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| Author(s)           | KHALIFA, Alaa Mohamed                                                                                                                                                                             |
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# 学 位 論 文 内 容 の 要 旨

博士の専攻分野の名称 博士(薬科学) 氏 名 Alaa Mohamed Khalifa

## 学 位 論 文 題 名

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)

### 1. Introduction:

Natural killer (NK) cells are effective in attacking tumor cells that escape from T cell attack [1]. 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 [2]. 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 [3]. 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 [4]. 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<sup>low</sup>CD27<sup>low</sup>, the most immature phenotype, then increasing the maturation as CD11b<sup>low</sup>CD27<sup>high</sup> then CD11b<sup>high</sup>CD27<sup>high</sup> till reaching the most mature stage of NK cells characterized as CD11b<sup>high</sup>CD27<sup>low</sup>. A brief stimulation with interleukin (IL)-12, IL-15 and IL-18 which 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 [2,5,6]. 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) [7-9] 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 [9]

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 [9]**

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 [3].

## **2.3. Protection against the development of melanoma lung metastasis [9]**

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 the 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 [9]**

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.

## **4. References:**

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