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Case Report: Feasibility and Safety of Autologous NK Cell Therapy in Patients with Cancer

Supansa Nilubol[1,2], Worawit Kitisakronnakorn[3], Pimjai Naigovit[1,2]

[1]LBM Stem Cell Research Unit, Life Balance Methodology Co., Ltd., Bangkok, Thailand
[2]National Institute of Health, Ministry of Public Health, Nonthaburi, Thailand
[3]Better Being Hospital, Bangkok, Thailand
Email: Info@lbmet.com

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Abstract

This study reported two cases of Thai cancer patients, including a 36-year-old female with thyroid cancer of more than 5 years and a 64-year-old male with lung and colon cancers of more than 10 years. The written informed consent was provided for autologous natural killer (NK) cell infusion at the anti-ageing and regenerative medicines clinic. Briefly, the blood was taken from the patient for NK cell count and their cytotoxic activity. Then, the patient's NK cells were expanded *in vitro*, characterized and then counted before being delivered to the same patient by a single intravenous infusion. The vital signs and general physical examinations were observed for 2 - 6 hours after the infusion. The patients were discharged if there were no adverse effects. The data showed the increasing number of NK cells and level of cytotoxic activity after the NK cell treatment, compared to the pre-treatment. In addition, the increasing total live cell concentration, as identified by the high percentage of $CD56^{dim}/CD16^{bright}$ cytotoxic NK cells, at day 21 of the NK cell expansion was consistent with the increasing cytotoxic activity of the patients after the treatment. Here, we demonstrated that this autologous NK cell therapy might be feasible; however, the study did not aim to evaluate the anti-cancer effect.

Keywords

Natural Killer Cells, NK Cell Therapy, Cancer, Mono-Treatment, Rejuvenation

1. Introduction

Natural Killer (NK) cells are accounted for 5% - 15% of lymphocytes in the peripheral blood. Besides, they reside in lymphoid and non-lymphoid organs such

as the spleen, lungs, and liver. The NK cells are characterized by the expression of $CD56^{bright}CD16^{dim/-}$ (accounting for less than 15% of total NK cells in the blood which are less mature and less potent cytokine-producing capacity) and the expression of $CD56^{dim}CD16^{+}$ (accounting for most NK cells which are more mature and more potent cytotoxicity) [1] [2] [3]. Although the NK cells are lymphocytes, they can function as an innate immune system that requires no prior sensitization [4] [5] [6].

Potential effects of NK cells against cancer have been demonstrated on several occasions, including hematopoietic stem cell transplantation (HSCTs), bone marrow transplantation and isolated NK cell infusion. In a previous report, the infusion of NK cells of HLA-haploidentical donors in a patient with aggressive acute myeloid leukemia (AML) could prohibit the disease relapse and metastasis [7] [8] [9]. In addition to hematological malignancies, the effects of NK cells against solid cancers, including metastatic colorectal cancer, glioblastoma, and ovarian cancer, have been demonstrated either in preclinical xenograft models or early clinical studies [10] [11] [12] [13]. Moreover, the safety of the NK cell-based therapy was demonstrated in both allogeneic haploidentical and autologous settings [9] [14] [15] [16].

In allogenic NK cell-based therapy, the contaminated T cells are of concern as they can trigger the potential adverse effects from Graft Versus Host Diseases (GVHD). On the contrary, the GVHD is less likely in autologous NK cell transfusion [17]. Recently, the methods for NK activation, expansion, and purification have been dramatically improved. Therefore, the NK cells could be purified before the cell expansion to minimize cell contamination. In addition, the cell expansion technique allowed the cell number to achieve the therapeutic level [17].

From research to clinical use, human NK cell activation and expansion kits are now available. Of note, FDA-approved Interleukin-2 (IL-2), the key cytokine for NK cell survival and cytotoxicity activation has been used for several cancer treatments [18] [19]. However, the efficiency of NK cell culture, in terms of survival period and yields of cells, highly depends on the quality and characteristics of the kits and the cell sources. For example, 1.59×10^{10} NK cells with 92.37% purity can be yielded from umbilical cord blood-derived NK progenitor expansion at 21 days [20]. In comparison, 21-day NK cultures from the placenta yielded only 1.2×10^{9} NK cells with 80% viability [21]. However, the recommended maximum tolerated dose, total dosage amount, and dosage intervals remained inconclusive [9] [15] [16] [22] [23] [24].

From several clinical studies, the feasibility of utilizing NK cells for cancer immunotherapy in both hematological and solid cancers has gained increasing evidence. Moreover, the clinical scale *ex vivo* NK cell activation and expansion kit are now commercially available. The NK cell-based therapy thus became an alternative therapeutic tool or an adjuvant to the standard cancer treatment such as surgery, radiation and chemotherapy. This study demonstrated an increasing

NK cell number with improving cytotoxic activity in two Thai patients with cancers after the infusion of autologous NK cell therapy. The data also showed the increasing concentration of total live cells at day 21 of NK cell expansion with a high percentage of $CD56^{dim}/CD16^{bright}$ cytotoxic NK cells, consistent with the increasing cytotoxic activity in the patient after treatment. We, therefore, suggested that this autologous NK cell therapy might be feasible. However, our study did not design to explore the anti-cancer effect.

2. Case Report

A 36-year-old Thai female patient with thyroid cancer of more than 5 years and a 64-year-old Thai male patient with lung and colon cancers of more than 10 years were included in the study. The patients provided written informed consent to receive the autologous natural killer cell infusion for cancer therapy. All processes of the NK cell therapy were performed under the physician supervision. Prior to the treatment, the patients were screened for infections of the human immunodeficiency virus (HIV), hepatitis B virus (HBV), and hepatitis C virus (HCV), systemic inflammation and liver function test. Moreover, complete blood count (CBC), natural killer cell (NK Cell) count, and NK cell activity were evaluated. A 3.0 ml EDTA blood (VACUETTE, Thailand) was collected for evaluating the complete blood count (CBC) and NK cell count. The cluster of differentiation (CD) marker expression as $CD56^{+}/16^{+}/3^{-}$ was used for NK cell characterization by the flow cytometer. A 10 - 12 ml lithium heparinized blood (VACUETTE, Thailand) was collected to evaluate the NK cell activity. In the NK activity assay, the NK cells from patient blood were used as the effector cells (E) while human tumor K562 cell lines (ATCC® CCL243™, USA) were used as the target cells (T). NK effector cells were incubated with K562 target cells at different E:T ratios (ranged from 50:1 to 12.5:1) in a 37˚C humidified incubator with 5% CO_2 for 4 hours. The NK cell activity was measured by a microfluorometer for the female patient and a flow cytometry-based NK cytotoxicity assay for the Thai male patient.

After the patient enrolment, the 50.0 - 100.0 ml heparinized blood samples were collected and then immediately processed to ensure the highest efficiency of isolated peripheral blood mononuclear cells (PBMCs). Briefly, the blood samples were centrifuged at 2000 rpm at 22˚C for 10 min to separate the top auto-plasma layer. Next, the bottom layer of the concentrated blood sample was diluted with RPMI 1640 medium (Corning®, USA). The diluted samples were carefully layered on the lymphocyte separation medium (Corning®, USA) and then centrifuged at 2000 rpm at 22˚C for 20 min. The PBMCs layer was collected and measured the cell number by dual-fluorescence dye using Luna automated cell counter (Logos Biosystems®, Korea). The density of PBMCs samples was adjusted to 1×10^6 cells/ml with KBM NK primary medium (Corning®, China) supplemented by 1.8 ml of KBM NK primary supplement and 10% of auto-plasma. The PBMCs were transferred into an antibody pre-coated 75 cm^2 cell

culture flask (Corning®, China) and humidified incubated at 37˚C under 5% CO_2. After 5 - 6 days of NK cells activation (cell density was more than 2×10^6 cells/ml), non-adherent NK cells were mixed with an appropriate volume of KBM NK Expansion medium (Corning®, China) containing 1000 IU/ml of IL-2 (StemCell Technologies®, United States) and 10% of auto-plasma. The cell mixture was then transferred into a gas-permeable culture bag (Corning®, China) and humidified incubated at 37˚C under 5% CO_2 for 21 days. NK cell samples were collected to measure NK cells number every 2 - 3 days by dual-fluorescence dye using Luna automated cell counter (Logos Biosystems®, Korea) for cell viability record. The high capacity of proliferation was observed in the female patient, as the NK cell number was increased to 6660.0×10^6 cells with 99.9% viability at 21 days, compared with 14.9×10^6 cells at day 3 of incubation (**Figure 1(a)**). In the male patient, the NK cell number increased from 19.0×10^6 cells to 8280.0×10^6 cells with 99.6% viability (**Figure 1(b)**).

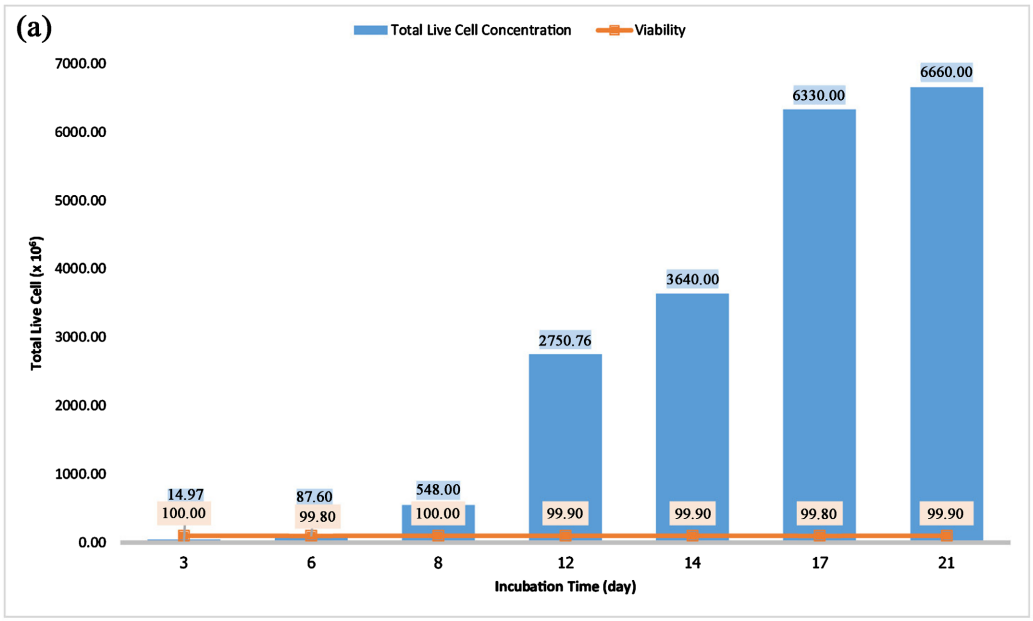
In addition, at day 20 of cell expansion, the NK cells were subjected for quality control tests including immunophenotyping ($CD56^+/3^-$ for the NK cell marker, $CD56^{dim}/CD16^{bright}$ for the cytotoxic NK cell, $CD56^{bright}/CD16^{+/-}$ for the cytokine-producing NK cell, and $CD3^+/8^+$ for the cytotoxic T cell), and sterility tests (including mycoplasma, endotoxin, bacteria, and fungi) before the batch release. The positive markers in the expanded NK cells included $CD56^+/3^-$ (61.9%), $CD56^{dim}/CD16^{bright}$ (43.3%), $CD56^{bright}/CD16^{+/-}$ (2.4%) and $CD3^+/8^+$ (19.0%) (**Figure 1(c)**) for Thai female patient and $CD56^+/3$ (73.6%), $CD56^{dim}/CD16^{bright}$ (65.5%), $CD56^{bright}/CD16^{+/-}$ (0.1%), and $CD3^+/8^+$ (0.1%) (**Figure 1(d)**) for Thai male patient.

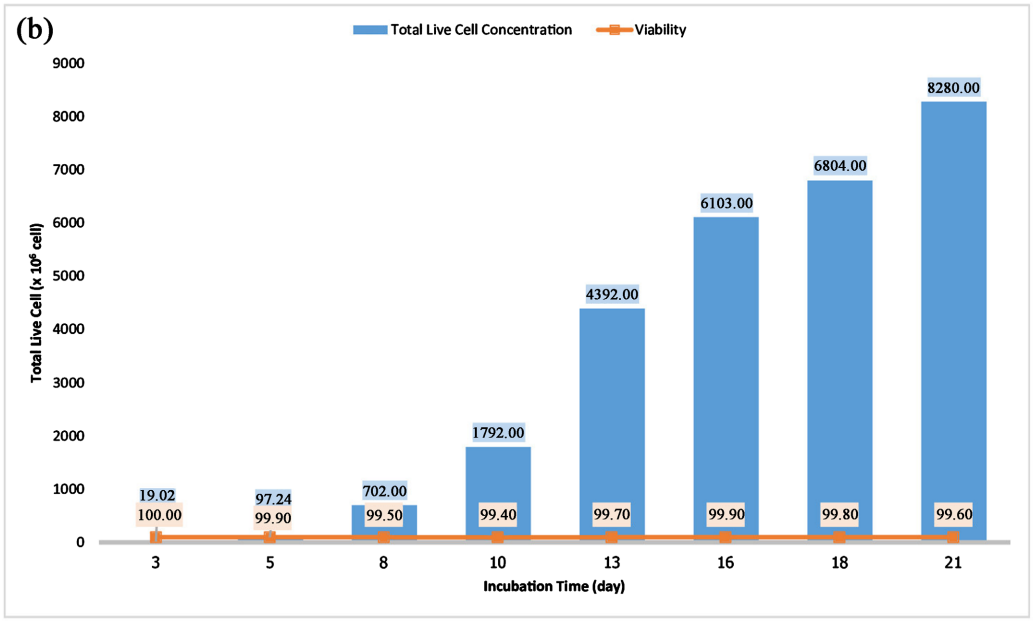
The allergy skin test was performed 30 minutes prior to the treatment. After the premedication with intravenous 10 mg chlorphenamine and 10 mg dexamethasone, all 21-day-cultured autologous NK cells were intravenously infused into the patient. The vital signs and general physical examination were monitored for 2 - 6 hours following the infusion, and the patients were discharged if there were no adverse effects.

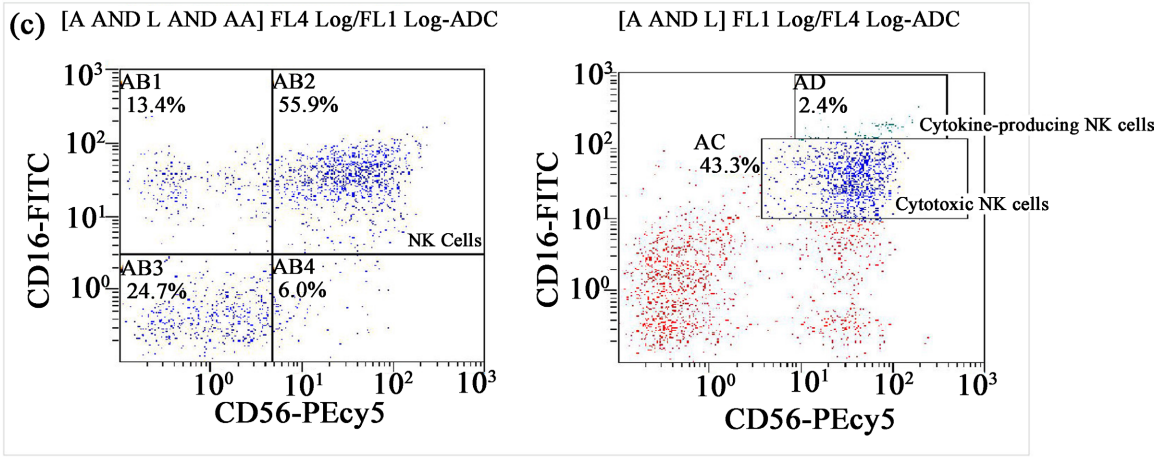
Three months after autologous NK infusion, patients were evaluated for CBC, NK cell count, and NK activity. The trend of both NK cell count and NK activity was increasing in both patients. NK cell count ($CD56^+/16^+/3^-$) increased in number from 122 cell/mm^3 to 486 cell/mm^3 in the female patient (**Figure 2(a)**) and from 598 cell/mm^3 to 803 cell/mm^3 in the male patient (**Figure 2(b)**). Consistently, NK activity also increased from 41% to 49% in the female patient (**Figure 2(c)**), which were from 10.30% to 67.10%, 10.80% to 59.50%, and 11.30% to 63.30% at the ratio of 50:1, 25:1, and 12.5:1, respectively in the male patient (**Figure 2(d)**).

3. Discussion

We demonstrated the feasibility and safety of autologous NK cell therapy in thyroid, lung and colon cancer patients using a commercial expansion method,


(a)
Total Live Cell Concentration
Viability
Total Live Cell (x 10⁶)
7000.00
6000.00
5000.00
4000.00
3000.00
2000.00
1000.00
0.00
14.97
100.00
87.60
99.80
548.00
100.00
2750.76
99.90
3640.00
99.90
6330.00
99.80
6660.00
99.90
3
6
8
12
14
17
21
Incubation Time (day)


(b)
Total Live Cell Concentration
Viability
Total Live Cell (x 10⁶ cell)
9000
8000
7000
6000
5000
4000
3000
2000
1000
0
19.02
100.00
97.24
99.90
702.00
99.50
1792.00
99.40
4392.00
99.70
6103.00
99.90
6804.00
99.80
8280.00
99.60
3
5
8
10
13
16
18
21
Incubation Time (day)


(c)
[A AND L AND AA] FL4 Log/FL1 Log-ADC
AB1
13.4%
AB2
55.9%
NK Cells
AB3
24.7%
AB4
6.0%
CD16-FITC
CD56-PEcy5
[A AND L] FL1 Log/FL4 Log-ADC
AD
2.4%
Cytokine-producing NK cells
AC
43.3%
Cytotoxic NK cells
CD16-FITC
CD56-PEcy5

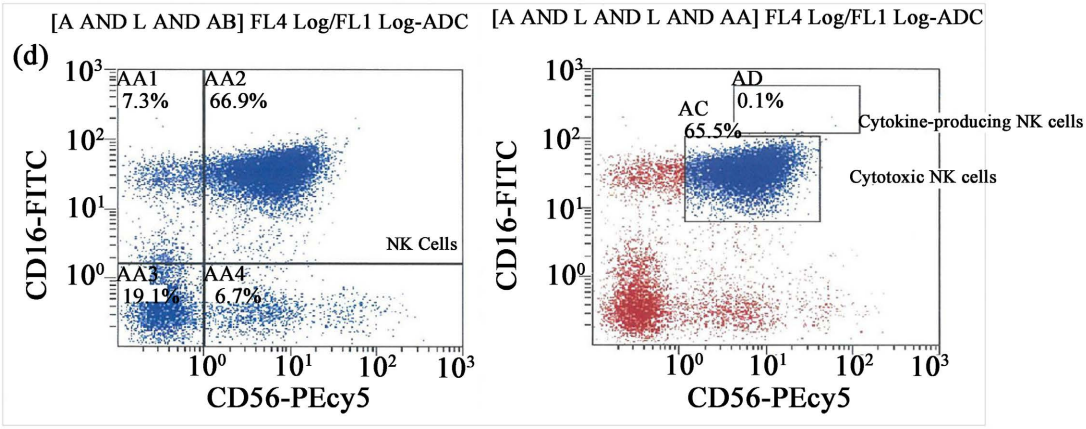
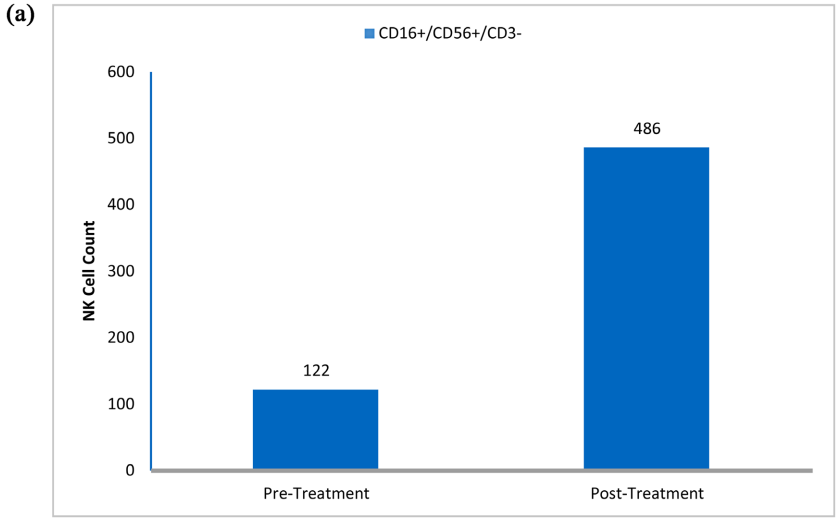
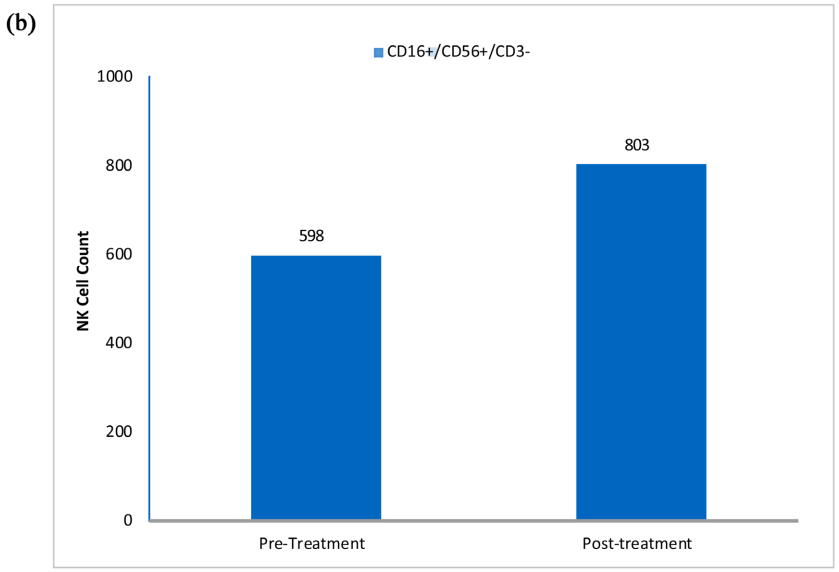


Figure 1. Cell viability and concentration during 21 days of cultivation show high percentage of viability and high capacity of proliferation in both patients. (a) Increasing number of cells up to 6660.0 × 10^6 cells with 99.9% viability at 21 days in Thai female patient. (b) Cell number increased from 19.0 × 10^6 cells to 8280.0 × 10^6 cells with 99.6% viability in Thai male patient. (c) Immunophenotype of NK cell population in Thai female patient and Thai male patient (d).





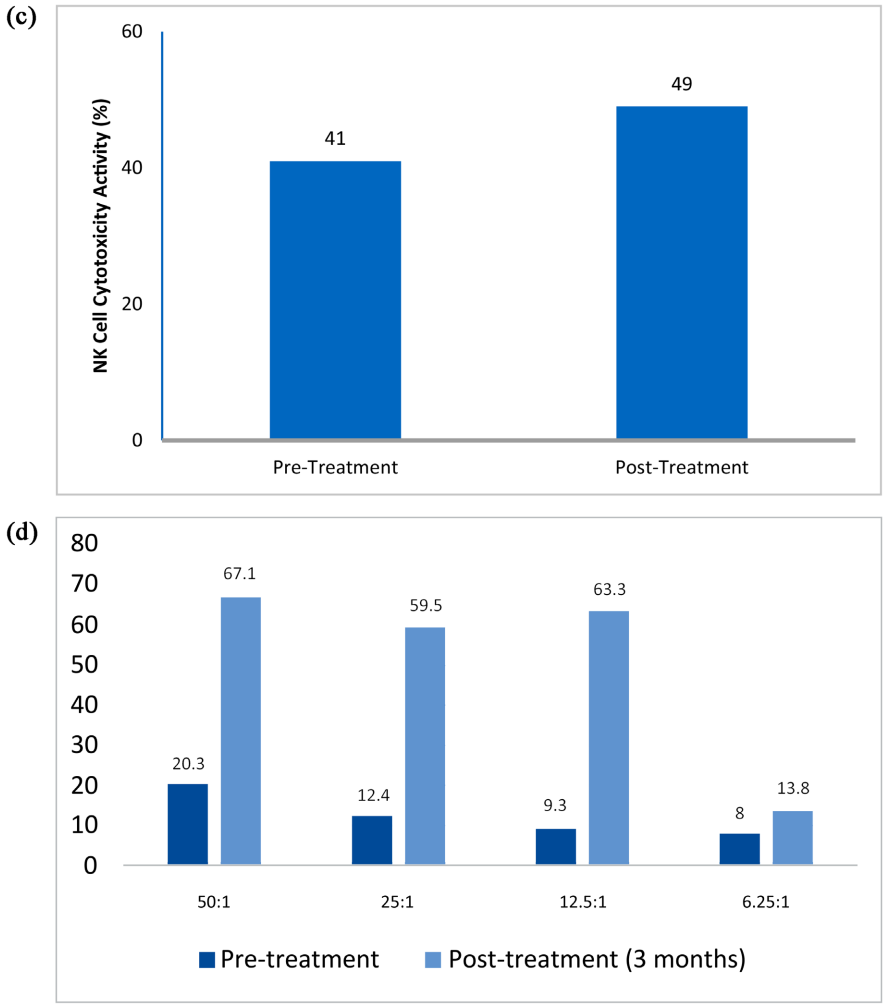


Figure 2. After three months of autologous NK infusion, NK cell count was increased in both female (a) and male (b) patients. Consistently, NK activity also increased in the female patient (c) using micro-fluorometer, and in the male patient (d) using flow cytometry-based NK cytotoxicity assay.

Corning® NK Expansion kit. With the kit, the PBMCs show rapid expansion (~100-fold proliferation) with high yield (~3 × 10^9 cells on day 14 from a 30 ml blood sample) and a high level of surface marker expression (83% of $CD56^+CD3^-$ on day 14). In addition, our report demonstrated the purity of the infused NK cells at the therapeutic level for cancer patients. After 21 days of NK cell expansion, total live NK cells were increased, and a high percentage of $CD56^{dim}/CD16^{bright}$ cytotoxic NK cells were observed. These immunophenotypes may be responsible for the increased NK cell cytotoxic activity of the patients after the NK cell infusion for 3 months. Micro-fluorometry and flow cytometry were used for evaluating NK cell cytotoxic activity in different patients. The NK cell activities were compared between pre-and post-treatment in the same patient. Though our report contains the data from only two patients, the quantitative measurement of the NK cell activities could imply the possible therapeutic effects of the NK cell therapy in other cancer patients. Moreover, our study outlined

the feasibility of NK cell therapy but did not aim to evaluate the anti-cancer effects.

NK cells in peripheral blood are generally found in a small proportion of the lymphocytes. In cancer patients, the NK cells are usually lower in number and have less cytotoxic activity. Therefore, an *ex vivo* NK cell expansion to reach sufficient cell numbers with improving killing efficiency could benefit cancer patients. Several studies aimed to generate a highly efficient protocol to expand the NK cells with high purity. However, the biggest obstacle is the limited number of pure NK cells without prior NK cell purification. In one study, the use of interleukin-2 (IL-2) can induce an NK cell expansion for up to 50 folds after 2 weeks. Another study revealed 1600-fold NK expansions after 20 days of the cultured NK cells using IL-2 and anti-CD3 induction from the blood of cancer patients, but NK cytotoxicity against K562 cells was lesser than 10% at 1:1 E/T ratio [25] [26] [27] [28] [29]. While Sakamoto *et al.* demonstrated the higher level of cytotoxicity 38.77% at 0.75:1 and 57.54% at 1.56:1 E/T ratio [14].

In the expansion kit, the cytokine cocktails mainly comprise IL-2, IL-12, and IL15, which can induce various intracellular signaling events, including Jak1, Jak2, Jak3, STAT1, STAT5, and STAT4 transcriptions, resulting in stimulating cell proliferation and enhancing cytotoxicity of the NK cells. In our cases, the NK cells were expanded to approx. 400 folds at day 21, with the high yield of cell numbers at approx. 6.6 and 8.2 × 10^9 cells and high level of $CD56^+CD3^-$ at 61.9% and 73.6%. In our cases, the relatively lower NK cell purity (83% of NK cells) may be due to the lower number of transferred cell numbers and the adjuvant chemotherapy and systemic IL-2 administration in other studies [30].

Using the flow cytometry, the expanded NK cells demonstrated a high level of CD16 surface marker, indicating the NK cell subtypes predominately involved in cytotoxicity and ADCC activation. Furthermore, this finding was correlated with the increasing NK cell cytotoxicity level against the K562 cells after the NK cell therapy. However, as the CD16 expression of the NK cells decreased approx. 30% after being thawed (data from Phase I clinical trial of autologous NK cell therapy), the fresh blood was used for NK expansion. Additionally, the freshly expanded NK cells were transfused to the patients. Thus, the protocol may explain the high level of $CD16^+$ NK cells and the increasing cytotoxic activity in our cases.

4. Conclusion

In our cases, we mainly focused on possible toxicities of NK cell therapy, including NK cell purity, cell number, and cytotoxicity activity, to evaluate the safety of the expanded NK cells using the commercial kit. The NK cell expansion can achieve a high level of purity and functionality. In addition, the relatively small amount of NK cells used in the infusion to our patients may guarantee safety. However, the efficacy of NK cells in cancer treatment requires further investigations.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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