



Original Article

Establishment of an efficient ex vivo expansion strategy for human natural killer cells stimulated by defined cytokine cocktail and antibodies against natural killer cell activating receptors

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ABSTRACT

Introduction: Cell-based immunotherapy is categorized as a regenerative therapy under the Regenerative Medicine Safety Act in Japan. Natural killer (NK) cell-based immunotherapy is considered a promising strategy for treating cancer, including glioblastoma (GBM). We previously reported an expansion method for highly purified human peripheral blood-derived NK cells using a cytokine cocktail. Here, we aimed to establish a more efficient NK cell expansion method as compared to our previously reported method.

Methods: T cell-depleted human peripheral blood mononuclear cells (PBMCs) were isolated from three healthy volunteers. The depleted PBMCs were cultured in the presence of recombinant human interleukin (rhIL)-18 and high-dose rhIL-2 in anti-NKp46 and/or anti-CD16 antibody immobilization settings. After 14 days of expansion, the purity and expansion ratio of CD3-CD56⁺ NK cells were determined. The cytotoxicity-mediated growth inhibition of T98G cells (an NK activity-sensitive GBM cell line) was evaluated using a non-labeling, impedance-based real-time cell analyzer.

Results: Anti-NKp46 stimulation increased the NK cell purity and expansion ratio as compared to the non-antibody-stimulated population. Anti-CD16 stimulation weakly enhanced the NK cell expansion ratio of the non-antibody-stimulated population and enhanced the NK cell purity and expansion ratio of anti-NKp46-stimulated populations. All NK cell-containing populations tested distinctly inhibited T98G cell growth. These effects tended to be enhanced in an NK cell purity-dependent manner. In some cases, anti-CD16 stimulation decreased growth inhibition of T98G cell compared to other conditions despite the comparable NK cell purity.

Conclusions: We established a robust large-scale feeder-free expansion system for highly purified human NK cells using a defined cytokine cocktail and anti-NK cell activating receptor antibodies. The expansion system could be feasible for autologous or allogeneic NK cell-based immunotherapy of GBM. Moreover, it is easily controlled under Japanese law on regenerative medicine.

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1. Introduction

In 2014, Japan enacted the Regenerative Medicine Promotion Act, followed by two other associated Acts on translational regenerative medicine. The first is the Pharmaceuticals, Medical Devices, and Other Therapeutic Products Act (PMDA), which created a new category for regenerative medical products in addition to the previously categorized pharmaceutical products, medical device products, quasi-drugs, and cosmetics. The PMDA regulates the

production and marketing of regenerative and cellular therapeutic products by firms. The second law is the Safety of Regenerative Medicine Act (RM Act), which established a framework for regenerative medicine in clinical research and clarified the measures necessary for ensuring patient safety. The RM Act specified the regulations that physicians, review committees, and cell processing facilities must adhere to when providing regenerative medicine in medical care not only in clinical research, but also in private practice [1]. In the RM Act, regenerative medicine technologies and products are defined as processed live human/animal cells intended for use in reconstructing, repairing, or forming structures or functions of the human body, or in treating or preventing human diseases [1]. Immune cells used in cancer immunotherapy are categorized under the above definition.

Immunotherapy has progressed greatly and has become a promising approach for treating several cancers in the last decade. T cell-based immunotherapy, including chimeric antigen receptor-expressing T cell (CAR-T) therapy, has been developed and successfully applied in the clinical treatment of leukemia. However, the effects are limited against solid tumors, including glioblastoma (GBM), as the loss of tumor antigens and heterogeneity cause immune escape [2,3]. Further, CAR-T therapy is subject to limitations such as the risk of cytokine release syndrome and neurotoxicity caused by immune cell-associated neurologic syndrome [4–8]. Therefore, there is a need to explore the availability of cells with antitumor effects apart from CAR-T.

Natural killer (NK) cell-based immunotherapy is considered a promising approach for treating a variety of solid tumors. NK cells directly kill virus-infected cells and transformed cells via the granzyme–perforin pathway without being sensitized [9–11]. Unlike T cells, NK cells recognize plural antigens of target cells via the NK cell activating and inhibitory receptors, namely a major histocompatibility complex (MHC)–T cell receptor-independent mechanism [12]. NK cells do not kill normal cells but kill virus-infected cells and cancer cells that have lost MHC expression or MHC-mismatched allogeneic cells. The “missing self” hypothesis states that NK inhibitory receptors bind MHC class I and inhibit NK cell functions [13]. Recently, the United States Food and Drug Administration (FDA) approved an investigational new drug for the use of placenta-derived NK cells against GBM, the most aggressive brain tumor. This success has encouraged many ongoing clinical investigations of NK cell-based cancer immunotherapy alone or in combination with other modalities [14]. [Clinicaltrials.gov](https://clinicaltrials.gov) currently lists more than 100 clinical trials of NK cell-based cancer immunotherapy. The safety and efficacy of allogeneic or autologous donor-derived NK cell-based immunotherapy for treating hematological malignancies, such as acute myeloid leukemia, have been well-established [15–17]. Naturally, clinical trials of NK cell-based therapy for solid tumors are ongoing. The Phase I or II clinical trials are evaluating the safety and efficacy of NK cells combined with nimotuzumab for treating late-stage malignancies (NCT0354889) and of NK cell infusion in patients with advanced malignant tumors following multi-line therapies (NCT03619954).

Several strategies for obtaining large numbers of peripheral blood-derived NK cells have been developed [18]. We previously reported a highly purified and efficient expansion method for NK cells, i.e., T cell-depleted peripheral blood mononuclear cells (PBMCs) were stimulated under defined cytokine cocktail conditions [19]. The expanded NK cells exhibited antitumor effects against GBM in vitro and in vivo, and against 3-dimensional GBM cell-derived spheroids [20,21]. In some cases, however, the NK cells demonstrated low expansion efficiency and purity based on the condition of the obtained PBMCs.

In the present study, we evaluated NK cell purity and expansion efficacy using specific culture conditions where CD3-depleted

PBMCs were stimulated with the defined cytokine cocktail in the presence of agonistic antibodies to NK cell activation receptors without feeder cells such as cancer cells as compared to our earlier method. The proposed method resulted in the robust expansion of highly purified cytotoxic human NK cells for adoptive immunotherapy of GBM.

2. Materials and methods

2.1. Ethics

Peripheral blood was collected from healthy volunteers with the approval of the Nara Medical University Ethics Committee (No. 1058) and in accordance with its guidelines. Informed consent was obtained according to the tenets of the Declaration of Helsinki.

2.2. Antibody-coated flask

Anti-human NKp46 antibody (clone 195314, R&D Systems, Minneapolis, MN, USA) and/or anti-human CD16 antibody (clone 3G8, Thermo Fisher Scientific, Waltham, MA, USA) (both 5 µg/mL) were prepared in phosphate-buffered saline (PBS; Kohjin Bio, Saitama, Japan) containing 0.1% human serum albumin (FUJIFILM Wako Pure Chemical, Tokyo, Japan). For antibody immobilization, 2 mL antibody solution was transferred to 6-well plates (Corning, Steuben, NY, USA) and incubated at 4 °C for >12 h. The antibody solution in the flask was removed, and the flask was washed with PBS and used for the human NK cell culture.

2.3. Ex vivo expansion of human NK cells

PBMCs were obtained from 24 mL heparinized peripheral blood from three healthy volunteers (30–42 years old). The CD3 fraction of the PBMCs was depleted by RosetteSep™ Human CD3 Depletion Cocktail (STEMCELL Technologies, Vancouver, Canada). The CD3-depleted PBMCs were placed in 6-well anti-NKp46 and/or anti-CD16 antibody immobilization plates containing AIM-V medium (Thermo Fisher Scientific) supplemented with 10% autologous plasma, 50 ng/mL recombinant human IL-18 (rhIL-18, Medical & Biological Laboratories, Nagoya, Japan), and 3000 IU/mL rhIL-2 (COREFRONT, Tokyo, Japan) at 37 °C in a humidified atmosphere containing 5% CO₂. AIM-V medium containing 3000 IU/mL rhIL-2 was replenished as necessary for 14 days. The expanded cells were frozen in CELLBANKER (Nippon Zenyaku Kogyo Co., Ltd., Fukushima, Japan). For evaluating the growth inhibitory effects on tumor cells, the frozen cells were recovered into AIM-V medium supplemented with 10% heat-inactivated fetal bovine serum (FBS; MP Biomedicals, Tokyo, Japan) and 3000 IU/mL rIL-2 and incubated for 2 days.

2.4. Human cell lines

A standard human GBM cell line, T98G (American Type Culture Collection, Manassas, VA, USA), which is NK activity-sensitive [19], was used in this study. The cells were maintained in Dulbecco's modified Eagle's medium (Thermo Fisher Scientific) supplemented with 10% heat-inactivated FBS, 100 U/mL penicillin, and 100 µg/mL streptomycin (Thermo Fisher Scientific) at 37 °C in a humidified atmosphere containing 5% CO₂.

2.6. Flow cytometry

The cells were stained with the appropriate antibodies and fixed in 1% paraformaldehyde containing PBS (FUJIFILM Wako Chemicals) at 4 °C for 30 min. Data were obtained using a BD FACSCalibur

flow cytometer (BD Biosciences, San Jose, CA, USA) and analyzed using FlowJo v10 (BD Biosciences). The following antibodies were used: PE/Cy5-labeled anti-CD3 antibody (clone HIT3a, BioLegend, San Diego, CA, USA) and Alexa 488-labeled anti-CD56 antibody (clone B159, BD Biosciences).

2.7. Cytotoxicity-mediated growth inhibition assays

The cytotoxicity-mediated growth inhibition of GBM cell by NK cell-containing populations was investigated using xCELLigence RTCA (real-time cell analysis) S16 and DP (dual purpose) instruments (ACEA Biosciences, San Diego, CA, USA) as described previously [22]. Briefly, complete medium (100 μL) was added to each well on E-plate 16 (ACEA Biosciences) and background impedance was measured at 37 °C in a humidified atmosphere containing 5% CO₂. T98G cells [2×10^4 /well (50 μL)] were seeded in each well as the target (T) cells and cultured for 20 h. Then, the NK cell-containing populations (50 μL) were added to each well as the effector (E) cells in E:T ratios of 1:1. The impedance measurement was recorded every 5 min for 48 h. The data were analyzed using RTCA 1.2 (ACEA Biosciences).

2.8. Statistical analysis

Statistical analyses were performed using Prism 8 (GraphPad Software Inc., San Diego, CA, USA). The data are reported as the mean ± standard deviation (SD). The statistical significance of differences was determined using the one- or two-way analysis of variance (ANOVA) followed by Tukey's test. We considered $p < 0.05$ statistically significant.

3. Results

3.1. Ex vivo expansion of human peripheral blood-derived NK cells stimulated by NK cell activating receptor antibodies

To evaluate the efficacy of the specific culture conditions for NK cell expansion, CD3-depleted PBMCs from three healthy donors were cultured ex vivo. Table 1 summarizes the data. After isolation, approximately $1-2 \times 10^7$ cells were collected from 24 mL peripheral blood and an average of $53.7 \pm 5.2\%$ CD3-CD56+ NK cells were obtained. Donor 1, 2, and 3 yielded 48.2%, 52.1%, and 60.7% CD3-CD56+ NK cells, respectively, where CD3+ cells comprised 0.1%, 0.4%, and 0.5%, respectively (Fig. 1). The donor-derived CD3-depleted PBMCs were expanded with a mixture of the cytokines rhIL-18 and high-dose rhIL-2 on anti-NKp46 and/or anti-CD16 antibody immobilization plates at a cell density of 5×10^5 cells/mL in 2 mL culture medium. The total and CD3-CD56+ NK cell

numbers were calculated on day 0 and 14 of expansion. On day 14, the viability of all expanded cells was >90%.

From Donor 1, the expansion yielded 214.0×10^6 , 243.7×10^6 , 353.3×10^6 , and 403.3×10^6 CD3-CD56+ NK cells in the control, anti-CD16 stimulation, anti-NKp46 stimulation, and anti-CD16/NKp46 stimulation, respectively, with 90.1%, 80.8%, 96.2%, and 96.9% purity, respectively. There were 192.8×10^6 , 196.9×10^6 , 339.9×10^6 , and 390.8×10^6 CD3-CD56+ NK cells, respectively. The CD3-CD56+ NK cells demonstrated an expansion ratio of 400.0-, 408.5-, 705.1-, and 810.8-fold, respectively (Table 1). Although NK cell purity in the anti-CD16 stimulation was decreased by 9.3%, the expansion ratio was 1.02-fold higher compared to that of the control. The NK cell purity in the anti-NKp46 stimulation was 6.1% higher and the expansion ratio was 1.76-fold higher compared to the control. The anti-CD16/NKp46 stimulation showed that NK cell purity was increased by 6.8% and the expansion ratio was 2.02-fold higher compared to the control. These results indicate that anti-NKp46 stimulation markedly enhanced the expansion ratio of the Donor 1 NK cells and that the additional anti-CD16 stimulation enhanced it.

From Donor 2, the expansion yielded 88.8×10^6 , 130.0×10^6 , 382.2×10^6 , and 412.2×10^6 CD3-CD56+ NK cells in the control, anti-CD16 stimulation, anti-NKp46 stimulation, and anti-CD16/NKp46 stimulation, respectively, with 95.2%, 97.4%, 97.6%, and 98.6% purity, respectively. The number of CD3-CD56+ NK cells was 84.5×10^6 , 126.6×10^6 , 373.0×10^6 , and 406.4×10^6 , respectively. The expansion ratio was 162.3-, 243.0-, 716.0-, and 780.1-fold, respectively. Although the NK cell purity was maintained at >95% in all conditions, the control demonstrated markedly low expansion efficiency compared to that of Donor 1 (400.0-fold). The purity of the anti-CD16-stimulated NK cells was increased by 2.2% and the expansion ratio was 1.49-fold higher compared to the control. The purity of the anti-NKp46-stimulated NK cells was increased by 2.4% and the expansion ratio was 4.41-fold higher compared to the control. NK cells stimulated by anti-CD16/NKp46 exhibited 3.4% increased purity and a 4.80-fold higher expansion ratio compared to the control. These results indicate that anti-NKp46 stimulation markedly enhanced the low expansion ratio of the Donor 2 NK cells and that anti-CD16 stimulation enhanced it.

From Donor 3, the expansion yielded 186.0×10^6 , 348.8×10^6 , 447.2×10^6 , and 478.4×10^6 CD3-CD56+ NK cells in the control, anti-CD16 stimulation, anti-NKp46 stimulation, and anti-CD16/NKp46 stimulation, respectively, with 45.9%, 35.0%, 83.2%, and 86.7% purity, respectively. There were 85.4×10^6 , 122.1×10^6 , 372.1×10^6 , and 414.8×10^6 CD3-CD56+ NK cells, respectively. The expansion ratio was 140.6-, 201.1-, 613.0-, and 683.3-fold, respectively. The control NK cells had markedly low expansion efficiency compared to that of Donor 1 and 2. The NK cell purity was markedly low compared to that of Donor 1 and somewhat low compared to

Table 1
Summary of NK cell purity and expansion ratio in the NK cell-containing populations.

Donor	1				2				3							
	PBS	αCD16	αNKp46	αCD16/ αNKp46	PBS	αCD16	αNKp46	αCD16/ αNKp46	PBS	αCD16	αNKp46	αCD16/ αNKp46				
Day 0	Total cell number (10 ⁶)				1.0				1.0							
	Frequency of CD3-CD56+ NK cell (%)				48.2				60.7							
	Cell number of CD3-CD56+ NK cell (10 ⁶)				0.48				0.61							
Day 14	Total cell number (10 ⁶)				214.0	243.7	353.3	403.3	88.8	130.0	382.2	412.2	186.0	348.8	447.2	478.4
	Frequency of CD3-CD56+ NK cell (%)				90.1	80.8	96.2	96.9	95.2	97.4	97.6	98.6	45.9	35.0	83.2	86.7
	Cell number of CD3-CD56+ NK cell (10 ⁶)				192.8	196.9	339.9	390.8	84.5	126.6	373.0	406.4	85.4	122.1	372.1	414.8
	Expansion ratio of CD3-CD56+ NK cell (fold)				400.0	408.5	705.1	810.8	162.3	243.0	716.0	780.1	140.6	201.1	613.0	683.3

CD3-CD56+ NK cell numbers were calculated by the cell number and percentage of CD3-CD56+ cells. The expansion ratios were determined by the day 14 CD3-CD56+ NK cell number divided by the day 0 CD3-CD56+ NK cell number.

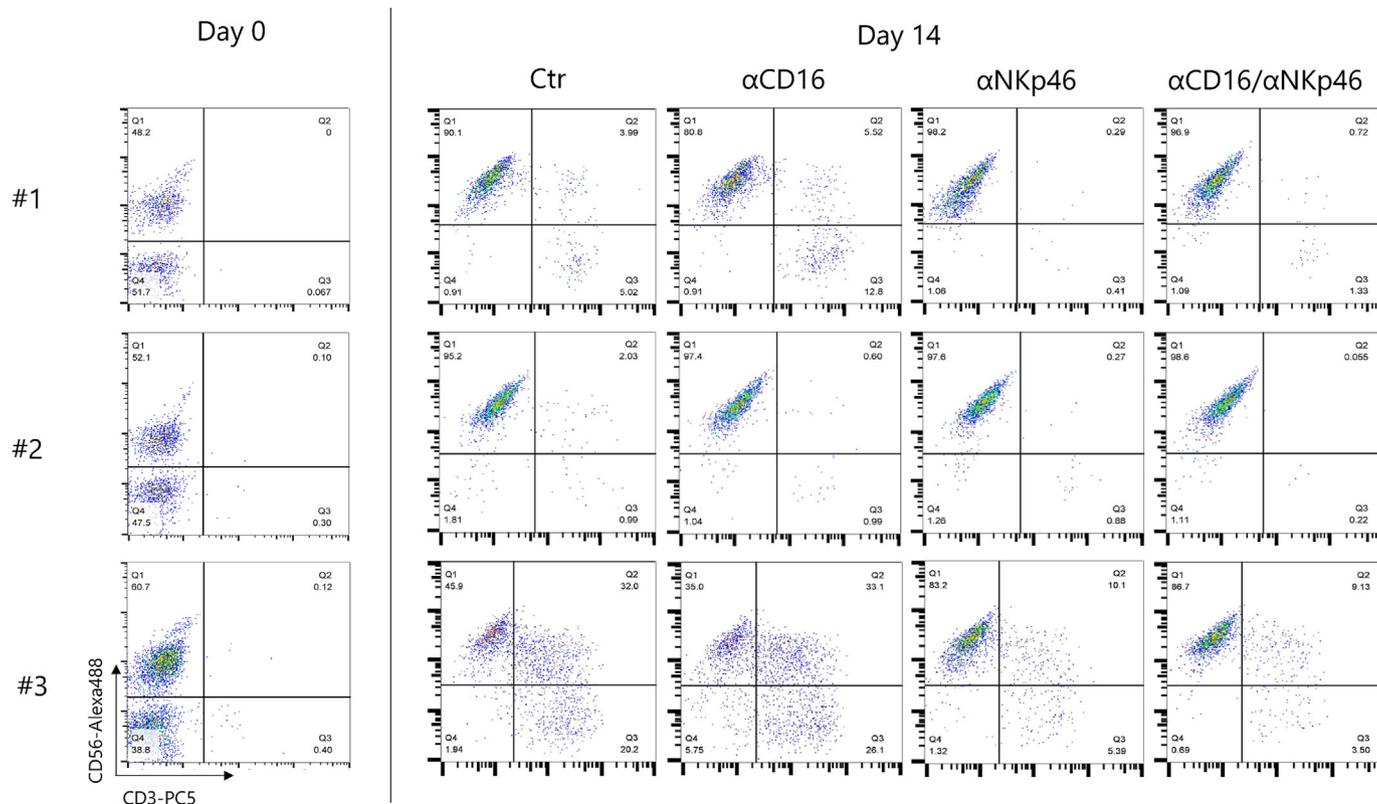


Fig. 1. Ex vivo expansion of human peripheral blood-derived NK cells by defined cytokine cocktail and NK cell activating receptor stimulation. Human CD3-depleted PBMCs were expanded with serum-free medium containing the defined cytokine cocktail and 10% autologous plasma on antibody immobilization plates. The dot plots depict the flow cytometric analysis data of CD3- and/or CD56-expressing cells. Day 0 indicates the CD3-depleted PBMCs. Day 14 indicates the 14-day expansion in the specific conditions. Ctr, non-immobilized; α CD16, anti-CD16 antibody; α NKp46, anti-NKp46 antibody; α CD16/ α NKp46, anti-CD16/NKp46 antibody. Top, middle, and bottom panels indicate Donor 1, 2, and 3, respectively.

that of Donor 2 (Donor 1, Donor 2, Donor 3 expansion efficiency: 400.0-, 162.3-, 140.6-fold, respectively). Compared to the control, the anti-CD16-stimulated NK cells had 10.9% lower purity and a 1.43-fold higher expansion ratio. The anti-NKp46-stimulated NK cells had 37.3% higher purity and a 4.35-fold higher expansion ratio. Anti-CD16/NKp46 stimulation increased NK cell purity by 40.8% and the expansion ratio was 4.86-fold higher than that of the control. These results indicate that anti-NKp46 stimulation markedly enhanced Donor 3 NK cell expansion efficacy and that anti-CD16 stimulation enhanced these effects.

Collectively, anti-NKp46 stimulation markedly enhanced the purity and expansion efficacy of NK cells from all donors. The additional stimulation by anti-CD16 enhanced these effects. In particular, anti-NKp46 stimulation, and not anti-CD16, greatly enhanced the NK cell purity of donors with low-purity NK cells. Anti-CD16 stimulation decreased the purity of NK cells from one donor. Therefore, T cell-depleted PBMCs stimulated by anti-CD16 and anti-NKp46 enabled the efficient expansion of highly purified NK cells ex vivo.

3.2. Evaluation of cytotoxicity-mediated inhibition of GBM cell growth by the expanded NK cell-containing populations

The cytotoxicity-mediated growth inhibitory effects of the expanded cellular populations against the standard T98G human GBM cell line (an NK-sensitive cell line) were evaluated using an RTCA system. T98G cells were seeded and cultured for 20 h. Then, the recovered 14-day expanded cells were added to each well of the E plate (16-well plates in the RTCA system) at an E:T cell ratio of 1:1. The viability of the recovered expanded cell populations was >90%.

Fig. 2 shows the clear resultant inhibition of T98G cell growth by all expanded cell populations. Donor 1 and 2 control or anti-NKp46-stimulated populations inhibited T98G cell growth but the two populations were not significantly different. The anti-CD16/NKp46-stimulated population also did not demonstrate different growth inhibitory effects on T98G cells compared to the control and anti-NKp46-stimulated populations. The anti-CD16-stimulated population significantly attenuated cytotoxicity-mediated inhibition of T98G cell growth compared to the other conditions. The Donor 3 anti-NKp46-stimulated population significantly inhibited T98G cell growth compared to the control and anti-CD16-stimulated populations. The anti-CD16/NKp46-stimulated population did not inhibit T98G cell growth differently compared to the anti-NKp46-stimulated population.

4. Discussion

Various NK cell sources, such as bone marrow (BM) and umbilical cord blood (UCB) mononuclear cells, induced pluripotent stem cells (iPSC) and embryonic stem cells (ESC), and PBMCs have been used for generating NK cells in vitro [23–25]. Although BM- and UCB-derived NK cells are promising [26,27], their sources and availability are limited. ESC- and iPSC-derived NK cells also hold promise but are subject to limited induction efficacy and the time-consuming induction duration (approximately 1–2 months) [28,29]. Furthermore, the culture is costly because the induction uses large amounts of cytokines and defined serum-free medium. The easy and stable expansion of PBMC-derived NK cells can overcome these problems. Previously, PBMC-derived NK cells were expanded by co-culture with the HFWT Wilms tumor cell line [30], gene-modified K562

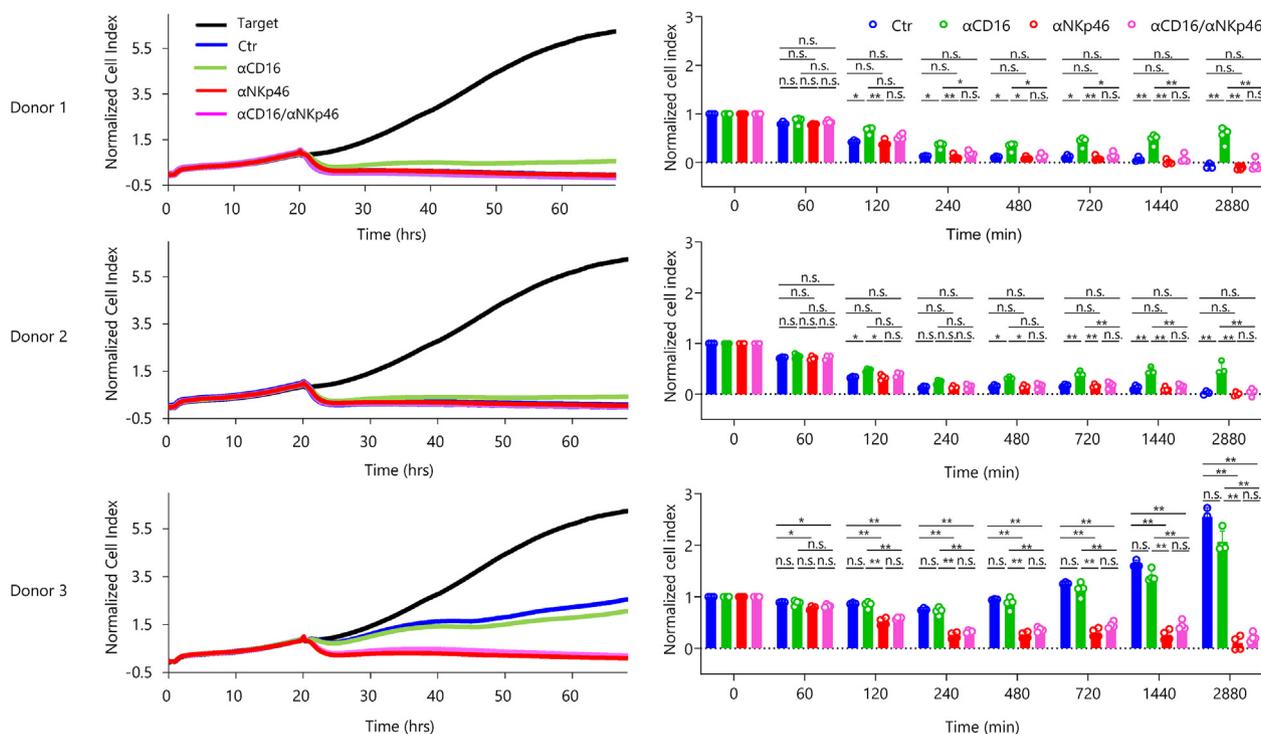


Fig. 2. Cytotoxicity-mediated growth inhibition of GBM cell by the expanded NK cell-containing populations. The NK cell-containing populations were co-cultured with attached T98G cells at an E:T cell ratio of 1:1. Left: Graphs depict representative data of real-time normalized cell index value. X-axis indicates time from target cell seeding, Y-axis indicates the normalized cell index calculated by the cell index of each point divided by the cell index of the co-culture start point. Right: Bar graphs illustrate the normalized cell index of each time point. Data are the mean \pm SD of two independent experiments ($n = 3-4$). Statistical differences were determined by two-way ANOVA followed by Tukey's test. ** $p < 0.01$, * $p < 0.05$, n.s.: not significant.

leukemia cells [31], irradiated PBMCs [32], or expanded T cells as feeder cells [33]. However, NK cell expansion using feeder cells is complicated and subject to infection by viruses or bacteria, including mycoplasma. While stimulation with OK432, which is dead *Streptococcus pyogenes*, has been reported, the system demonstrated varied expansion efficiency and purity [34,35]. Confirming sterility in this system is difficult because OK432 made it difficult to determine whether viable or dead bacteria exist when the culture is performed. If a large amount of PBMC-derived NK cells can be expanded safely and stably under feeder-free conditions, it could be the best strategy for the clinical application of NK cell-based cancer immunotherapy. Miller et al. used the strategy of depleting CD3 in PBMCs from haploidentical donors, followed by a brief 8–16-h culture with rhIL-2 (1000 U/mL). Subsequently, the activated cells were infused into patients after haploidentical stem cell transplantation or no transplantation [15]. We developed this system further using rhIL-18 and high-dose rhIL-2 (3000 IU/mL) stimulation and expansion [19]. In the present study, we evaluated the impact of anti-NKp46 and anti-CD16 stimulation on our previously reported NK expansion method. We previously demonstrated that T cell-depleted PBMCs stimulated by IL-2 or IL-18 exhibited >90% NK cell purity and 440-fold NK cell expansion ($n = 10$) [19]. However, in the present study, three donors were evaluated: two donors demonstrated low NK expansion efficacy (140.6- and 162.3-fold, respectively) and one donor demonstrated low NK cell purity (45.9%). These data demonstrate that rhIL-2 or rhIL-18 stimulation remains insufficient for the efficient and highly purified expansion of peripheral blood-derived NK cells. Our findings reveal that additional anti-NKp46 and anti-CD16 stimulation not only enhanced the expansion effect of the NK cells but also enhanced their purity, even in donor-derived blood, where efficiency and purity are typically low following rhIL-2 or rhIL-18 stimulation alone.

We have reported that CD3-CD56+ NK cells derived from CD3-depleted PBMCs were cytotoxic against GBM cell lines (T98G, U87MG, LN-18) via apoptosis [19]. In the present study, there was clear cytotoxicity-mediated growth inhibition of T98G cells in all cases that tended to depend on NK cell purity. In one donor, however, anti-CD16 stimulation attenuated the cytotoxicity-mediated growth inhibition of rhIL-2- or rhIL-18-stimulated populations despite the increased NK cell purity. These results demonstrate that anti-CD16 stimulation does not necessarily increase the cytotoxicity of rhIL-2- or rhIL-18-stimulated cells. Anti-CD16 stimulation enhanced NK cell expansion but did not affect the cytotoxicity-mediated growth inhibition by the anti-NKp46-stimulated populations. Collectively, the addition of anti-NKp46 and anti-CD16 stimulation to our previously reported NK expansion method enabled stable expansion of a large number of highly purified NK cells without reducing their cytotoxic activity.

Due to advances in understanding NK cell receptors and their ligands, NK cell-based immunotherapy has emerged as a promising therapeutic approach for solid tumors and hematological malignancies [14,25]. Furthermore, it should be determined whether autologous or allogeneic NK cells should be used in NK cell-based immunotherapy. Allogeneic NK cell therapy exerts a strong antitumor effect compared to autologous NK cell therapy due to the “missing self” hypothesis that states that NK cells can sense the absence of self MHC class I on target cells [13] while autologous NK cell activity is inhibited in patients with cancer largely due to KIR-HLA (killer cell immunoglobulin-like receptor–human leukocyte antigen) matching [36]. In addition, NK cell inhibitory receptors, the so-called checkpoint receptors, such as PD-1, LAG-3, TIM-3, TIGIT, and TACTILE, inhibit NK cell function in tumor cells [37]. That is, NK cell inhibitory receptor blockade is crucial for enhancing the antitumor effect of autologous NK cell immunotherapy for

GBM. KIR blocking antibody enhanced the antitumor effect of NK cells against leukemia [38]. We and others have reported that CRISPR/Cas9 disruption of NK cell inhibitory receptor genes in human NK cells enhanced GBM cell growth [39,40]. We believe that obtaining antitumor effects in GBM in the clinical setting requires the addition of these techniques to autologous NK cell-based immunotherapy. The present NK expansion method could be a robust platform for obtaining large amounts of NK cells and genome editing human NK cells.

Allogeneic NK cell therapy elicits strong antitumor effects via graft-versus-leukemia effects but includes the risk of graft-versus-host disease (GVHD). Approximately 7% of patients receiving KIR ligand-mismatched allogeneic NK cell treatment experienced GVHD. However, this increased GVHD occurrence correlated positively with higher CD3 chimerism, suggesting that the cell preparations contained mixed T cells and triggered a GVHD response [41,42]. Increasing the purity of NK cells would reduce GVHD occurrence. We expect that the highly purified NK cells expanded using our present method will contribute to both autologous and allogeneic NK cell-based immunotherapy.

GBM is the most common and aggressive primary brain tumor classified as grade IV by the World Health Organization [43]. The median overall survival is only 15–17 months under standard treatment, which includes surgical resection followed by radiotherapy and chemotherapy [44]. Patients with GBM need new treatment strategies and immunotherapy could be a promising adjuvant treatment. We have reported that the expanded PBMC-derived NK cells elicited antitumor effects against GBM in vitro, in vivo, and against 3-dimensional GBM cell-derived spheroids [20,21]. The sophisticated NK cell expansion method in the present study could yield a stable supply of therapeutic NK cells with antitumor effects against GBM.

The limitations of this research are as follows: First, we evaluated the antitumor effect of NK cells in allogeneic GBM settings. Further evaluation of autologous GBM settings is needed. However, allogeneic NK cells exhibited strong antitumor effects against GBM in in vivo xenograft experiments and did not cause GVHD [20]. Further, based on the accumulation of clinical trials [14], allogeneic NK cell therapy could be a safe and effective treatment for patients with GBM. Second, we used peripheral blood from healthy volunteers. Typically, NK cell expansion from the blood of patients with GBM is challenging because of the possibility of the patients having an immune function disorder [33]. Alkylating agents of cancer treatment such as temozolomide generally inhibit hematopoietic stem cell proliferation and limit peripheral lymphocyte numbers [45]. Moreover, steroids are commonly used for managing brain edema in patients with GBM who have been treated with surgery and concomitant chemoradiotherapy. The adverse effects of glucocorticoids include lymphopenia, hyperglycemia, and risk of infection caused by immunosuppressive effects [46]. In this situation, NK cells may also demonstrate decreased reactivity. The next step is to investigate whether it is possible to induce NK cells using blood from patients with GBM. Currently, we are planning to perform clinical research on the expansion of NK cells from the blood of patients with GBM.

5. Conclusion

We established an efficient, large-scale, feeder-free expansion system for highly purified NK cells derived from human peripheral blood using defined cytokine cocktail and anti-NK cell activating receptor antibodies. The expansion system could be feasible for NK cell-based immunotherapy for cancer, including GBM, in allogeneic and autologous settings. This is easily controlled under Japanese law on regenerative medicine.

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None.

Declaration of competing interest

Tsutomu Nakazawa is registered with Nara Medical University as a postdoctoral fellow member paying registration fees.

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