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Autologous NK cell therapy in combination with anti-PD-1 antibody in non-small cell lung cancer

비소세포폐암에서 항 PD-1 항체와 병용하는 자가 NK 세포 요법

울 산 대 학 교 대 학 원

의 학 과

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Autologous NK cell therapy in combination with anti-PD-1 antibody in non-small cell lung cancer

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이 논문을 의학박사 학위 논문으로 제출함

2022 년 8 월

울 산 대 학 교 대 학 원

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Abstract

Background: Although the new development of immune checkpoint inhibitors (ICIs) has led to advances in non-small cell lung cancer (NSCLC) treatment, the low response rates to ICIs are still a problem. Since natural killer (NK) cells participate in the immune response against cancer cells, the development of NK cell-mediated immunotherapies has the potential to be a strategy to increase the efficacy of current T cell-mediated immunotherapies. This study was conducted to evaluate the role of NK cells in anti-PD-1 therapy and assess the efficacy of autologous NK cell therapy in combination with anti-PD-1 antibody (Ab) in NSCLC.

Materials and Methods: Non-genetically modified, ex-vivo culture expanded human NK cells (SNK01) were generated through an NK cell activation process. The role of NK cells in the antitumor immune response elicited by anti-PD-1 therapies and the in vivo efficacy of SNK01 and anti-PD-1 Ab combination therapy compared to anti-PD-1 monotherapy were evaluated in a mouse model. Based on these preclinical results, we analyzed immune cell counts and cytokine levels according to treatment response, timepoint (pre- vs. post-NK cell therapy) and treatment group to evaluate the mechanisms underlying anti-PD-1 Ab and SNK01 combination therapy.

Results: In a mouse model with PD-L1-overexpressing mouse NSCLC cells, when mice were depleted of NK or T cells, anti-PD-1 Ab treatment was ineffective, while the treatment was effective in undepleted mice. This result suggests that PD-1 blockade mobilizes an NK cell and T cell response, and NK cells contribute to the therapeutic effect of PD-1 blockade. SNK01 and anti-PD-1 Ab combination therapy resulted in enhanced growth inhibition of xenograft tumors of human NSCLC cells in NOD-rag1$^{-/-}$Il2rg^{null} (NRG) mouse models, regardless of PD-L1 expression status, suggesting that combination therapy would be also effective in PD-L1–negative NSCLC. In the clinical study with NSCLC patients, treatment responders (n=5) showed a tendency towards higher baseline NK activity than non-responders (n=13; p=0.01). Otherwise, there were no significant differences in immune cell counts and cytokine levels according to treatment group, treatment response or timepoint (pre- vs. post-treatment).

Conclusion: Based on the findings of this study, the NK cell therapy combined with anti-PD-1 Ab may be considered as a treatment option for NSCLC.

Keywords: Non-small cell lung cancer, NK cell, pembrolizumab, combination therapy

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Introduction

In the past few years, the incidence of non-small cell lung cancer (NSCLC) has drastically increased, and NSCLC remains one of the leading causes of cancer-related death worldwide [1]. Although platinum-based chemotherapies have been used as the first-line treatment for NSCLC, the clinical benefit of these therapies are limited to only a small portion of patients and accompanied by a plateau [1, 2]. Recently, the new development of immune checkpoint inhibitors (ICIs) has led to advances in NSCLC treatment. Pembrolizumab has replaced chemotherapy as the first-line treatment for NSCLC patients with a PD-L1 tumor proportion score (TPS) ≥50% [3, 4]. However, the low response rate to ICIs among NSCLC patients is still a problem encountered in current immunotherapy regimens.

The ICIs developed so far, including pembrolizumab and nivolumab, blockade the binding of PD-L1/PD1 or B7/CTLA4 from tumor cells/T cells, which interferes with antitumor activity mediated by T cell activation, thereby activating the antitumor immune response [5].

To improve the efficacy of ICIs, many studies have focused on identifying predictive biomarkers for tumor response and novel combination approaches. Chemotherapy, radiation therapy, molecular targeted therapy, and cell therapy have been considered for combination regimens. Currently, ICI combination therapies such as ICI/chemotherapy, ICI/anti-VEGF therapy/chemotherapy and ICI/ICI therapy are in use for NSCLC treatment [3, 6, 7]. However, many issues, including safety and efficacy, must be taken into consideration when designing the ICI-based combination regimens because the other therapies ICIs are combined with may have significant influence on host immunity or the tumor microenvironment.

In addition, many new ICIs have been developed and clinical trials are currently being conducted for each of the interactions between the various receptors and ligands that occur in the tumor microenvironment, including those on natural killer (NK) cells and macrophages, as well as the interactions between T cells and tumor cells [8].

NK cells play an essential role in tumor immunosurveillance by orchestrating the innate immunity in the heterogeneous tumor microenvironment [9, 10]. NK cells directly lyse cancer cells through the secretion of perforin or granzyme as effector cells or indirectly induce cancer cell death by activating T cells through the secretion of various cytokines and chemokines [11].

Moreover, unlike T cells, NK cells can recognize and attack cancer cells without neoantigen or MHC expression. NK cells are activated through three main mechanisms, 1) when cancer cells fail to express MHC1, 2) by ligands that are often upregulated with oncogenic stress, or 3) when CD16 recognizes antibodies that are specific to tumor-associated antigens, which can lead to antibody-dependent cell-mediated cytotoxicity (ADCC) [9, 12]. Therefore, the development of NK cell-mediated immunotherapies could be an ideal strategy to increase the efficacy of current T cell-mediated immunotherapies. Since it has been reported that the presence of tumor-infiltrating NK cells is a positive prognostic marker for multiple malignancies, the interest in clinical cancer immunotherapies that target NK cells has increased [13-15].

NK cell-based immunotherapy has been attempted in various ways, and the approaches to the NK cell-based immunotherapy studied so far can be divided into three main categories: 1) injecting monoclonal antibodies that act on cytokines or receptors involved in NK cell activation as immune stimulants, 2) direct adoptive transfer of NK cells, and 3) separating and transferring extracellular vesicles containing various degranulated substances obtained from NK cell cultures [11, 16]. Since NK cells do not require a specific antigen such as MHC for activation, adoptive transfer of NK cells is possible. Adoptive transfer of NK cells is again divided into four major approaches depending on the source of the NK cells: autologous NK cell transfer, allogeneic NK cell transfer, production/proliferation/administration of an NK cell line, and chimeric antigen receptor (CAR)-NK cell therapy. Except for the NK cell line approach, the expansion of NK cells ex vivo is a fundamental step for adoptive transfer of NK cells.

In the previous studies, adoptive transfer of autologous NK cells has been shown to have limitations in terms of relatively low efficacy and technical difficulties in expanding NK cells with high purity [16-18]. Recently, a strategy which combines NK cell therapy and immune checkpoint inhibitors has been proposed [19], based on growing evidence that suggests not only T cells, but also NK cells, contribute to the antitumor immune response mediated by PD-1 blockade [20]. Results from several in vivo and in vitro studies suggest that some NK cells express PD-1 in the process of interacting with tumor cells, the interaction between PD-1 and

PD-L1 interferes with NK cell activation, and thus the administration of anti-PD-1/L1 antibody (Ab) kills tumor cells through NK cell activation [9, 21, 22]. Therefore, it has been assumed that PD-1 acts as an inhibitory regulator in NK cells and that a combination of NK cell therapy and PD-1 blockade could be an effective immunotherapy approach.

In this study, we used the newly generated, non-genetically modified and ex-vivo culture expanded human NK cells (SNK01) with enhanced cytotoxicity through an NK cell activation process to evaluate the role of NK cells in anti-PD-1 Ab immunotherapy and to assess the safety and efficacy of autologous NK cell therapy in combination with anti PD-1 Ab in NSCLC mouse models. Here, we present the study results from mouse models and immune cell profiling/cytokine analysis in NSCLC patients.

Materials and Methods

1. Isolation, expansion, and characterization of the NK cells

Ex-vivo culture expanded human NK cells, SNK01 were used in mouse experiments and the clinical trial involving NSCLC patients. All the manufacturing and testing procedures used to produce SNK01 were performed under good manufacturing practice (GMP) conditions (NKMAX Co., Ltd, Seongnam, South Korea). Peripheral blood mononuclear cells (PBMCs) were collected from the leukapheresis products of enrolled patients in the treatment group and then used for NK cell expansions as described previously with some modification [23]. The phenotype of culture-expanded NK cells was determined via flow cytometric analysis. For assessing NK cell activity, cytotoxicity and degranulation assays were performed. The detailed methods and results of these assays are described in a previous report [24].

2. Response to anti-PD-1 Ab in NK cell- and/or T cell-depleted mouse models with PD-L1-overexpressing mouse lung cancer cells

All animal studies were performed in accordance with the guidelines of the Korean Food and Drug Administration. Protocols were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of Asan Medical School. The C57BL/6 and SCID/g (*NOD-Prkdc*^{scidtm1Baek}) mice were obtained from GemBiosciences (Chungbuk, Korea).

To evaluate the role NK cells and T cells in the anti-PD-1-mediated antitumor immune response, we tested the in vivo efficacy of PD-1 blockade in immune cell-depleted mice. Five-week-old C57BL/6N mice (N=16) were injected subcutaneously in the dorsal flank with LLC-1 mouse lung carcinoma cells (2 x 10^5 cells/mouse) in 50 μl of ice-cold phosphate-buffered saline (PBS, Gibco). In half the mice, LLC-1 cell lines were transduced with a PD-L1 expression vector, and in the other half, the LLC-1 cell lines were left untransduced. Five days after injection of LLC-1, the mice were randomly divided into two groups and assigned to receive 4 intraperitoneal (IP) injections of either 200 μg of monoclonal PD-1 Ab (Invivogen, #BE0146) or control IgG1 (Invivogen, #BE0089) at intervals of 2-3 days. Tumor volume was

monitored for twenty days. The outcomes were evaluated in terms of tumor volume and intratumoral immune cell infiltration.

To deplete mice of $CD8^+$ T cells, they were given IP injections of 250 μg CD8α monoclonal antibodies, 2 and 1 days before LLC-1 injection. $CD4^+$ T cells were depleted by IP injections of 500 μg of GK1.5 monoclonal antibodies on 3 and 1 days before LLC-1 injection. To deplete mice of NK cells, IP injections of 250 μg of PK136 (specific for NKR-P1C) 2 and 1 days before LLC-1 injection.

3. Xenograft model using human lung cancer cells in NOD-rag1$^{-/-}$Il2rg^{null} (NRG) mice

To evaluate the in vivo efficacy of the combination of SNK01 and PD-1 blockade, the dose-response relationship for SNK01 and the effect of tumor PD-L1 positivity, 5-week-old NRG mice (N=60) were injected subcutaneously in the dorsal flank with H460 or H460-PD-L1 knockout human lung adenocarcinoma cells (5×10^5) in 50 μl of ice-cold PBS. Immunodeficient mice were used because they were to be injected with human NK cells instead of mouse NK cells, which were difficult to produce. Five days after tumor cell injection, the mice were randomly divided into two groups to receive either 200 μg of monoclonal PD-1 Ab (Invivogen, #BE0193) or control IgG1 (Invivogen, #BE0297) by IP injection 5 times (at 2- or 3-day intervals). SNK01 was also injected into the tail vein 6 times (at 2- or 3- day intervals, Figure 2A). To evaluate the dose-response relationship, SNK01 was administered at $2x10^6$ cells/dose in half and at $2x10^7$ cells/dose in half. Tumor growth was monitored for twenty days. Outcomes were evaluated in terms of tumor volume and intratumoral infiltration of human NK cells, including PD-1 positive NK cells, which were identified using immunohistochemistry (IHC).

4. Immunohistochemistry (IHC)

4-μm paraffin-embedded tissue samples were incubated with 10mM sodium citrate buffer (pH 6.0) and autoclaved for 15 min for antigen retrieval. The samples were then blocked with a PBS-based mixture of 5% bovine serum albumin (BSA; Affymetrix, CA) and 1% normal horse serum (NGS, Vector Laboratories, CA) for 30 min. After blocking, sections were incubated with anti-human PD-1 (R&D systems, AF1086, 1:100) and anti-human NKp46

(R&D systems, MAB1850, 1:100) primary antibodies overnight at 4 ℃, followed by incubation with an anti-Rabbit IgG polymer kit (Vector Laboratories, MP-7401) or with an anti-goat IgG polymer kit (Vector Laboratories, MP-7405) for 1 h at room temperature. The tissues were stained with 3, 3′-diaminobenzidine (DAB; Vector Laboratories, SK-4100) and then counterstained with Mayer's Hematoxylin (abcam, ab220365) and mounted with Permount medium (Fisher chemical, SP15-100). All incubations were conducted in wet chambers. Positive signals were visualized using a TE-2000U microscope (Nikon, Japan).

5. Clinical Study

Based on the preclinical results, we investigated the safety and efficacy SNK01 when administered in combination with pembrolizumab in patients with NSCLC. Full details of the clinical study design have been reported previously [24]. The aim of this randomized, open-label, single-center study was to evaluate the safety, tolerability, and anti-tumor activity of SNK01 in combination with pembrolizumab in patients with advanced NSCLC (PD-L1 TPS ≥1%) who had a history of failed frontline platinum-based therapy. The primary endpoint was safety, and the secondary endpoints included efficacy, represented by objective response rate, progression-free survival, overall survival, time to progression, and quality of life. This prospective clinical trial was approved by the institutional review board (IRB, 2018-1479) of Asan Medical Center (Seoul, South Korea) and registered at the Clinical Research Information Service (CRIS, KCT0003463). Informed consent was obtained from all participants prior to enrollment. The trial was designed and conducted in accordance with the Helsinki Declaration and the Ethical Guidelines for Clinical Studies.

Eighteen patients were randomized (2:1) to receive pembrolizumab 200mg every 3 weeks +/- 6 weekly infusions of SNK01 at either 2 x 10^9 or 4 x 10^9 cells per infusion (pembrolizumab monotherapy vs. SNK combination).

6. Immune cell profiling and cytokines in NSCLC patients receiving SNK01/anti-PD-1 Ab combination

In the patients enrolled for the clinical study, immune cell counts and cytokine levels

according to each treatment group, treatment response and timepoint (pre- vs. post-NK cell therapy) were analyzed to evaluate the mechanisms underlying anti-PD-1 Ab and autologous NK cell combination therapy.

NK cell activity by detection of NK cell-secreted interferon-γ level (via NK Vue® Kit, NKMAX Co., Ltd.), immune cell (total T cell, CD 4/8+ T cell [naïve, central memory, effector memory, terminal effector T cell, respectively], Treg cell, NK cell [early, late], B cell, monocyte, dendritic cell, granulocyte) profiles from PBMC by flow cytometry, and serum cytokine/chemokine (IL-2, TNF-a, MCP-1, IL-10, IL-12p70, GM-CSF, TGF-b1, IFN-a, IL-15) levels by enzyme-linked immunosorbent assay (ELISA) were evaluated during the screening period; at weeks 4, 7, 10; and then every 6 weeks until the end of the clinical study.

7. Statistical analysis

Pearson's chi-square test and Fisher's exact test were used for data comparisons, and the Mann-Whitney U test for comparisons of the nonparametric variables. Survival was estimated using the Kaplan-Meier method, and the log-rank test was used to determine the significance of any differences in survival curves. All tests were two-sided, and a p value of < 0.05 was considered statistically significant. The SPSS 25.0 software (IBM SPSS Inc., Chicago, IL) and SAS 9.4 software were used for the analyses.

Results

1. Response to anti-PD-1 Ab in NK cell- and/or T cell-depleted mouse models with PD-L1-overexpressing mouse lung cancer cells

To investigate whether PD-1/PD-L1 blockade elicits an effective response against tumors, we injected LLC-1 mouse lung carcinoma cells into C57BL/6N mice and, after 5 days, treated the mice with anti-PD-1 Ab or control IgG1. We observed that LLC-1 cells show relatively low PD-L1 expression through western blot analysis (data not shown). Thus, LLC-1 cell lines were transduced with a mouse PD-L1 expression vector, and PD-L1 overexpressing cell lines were selected by flow cytometry. The PD-L1 overexpressing LLC-1 cell lines were injected into half of the mice.

In mice treated with anti-PD-1 Ab, tumor growth was significantly restricted (Fig. 1A, B). In addition, among mice treated with control IgG1, tumor growth was much more aggressive in those injected with the PD-L1 overexpressing LLC-1 cell lines than in those injected with the untransduced LLC-1 cell lines. Overall, tumor development was the most effectively restricted in mice injected with the PD-L1 overexpressing LLC-1 cell lines and treated with anti-PD-1 Ab (Fig. 1A, B). This result indicates that overexpression of PD-L1 by tumor cells inhibits the antitumor immune response, and anti-PD-1 Ab therapy rescues the antitumor response, demonstrating the therapeutic effect of PD-1 blockade.

To determine which immune cells were susceptible to PD-1-mediated inhibition, groups of mice were depleted of NK cells, CD8+ T cells, or both before being challenged with tumor cells. Since tumor growth was restricted anti-PD-1 Ab even in mice injected with the untransduced LLC-1 cell lines, the following analyses were performed using experimental data from the untransduced LLC-1 cell lines (N=64). Concurrent depletion of NK and CD8+ T cells significantly accelerated tumor growth, whereas depletion or NK or CD8+ T cells separately did not completely accelerate tumor growth (Fig.1C, I). This may suggest that both NK cells and CD8+ T cells participate in the antitumor immune response at least partially independently and that without both, the antitumor immune response is significantly suppressed. When mice were depleted of NK cells or T cells before tumor cell injection, anti-PD-1 Ab treatment was ineffective, whereas it was effective in undepleted mice (Figure 1D-H), showing that PD-1

blockade mobilizes an NK cell as well as a T cell response. Therefore, NK cells in addition to CD8+ T cells contribute to the therapeutic effect of PD-1 blockade.

These data show that the efficacy of PD-1 blockade in mouse lung carcinoma cells and control of cancer development depend on NK cell as well as CD8+ T cell activity. In addition, both NK cells and T cells are inhibited by PD-1/PD-L1 interactions, and that inhibition is rescued by PD-1/PD-L1 blockade. Therefore, in this mouse model, PD-1 blockade seems to reinvigorate the NK response, which results in tumor growth restriction.

2. Xenograft model using human lung cancer cells in NRG mice

We investigated whether PD-1–mediated inhibition of NK cell response is physiologically relevant when T cells participate in the antitumor immune response. Initially, we employed a xenograft tumor model based on subcutaneous injection of H460 cells into NRG mice. We observed that H460 cells show relatively high PD-L1 expression using western blot analysis (data not shown). To examine the role of PD-1 inhibition in this xenograft tumor model, PD-L1-knockout H460 cell lines were also created. PD-L1-knockout H460 cell lines were subcutaneously injected into half of the NRG mice.

Tumor growth was significantly suppressed when NK cells were administered regardless of PD-L1 knockout status (Figure 2B-C). In the H460 cell lines without PD-L1 knockout, combined administration of anti-PD-1 Ab and NK cells significantly restricted tumor growth compared to anti-PD-1 Ab or NK cell therapy alone. A similar trend was also observed in the PD-L1 knockout H460 cell lines, but combined administration of anti-PD-1 Ab and NK cells did not significantly restrict tumor growth compared to NK cell therapy alone. This was probably because tumor growth restriction due to anti-PD1 Ab was limited in the PD-L1 knockout H460 cell lines. NK cell therapy combined with anti-PD-1 Ab significantly restricted tumor growth and resulted in significantly greater NK cell infiltration into tumor tissue than NK cell therapy alone in H460 cell lines without PD-L1 knockout, whereas there was no significant difference in tumor growth or NK cell infiltration between combination therapy and NK cell therapy alone in the PD-L1 knockout cell lines (Figure 2B-D). This may suggest that PD-1/PD-L1 blockade affects the antitumor immune response of NK cells.

When comparing the group administered with $2x10^6$ or $2x10^7$ of NK cells, a dose-response

relationship in which tumor growth was more effectively restricted in the group administered with a higher number of NK cells was observed (data not shown).

3. Immune cell profiling and cytokine levels in NSCLC patients treated with SNK01/anti-PD-1 Ab combination

From the clinical study evaluating SNK01 in combination with pembrolizumab in patients with advanced NSCLC (PD-L1 TPS ≥1%) who had a history of failed frontline platinum-based therapy, 18 patients were included in the analysis. Table 1 summarizes the baseline characteristics of study patients [24]. The median age was 59 years (range, 31 to 73 years), and the most common histologic type of tumor was adenocarcinoma, accounting for all the tumors except for one pleomorphic carcinoma. The baseline characteristics including the PD-L1 expression status were balanced between the two groups, except for EGFR status and previous lines of chemotherapy.

Treatment responders (n=5) showed a tendency towards higher baseline NK activity than non-responders (n=13; p=0.01, Figure 3). In the peripheral blood of non-responder group, the frequencies of the B cells and NK cells increased significantly after immunotherapy (with or without SNK01) (Figure 4). They also increased in the responder group but did not show statistical significance probably due to the small number of patients. In the responder group, the frequency of the monocytes decreased significantly after immunotherapy (with or without SNK01), but this decrease was not shown in the non-responder group. In addition, unlike the non-responder group, in which the frequency of the terminal effector CD4 T cell subpopulation did not significantly increase after immunotherapy (with or without SNK01), the significant increase was observed in the responder group. However, when the pembrolizumab alone group and the pembrolizumab and SNK01 combination group were analyzed respectively, this tendency was observed only in the pembrolizumab alone group (Figure 5). The IL-10 cytokine level showed a tendency to increase after treatment in the pembrolizumab and SNK01 combination group, unlike the pembrolizumab alone group (p=0.07), but there was no statistical significance (Figure 6).

Otherwise, the trend in immune cell (Total T cell, CD 4/8+ T cell, Treg cell, NK cell, B cell,

monocyte, dendritic cell, granulocyte) counts and cytokine (IL-2, TNF-a, MCP-1, IL-10, IL-12p70, GM-CSF, TGF-b1, IFN-a, IL-15) levels did not show any significant differences according to treatment group, treatment response or timepoint (pre- vs. post-NK cell therapy; Figure 3, Figure 4, Figure 5 and Figure 6).

Discussion

In the present study, we observed that NK cells as well as T cells contribute to the therapeutic effect of PD-1/PD-L1 axis blockade in NK cell-, T cell-, and NK cell- and T cell-depleted mouse models injected with PD-L1 overexpressing mouse lung cancer cells, consistent with a previous study [9], indicating that NK cells may a play role in inducing a T cell response and/or kill tumor cells directly. In the xenograft model using human lung cancer cells in NRG mouse, NK cell therapy combined with anti-PD-1 Ab significantly restricted tumor growth and promoted NK cell infiltration into tumor tissue compared to NK cell therapy alone in cell lines with high PD-L1 expression, whereas combination therapy was not significantly different from NK cell therapy alone in the PD-L1 knockout cell lines. PD-1/PD-L1 blockade affects the antitumor immune response of NK cells. Given our in vivo immune cell depletion experiment results, which show NK cells participate in the therapeutic effects of the PD-1 blockade, and the cytotoxic effects of the NK cells on MHC- and neoantigen-deficient cancer cells [25, 26], we determined the therapeutic effects of the combination therapy of NK cell and PD-1 blockade for treating NSCLC patients [24]. In the immune cell profiling and cytokine analysis in NSCLC patients given autologous NK cell/anti-PD-1 Ab combination therapy, the trend in immune cell counts and cytokine levels did not show significant difference according to treatment group, treatment response and timepoint (pre- vs. post-NK cell therapy).

Among the approaches to NK cell-based immunotherapy, various phase I/II studies of immune stimulants targeting NK cell receptors or CAR-NK cells in mainly hematologic malignancies are ongoing [11, 16]. Adoptive transfer using autologous NK cells or NK cell lines has shown limited efficacy in previous studies [17]. It has been hypothesized that, due to matching MHC I, autologous NK cells could recognize tumor cells as self, so NK cell activation is limited, and that NK cells obtained from immunosuppressed cancer patients are not sufficiently effective. For adoptive transfer using NK cell lines, it is hypothesized that either ADCC does not occur due to poor CD16 expression, or irradiation performed prior to adoptive transfer shortens the lifespan of NK cells in vivo. For adoptive transfer using allogeneic NK cells, efficacy data is mainly from studies involving intensive chemotherapy or bone marrow transplantation in hematologic malignancies, and data from several studies

are available involving solid tumors, including one large study recently conducted on NSCLC patients [19]. Allogeneic NK cell transfer requires an NK cell donor, and if the HLA type of the donor matches that of the patient, the killer cell Ig-like receptor (KIR) of NK cells matches with MHC I and the NK cells are inhibited. Thus, HLA typing of the patient and donor is required when selecting an NK cell donor. On the other hand, autologous NK cells can be produced relatively simply using the patient's own blood. However, there have been technical difficulties in expanding autologous or allogeneic NK cells with high purity. In this study, non-genetically modified and ex-vivo culture expanded human NK cells (SNK01) that exhibit superior purity, cytotoxicity, and activity were used to overcome the technical difficulties.

It is known that the effects of immunotherapy are mainly mediated by the activation of T cells. According to previous reports, PD-1 blockade was efficacious even in MHC I-deficient tumors. Therefore, it was hypothesized that NK cells also contribute to the antitumor activity of PD-1 blockade. Recent in vivo and in vitro studies showed that NK cells can express PD-1, and therefore anti-PD-1 Ab increases the antitumor immune response of NK cells. In addition, in mouse models in which NK cells were depleted, tumor growth inhibition by NK cells was suppressed [21, 22]. Thus, it was suggested that PD-1 acts as an inhibitory regulator in NK cells, and that combination with PD-1 blockade could be an effective strategy for immunotherapy using NK cells.

Unlike previous studies, in this study, depletion of NK or CD8+ T cells separately did not completely accelerate tumor growth, whereas concurrent depletion of NK and CD8+ T cells significantly accelerated the tumor growth. This may suggest that both NK cells and CD8+ T cells participate in the antitumor immune response at least partly independently, and that without both, the antitumor immune response is significantly suppressed. While a previous study by Hsu et al. emphasized the role of NK cells alone [9], this study showed that both NK cells and T cells play an important role in the antitumor immune response and anti-PD-1 Ab response. Unlike the cell lines used by Hsu et al., the LLC-1 NSCLC cell lines used in our study expressed a low level of PD-L1, and this may have caused the differences in our study results. Moreover, this study is meaningful in that it additionally evaluated the effectiveness of the combination of NK cell therapy and anti-PD-1 Ab in NSCLC cell lines.

Recent studies have reported that the level of PD-L1 expression, antigenicity and microenvironment of a tumor can dynamically change depending on the tumor stage and the types and timing of the preceding or current treatments [27-29]. Therefore, in this study, proportions of immune cells and cytokine levels were analyzed during SNK01 and pembrolizumab combination therapy so that we could better understand the mechanisms underlying the combination therapy. We found that after immunotherapy (with or without SNK01), the frequency of the terminal effector CD4 T cell subpopulation significantly increased in the peripheral blood of the responder group, especially in pembrolizumab alone group. This was different from the results of the previous paper, which observed that the subproportions of CD4+ T cells were not altered by immunotherapy [30]. The possible hypothesis is that when immune cells are activated after immunotherapy, the differentiated CD4+ T cell subset may increase as the naïve T cell subset decrease. However, since the number of studied patients was small, further studies are needed.

In a previous study that evaluated the immune parameters of 109 NSCLC patients receiving combination therapy with allogeneic NK cells and pembrolizumab as well as the efficacy and safety of the therapy, the proportion of NK cells and the levels of Th1 cytokines, including IL-2, TNF-β, and IFN-γ, increased after combination therapy [19]. However, we did not find any significant changes in the proportion of NK cells and the levels of Th1 cytokines in the present study. This is probably because the number of patients enrolled to the study was too small to detect statistical significance. In particular, the number or proportion of NK cells did not significantly increase even after NK cell therapy. There are two possible main reasons for this: First, the half-life of NK cells in the human body after administration of autologous NK cell therapy is short, less than one week. Second, once injected, mature and terminally differentiated NK cells with enhanced effector function become widely distributed in target organs or tissues such as the bone marrow, spleen, or lungs in addition to the bloodstream [31, 32]. It cannot be excluded that these limitations affected the difference in efficacy between autologous and allogeneic NK cell therapy.

This study had limitations. In the xenograft model using human lung cancer cells in NRG mouse, we wanted to evaluate the efficacy of autologous NK cell combination therapy, but because it was difficult to produce NK cells for mice, human NK cells were used instead,

which were the xenograft NK cells. Therefore, immunodeficient mice were used for the experiment, resulting in difficulty in observing antitumor immune responses other than that of administered NK cells and in evaluating the interaction between the NK cells and T cells or other components of tumor microenvironment.

Despite of these limitations, this study is meaningful in that it was an experimental study that confirmed the rationale for autologous NK cell combination therapy, which is relatively safe and is simple to implement, with mouse lung cancer models to overcome the limitations of immune checkpoint inhibitor therapy in NSCLC. Moreover, the present study served as the basis for a clinical trial of autologous NK cell and pembrolizumab combination therapy in NSCLC patients [24], and described the dynamic changes in NK cell activity, immune cell profile, and cytokine levels in these patients. This study also provides a basis for performing large-scale phase clinical trials of pembrolizumab and autologous NK cell combination therapy in the future.

Conclusion

NK cells play an important role in the antitumor immune response, and in particular, are assumed to be involved in the PD-1/PD-L1 axis and the mechanism of action of immune checkpoint inhibitors, making them an attractive new target for cancer immunotherapy. NK cell therapy combined with anti-PD-1 Ab may be considered as a treatment option for NSCLC.

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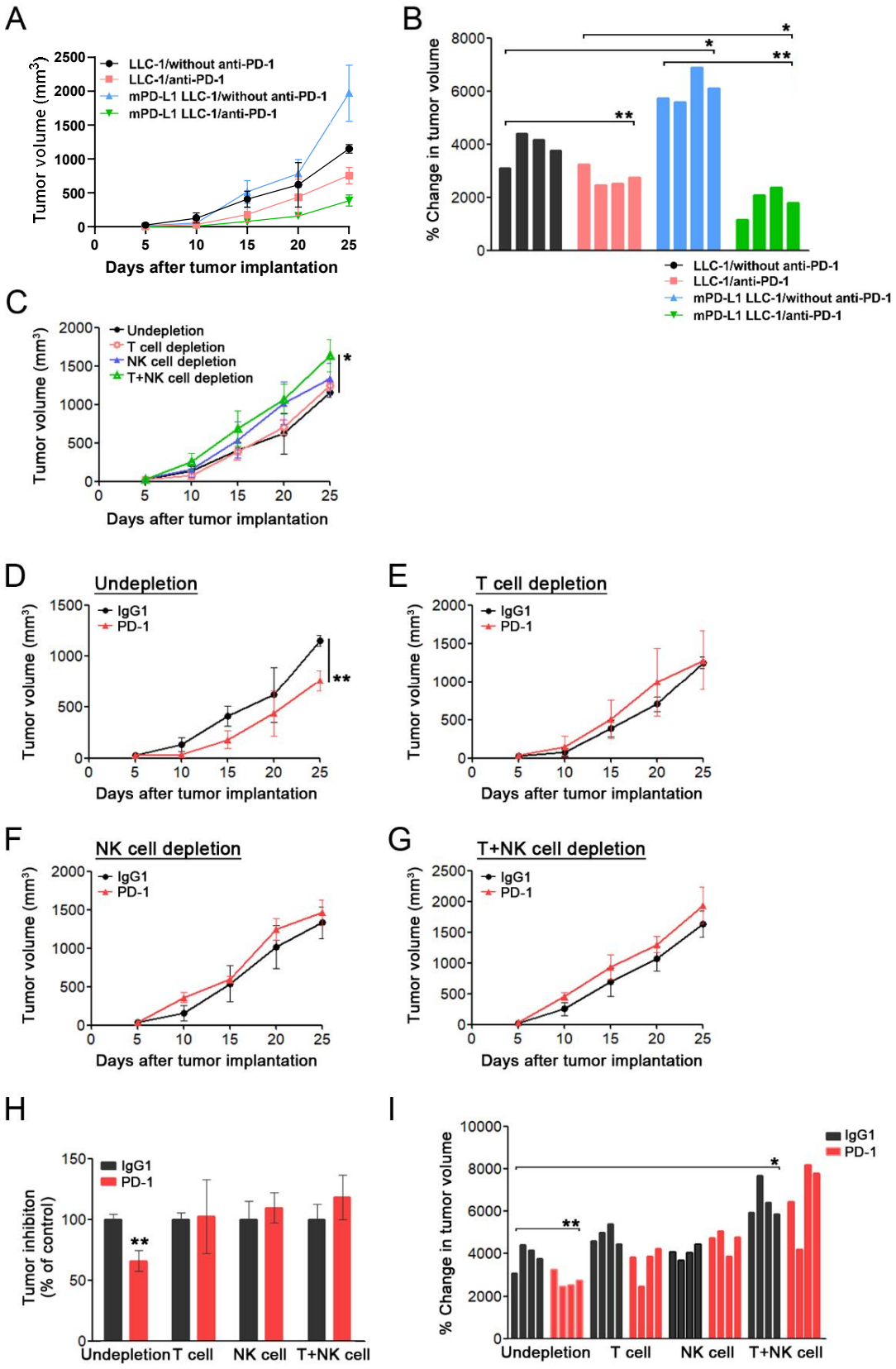
Table 1. Baseline clinical characteristics of study patients (N=18)

Characteristic	Pembrolizumab Monotherapy (%, N=6)	SNK Combination (%, N=12)	*p*-value
Age (years)			
Median, range	56.5, 49-70	60, 49-73	
≥ 65	1 (16.7%)	5 (41.7%)	0.21
Sex			0.14
Male	2 (33.3%)	9 (75.0%)	
Female	4 (66.7%)	3 (25.0%)	
Smoking status			0.14
Current smoker	0 (0%)	0 (0%)	
Ex-smoker	4 (66.7%)	9 (75.0%)	
Never smoker	2 (33.3%)	3 (25.0%)	
ECOG performance status			
0	0 (0%)	0 (0%)	
1	6 (100.0%)	12 (100.0%)	
Histology			0.99
Adenocarcinoma	6 (100.0%)	11 (91.7%)	
Squamous cell carcinoma	0 (0%)	0 (0%)	
Pleomorphic carcinoma	0 (0%)	1 (8.3%)	
PD-L1 22c3 TPS			
Median, range (%)	1, 1-15	17.5, 1-100	
≥ 50%	0 (0%)	5 (41.7%)	0.08
EGFR status			0.04
Wild type	1 (16.7%)	9 (75.0%)	

Mutant	5 (83.3%)	3 (25.0%)	
ALK translocation			
No	6 (100.0%)	12 (100.0%)	
Yes	0 (0%)	0 (0%)	
Previous lines of chemotherapy			0.02
1	0 (0%)	8 (66.7%)	
2	2 (33.3%)	2 (16.7%)	
≥ 3	4 (66.7%)	2 (16.7%)	

ECOG; Eastern Cooperative Oncology Group, TPS; tumor proportion score

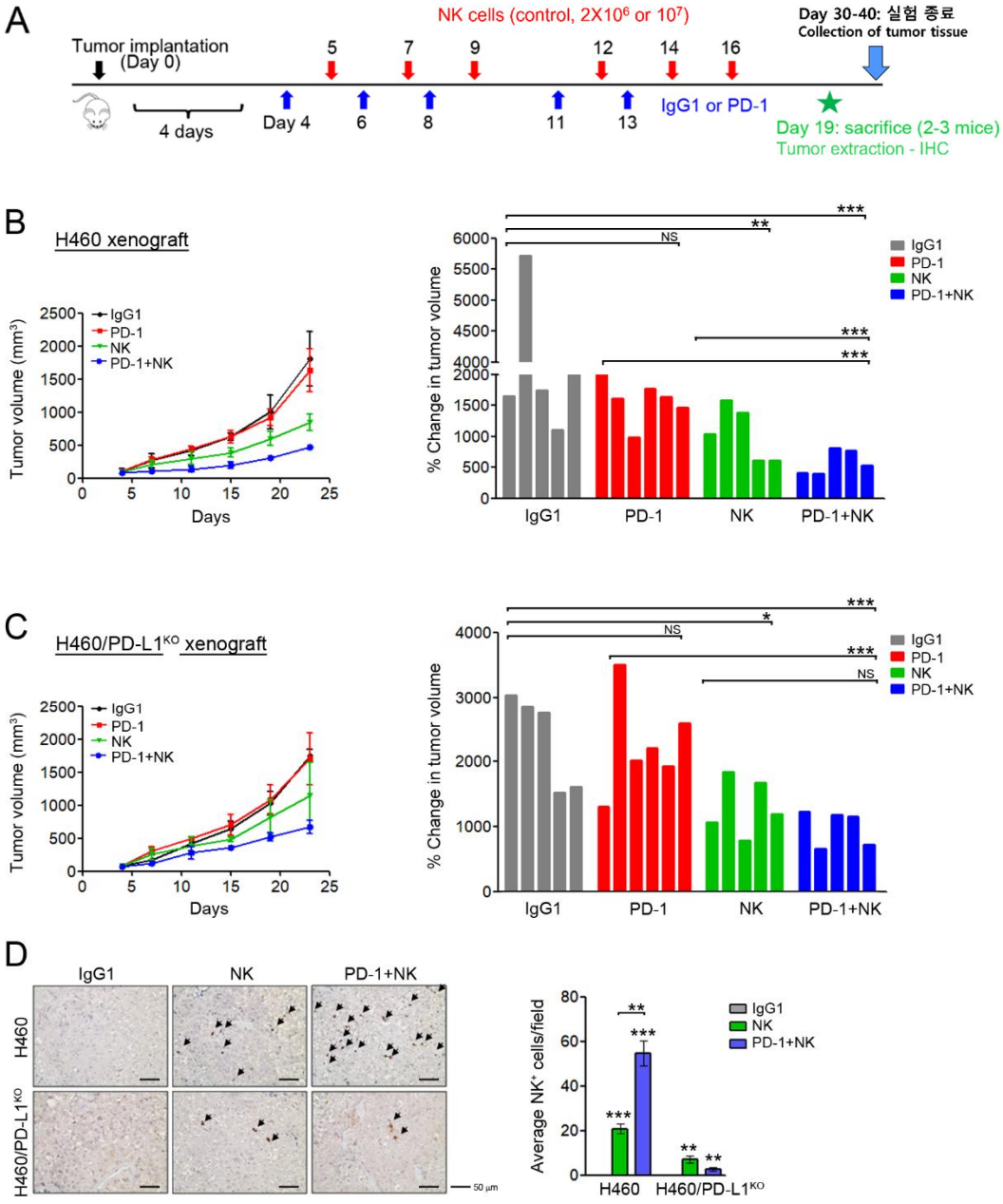
Figure 1. The role of T cells and NK cells in the therapeutic effect of anti-PD-1 antibody†



(A) C57BL/6N mice were injected with 2 x 10^5 of untransduced or PD-L1 expression vector-transduced (mPD-L1) LLC-1 mouse lung carcinoma cells and treated with 10 mg/kg of anti-PD-1 Ab or control IgG. N=4 mice/group. Tumor volume was monitored and measured on the indicated days. (B) Tumor growth was assessed by comparing the initial and final tumor volumes. (C-I) The mice were injected with 2 x 10^5 of LLC-1 mouse lung carcinoma cells. Mice were divided into groups and depleted of T cells by intraperitoneal (IP) injection of 12.5 mg/kg anti-CD8α monoclonal antibodies and 25 mg/kg GK1.5 monoclonal antibodies, NK cells by IP injection of 12.5 mg/kg μg PK136, or both before tumor cell injection (n=4 mice/group). (C) The effects of T cell-, NK cell-, and both T cell- and NK cell-depletion on therapeutic effect of anti-PD-1 Ab were determined by measuring the tumor volumes of undepleted mice (D), T cell-depleted mice (E), NK-depleted mice (F), and both T cell- and NK cell-depleted mice (G). (H) PD-1 efficacy was determined by comparing the tumor volumes of IgG1-treated and anti-PD-1 Ab-treated mice according to depletion status. (I) Tumor growth was assessed by comparing the initial and final tumor volumes. *$P < 0.05$ and **$P < 0.005$, by a paired two-tailed Student's t-test.

†Some of the data included in this figure were also included in the supplementary data of the paper [24] published by the author.

Figure 2. The efficacy of combination therapy with anti-PD-1 antibody and NK cells†

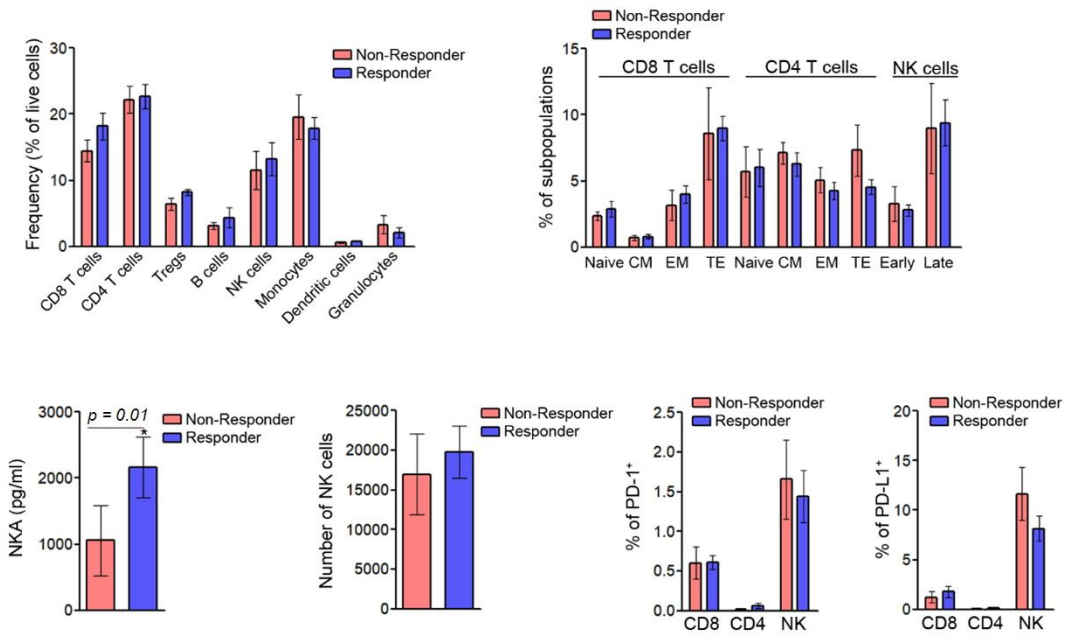


(A) Drug treatment schedule. NOD-rag1$^{-/-}$Il2rg^{null} mice were injected with 10 mg/kg of control IgG, anti-PD-1, or 2 x 10^6 or 10^7 NK cells at the indicated times. (B and C) The mice were injected with 1 x 10^6 H460 or PD-L1 knockout H460 cells before therapy. Tumor volume was monitored and measured on the indicated days. The panels on the right panel show the final tumor volume. (D) The mice were sacrificed at 15 days after therapy and the tumors were harvested from tumor-bearing mice. Tumor-infiltrating NK cells were identified via

immunohistochemical analysis. *$P < 0.05$, **$P < 0.005$, and ***$P < 0.0005$, by a paired two-tailed Student's t-test.

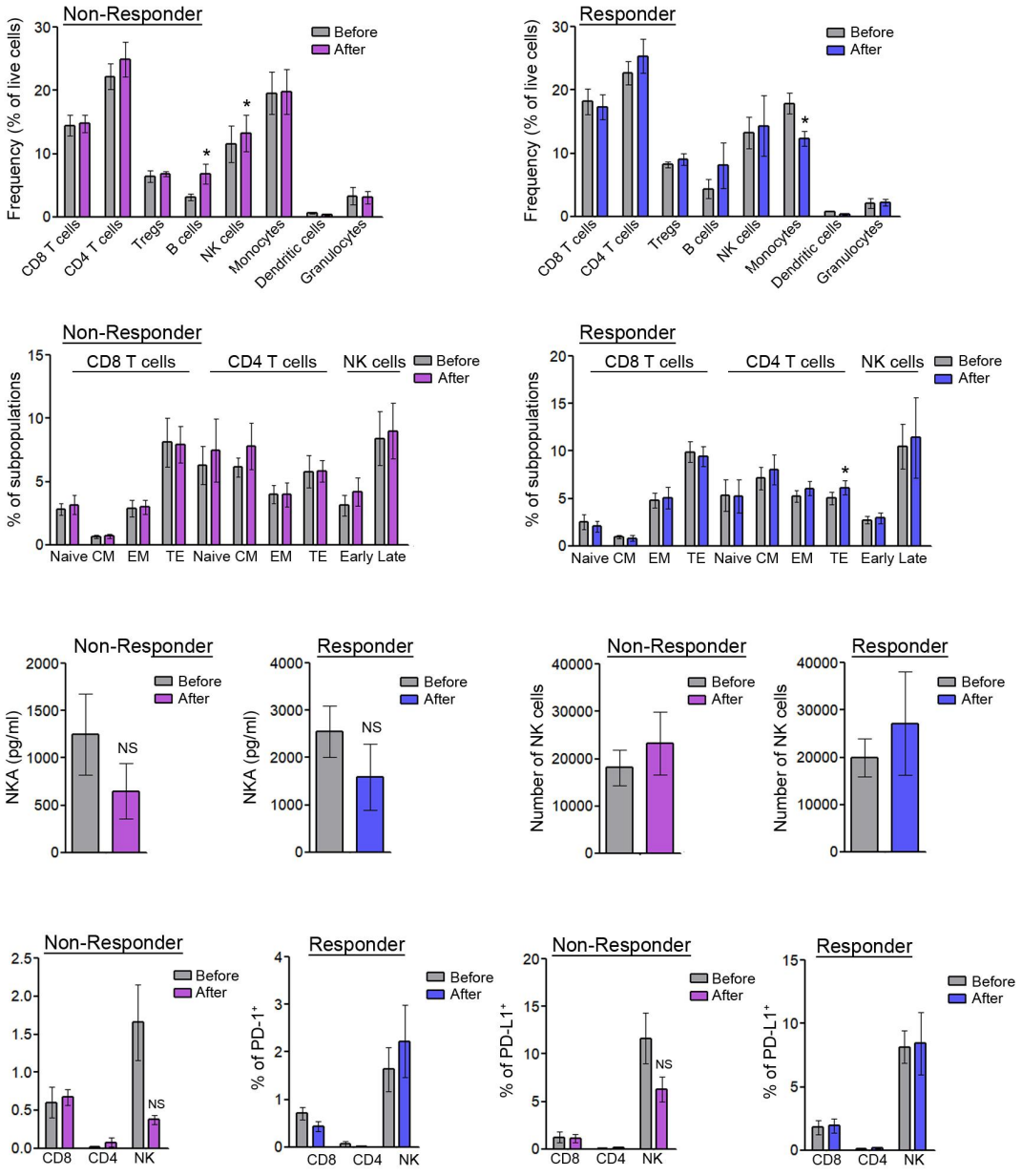
†Some of the data included in this figure were also included in the supplementary data of the paper [24] published by the author.

Figure 3. Immune cell profiling in responder vs non-responder NSCLC patients treated with autologous NK cell/anti-PD-1 antibody combination at visit 1 (pre-treatment)



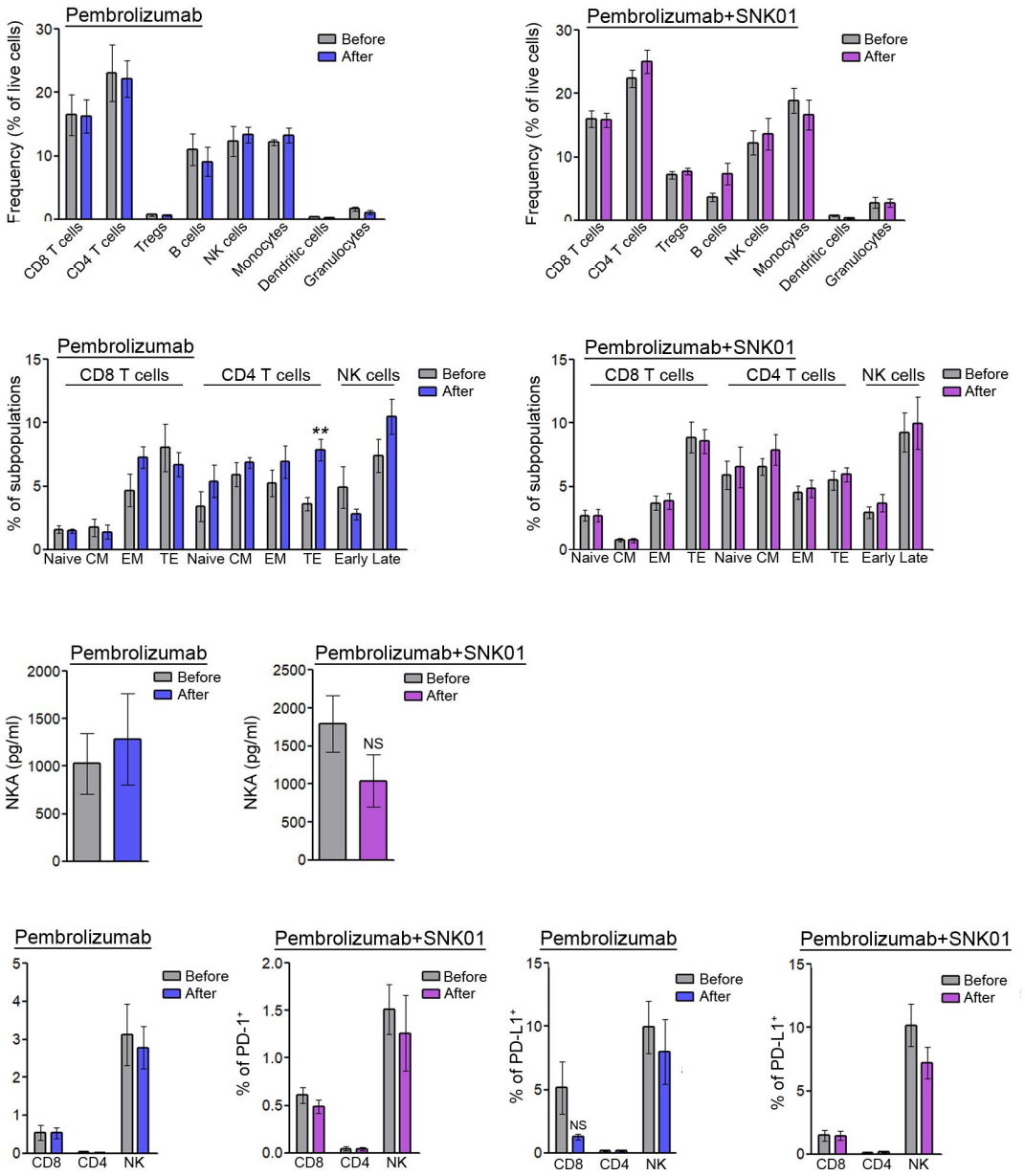
CM, central memory; EM, effector memory; TE, terminal effector; Memory phase, CM+EM;

Figure 4. Immune cell profiling in responder/non-responder NSCLC patients treated with autologous NK cell/anti-PD-1 antibody combination before vs. after treatment



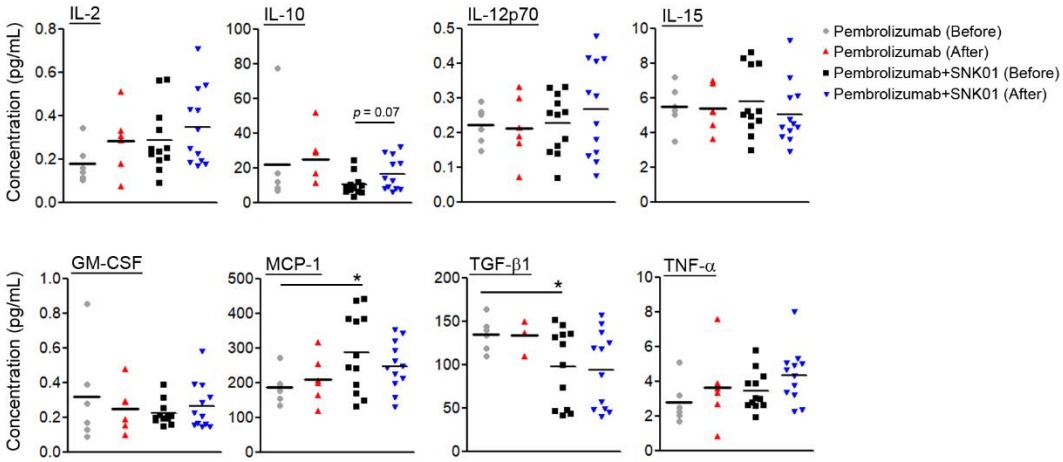
CM, central memory; EM, effector memory; TE, terminal effector; Memory phase, CM+EM; *$P < 0.05$, **$P < 0.005$, and ***$P < 0.0005$ by a paired two-tailed Student's t-test.

Figure 5. Immune cell profiling in NSCLC patients treated with autologous NK cell (SNK01)/anti-PD-1 antibody (pembrolizumab) combination or anti-PD-1 antibody alone before vs. after treatment



CM, central memory; EM, effector memory; TE, terminal effector; Memory phase, CM+EM; *$P < 0.05$, **$P < 0.005$, and ***$P < 0.0005$ by a paired two-tailed Student's t-test.

Figure 6. Cytokine analysis in NSCLC patients treated with autologous NK cell (SNK01)/anti PD-1 antibody (pembrolizumab) combination vs. anti-PD-1 antibody alone before vs. after treatment



*$P < 0.05$, **$P < 0.005$, and ***$P < 0.0005$ by a paired two-tailed Student's t-test.

국문 요약

배경: 면역관문억제제가 개발 및 적용되면서 비소세포폐암(NSCLC)의 치료에 큰 발전이 있었지만, 여전히 면역관문억제제의 낮은 반응률은 당면문제 중 하나이다. 자연살해(NK) 세포가 암세포에 대한 면역반응에 참여하기 때문에 NK 세포 매개 면역치료제의 개발은 현재 널리 사용되는 T 세포 매개 면역치료제의 제한적인 효능을 극복하는 전략이 될 가능성이 있다. 본 연구는 항 PD-1 항체 치료시 NK 세포의 역할을 평가하고 NSCLC 에서 항 PD-1 항체와 병용한 자가 NK 세포 요법의 효능을 평가하기 위해 수행되었다.

대상 및 방법: NK 세포를 활성화시키는 일련의 과정을 통해 유전자 변형되지 않은, 생체 외에서 배양/증식시킨 사람 NK 세포(SNK01) 제제가 생산되었다. 항 PD-1 항체 치료에 의해 유도된 항종양 면역반응에서 NK 세포의 역할 및 항 PD-1 항체 단독요법과 비교하여 SNK01 및 항 PD-1 항체 병용요법의 생체내 효능을 마우스 모델에서 평가하였다. 이러한 전임상 결과를 바탕으로 항 PD-1 항체와 SNK01 병용요법의 작용기전을 이해하기 위해 치료에 대한 반응, 시점 (면역치료 전/후) 및 치료군에 따른 면역세포의 수 및 사이토카인 수치를 분석하였다.

결과: PD-L1 과발현 마우스 NSCLC 세포를 주입한 마우스 모델에서, NK 또는 T 세포가 인위적으로 고갈시켰을 때 항 PD-1 항체요법은 효과가 없었지만, 면역세포를 고갈시키지 않은 마우스에서는 효과적이었다. 이러한 결과는 PD-1 차단이 NK 세포 및 T 세포 반응을 동원하고, NK 세포가 PD-1 차단의 치료적 효과에 기여함을 시사한다. SNK01 및 항 PD-1 항체의 병합요법은 PD-L1 발현 여부와 관계없이 NOD-rag1$^{-/-}$Il2rg^{null}(NRG) 마우스 모델에서 사람 NSCLC 세포 이종이식 종양의 성장을 억제시켰으며, 이 병용요법이 PD-L1 음성 NSCLC 환자에게도 효과적일 가능성을 시사하였다. NSCLC 환자를 대상으로 한 임상 연구에서 반응군 (n=5)의 환자들은 비반응군 (n=13)의 환자들에 비해 치료전 NK 활성도가 더 높은 경향을 보였다 (p=0.01). 이외에는 치료군, 치료 반응 또는 시점 (치료 전 vs. 후)에 따른 면역세포 수와 사이토카인 수치에는 유의한 차이가 없었다.

결론: 본 연구의 결과에 따르면 항 PD-1 항체와 자가 NK 세포의 병합요법은 NSCLC 의 새로운 치료 옵션으로 고려될 수 있다.