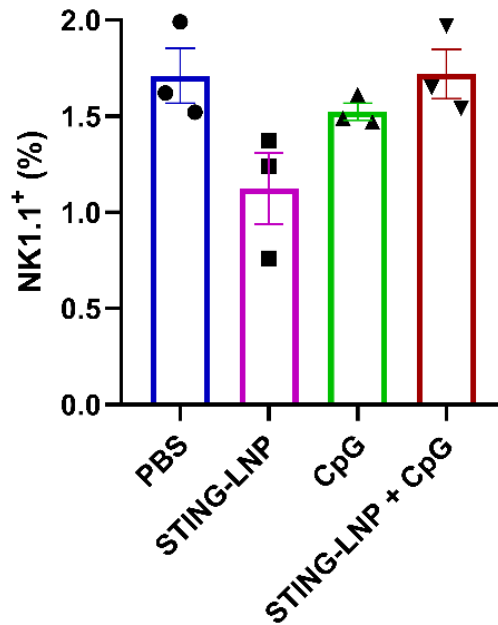


**Fig. 16. Combining STING-LNP and CpG-ODN can generate memory-like NK cells.** Mice were intravenously injected with B16-F10-Luc2 cells ( $2 \times 10^5$ ). PBS, STING-LNP (4  $\mu$ g/mouse of c-di-GMP), CpG-ODN (250  $\mu$ g) or a combination thereof were intravenously injected. **(A)** The FI (Median) of CD11b, CD27, CD43 and KLRG1 expression in NK cells. **(B)** Dot plots show

the expression of CD11b and CD27 on splenic NK cells (gated on CD3 $\epsilon$ <sup>-</sup>NK1.1<sup>+</sup>) on day 14. Numbers indicate percentages. **(C)** The percentages of DN (CD11b<sup>-</sup> CD27<sup>-</sup>), CD27<sup>+</sup> SP (CD11b<sup>-</sup> CD27<sup>+</sup>), DP (CD11b<sup>+</sup> CD27<sup>+</sup>) and CD11b<sup>+</sup> SP (CD11b<sup>+</sup> CD27<sup>-</sup>) NK cells. The values represent the mean  $\pm$  SEM (n = 3), \*\*\*\*p<0.0001; \*\*\*p<0.001; \*\*p<0.01; \*p<0.05). DN, double negative; SP, single positive; DP, double positive.



**Fig. 17. Combining STING-LNP and CpG-ODN can induce the maturation and activation of NK cells.** Mice were intravenously injected with  $2 \times 10^5$  B16-F10-Luc2 cells. PBS, STING-LNP (4  $\mu$ g/mouse of c-di-GMP), CpG-ODN (250  $\mu$ g) or combination of both were intravenously injected. The percentages of CD11b<sup>+</sup> NK cells, CD27<sup>+</sup> NK cells, CD43<sup>+</sup> NK cells and KLRG1<sup>+</sup> NK cells in the spleen. The values represent the mean  $\pm$  SEM (n = 3, \*\*\*\*p<0.0001; \*\*\*p<0.001; \*\*p<0.01; \*p<0.05).

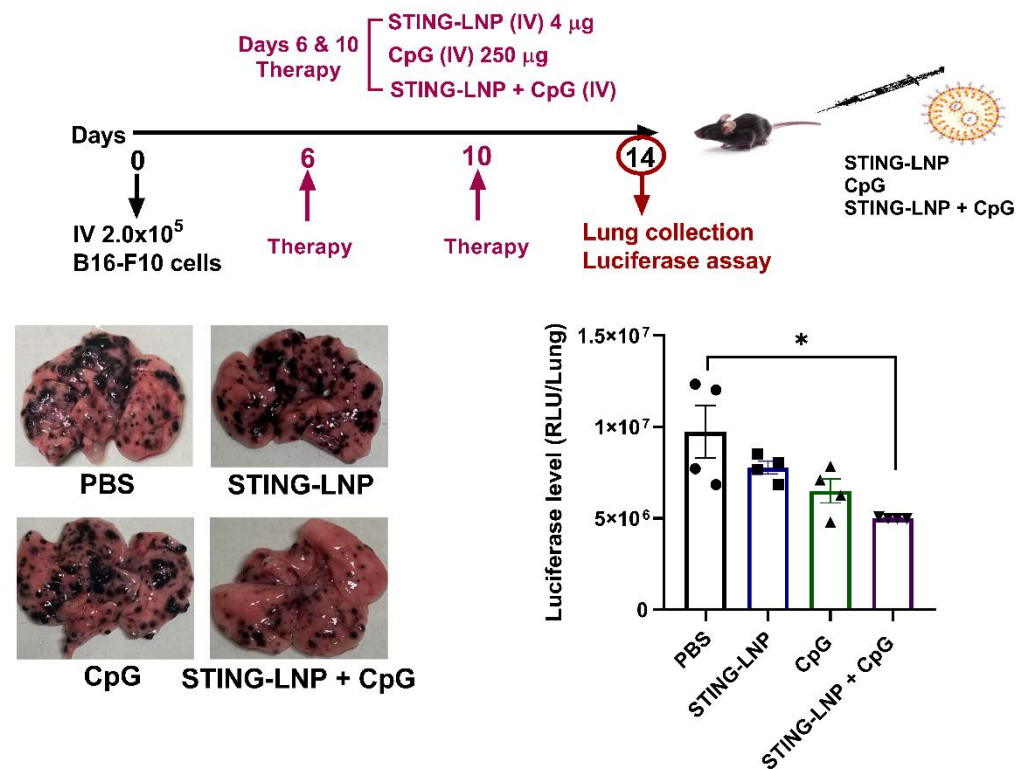


**Fig. 18. NK cell percentage among the four treatment groups.** Mice were intravenously injected with  $2 \times 10^5$  B16-F10-Luc2 cells. PBS, STING-LNP (4  $\mu$ g/mouse of c-di-GMP), CpG-ODN (250  $\mu$ g) or combination of both were intravenously injected. The percentages of NK1.1 cells in the spleen. The values represent the mean  $\pm$  SEM (n = 3).

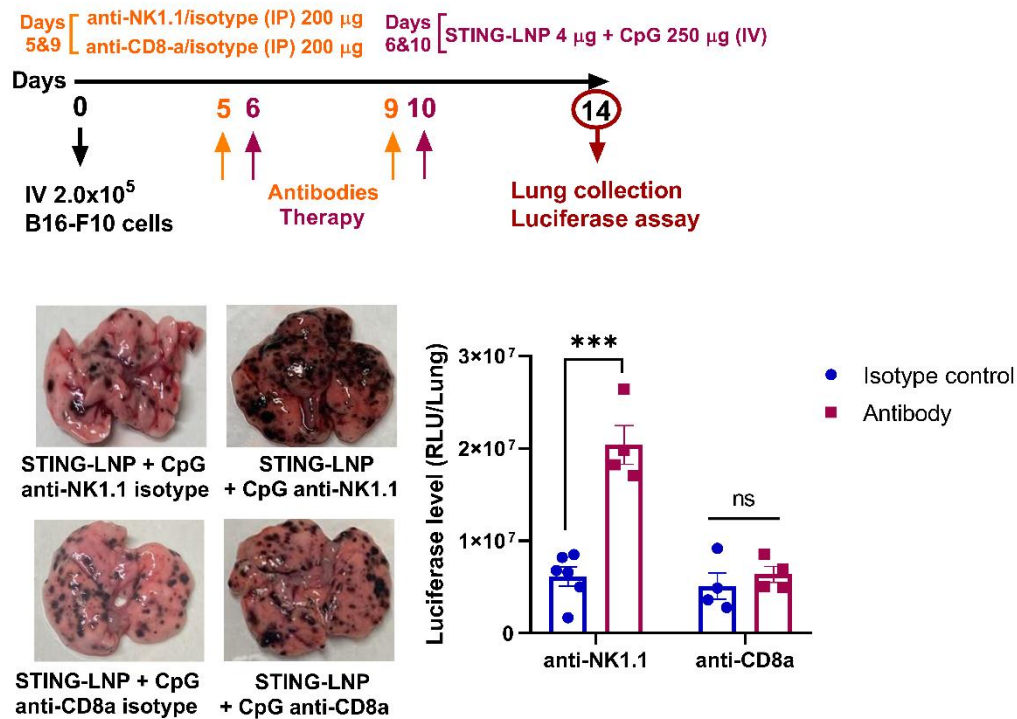
### **NK cells play a significant role in the enhancement of the antitumor effect of STING-LNP and CpG-ODN**

Next, we assessed the therapeutic efficacy of the combination therapy against a B16-F10 lung metastasis model. Mice with melanoma lung metastasis were intravenously administered with PBS, the STING-LNP, CpG-ODN or a combination of both for two cycles on days 6 and 10 as shown in Fig. 19A. This was followed by Lung collection on day 14 for checking the antitumor effect. The combination therapy clearly caused a reduction in the number of tumor colonies in the lung (Fig. 19A). Moreover, the combination therapy significantly reduced the luciferase activity, indicating a reduction in the tumor cell numbers (Fig. 19A). To determine the contribution of NK cells and CD8<sup>+</sup> T cells to the antitumor effect of the combination therapy, we used specific antibody treatments. The antitumor effect was abrogated by anti-NK1.1 antibody treatment, while it was maintained following anti-CD8a antibody treatment (Fig. 19B). This indicates that the antitumor effect of the combination therapy is primarily dependent on NK cells.

**A**



**B**



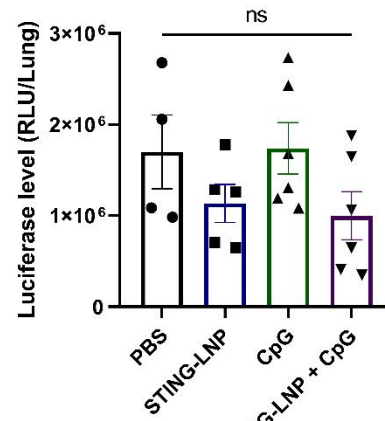
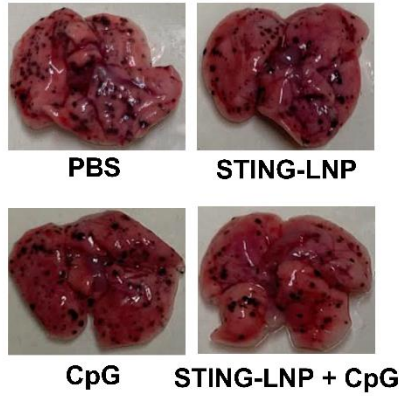
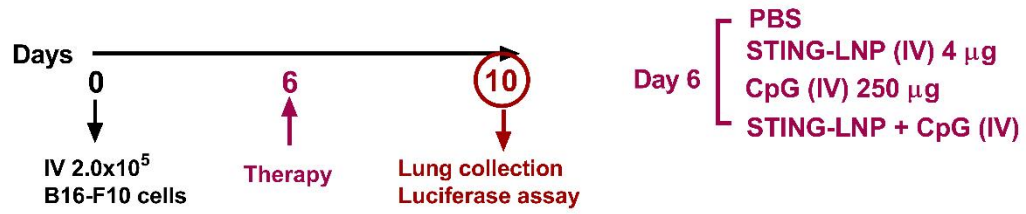
**Fig. 19. NK cells play a significant role in the enhancement of the antitumor effect of STING-LNP and CpG-ODN. (A)** Antitumor effect of monotherapies and combination therapy against B16-F10 lung metastasis. Mice were intravenously injected with B16-F10-Luc2 cells ( $2 \times 10^5$ ). PBS, STING-LNP (4  $\mu$ g/mouse of c-di-GMP), CpG-ODN (250  $\mu$ g) or a combination thereof were intravenously injected for two cycles. The images depict the lungs of various treatment groups along with the quantitative analysis of lung metastasis based on luciferase activity levels. The values represent the mean  $\pm$  SEM (n = 4, \*P<0.05). **(B)** The effect of the depletion of NK cells or cytotoxic T lymphocytes (CTL) on antitumor activity by combination therapy. B16-F10 lung metastasis bearing mice were intraperitoneally injected with anti-NK1.1 (200  $\mu$ g) or anti-CD8a (200 $\mu$ g) or their isotype control at similar doses. The images show the lungs of various treatment groups alongside the quantitative analysis of lung metastasis determined by luciferase activity levels. The RLU per whole lung for the PBS-treated mice was normalized to 1. The values represent the mean  $\pm$  SEM (n = 4-6, \*P<0.05). ns, non-significant.

### **Two treatment cycles are essential for tumor reduction and CIML NK cell induction**

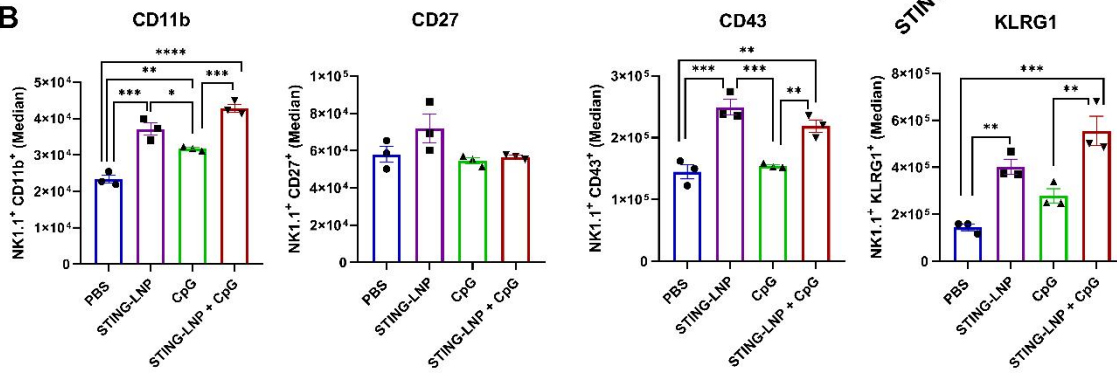
To determine whether two cycles of combination therapy are necessary for significant antitumor effects and CIML NK cell induction, or one cycle might be sufficient. Mice with B16-F10 lung metastasis were administered PBS, STING-LNP, CpG-ODN, or a combination of both on day 6. Then lungs were collected on day 10 and not 14, maintaining the same four-day interval between treatment and termination as in the two-cycle protocol (Fig. 20A). Following

one cycle administration, no significant reduction in tumor burden was observed, though slight reductions were noted following the STING-LNP and combination therapy (Fig. 20A). To assess whether one cycle could activate NK cells and induce their differentiation into memory-like cells. A similar protocol was followed, then the spleens were collected, and surface markers were analyzed by FCM. The levels of expression and the percentage of CD11b and KLRG1 following the STING-LNP and the combination groups, were drastically elevated and reached similar levels to that after two cycles of the combination therapy (Fig. 20B and 21). We further analyzed the surface density of CD11b and CD27 on NK cells. The percentage of DN and CD27<sup>+</sup> SP populations was significantly reduced in the STING-LNP and combination therapy groups (Fig. 20C). In addition, the percentage of DP population was significantly elevated following the STING-LNP and combination therapy (Fig. 20C). However, the percentage of the CD11b<sup>+</sup> SP population was comparable among all groups (Fig. 20C). Collectively, the percentage of DN and CD27<sup>+</sup> SP populations were similar following one and two cycles of the combination therapy. Although DP was the highest population following one cycle, it did not reach the levels of CD11b<sup>+</sup> SP population which was drastically increased following the two-cycle administration (Fig. 22). These results indicate that one cycle of the combination therapy can enhance NK cell maturation and activation, but it was insufficient for inducing the most potent phenotype of NK cells which express CD11b<sup>high</sup>CD27<sup>low</sup>, that we speculate to be memory-like NK cells.

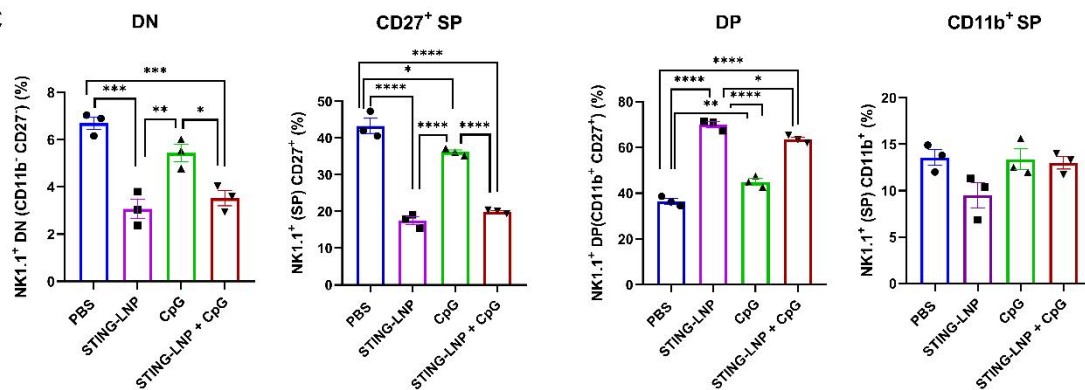
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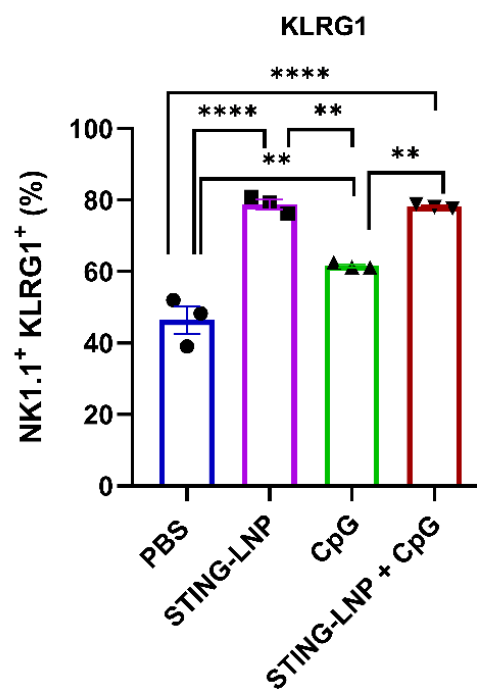
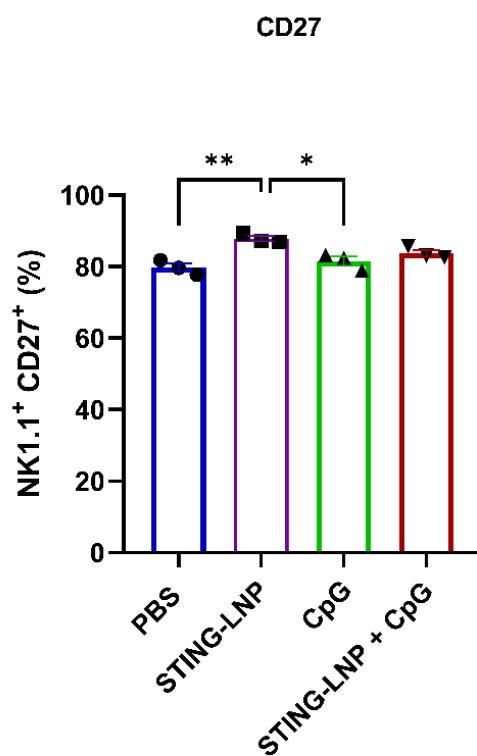
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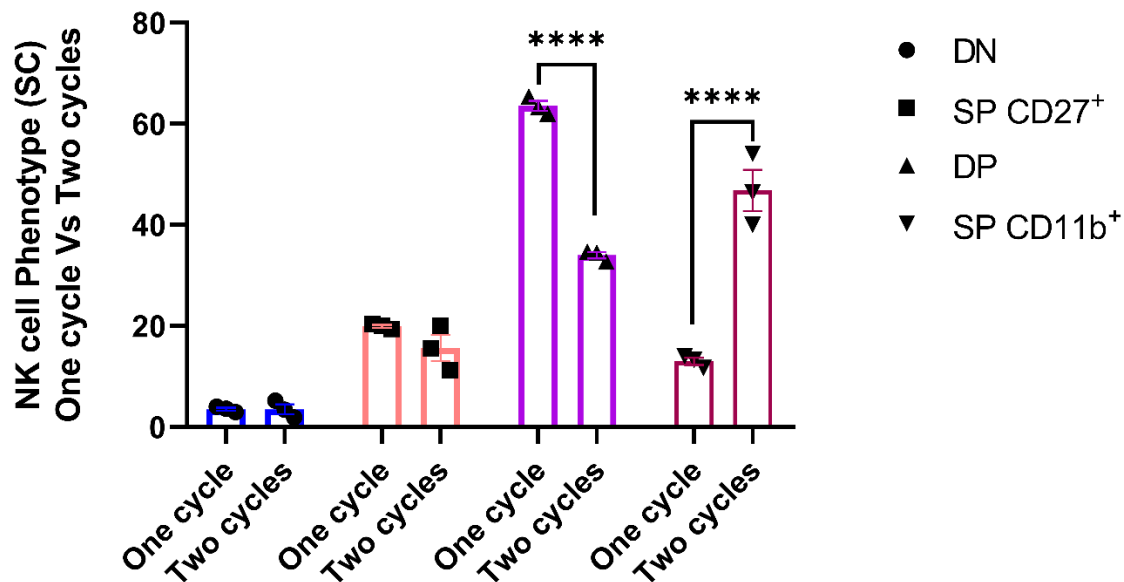
**C**



**Fig. 20. Two cycles of STING-LNP and CpG-ODN are indispensable for tumor reduction and memory-like NK cell induction. (A)** Antitumor effect of monotherapies and combination therapy against B16-F10 lung metastasis. Mice were intravenously injected with B16-F10-Luc2 cells ( $2 \times 10^5$ ). PBS, STING-LNP (4  $\mu$ g/mouse of c-di-GMP), CpG-ODN (250  $\mu$ g) or a combination thereof were intravenously injected for one cycle. Images represent the lungs of different treatment groups and quantitative analysis of lung metastasis based on the values of the luciferase activities. The values represent the mean  $\pm$  SEM (n = 4-6). ns, non-significant. **(B)** The FI (Median) of CD11b, CD27, CD43 and KLRG1 expression in NK cells. **(C)** The percentages of DN (CD11b<sup>-</sup> CD27<sup>-</sup>), CD27<sup>+</sup> SP (CD11b<sup>-</sup> CD27<sup>+</sup>), DP (CD11b<sup>+</sup> CD27<sup>+</sup>) and CD11b<sup>+</sup> SP (CD11b<sup>+</sup> CD27<sup>-</sup>) NK cells (gated on CD3 $\epsilon$ <sup>-</sup> NK1.1<sup>+</sup>) on day 10. The values represent the mean  $\pm$  SEM (n = 3, \*\*\*\*p<0.0001; \*\*\*p<0.001; \*\*p<0.01; \*p<0.05). DN, double negative; SP, single positive; DP, double positive.



**Fig. 21. One cycle of combination therapy can induce the maturation and activation of NK cells.** Mice were intravenously injected with  $2 \times 10^5$  B16-F10-Luc2 cells. PBS, STING-LNP (4  $\mu$ g/mouse of c-di-GMP), CpG-ODN (250  $\mu$ g) or combination of both were intravenously injected. The percentages of CD11b<sup>+</sup> NK cells, CD27<sup>+</sup> NK cells, CD43<sup>+</sup> NK cells and KLRG1<sup>+</sup> NK cells in the spleen. The values represent the mean  $\pm$  SEM (n = 3, \*\*\*\*p<0.0001; \*\*p<0.01; \*p<0.05).



**Fig. 22. Two cycles are indispensable for the induction of memory-like NK cells.** A collective chart illustrating the differences between the four developmental stages of NK cells following one and/or two cycles of STING-LNP and CpG-ODN administration. Mice were intravenously injected with  $2 \times 10^5$  B16-F10-Luc2 cells. PBS, STING-LNP (4  $\mu$ g/mouse of c-di-GMP), CpG-ODN (250  $\mu$ g) or combination of both were intravenously injected. The

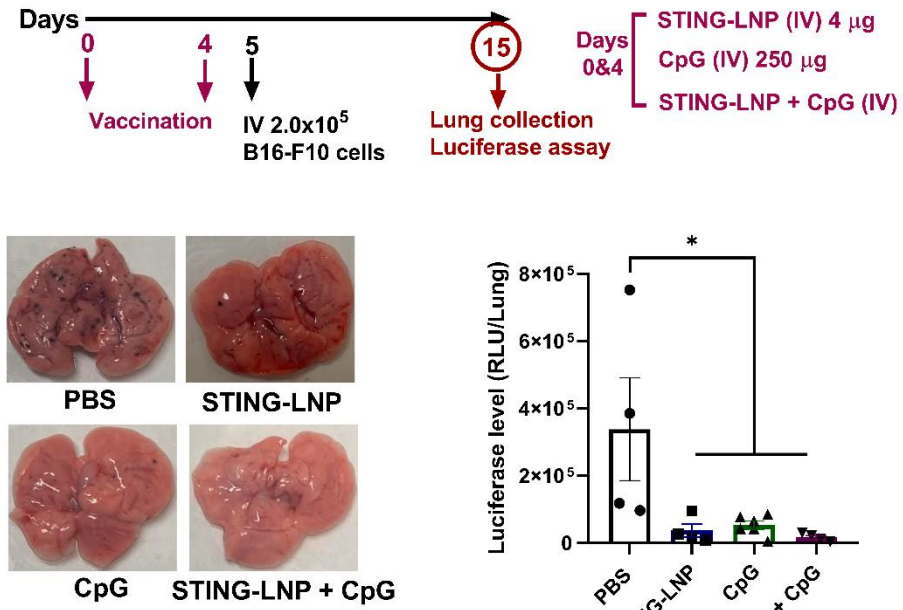
percentages of DN (CD11b<sup>-</sup> CD27<sup>-</sup>), CD27<sup>+</sup> SP (CD11b<sup>-</sup> CD27<sup>+</sup>), DP (CD11b<sup>+</sup> CD27<sup>+</sup>) and CD11b<sup>+</sup> SP (CD11b<sup>+</sup> CD27<sup>-</sup>) NK cells (gated on CD3 $\epsilon$ <sup>-</sup>NK1.1<sup>+</sup>). The values represent the mean  $\pm$  SEM (n = 3, \*\*\*\*p<0.0001). DN, double negative; SP, single positive; DP, double positive; SC, STING-LNP + CpG-ODN; Vs, versus.

### **Long-term vaccination effect through the sustained persistence of memory-like NK cells induced by STING-LNP and CpG-ODN**

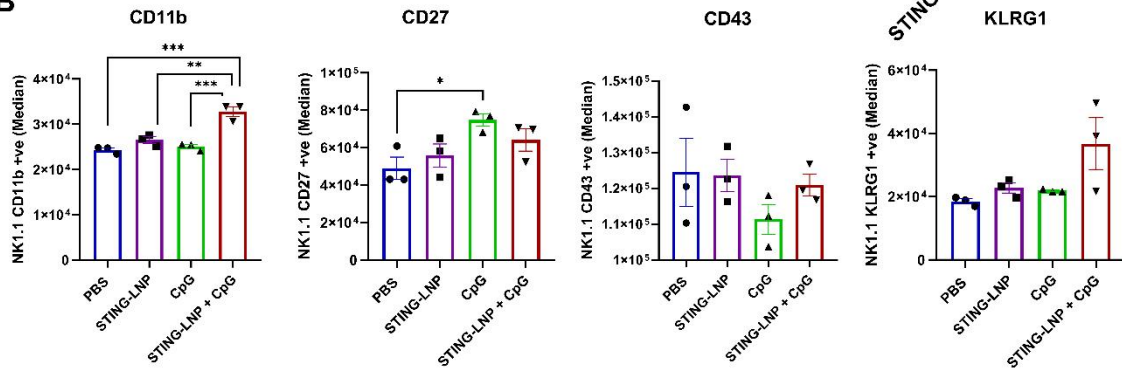
We investigated whether the combination therapy could induce a prophylactic effect against the B16-F10 lung metastasis model, aiming to confirm that CD11b<sup>high</sup> CD27<sup>low</sup> NK cells are, in fact, memory-like NK cells. In the prophylactic protocol, mice were administered PBS, STING-LNP, CpG-ODN, or a combination of both intravenously on days 0 and 4, followed by tumor inoculation on day 5 as shown in Fig. 23A. All treatment groups showed a drastic reduction in the tumor burden (Fig. 23A). This suggests that the anti-tumor activity may be due to the immediate activation of the innate immune response, independent of memory-like NK cell responses. We then examined the phenotype of the NK cells on day 15 following a similar protocol but using spleen samples instead. The expression level of CD11b and the percentage of CD11b<sup>+</sup> NK cells was drastically induced only after the combination therapy (Fig. 23B and Fig. 24). Moreover, the percentage of CD27<sup>+</sup> NK cells were significantly decreased only following the combination therapy (Fig. 24). The expression and percentage of KLRG1 were elevated following the combination therapy (Fig. 23B and Fig. 24). We further analyzed the simultaneous surface density expression of CD11b and CD27 on the NK cells per group. The DN

population percentage was similar among the groups except for a slight reduction following the combination group (Fig. 23C). Additionally, the percentage of CD27<sup>+</sup> SP was significantly decreased following the combination therapy when compared to each monotherapy group (Fig. 23C). Furthermore, the percentage of the DP population was drastically reduced following the administration of CpG-ODN, and the combination compared to the PBS group (Fig. 23C). Most notably, the percentage of the CD11b<sup>+</sup> SP population significantly increased only after the combination therapy when compared to all other groups (Fig. 23C).

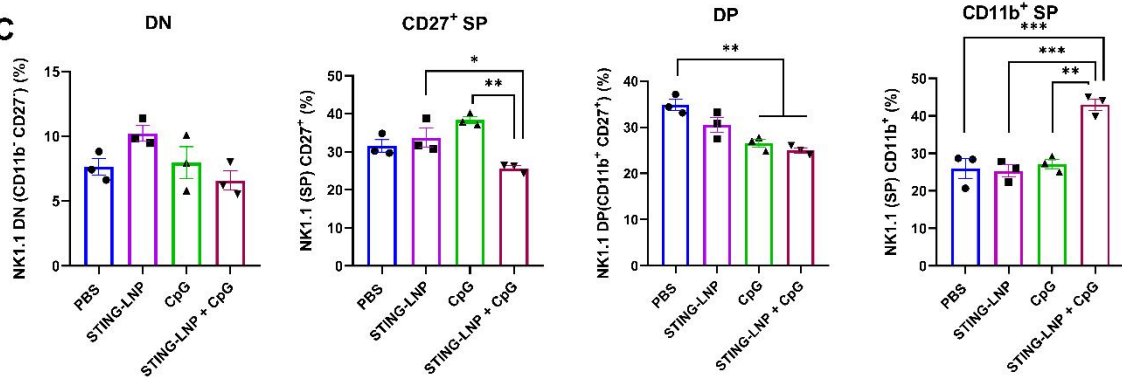
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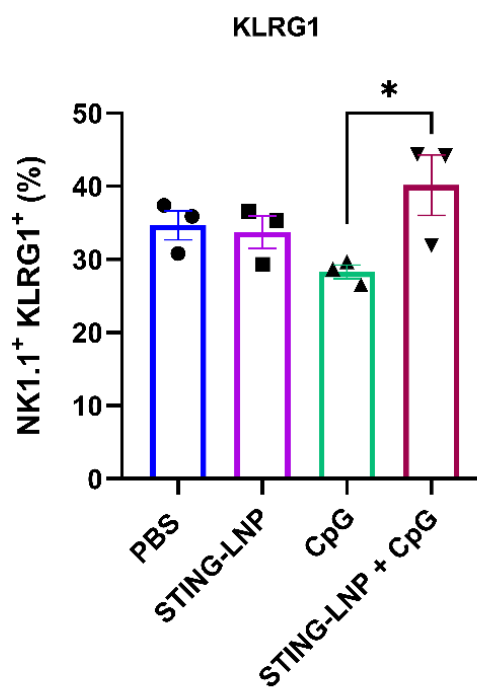
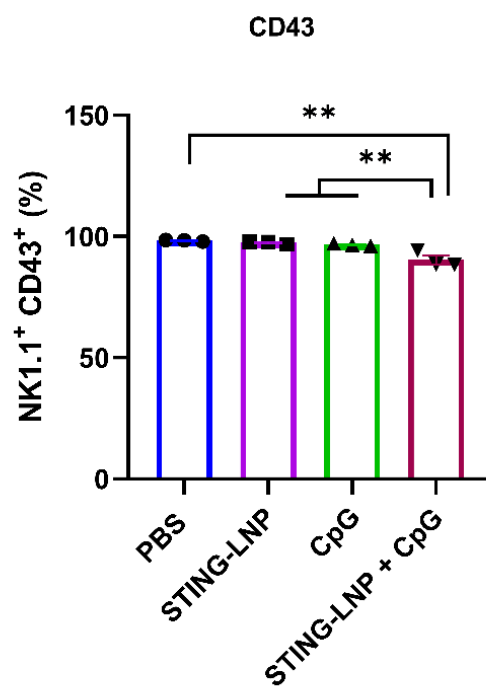
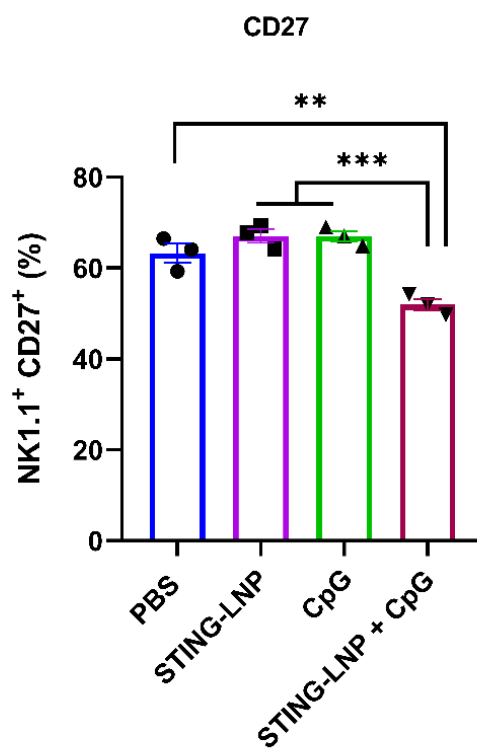
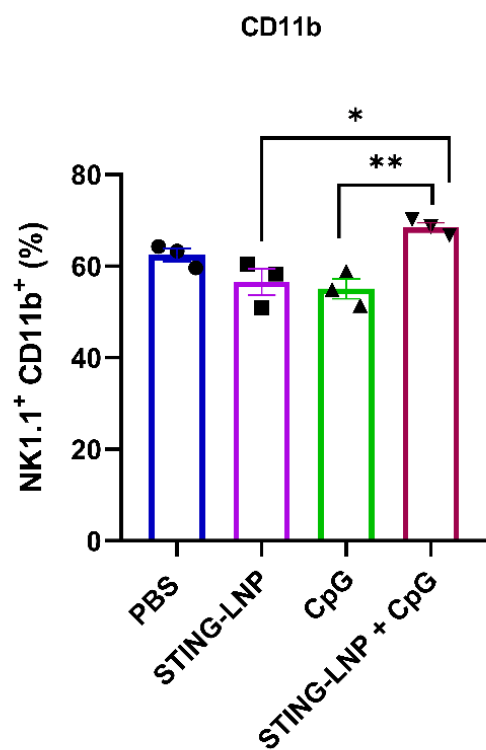
**B**



**C**



**Fig. 23. Antitumor effect is based on the innate activation of NK cells. (A)** Antitumor effect of monotherapies and the combination therapy against B16-F10 lung metastasis following a prophylactic protocol. Mice were intravenously injected with PBS, STING-LNP (4  $\mu$ g/mouse of c-di-GMP), CpG-ODN (250  $\mu$ g) or combination thereof for two cycles. After one day, the mice were intravenously injected with  $2 \times 10^5$  B16-F10-Luc2 cells. Representative photos of the lungs of different treatment groups and quantitative analysis of lung metastasis based on the values of luciferase activities. The values represent the mean  $\pm$  SEM (n = 4-6, \*P<0.05). **(B)** The FI (Median) of CD11b, CD27, CD43 and KLRG1 expression in NK cells. **(C)** The percentages of DN (CD11b<sup>-</sup> CD27<sup>-</sup>), CD27<sup>+</sup> SP (CD11b<sup>-</sup> CD27<sup>+</sup>), DP (CD11b<sup>+</sup> CD27<sup>+</sup>) and CD11b<sup>+</sup> SP (CD11b<sup>+</sup> CD27<sup>-</sup>) NK cells. The values represent the mean  $\pm$  SEM (n = 3, \*\*\*\*p<0.0001; \*\*\*p<0.001; \*\*p<0.01; \*p<0.05). DN, double negative; SP, single positive; DP, double positive.

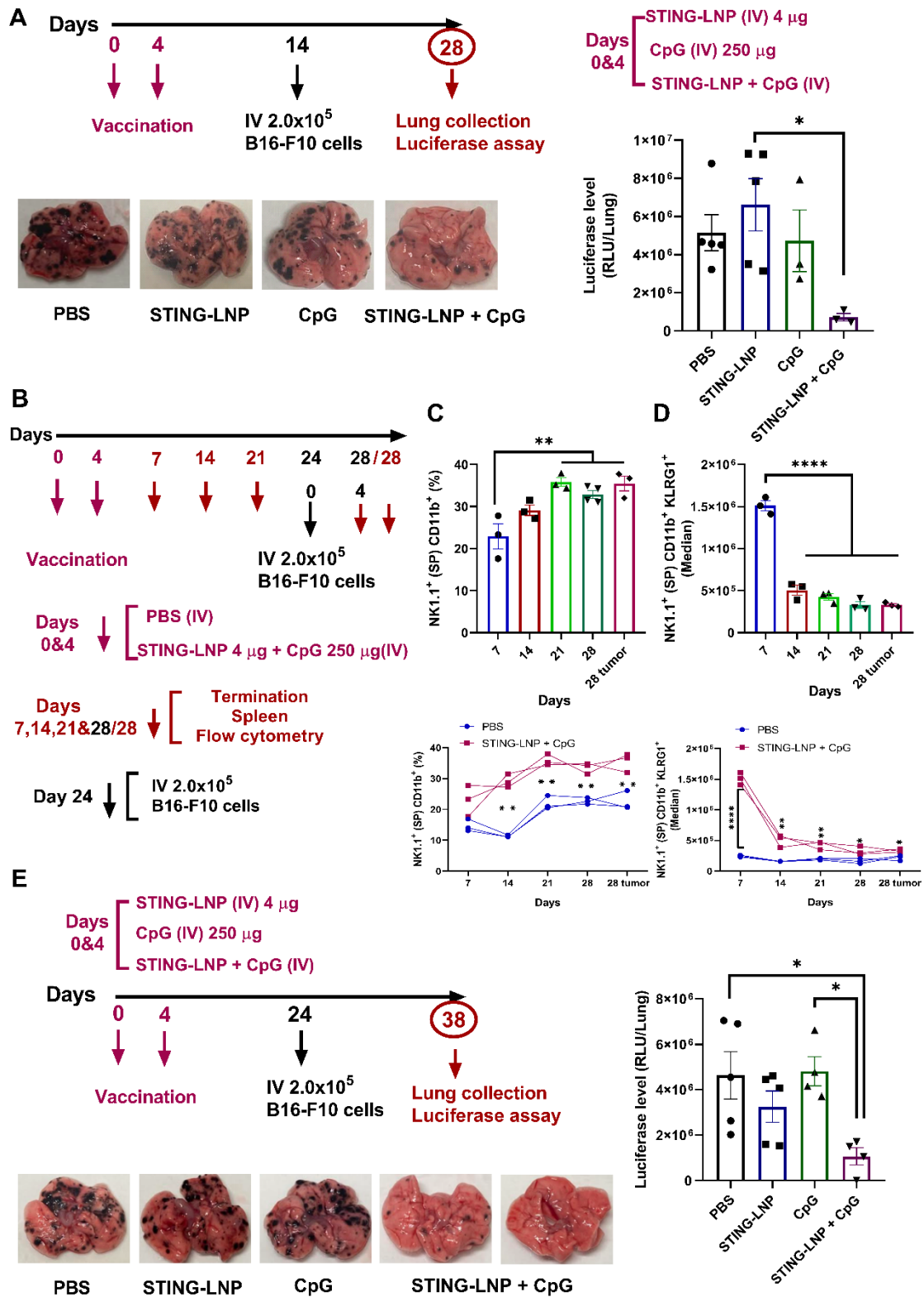


**Fig. 24. Prophylactic administration of STING-LNP and CpG-ODN induce the activation and maturation of NK cells.** PBS, STING-LNP (4 µg/mouse of c-di-GMP), CpG-ODN (250 µg) or a combination of both were intravenously injected. Followed by intravenous injection of the vaccinated mice with  $2 \times 10^5$  B16-F10-Luc2 cells. The percentages of CD11b<sup>+</sup> NK cells, CD27<sup>+</sup> NK cells, CD43<sup>+</sup> NK cells and KLRG1<sup>+</sup> NK cells in the spleen. The values represent the mean  $\pm$  SEM (n = 3, \*\*\*p<0.001; \*\*p<0.01; \*p<0.05).

To evaluate the memory function of CD11b<sup>high</sup>CD27<sup>low</sup> NK cells retained on day 15 after two vaccination cycles, we implemented a modified prophylactic protocol. In this prophylactic protocol, mice received intravenous administrations of PBS, STING-LNP, CpG-ODN, or a combination of both on days 0 and 4, followed by tumor inoculation on day 14. Lungs were collected on day 28, and luciferase activity was measured among the groups (Fig. 25A). Notably, only the combination therapy showed a dramatic preventative effect, while the other groups were comparable (Fig. 25A). This finding confirmed that the CD11b<sup>high</sup> CD27<sup>low</sup> phenotype corresponds to memory-like NK cells, marking the first confirmation of this phenotype.

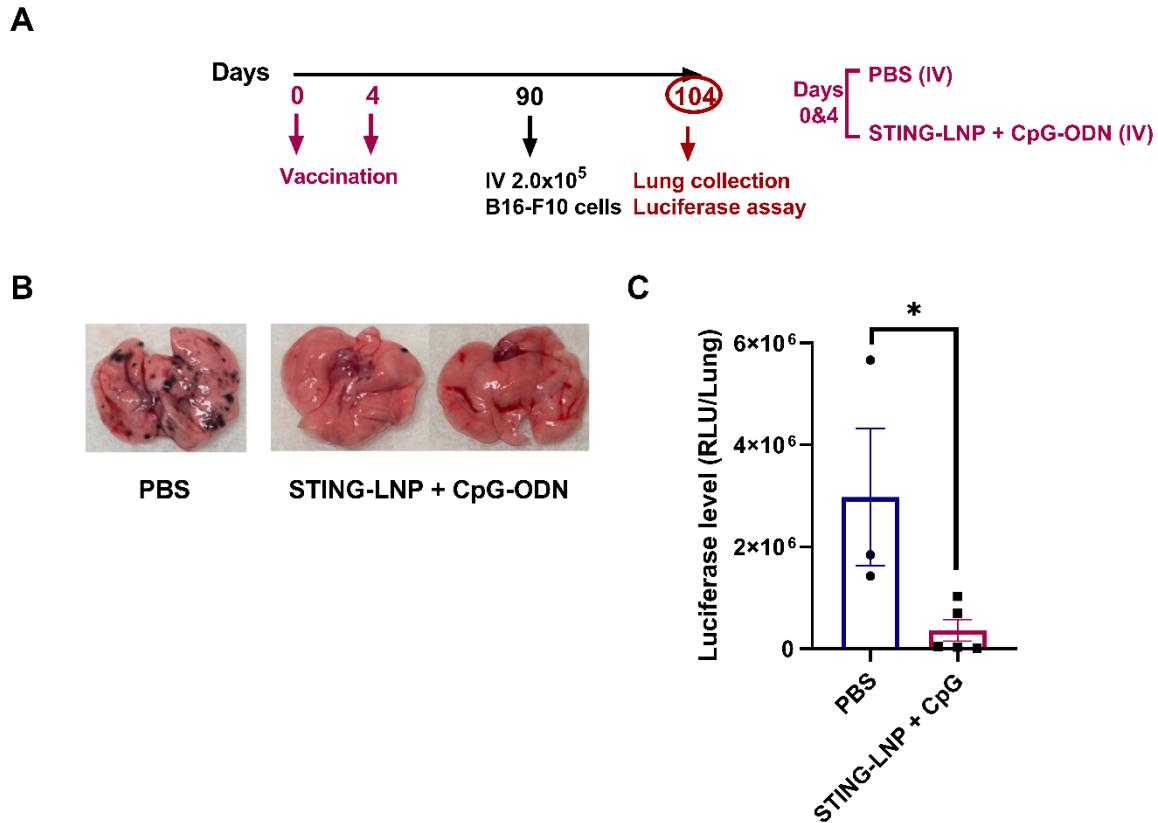
To track the induction and prolonged existence of memory-like NK cells following the combination therapy with and without tumor inoculation, we used a prophylactic protocol where two cycles of PBS or a combination of STING-LNP and CpG-ODN were administered intravenously on days 0 and 4 (Fig. 25B). Then the phenotype of the NK cells in the spleen was checked by FCM on days 7, 14, 21 and 28. On day 24, another group of mice was challenged with B16-F10 cells, and the phenotype of memory-like NK cell was assessed 4 days

post-inoculation. The SP CD11b<sup>+</sup> NK cell population was induced following the vaccination starting from day 7, significantly increasing compared to the PBS group on day 14, peaking on day 21, and persisting thereafter (Fig. 25C). Additionally, the expression of KLRG1, a differentiation marker, was tracked among the different days. The SP CD11b<sup>+</sup> KLRG1<sup>+</sup> expression level was significantly elevated on day 7 just after the two-cycle vaccination when compared to the other days and the PBS group (Fig. 25D). Unexpectedly, challenging mice with tumor cells did not affect the SP CD11b<sup>+</sup> NK cells or the SP CD11b<sup>+</sup> KLRG1<sup>+</sup> expression levels (Fig. 25C and 25D). To examine the prolonged prophylactic effect of the extended lifespan of memory-like NK cells induced by the combination therapy, we administered two cycles of PBS, STING-LNP, CpG-ODN, or a combination of both on days 0 and 4, followed by tumor inoculation on day 24. Lungs were collected on day 38, and luciferase activity was measured among the groups (Fig. 25E). Only the combination of STING-LNP and CpG-ODN resulted in a significant preventative antitumor effect, with one sample appearing completely protected against tumor induction (Fig. 25E). This prolonged prophylactic effect persisted up to 3 months post-vaccination (Fig. 26). These findings indicate that the sustained existence of CD11b<sup>high</sup> CD27<sup>low</sup> memory-like NK cells induced by the STING-LNP and CpG-ODN vaccination results in a prolonged prophylactic effect against the melanoma lung metastasis.



**Fig. 25. The sustained existence of the memory-like NK cells induced by the combination of STING-LNP and CpG-ODN resulted in a prolonged protection against the development of melanoma lung metastasis. (A)** Prophylactic effect of monotherapies and combination therapy against B16-F10 lung metastasis. Mice were intravenously injected with PBS, the STING-LNP (4 µg/mouse of c-di-GMP), CpG-ODN (250 µg) or a combination thereof for two cycles. After 10 days, mice were intravenously injected with  $2 \times 10^5$  B16-F10-Luc2 cells. Representative photos of the lungs of different treatment groups and quantitative analysis of lung metastasis based on the values of luciferase activities. The values represent the mean  $\pm$  SEM (n = 3-5, \*P<0.05). **(B)** The sustained persistence of the memory-like NK cells induced by STING-LNP and CpG-ODN vaccination. Mice were intravenously injected with PBS or a combination of STING-LNP (4 µg/mouse of c-di-GMP) and CpG-ODN (250 µg) for two cycles. After 20 days one group of mice was intravenously injected with ( $2 \times 10^5$ ) B16-F10-Luc2 cells. **(C)** The percentage of CD11b<sup>+</sup> SP (CD11b<sup>+</sup> CD27<sup>-</sup>) NK cells on the indicated days after vaccination. Upper graph: comparison of vaccinated group SP CD11b<sup>+</sup> NK cell percentage among days. Bottom graph: comparison of SP CD11b<sup>+</sup> NK cell percentage between PBS and vaccinated group on each termination day. The values represent the mean  $\pm$  SEM (n = 3-4, \*\*p<0.01). **(D)** The FI (Median) of KLRG1 expression on CD11b<sup>+</sup> SP (CD11b<sup>+</sup> CD27<sup>-</sup>) NK cells on the indicated days after vaccination. Upper graph: comparison of vaccinated group KLRG1 expression level on SP CD11b<sup>+</sup> NK cells among days. Bottom graph: comparison of KLRG1 expression level on SP CD11b<sup>+</sup> NK cell between PBS and vaccinated group on each termination day. The values represent the mean  $\pm$  SEM (n = 3,

\*\*\*\* $p < 0.0001$ ; \*\* $p < 0.01$ ; \* $p < 0.05$ ). **(E)** Prolonged vaccination effect following two cycles of the STING-LNP and CpG-ODN. Mice were intravenously injected with PBS, the STING-LNP (4  $\mu\text{g}/\text{mouse}$  of c-di-GMP), CpG-ODN (250  $\mu\text{g}$ ) or a combination thereof for two cycles. After 20 days, mice were intravenously injected with ( $2 \times 10^5$ ) B16-F10-Luc2 cells ( $2 \times 10^5$ ). Representative photos of the lungs of different treatment groups and quantitative analysis of lung metastasis based on the values of luciferase activities. The values represent the mean  $\pm$  SEM ( $n = 4-5$ , \* $P < 0.05$ ).



**Fig. 26. Effect of vaccination by the combination of STING-LNP and CpG-ODN against the development of melanoma lung metastasis after three months.** **(A)** Prophylactic effect of combination therapy against B16-F10 lung metastasis. Mice were intravenously injected with PBS or a combination of STING-LNP (4  $\mu$ g/mouse of c-di-GMP) and CpG-ODN (250  $\mu$ g) for two cycles. After 90 days, mice were intravenously injected with  $2 \times 10^5$  B16-F10-Luc2 cells. **(B)** Representative photos of the lungs of different treatment groups **(C)** Quantitative analysis of lung metastasis based on the values of luciferase activities. The values represent the mean  $\pm$  SEM (n = 3-5, \*P<0.05).

# *Discussion*

## ***Discussion***

The concept of inducing memory-like NK cells for cancer immunotherapy has garnered considerable interest among researchers. To date, existing research has focused on either a pathogen-associated molecular pattern (PAMPS) or a combination of cytokines for the in vitro induction of the memory-like NK cells (32, 48). Our study is the first report on the in vivo induction and phenotypical characterization of memory-like NK cells demonstrating a memory effect in cancer immunotherapy. The findings reported in this study and summarized in figure 27, reveal that two cycles of the combined STING-LNP and CpG-ODN are essential and tend to be sufficient for the cytokine release which are crucial for the induction of memory-like NK cells. These memory-like NK cells are essential for the prevention of and combating melanoma lung metastasis. Our findings could pave the way for developing immunotherapies that harness NK cells' potential for treating and preventing cancer metastasis.

Efficient NK cell-based immunotherapy or cancer vaccination requires an immune adjuvant that can induce cytokines essential for memory-like NK cell induction. STING activation robustly induces type 1 IFNs and lower levels of type-1 immune responses alongside type 2 immune responses (19, 81). Conversely, CpG-ODN induces high levels of type-1 immune response but only weakly induces IFNs (82, 83). To address this limitation, we combined STING-LNP and CpG-ODN to induce type-1 immune responses, particularly IL-12, IL-18, and IFN- $\beta$ . It is notable from the data in figure 14 that when CpG-ODN was combined to STING-LNP the IFN- $\beta$  release was decreased in a dose dependent manner. In contrast, IL-12 was drastically enhanced in a dose dependent manner

when CpG-ODN was combined to STING-LNP. Which can confirm the importance of STING-LNP and CpG-ODN for the induction of type 2 and type 1 immune response, respectively. Trained immunity relies on epigenetic and metabolic modifications, such as DNA methylation, histone modification, and chromatin opening, which enhance gene transcription and translation (84, 85). These epigenetic changes are inherited from parent to daughter cells (86). Regarding the metabolic changes, the CIML NK cells tend to exhibit elevated expressions of nutrient transporters and a glycolysis-skewed metabolic profile (87). The glycolytic pathway was reported to be crucial for the IFN- $\gamma$  and granzyme B production for achieving efficient cytotoxicity (88). Additionally, blocking negative regulators like killer immunoglobulin receptors (KIR) and transforming growth factor- $\beta$  (TGF- $\beta$ ) and enhancing CD25 expression (an IL-2 receptor) is vital for NK cell survival and proliferation (89). Accordingly, we predicted that the first stimulus of this combination would activate myeloid cells to secrete cytokines which would result in the activation of the NK cells. Upon the restimulation, the NK cells would acquire memory-like properties through epigenetic and metabolic modifications resulting in a robust and quicker release of specific inflammatory cytokines and effector molecules thus potentiating the effect (85). In addition, we do believe that this effect is enhanced by the delivery system utilized in this study. The delivery system utilized here plays an essential role in enhancing memory-like NK cells induction, effector molecules and hence the preventative effect. Therefore, further investigation into the mechanism, including genetic alterations and the role of the delivery system, is planned.

CIML NK cells have shown enhanced antitumor responses, particularly against melanoma cell lines (54). The exact mechanism remains unclear, but CIML NK cells were reported to increase the production of IFN- $\gamma$ , CD107a, and TNF (44, 54). In addition to the upregulation of the activating receptors such as NKG2D and NKP46 and DNAM-1 which are known to control NK cell cytokine production and cytotoxicity (44, 90). The ligands of these receptors are present on melanoma cell lines, allowing cytokine induction and degranulation in response to melanoma targets (91). Moreover, memory-like NK cells can block inhibitory regulatory receptors and ignore their signals (44, 89). Therefore, we concluded that once the resting self-renewing memory-like NK cells are sensitized by the tumor cells, they will become capable of killing them and thus, preventing metastasis.

Our study demonstrates that two cycles, not one, of STING-LNP and CpG-ODN are sufficient to induce memory-like NK cells and achieve a significant antitumor effect. This aligns with previous reports in which the first stimulus activates innate immune cells, followed by a resting state until the second stimulus, that is essential for trained immune cell induction (92). Romee et al. found that the functional activity of in vitro IL-12 + IL-18-preactivated NK cells significantly increased compared to the control NK cells after restimulation (47). Our prior research also showed that two cycles of STING-LNP and PD-1 antibody were essential and sufficient for inducing an antitumor effect (24). Our findings indicate that two cycles, but not one, of the combination therapy result in significant tumor control following the induction of memory-like NK cells. One cycle of STING-LNP and the combination therapy induced slight tumor control (Fig. 20A). Mechanistic findings showed

high expression levels of CD11b, CD43, and KLRG1, indicating higher NK cell maturation and activation following one stimulus with STING-LNP and the combination group (Fig. 20B). Although one cycle of either the STING-LNP or the combination therapy allowed NK cells to differentiate and to reach the DP population, a CD11b<sup>+</sup> SP population (memory-like NK cells) was not achieved (Fig. 20C). This underscores the importance of two cycles of STING-LNP and CpG-ODN for inducing CD11b<sup>high</sup> CD27<sup>low</sup> memory-like NK cells and hence, effective tumor control. It has been reported that if restimulating innate immune cells leads to enhanced responses compared to the initial stimulus, these cells can be considered to have acquired trained immunity (31).

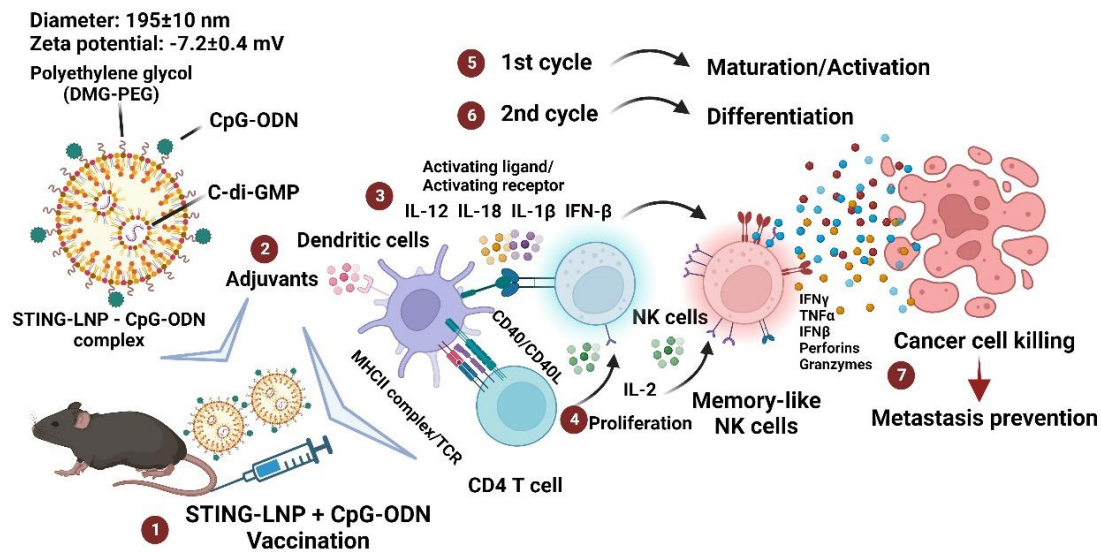
Although long-lived memory or memory-like NK cells have been documented, the prolonged effect of these persistent cells has not yet been confirmed. Previously, murine cytomegalovirus (MCMV) memory NK cells were shown to have lifetimes of around 90 days (40). Additionally, in vitro preactivated CIML NK cells could still be detected three weeks after adoptive transfer (93). However, despite their prolonged lifetimes, these memory or memory-like NK cells were detected at very low percentages compared to their initial induction (40, 43). In this study, we report that memory-like NK cells induced in vivo through a combination of immunotherapeutic drugs persist for at least one month at their peak percentage without any reduction, resulting in a prolonged prophylactic effect (Fig. 25C). The long-term persistence of these memory-like NK cells is primarily controlled by metabolic and epigenetic changes induced by cytokines. IL-12, IL-18, and IL-15 cause CIML NK cells to shift their metabolism towards glycolysis, persisting even after cytokine removal, leading to enhanced proliferation, long-term persistence, and recall

function of these NK cells (94). Additionally, IL-15 and IL-2 are crucial for the proliferation, homeostasis, survival, expansion, and persistence of NK cells. Terren et al. reported that the effector function, specifically IFN- $\gamma$  production, of CIML NK cells was significantly impaired following glycolysis inhibition (95). Similarly, glucose metabolism inhibition impaired NK cell proliferation and cytotoxicity in vitro, resulting in susceptibility to MCMV infection in vivo (88). In addition, previous studies have shown that memory-like NK cells have stability and heritable properties (43, 86, 96). Differentiation into memory-like NK cells through permanent or heritable epigenetic or metabolic changes could result in a self-renewing population of cells regardless of continuous stimuli (43). Our findings on the prolonged persistence and long-term vaccination effect of memory-like NK cells, maintaining their enhanced functionality, can be attributed to their heritable properties.

Vaccines have historically been highly effective in preventing infections or reducing their onset. Many vaccines are available for preventing various viral and bacterial infections; however, developing cancer vaccines has been challenging. The concept of trained immunity inspired us to undertake this research, promising to pave the way for developing prophylactic cancer vaccines. BCG vaccination has been reported to decrease susceptibility to many infections through long-term boosting of innate immune cell formation. Recently, BCG has been shown to protect against various cancers and COVID-19 by inducing memory-like NK cells (97, 98). Moreover, recent studies have demonstrated that reengineering BCG with a STING-agonist enhances trained immunity and antitumor effects (99, 100). Despite these positive results in controlling tumor burden via trained immunity, little effort has been directed

towards developing a prophylactic tumor vaccine that can protect against cancers. In this study, we demonstrate that combining STING-LNP and CpG-ODN can protect against melanoma lung metastasis by inducing memory-like NK cells and extending their persistence (Fig. 25 and Fig. 26). This substantial control of tumor metastasis and prevention confirms the induction and phenotypical characterization of memory-like NK cells as CD11b<sup>high</sup> CD27<sup>low</sup> and KLRG1<sup>high</sup> NK cells.

Our findings promise to pave the way for developing efficient cancer immunotherapies by harnessing the potential of producing memory-like NK cells in vivo instead of in vitro pre-activation and adoptive transfer. The phenotypical characterization of memory-like NK cells performed and confirmed here will facilitate their identification in future studies. Additionally, in vivo induction of memory-like NK cells may help avoid complications associated with the infusion of memory NK cells in clinical trials, such as the deaths reported in 3 out of 13 patients due to bacteremia with septic shock (44). In addition, the in vivo induction also potentially protects against the inflammation caused by the presence of foreign cells, making it a safer, more time-saving, and cost-effective alternative to adoptive transfer.



**Fig. 27. Summary for the mechanism behind the induction of memory-like NK cells and their therapeutic/prophylactic effect following STING-LNP and CpG-ODN in the lung metastasis model.** STING-LNP and CpG-ODN complex preparation and characterization followed by sensitizing the mice with this vaccination intravenously is expected to activate the innate monocytic cells. Which in return will release the cytokines essential for the maturation and activation of NK cells after the first cycle. However, upon the second cycle administration the activated NK cells will be differentiated to memory-like ones that will effectively destroy the cancer cells and prevent against its metastasis.

# *Conclusion*

## ***Conclusion***

In summary, our study offers new insights into the in vivo induction and phenotypic characterization of memory-like NK cells, identified as CD11b<sup>high</sup> CD27<sup>low</sup> and KLRG1<sup>high</sup> NK cells. By employing a combination of immune adjuvants, we underscored the critical role of these cells in defending against lung metastasis. Administering two cycles of STING-LNP and CpG-ODN resulted in the production of cytokines necessary for the activation and differentiation of NK cells into memory-like CD11b<sup>+</sup> SP NK cells, which may have extended lifespans. This led to a strengthened antitumor response and protection against the development of lung metastasis.

# *Declaration*

## ***Declarations***

**Ethics approval:** All animal experiments were approved by the Ethics of Pharmaceutical Science Animal Committee of Hokkaido University (approval number: 20-0126). All experiments were carried out in accordance with the National University Corporation Hokkaido University Regulations on Animal Experimentation.

A patent application for the intellectual property associated with this work included in this dissertation has been submitted to Japan Patent Office, Tokyo, Japan (Application number: 特願 2023-145994). Any reproduction of the results or strategies revealed in this dissertation should be done in agreement with the inventors.

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# *Dedication*

***Dedication:***

This work is dedicated to my precious family; father; Prof. Mohamed Khalifa, mother; Prof. Ghada Youssef and brothers; Dr. Ahmed Khalifa, Dr. Amr Khalifa, without their patience, understanding and encouragement it would never have been completed.