

Improving cytokine-induced killer cell expansion using a gas-permeable culture method for clinical-scale production

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Abstract

Background: Cytokine-induced killer (CIK) cells are a heterogeneous group of immune cells that exert potent MHC-unrestricted cytotoxicity toward various cancer cells in both solid and hematological malignancies.

Objective: The purposes of this study were to compare the expansion and characteristics of cytokine-induced killer cells between a standard culture method and a gas-permeable culture method and to develop a clinical-scale expansion protocol for cytokine-induced killer cells using a gas-permeable culture method.

Methods: We compared the absolute cell number, fold change, cell subsets, activation markers, cytokine concentrations, and cytotoxicity toward myeloid leukemia cell lines between cytokine-induced killer cells expanded using two different culture methods. Then, we determined the ability to achieve clinical-scale expansion of cytokine-induced killer cells using the gas-permeable culture method.

Results: Cytokine-induced killer cells in the gas-permeable culture method group exhibited significantly better expansion but maintained similar cell subsets, activation markers, and cytotoxicity to those in the standard culture method group. In addition, we successfully manufactured cytokine-induced killer cells for clinical use using the gas-permeable culture method. We also showed the clinical efficacy of allogeneic cytokine-induced killer cells produced by the gas-permeable culture method in a patient with acute myeloid leukemia that relapsed after allogeneic hematopoietic stem cell transplantation. This patient maintained ongoing disease remission for 2 years with minimal side effects after cytokine-induced killer cell infusion.

Conclusion: We successfully developed a simple and effective protocol for the ex vivo expansion of cytokine-induced killer cells using the gas-permeable culture method for clinical application.

Key words: Cytokine-induced killer cell, gas-permeable culture, acute myeloid leukemia, adoptive cellular therapy, clinical-scale expansion

Citation:

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Introduction

Cytokine-induced killer (CIK) cells are a heterogeneous group of immune cells that are activated *ex vivo* and expanded from peripheral blood mononuclear cells (PBMCs), bone marrow, or cord blood by specifically timed exposure to a combination of cytokines, including recombinant human interferon-gamma (rhIFN- γ), anti-CD3 monoclonal antibody, and recombinant human interleukin 2 (rhIL-2) for 2–4 weeks.¹ CIK cells consist of two main subpopulations: CD3⁺CD56⁻ cells (T cells) and CD3⁺CD56⁺ cells (NK-T cells).^{1–3} CD3⁺CD56⁺ cells are considered to be the major effector cells in the CIK cell population,⁴ and they exhibit potent MHC-unrestricted^{5–6} cytotoxicity toward various cancer cells in both solid and hematological malignancies through activation of receptors such as LFA-1, DNAM-1, Nkp46, NKG2D, and Nkp30.^{4,6–8} Moreover, several *in vitro* studies have shown that CIK cells exhibit decreased alloreactivity, which could alleviate the risk of graft-versus-host disease (GVHD).^{9–10} Several clinical studies have shown the safety and promising efficacy of CIK cells in the treatment of solid and hematological malignancies in both autologous and allogeneic settings.^{9,11–14}

According to the International Registry on Cytokine-Induced Killer Cells, at least 1×10^9 CIK cells are required to attain clinically meaningful therapeutic efficacy.^{11,15} There are several limitations to using the standard CIK cell culture method in a standard cell culture plate or T-flask to achieve clinical-scale cell expansion for clinical applications, including the process being time-consuming and requiring frequent changes of the culture medium to maintain an appropriate cell concentration, which could increase the risk of culture contamination.^{16–17}

A gas-permeable culture method allows cells to grow with an increased oxygen supply and excess nutrients, which promote cell proliferation. The gas-permeable rapid expansion culture device (G-Rex) can contain a larger volume of culture medium to provide extra nutrients and dilute waste due to the gas-permeable membrane at the bottom of the device, which allows gas exchange throughout the depth of the culture medium.^{18–19} Clinical-scale expansion of immune cells for adoptive cellular therapy, such as T cells,^{19–20} NK cells,^{21–22} and tumor-infiltrating lymphocytes (TILs),^{23–24} has been optimized using the G-Rex culture device to improve cell expansion.

In this study, we compared the expansion and characteristics of CIK cells in the standard culture method using a conventional 24-well cell culture plate and the gas-permeable culture method using a G-Rex 24-well plate.

Moreover, we confirmed the validity of the gas-permeable culture method for the clinical-scale expansion of CIK cells in the G-Rex 6-well plate. We also demonstrated the clinical efficacy of CIK cells manufactured according to our clinical-scale expansion protocol using the gas-permeable culture method in a patient with acute myeloid leukemia (AML) that relapsed after allogeneic hematopoietic stem cell transplantation (allo-HSCT).

Methods**Cell lines**

Human myeloid leukemia cells, K562 (chronic myeloid leukemia cell) and Kasumi (acute myeloid leukemia cell), were purchased from ATCC (Rockville, MD, USA) and maintained in RPMI-1640 (Hyclone) with 10% fetal bovine serum (FBS, Gibco), in accordance with the manufacturer's instructions.

Generation of CIK cells: standard culture method

After approval by the institutional review board of the Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand (COA# 774/2017) and written informed consent from a healthy donor were obtained, PBMCs were isolated from the peripheral blood of the healthy donor ($n = 3$) by standard Ficoll separation using Ficoll-Paque Plus density gradient medium (GE Healthcare). Then, the PBMCs were plated in a 24-well cell culture plate (Thermo Fisher) at 1×10^6 cells per well in 2 ml (0.5×10^6 cells/ml) of culture medium consisting of RPMI-1640 medium (Hyclone) containing 10% FBS (Gibco) with 1,000 IU/ml recombinant human IFN- γ (rhIFN- γ) (Miltenyi) and incubated at 37°C in a humidified atmosphere containing 5% CO₂. After 24 h of incubation, 50 ng/ml anti-CD3 antibody (Miltenyi) and 500 IU/ml rhIL-2 (R&D Systems) were added to the culture medium. On days 4, 7, and 11 of culture, RPMI 1640 medium containing 10% FBS and 500 IU/ml rhIL-2 was added to maintain the cell density at 1×10^6 cells/ml, and the cells were plated in a 24-well cell culture plate at a maximum density of 2 ml/well. On day 14 of culture, CIK cells were harvested for further analysis.

Generation of CIK cells: gas-permeable culture method

The PBMCs were plated in a G-Rex 24-well plate (2 cm² surface area) (Wilson Wolf) at 1×10^6 cells per well (0.5×10^6 cells/cm² surface area) in 8 ml of culture medium using RPMI-1640 medium containing 10% FBS (Gibco) with 1,000 IU/ml rhIFN- γ (Miltenyi) and incubated at 37°C in a humidified atmosphere containing 5% CO₂. After 24 h of incubation, 50 ng/ml anti-CD3 antibody (Miltenyi) and 500 IU/ml rhIL-2 (R&D system) were added to the culture medium. On days 4, 7, and 11 of culture, 500 IU/ml rhIL-2 was added, and the culture medium was changed once on day 7 by replacing 6 ml of the old medium with fresh culture medium. On day 14 of culture, CIK cells were harvested for further analysis.

Immuno-phenotype analysis using flow cytometry

Cells in suspension were stained with different fluorochrome monoclonal antibodies specific to human antigens, in accordance with the manufacturer's instructions, and flow cytometry was performed using BD FACS ARIA II (BD Biosciences) with FlowJo software. All antibodies were purchased from Biolegend. The following monoclonal antibodies were used: anti-CD3 PE (OKT-3), anti-CD56 PerCPy5.5 (HCD56), anti-NKG2D-FITC (1D11), anti-DNAM-1-FITC (TX-2), anti-NKp30-PE (P30-15), and anti-LFA-1-APC (m24).

Cytotoxicity assay

K562 and Kasumi were used as target cells for CIK cytotoxicity. Briefly, target cells were labeled with 0.25 μM carboxyfluorescein succinimidyl ester (CFSE; Biolegend). Then, effector cells were co-cultured with CFSE-labeled target cells at effector:target ratios of 5:1, 10:1, 50:1, and 100:1. Target cells without effector cells were used to represent spontaneous cell death. After 4 h of incubation, the cells were collected and stained with 4 μl of propidium iodide (Biolegend). Data analysis was performed using BD FACS ARIA II flow cytometry with FlowJo software, and the percent cytotoxicity was calculated.

Cytokine quantification

On day 14, culture media supernatants were collected and centrifuged at 2000 rpm for 5 min and then stored at -20°C . Cytokine levels in culture medium supernatants were measured with a flow cytometer using Cytometric Bead Array kits for Human Th1/Th2 Cytokine Kit II (BD Bioscience) to determine the levels of IFN- γ , interleukin-2, and interleukin-10.

Clinical-scale expansion of CIK cells using the gas-permeable culture method

PBMCs from three healthy donors were isolated from cells remaining in platelet apheresis tubing sets after single-donor platelet donation by standard Ficoll separation using Ficoll-Paque Plus density gradient medium (GE Healthcare). The PBMCs were plated in a G-Rex 6-well plate (10 cm^2 surface area) (Wilson Wolf) at 5×10^6 cells per well (0.5×10^6 cells/ cm^2 surface area) in 30 ml of culture medium consisting of RPMI-1640 medium (Hyclone) containing 10% FBS (Gibco) with 1,000 IU/ml rhIFN- γ (Miltenyi) for a total of 6 wells (total starting cells 30×10^6 cells) and incubated at 37°C in a humidified atmosphere containing 5% CO_2 . After 24 h of incubation, 50 ng/ml anti-CD3 antibody (Miltenyi) and 500 IU/ml rhIL-2 (R&D Systems) were added to the culture medium. On days 4, 7, and 11 of culture, 500 IU/ml rhIL-2 was added. On day 14 of culture, CIK cells were harvested for further analysis.

Cell number, cell viability, cytotoxicity, gram staining, aerobic and anaerobic culture (BD BACTEC), fungal culture (BD BACTEC), mycoplasma detection (Lonza MycoAlert), and endotoxin levels (Charles River Endosafe) were determined in the final product of clinical-scale expansion of CIK cells for quality control.

Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 software (GraphPad Software, Inc.). The independent samples were evaluated by Student's *t*-test. A *p*-value less than 0.05 was considered statistically significant.

Results

CIK cells expanded by the gas-permeable culture method exhibit greater expansion than those expanded by the standard culture method

CIK cell culture was initiated with 1×10^6 PBMCs in a 24-well cell culture plate for the conventional method and in a G-Rex 24-well plate for the gas-permeable culture method. On day 0, PBMCs were primed with IFN- γ , and anti-CD-3 antibody and rhIL-2 were added 24 h later. In the standard culture method group, fresh medium was supplemented with FBS, and rhIL-2 was exchanged on days 4, 7, and 11 to maintain the final cell concentration at 1×10^6 cells/ml (**Figure 1A**). In the gas-permeable culture method group, rhIL-2 was added to the culture medium every 3-4 days, but fresh culture medium with FBS was exchanged once on day 7. Cell number, cell subsets, and fold expansion were evaluated on days 0, 7, and 14 (**Figure 1B**).

After 14 days of culture, the gas-permeable cell culture method had a significantly higher absolute total cell number and absolute NK-T cell number than the standard method. Absolute total cell numbers were $110 (\pm 9) \times 10^6$ cells and $73 (\pm 10) \times 10^6$ cells in the gas-permeable and conventional methods, respectively ($p = 0.01$). The corresponding absolute NK-T cell numbers were $17 (\pm 2) \times 10^6$ cells and $9 (\pm 3) \times 10^6$ cells, respectively ($p = 0.01$) (**Figure 1C**).

The gas-permeable cell culture method also achieved higher expansion of both total cells and NK-T cells compared with the standard culture method after 14 days of culture. The total cell number increased 110 (± 9) and 73-fold (± 10) in the gas-permeable and standard culture methods, respectively ($p = 0.01$). Meanwhile, NK-T cells increased 982 (± 296) and 445-fold (± 42) in the gas-permeable and conventional methods, respectively ($p = 0.01$) (**Figure 1D**).

CIK cells cultured by the gas-permeable culture method showed similar cell subsets, activation marker levels, and cytotoxicity to those cultured by the standard culture method

Flow cytometry was performed on days 7 and 14 of cell culture to define cell subsets using anti-CD-3 and anti-CD56 antibodies to determine the percentages of T cells ($\text{CD}3^+\text{CD}56^-$), NK cells ($\text{CD}3^-\text{CD}56^+$), NK-T cells ($\text{CD}3^+\text{CD}56^+$), and other cells ($\text{CD}3^-\text{CD}56^-$) (**Figure 2A**). There were no significant differences in the percentages of each cell subset between the gas-permeable and standard culture methods on either day 7 or day 14 of culture. The percentages of NK-T cells on day 7 were 3.43% ($\pm 1.27\%$) and 4.30% ($\pm 2.17\%$) in the gas-permeable and standard culture methods, respectively ($p = 0.9816$). Moreover, the percentages of NK-T cells on day 14 were 15.97% ($\pm 2.55\%$) and 12.70% ($\pm 5.46\%$) in the gas-permeable and standard culture methods, respectively ($p = 0.4985$) (**Figure 2B and C**).

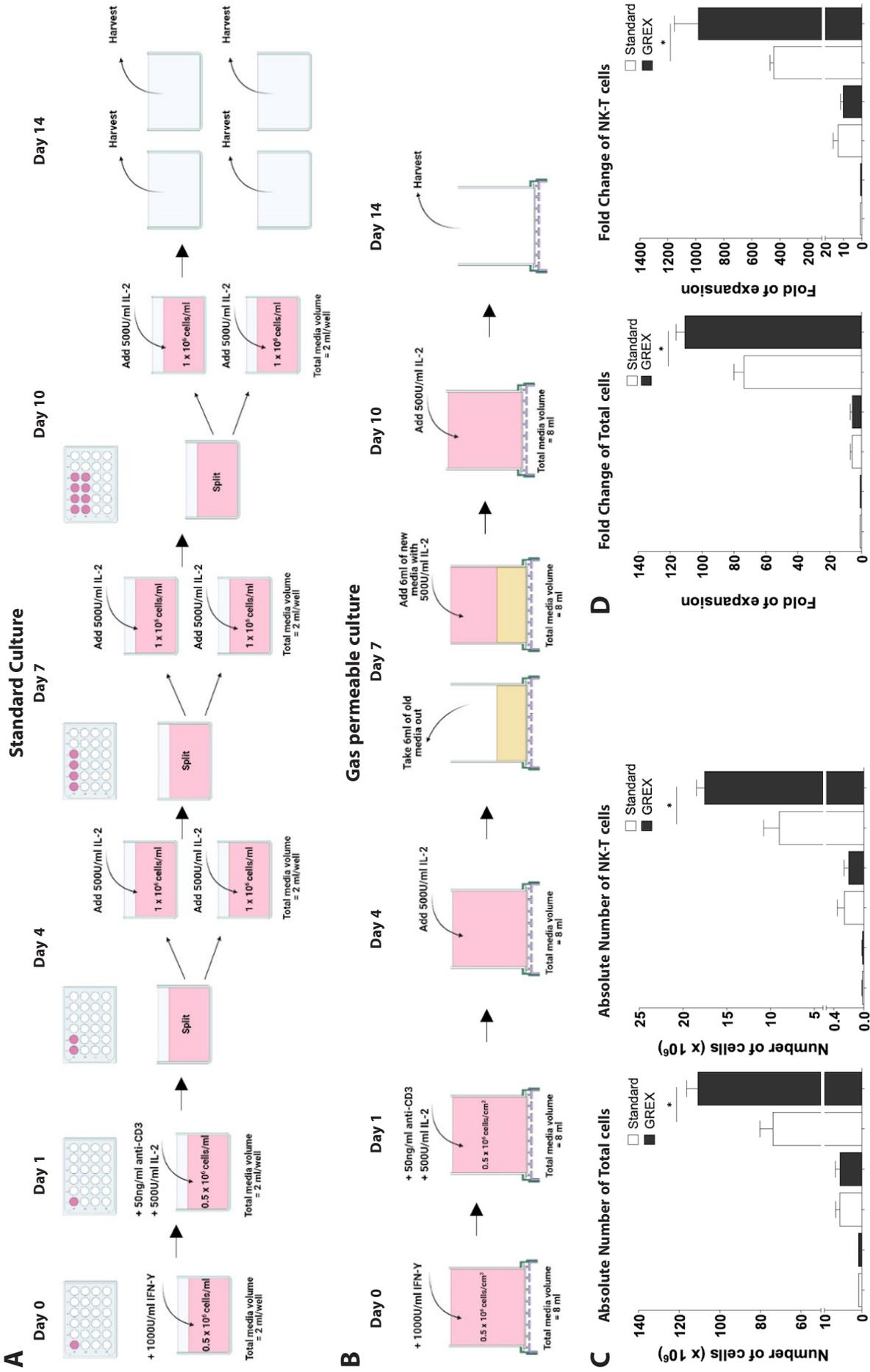


Figure 1. CIK cell expansion using the standard culture method versus the gas-permeable culture method. (A) Diagram of the standard culture method and (B) gas-permeable culture method upon the addition of cytokines and change of medium on days 0, 1, 4, 7, 11, and 14. PBMCs from three different donors were seeded in a conventional 24-well cell culture plate and a G-Rex 24-well plate. (C) Total cells (left) and NK-T cells (right) were counted and calculated on days 0, 7, and 14. (D) Expansion of total cells (left) and NK-T cells (right) on days 0, 7, and 14 of culture. CIK cells cultured with the gas-permeable method (black) showed greater expansion than those cultured with the standard culture method, with significant differences in both absolute number and fold change on day 14. The data shown are mean \pm S.E.M. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

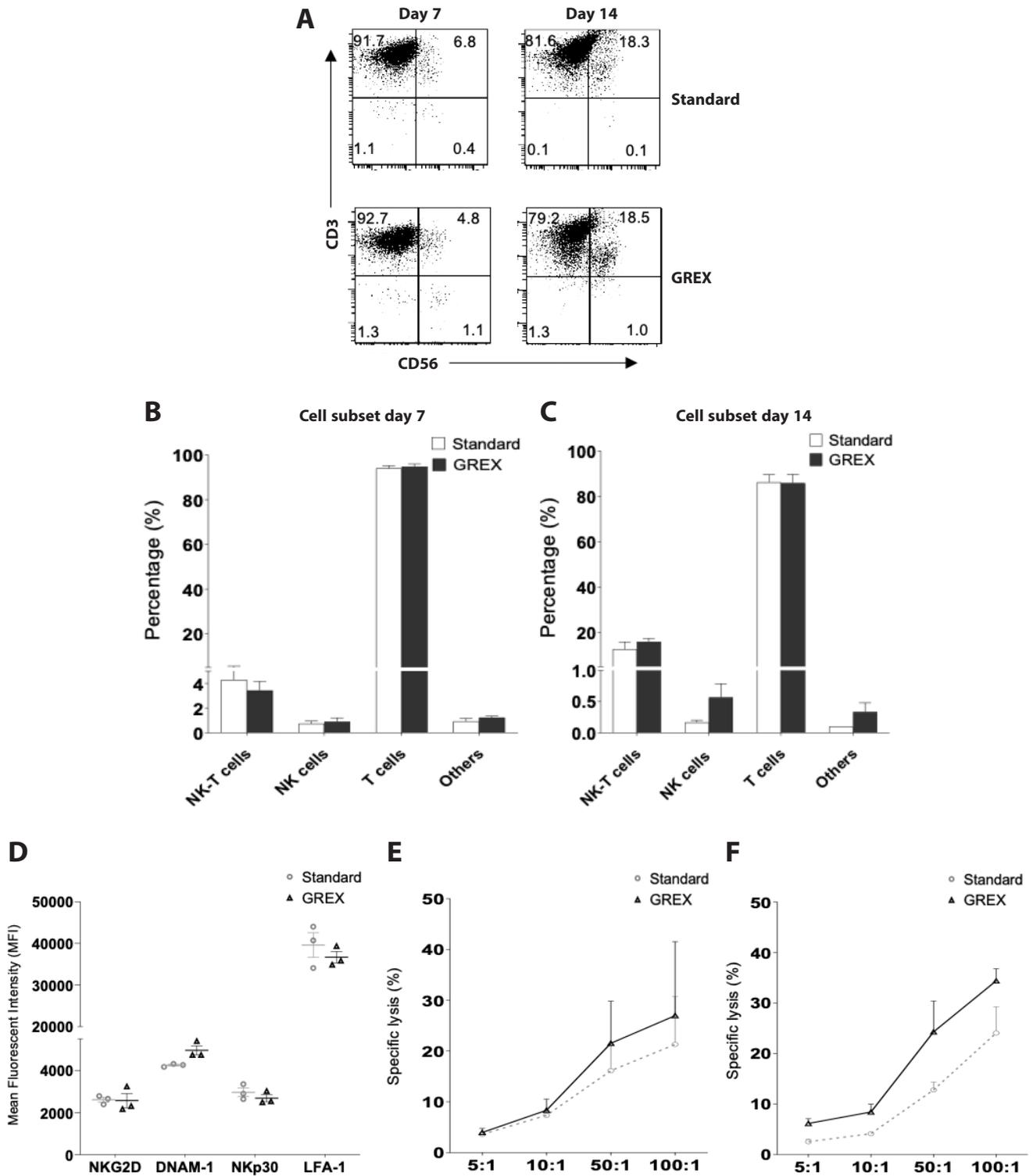


Figure 2. Comparing CIK cell characteristics between the standard culture method and the gas-permeable culture method. (A) Representative flow cytometry plots on days 7 and 14 of the standard culture method compared with the gas-permeable culture method. (B) Cell subsets of CIK cells on days 7 (C) and 14 were calculated, and the percentages of NK-T cells (CD3⁺CD56⁺), T cells (CD3⁺CD56⁻), NK cells (CD3⁻CD56⁺), and other cells (CD3⁻CD56⁻) are shown. (D) Activating markers on day 14 from the standard culture method (circle) and the gas-permeable culture method (triangle) are shown and plotted in MFI. Cytotoxicity of CIK cells against K562 (E) and Kasumi (F) cells co-cultured at effector-to-target ratios of 5:1, 10:1, 50:1, and 100:1 with CIK cells. Specific lysis was assessed by flow cytometry after 4 h. (G) Quantification of flow cytometric analysis of cytokine concentrations (IFN- γ , IL-2, and IL-10) in culture supernatants on day 14. The data shown are means \pm S.E.M. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; Standard = standard culture method, G-Rex = gas-permeable culture method.

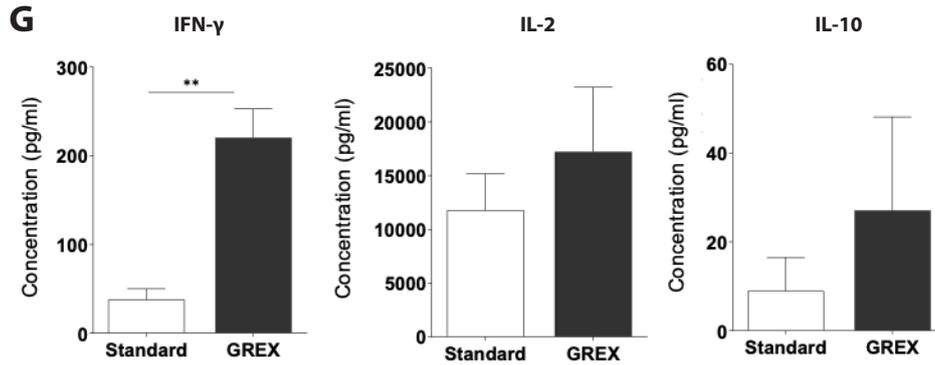


Figure 2. (Continued)

On day 14 of culture, activation marker expression was analyzed by flow cytometry using anti-LFA-1, anti-NKp-30, anti-DNAM-1, and anti-NKG2D antibodies. There was no significant difference in the levels of expression of any activation marker between CIK cells from the gas-permeable and standard culture methods (Figure 2D).

CIK cell cytotoxic activity toward myeloid leukemia cell lines was measured using the K562 (chronic myeloid leukemia) and Kasumi cell lines (AML) at E:T ratios of 5:1, 10:1, 50:1, and 100:1. CIK cells exhibited dose-dependent cytotoxicity toward both K562 (Figure 2E) and Kasumi (Figure 2F). However, there was no significant difference in cytotoxic activity toward the myeloid leukemia cell lines between CIK cells from the gas-permeable and standard culture methods.

The gas-permeable culture method increased the secretion of IFN- γ , IL-2, and IL-10 in cultured CIK cells

The concentrations of IFN- γ , IL-2, and IL-10 were measured by flow cytometry using the Cytometric Bead Array assay in the supernatants of CIK cells on day 14 of culture. IFN- γ concentrations were significantly higher in the gas-permeable culture method than in the standard culture method [220 (\pm 32) pg/ml versus 37 (\pm 12) pg/ml, respectively; $p = 0.0062$]. However, although IL-2 and IL-10 concentrations appeared to be higher in the gas-permeable culture method than in the conventional method, these did not reach statistical significance (Figure 2G).

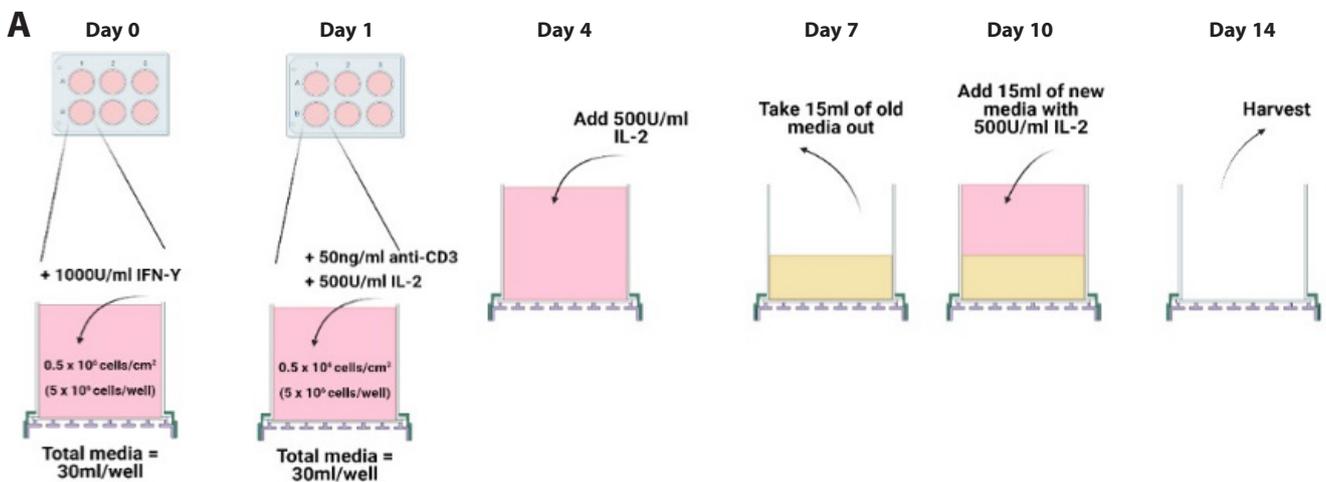


Figure 3. Clinical-scale expansion of CIK cells using the gas-permeable culture method.

(A) Diagram of the clinical-scale expansion of CIK cells using the gas-permeable culture method in a G-Rex 6-well plate. A total of 30×10^6 PBMCs (5×10^6 PBMCs per well) from three different donors were seeded in a G-Rex 6-well plate on day 0. Cells were cultured for 14 days in a G-Rex plate with the addition of cytokines on days 0, 1, 4, 7, and 10 as per the protocol, and with a change of culture medium once on day 7. Cells were counted on days 0, 7, and 14 of culture. Absolute total cells (B) and NK-T cells (C) were counted and plotted on days 0, 7, and 14. (C) Expansion of total cells (left) and NK-T cells (right) on days 0, 7, and 14 of culture. (D) Representative flow cytometry plots on days 0, 7, and 4. (E) Cell subsets of CIK cells on day 14 from three different donors are plotted, and the percentages of NK-T cells ($CD3^+CD56^+$), T cells ($CD3^+CD56^-$), NK cells ($CD3^-CD56^+$), and other cells ($CD3^-CD56^-$) are shown. The data shown are mean \pm S.E.M.

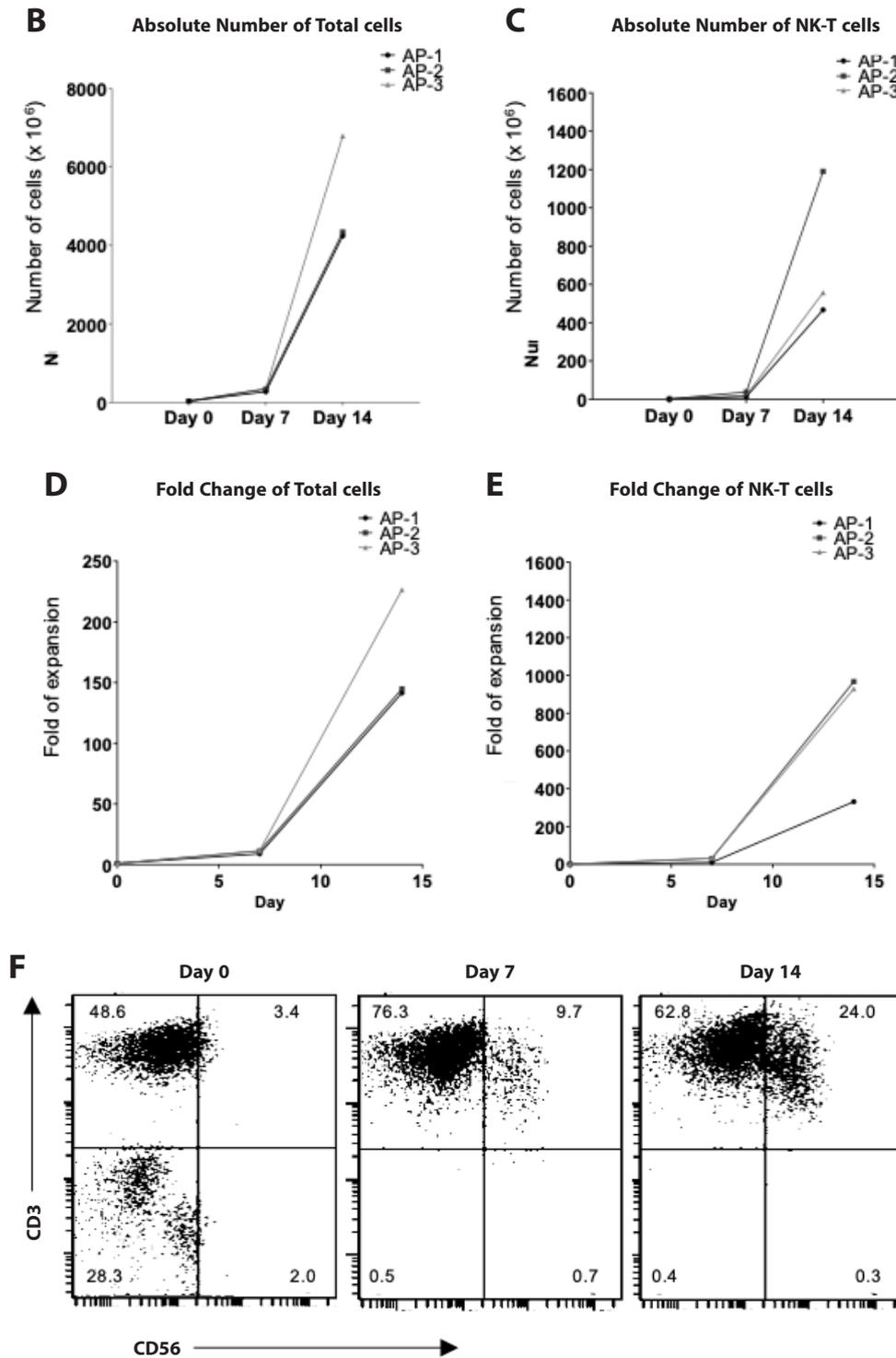


Figure 3. (Continued)

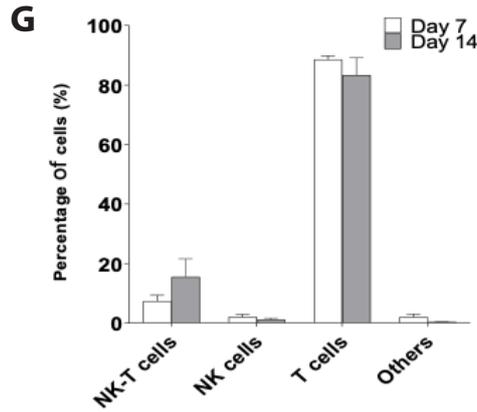


Figure 3. (Continued)

Table 1. Institutional release criteria for clinical use of CIK cells.

| Test | Specification | AP-1 | AP-2 | AP-3 |
|------------------------------------|-------------------------|-------------------------|-------------------------|-------------------------|
| Cell number ($\times 10^9$ Cells) | ≥ 1 | 4.2 | 4.3 | 6.8 |
| Cell viability (%) | ≥ 80 | 99 | 97 | 99 |
| Cytotoxicity (%) | ≥ 20 | 20 | 20 | 25 |
| Gram stain | Negative | Negative | Negative | Negative |
| Aerobic and anaerobic culture | No growth after 7 days |
| Mycoplasma | No detection | No detection | No detection | No detection |
| Fungal culture | No growth after 30 days |
| Endotoxin level (IU/ml) | < 5 | < 5 | < 5 | < 5 |

Clinical-scale expansion of CIK cells using the gas-permeable culture method

Clinical-scale expansion was performed to evaluate the capacity for CIK cell expansion using the gas-permeable culture method in G-Rex 6-well plates. PBMCs were isolated using Ficoll separation from cells remaining in the platelet apheresis tubing set to collect a high number of PBMCs. CIK cells were then expanded from 30×10^6 PBMCs in a G-Rex 6-well plate [5×10^6 cells/well (0.5×10^6 cells/cm² surface area) \times 6 wells] for a total of 14 days (Figure 3A).

The gas-permeable culture method could expand cultured CIK cells in all samples to more than 1×10^9 cells from only 30×10^6 PBMCs after 14 days. By the end of the culture, the absolute total cell number was $5.1 (\pm 0.85) \times 10^9$ cells (Figure 3B) and that of NK-T cells was $7.33 (\pm 2.33) \times 10^8$ cells (Figure 3C), which involved 171 (± 29) and 743 (± 185) -fold expansion (Figure 3D and E) of total cells and NK-T cells, respectively. The cell subsets upon the clinical-scale expansion of CIK cells from three apheresis products are shown in Figure 3F and 3G. Quality control testing in line with institutional release criteria for the clinical use of cellular therapy products was performed on the final CIK cell product, as summarized in Table 1.

Case presentation

A 52-year-old woman with AML suffered a relapse in the bone marrow before receiving an allogeneic CIK cell infusion. She had been diagnosed with high-risk AML 4 years earlier due to induction failure. Related-donor-matched HSCT was performed after three cycles of induction chemotherapy with 5% residual blasts in her bone marrow. The conditioning regimen consisted of busulfan and fludarabine with methotrexate and cyclosporine as a GVHD prophylaxis regimen. The patient tolerated the HSCT well and achieved complete remission 30 days later. There was no evidence of GVHD, and cyclosporine was gradually decreased as per the protocol. Six months after HSCT, she was evaluated for pancytopenia and found to have a hematological relapse, with evidence of 10% blasts in the bone marrow. Before the procedure, the patient was fully informed about the risks and benefits of the procedure, and she signed a written informed consent form. She was then enrolled to receive an allogeneic CIK infusion for relapsed AML after HSCT.

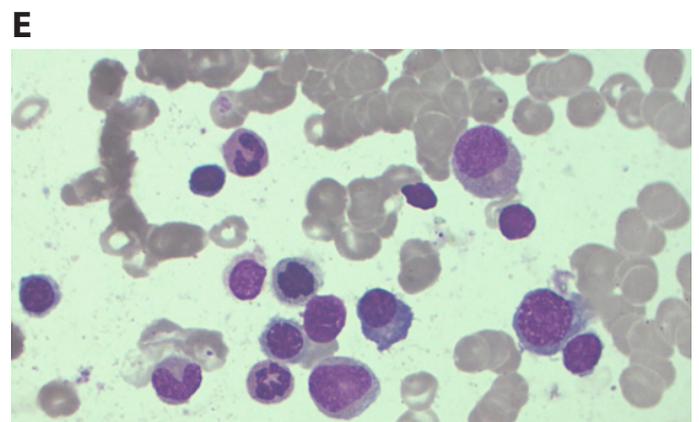
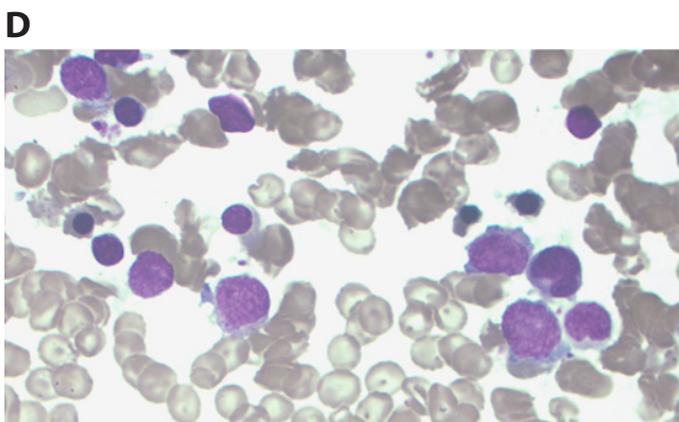
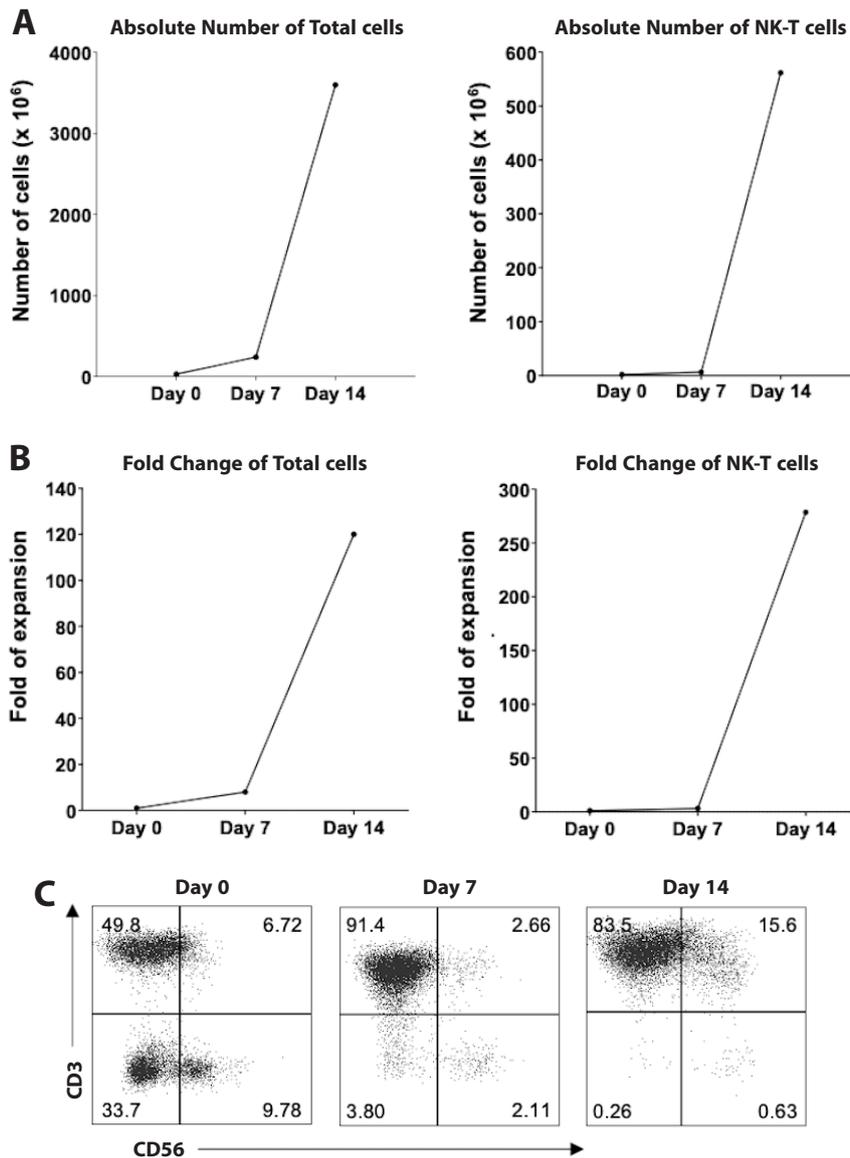


Figure 4. Allogeneic CIK cell expansion using the gas-permeable culture method for clinical use in AML patients.

A total of 30×10^6 PBMCs from the donor were seeded in a G-Rex 6-well plate on day 0 and cultured in accordance with the protocol for 14 days. (A) Absolute total cells (left) and NK-T cells (right) were counted on days 0, 7, and 14 and plotted. (B) Expansion of total cells (left) and NK-T cells (right) on days 0, 7, and 14 of culture was calculated and plotted. (C) Flow cytometry of CIK cell subsets on days 0, 7, and 14. Bone marrow aspiration before allogeneic CIK cell infusion (D) showed an increase of myeloblasts to 10%, and then (E) bone marrow aspiration showed no evidence of disease 30 days after cell infusion.

Clinical-scale CIK cells were manufactured from her HSCT donor using the gas-permeable culture method in the Good Manufacturing Practice (GMP)-compliant Cell Processing Facility at the Faculty of Medicine, Chulalongkorn University. The final product yielded a total of 3.6×10^9 cells with 15.6% CIK cells from 3×10^7 PBMCs cultured in a G-Rex 6-well plate using GMP-compliant reagents and materials (Figure 4A–C). The final CIK product was negative for gram staining, aerobic bacteria culture, anaerobic bacteria culture, fungal culture, mycoplasma, and endotoxin with an *in vitro* cytotoxicity of 35% against the K-562 cell line. Allogeneic CIK cells at a dose of 5×10^6 CD3-positive cells/kg (3×10^8 CD3-positive cells) were given after a lymphodepletion regimen with fludarabine and cyclophosphamide for 3 days. The patient tolerated the CIK cell infusion well with no evidence of transfusion-related side effects, tumor lysis syndrome, and cytokine releasing syndrome. Disease evaluation on day 30 after CIK infusion was consistent with complete hematological remission, with no evidence of excess blasts in bone marrow aspiration (less than 5% blasts in the bone marrow) (Figure 4D and E). Three months after the CIK cell infusion, she developed localized oral chronic GVHD, which was controlled well with only steroid mouthwash. She maintained complete remission for 2 years after the CIK cell infusion until she developed a bone marrow relapse and soon passed away from leukemia.

Discussion

We report a simple and effective protocol for the *ex vivo* expansion of CIK cells using a gas-permeable culture method. We successfully increased the expansion of CIK cells for clinical-scale production in 14 days using a G-Rex. In our study, we were able to generate at least 1×10^9 CIK cells from initial PBMCs numbering only 30×10^6 using the gas-permeable culture method, which could support the infusion of a high cell dose for the clinical use of CIK cells.

In the standard culture method, the input volume of culture medium is restricted to the depth through which O₂ can diffuse, which is about 1 ml/cm². However, G-Rex allows an excess volume of culture medium (4 ml/cm²) due to the gas-permeable membrane at the bottom of the device, which allows gas exchange to occur throughout the depth of the device, leading to excess nutrients and waste dilution. We could thus increase the maximum cell density from the initial cell density of 0.5×10^6 cells/cm² to 50×10^6 /cm² in the gas-permeable culture method by changing the culture medium once in 14 days of culture. In conventional cell culture, the maximum cell density is only $1\text{--}2 \times 10^6$ cells/cm², and the culture medium is changed every 2–3 days. The gas-permeable culture method could produce a high number of CIK cells with minimal cell culture manipulation, which could reduce the time-consuming nature of the process, the cost of production, and the risk of culture contamination.

Interestingly, our study showed that CIK cells in gas-permeable culture had a much higher concentration of IFN- γ with no significant differences in IL-2 and IL-10 concentrations. IFN- γ plays a critical role in the proliferation and cytotoxic function of CIK cells. *Ex vivo* generation of CIK cells requires IFN- γ priming before mitogenic stimulation

with OKT3 and IL-2 for maximal cytotoxic function.²⁵ It has been shown that IFN- γ stimulates and increases the amount of IL-2 receptor expression, which enhances the cell response to IL-2 stimulation.^{26–28} A higher IFN- γ concentration could directly promote the proliferation of CIK cells in the gas-permeable culture method compared with the conventional method and indirectly improve the IL-2 response. This would lead to greater expansion with the same amount of IL-2 secretion from CIK cells in the gas-permeable culture method compared with the standard culture method.

Despite the improvement of HSCT strategies in AML, more than half of AML patients still relapse after allo-HSCT. The prognosis of relapsed AML after allo-HSCT is generally poor due to the increased morbidity and mortality or refractoriness of the disease to salvage chemotherapy. Even with donor lymphocyte infusion or second HSCT in suitable patients, the 2-year overall survival rate of AML patients who have relapsed after allo-HSCT is usually less than 20%.²⁹ Several clinical studies have shown that donor-derived CIKs are well tolerated and produce a clinical response in relapsed AML after allo-HSCT.^{30–32} Our patient with relapsed AML after allogeneic HSCT received allogeneic CIK cells from her HSC donor in a compassionate use manner. We demonstrated the achievement of complete remission in this patient, who could maintain complete remission of the disease for 2 years after the infusion of allogeneic CIK cells manufactured by the gas-permeable culture method. Our study showed that CIK cell expansion using the gas-permeable culture method can achieve the number of cells needed for the clinical use of CIK cells and exhibit clinical efficacy in a patient with relapsed AML. The gas-permeable culture method using the G-Rex device can be employed as a manufacturing method for producing CIK cells in compassionate use settings or clinical trials for patients with relapsed or refractory AML.

Furthermore, our results support the findings of a recent study by Palmerini et al. on the feasibility of using a gas-permeable culture method in the *ex vivo* expansion of CIK cells for clinical applications. Although both studies demonstrated the superiority of CIK expansion in a gas-permeable culture method over the conventional culture flask, the details of the cultural protocols are slightly different. Palmerini et al demonstrated that the culture of CIK cells in X-VIVO serum-free media using G-Rex 6-well, and/or G-Rex 6M well plates at a seeding cell density of 2.5×10^6 cells per well led to approximately 100-fold growth on day 14.³³ In our clinical-scale expansion, CIK cells were cultured in RPMI media supplemented with 10% FBS using G-Rex 6-well plates at a seeding density of 5×10^6 cells per well. This protocol resulted in a more than 150-fold expansion of total cells and a more than 700-fold expansion of CD56⁺CD3⁺ cells, the effector population of CIK cells. Together, both studies demonstrated that using a gas-permeable culture method enabled rapid expansion of CIK cells with minimal culture manipulation from a small number of starting PBMCs to reach the desired clinical dose as soon as 14 days after cell culture was initiated in the G-Rex device.

Conclusion

We have developed a protocol for the ex vivo expansion of CIK cells using the gas-permeable culture method in the G-Rex device to achieve superior cell growth while still maintaining an immunophenotype and anti-tumor activity similar to those obtained with the standard culture method. The protocol has been optimized to achieve better cell expansion with minimal culture manipulation, which can be easily translated to cell production in a GMP environment. Overall, this protocol can be used as a method of producing CIK cells for use in clinical trials and for compassionate purposes.

Ethics approval and consent to participate

All experimental procedures were approved by the Institutional Review Board of the Faculty of Medicine, Chulalongkorn University (approval number: 774/2017, date of approval, August 26, 2017). All participants signed informed consents.

Authors' contributions

- ST designed the study, analyzed the data, and drafted the manuscript.
- ST and RT performed the experiments and drafted the manuscript.
- TA, KW, and CC conducted compassionate clinical use and drafted the manuscript.
- NH and UB contributed study concepts and coordinated technical and clinical support.
- KS contributed materials, study design, data interpretation, and revisions to the manuscript.
- All authors read and approved the final manuscript.

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