Perfusion culture of human natural killer cells in the WAVE Bioreactor 2/10 system

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CY14792-25Jun20-AN

Application note 28-9936-25 AA

Perfusion culture of human natural killer cells in the WAVE Bioreactor™ 2/10 system

This application note presents methods and results from three independent large-scale 21-day cultures of activated natural killer (NK) cells using the WAVE Bioreactor 2/10 system. The described method supported significant expansion of activated NK cells although large donor-to donor variability was observed. The WAVE Bioreactor 2/10 system requires minimal hands-on manipulation and the cost for consumables is reduced compared to typical static culture systems such as gas-permeable bags or tissue culture flasks. The rocking motion and the automated perfusion of fresh culture media provide a uniform culture environment for high cell-density culture, maintain crucial parameters such as pH, CO_2, glucose, and glutamine levels within an optimal range, and reduce the concentration of toxic metabolites. Handling only one bag also reduces the risk of cross-contamination as well as the time and effort to concentrate and harvest the cells at the end of culture.

Introduction

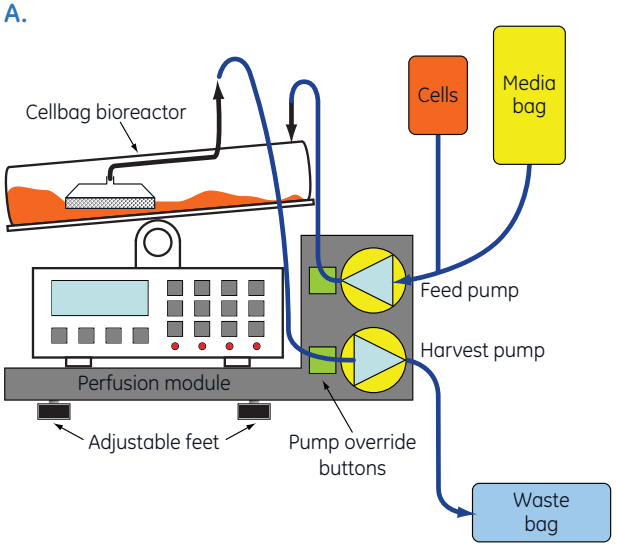
Natural killer (NK) cells are CD3-negative ($CD3^-$) cytolytic lymphocytes that mediate the first line of defense against virally infected cells or tumor cells and comprise 5% to 20% of human peripheral blood mononuclear cells (PBMC). NK cells can kill target cells without the need for prior sensitization, an effect that is regulated by a balance of stimulatory and inhibitory signals. Most NK cells express at least one inhibitory killer-cell immunoglobulin-like receptor (KIR) that is specific for a self-MHC class I allele and will kill cells that lack self-MHC class I molecules (1). In addition to sensing the absence of self-MHC class I antigens, NK cells also need to be activated by binding to target–cell ligands via specific activating receptors and can also be stimulated by several types of cytokines. Key receptors involved in triggering NK cell lysis of tumor cells are the NK cell-specific natural cytotoxicity receptors (NCR); NKp46 (NCR1, CD335), NKp44 (NCR2, CD336), and NKp30 (NCR3, CD337). Whereas NKp46 and NKp30 are expressed on both resting and activated NK cells, NKp44 is only expressed on activated NK cells. Activation of NK cells leads to induced proliferation, cytokine production, and/or cytolytic activity.

NK cells are divided into two major subsets according to functional and phenotypic differences (1,2). $CD56^{bright}CD16^-$ NK cells are a minor subset in blood, have predominantly immunoregulatory properties, and a potent cytokine (mainly IFN-γ) producing capacity. These cells also lack expression of KIRs, are phenotypically characterized by being almost free of granules, and only acquire cytotoxicity after prolonged activation. In contrast, $CD56^{dim}CD16^+$ NK cells secrete low levels of cytokines but have a marketed cytolytic function. The high expression of CD16/FcγRIII, the IgG Fc receptor, also enables antibody-dependent cell-mediated cytotoxicity (ADCC).

The tumor killing potential of NK cells has generated major interest in expanding high numbers of these cells *in vitro*. Several protocols for NK cell activation and/or expansion have been reported and the effects of many different cytokines (e.g., IL-2, IL-15, IL-7, IL-4, and IL-12) on proliferation and NK cell-mediated cytotoxicity have been studied (3). One of many ways to activate and induce NK cell proliferation is by co-culturing PBMCs with beads co-immobilized with anti-CD2 and anti-CD335/NKp46 monoclonal antibodies (NK Cell Activation and Expansion Kit from Miltenyi). In response to such activation, NK cells are induced to proliferate, secrete cytokines, degranulate, up-regulate various surface expression markers (e.g., NKp46), and specifically kill MHC class 1 negative target cells (e.g., K562 cells, which are often used in NK cell-specific cytotoxicity assays).

NK cells are typically cultured in static bags or tissue culture flasks but cell numbers above 2 × 10^9 require more than one bag/flask, increasing the amount of consumables and labor. Thus, to meet the need for large-scale expansion of functional NK cells a more scalable, cost-efficient, and simple production process is required. The WAVE Bioreactor 2/10 system (Fig 1A) can be used for cultivation of human white blood cells, and cell densities of more than 10 × 10^6 cells/mL at very high viability can be readily achieved (4–7). The cell culture medium and cells are only in contact with a presterile disposable chamber, the Cellbag™ bioreactor, which is placed on a special rocking platform. The rocking motion of this platform induces waves in the cell culture fluid that provide mixing and oxygen transfer, resulting in an optimal environment for cell growth. Because Cellbag bioreactors are disposable, presterilized, and completely contained, they are ideally suited for applications where the prevention of cross-contamination is critical. Using the perfusion capabilities of the WAVE Bioreactor system, very high cell densities can be generated while maintaining a closed system. This application note focuses on expansion of NK cells using the WAVE Bioreactor 2/10 system with a perfusion module and does not include selection, harvesting, or washing of the cells.

A.



B.

Fig 1. (A) WAVE Bioreactor 2/10 system with Perfusion Controller. **(B)** Custom-made transfer flask; glass bottle with two spouts; one is coupled to a sterile filter and the other to transfer tubing.

Materials and methods

Activation of NK cells in static culture

Approximately 200 × 10^6 freshly prepared (< 48 h old) density gradient isolated human PBMCs (3H Biomedical) were counted and characterized by flow cytometry as described below. The total number of NK cells ($CD16^+$ and/or $CD56^+$) was then calculated and cells were loaded at a concentration of 1 × 10^6 PBMCs/mL into 175 cm^2 flasks (Nunc) with preheated NK cell media consisting of CellGro™ SCGM (CellGenix) supplemented with 10% human serum, and 500 U/mL interleukin-2 (IL-2; Proleukin™; Novartis Pharmaceuticals). Anti-Biotin MACSiBead™ Particles were loaded with CD335 (NKp46)-Biotin and CD2-Biotin, according to the manufacturer's instruction (Miltenyi). Beads were then washed with NK cell media and added to the PBMCs at a ratio of 1:2 (beads:NK cells). Cells were cultured with beads for 5 to 7 days at 37°C with a 5% CO_2 atmosphere. This period ensures close contact between beads and cells and allows the NK cells to become activated and reach sufficient number before being transferred to the WAVE Bioreactor 2/10. From day 5, the cells were counted daily and media was added to adjust the cell concentration to 1 × 10^6 cells/mL. When the cells were actively dividing and the minimal cell number of 200 × 10^6 cells was reached, the cells were transferred to the Cellbag bioreactor (typically on days 5 to 7).

Preparing perfusion tubing

Each culture requires two perfusion-tubing assemblies. One is connected to the feed line of the Cellbag bioreactor and the other to the harvest line. The tubing assemblies were made as follows: Male Luer lock (MLL) fittings were installed on both ends of two 50 to 70 cm long, 3.2/6.4 mm (ID/OD) ASTP-ELP silicone tubings (Gore/Saniflex AB), and secured in place using zip-ties. The tubing assemblies were autoclaved and one was coupled to an autoclaved transfer flask (Fig 1B) via PVC extension tubing with a female Luer lock [FLL] and MLL (Sarstedt), and the feed line of the Cellbag bioreactor. The other silicone tubing assembly was connected to the Cellbag harvest line and a waste bag (M*BAG-20L) via PVC extension tubing.

Preparing the WAVE Bioreactor 2/10 system

A WAVE Bioreactor 2/10 system (software version 2.62) with Perfusion Controller (PERFCONT2E) was set up according to the user manual. All connections between the Cellbag bioreactor, perfusion-tubing assemblies, and waste bags were performed inside a tissue culture hood or by sterile welding (SCBII; Terumo Medical Corporation). NK cell media supplemented with 0.02% Pluronic™ surfactant (Invitrogen) was added to a transfer flask, placed inside the tissue culture hood, and connected to WAVE Bioreactor 2/10 via the feed perfusion assembly. The Cellbag bioreactor, with all the tubing fittings and filter heater in place, was filled with 5% CO_2 using the external CO_2 Controller

(CO2MIX20) and the weight of the empty Cellbag bioreactor was tared. At least 150 mL of media was transferred by gravity to the Cellbag bioreactor and heating and rocking were started. The temperature (37ºC) and pH were allowed to equilibrate for 2 h using a rocking speed of 6 rocks/min (rpm) at an angle of 6º, first ensuring that there was good contact between the temperature probe on the WAVE Bioreactor 2/10 and the Cellbag bioreactor.

NK cell culture in the WAVE Bioreactor 2/10 system

Cells from culture flasks were centrifuged and re-suspended in 50 to 100 mL fresh media. The CO_2 Controller was turned off and the inlet and outlet air filters clamped before the cells were added to the transfer flask and transferred to the Cellbag bioreactor by gravity flow. Fresh media was used to wash out the remaining cells before the transfer flask was sealed off. The inlet and outlet air filters were opened and rocking started. The following conditions were used throughout the culture: temperature 37°C, CO_2 5%, airflow 0.1 to 0.2 Lpm, angle of 6º, and rocking speed of 6 rpm. A 5 L bag (Hyclone™ HyQtainer™; Thermo Scientific), with NK cell media supplemented with 0,02% Pluronic surfactant, was connected to the extension tubings of the feed perfusion assembly and hung above the WAVE Bioreactor 2/10. Media, prepared fresh every second day, was added to maintain a cell density of approximately 1×10^6 cells/mL and the media bags were exchanged by sterile welding to maintain a closed system. Glucose, lactate, ammonia, and glutamine levels were measured daily on a BioProfile™ 100+ analyzer (Nova Biomedical) and pH, CO_2, and O_2 levels were measured on an ABL5 blood gas analyzer (Radiometer) according to the manufacturer's instructions. Viable cell counts were performed every 1 to 2 days using the standard Trypan Blue exclusion method (8) and absolute cell counts were calculated by multiplying the total cell number with the percentage of cell subsets determined by flow cytometry. Perfusion was started when the maximum culture volume of 1 L and a cell density of 3 to 5×10^6 cells/mL were reached. For the first days of perfusion, a feed rate of 300 mL/day and shot volume of 50 mL were used and the perfusion rate was then gradually increased to maintain the glutamine levels above 1 mM and the glucose levels above 1.5 g/L. Typically, the perfusion was increased to 500 mL/day when the cells had reached a density of ~7×10^6 cells/mL, 750 mL/day at ~10×10^6 cells/mL, and 1000 mL/day at ~25×10^6 cells/mL. Cells were sampled daily through the needleless sample port; first detaching cells sticking to the plastic by tapping the bottom of the bag and mixing the content of the bag thoroughly to ensure collection of a representative sample.

K562 culture

The K562 target cell line (MHC class I-negative erythroleukemia cells) was cultured according to the distributor's instructions (ATCC) in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 10% fetal bovine serum (FBS) (Hyclone).

Phenotypic analysis

Cellular subpopulations were analyzed weekly by flow cytometric analysis using standard procedures. In brief, 1×10^6 cells were washed and stained for 20 min in the following antibody cocktails: CD45 PerCP-Cy™5.5 (332784), CD3 FITC (345763), CD16 PE (332779), CD56 PE-Cy7, and CD19 APC (345791); CD45 PerCP-Cy5.5 and CD14 PE (345785); CD45 PerCP-Cy5.5, CD56-PE-Cy7 and CD336 (NKp44) PE (IM3710, Beckman Coulter); or CD45 PerCP-Cy5.5, CD56-PE-Cy7, and CD335 (NKp46) PE (IM3711, Beckman Coulter) and analyzed on a FACSCanto™ flow cytometer using FACSDiva™ software. Reagents, instrument, and software were all from BD Biosciences unless otherwise stated.

Cytokine production

Cytokine release was measured by a flow cytometry based method. The expanded cell suspension product was centrifuged, and re-seeded in 500 µL of fresh culture media at 1.5×10^7 cells/mL. The cells were left unstimulated or mixed with target K562 cells at a ratio of 10:1 (expanded effector cells:target K562 cells) for 5 h. The supernatants were collected and the level of cytokine production was determined using the CBA Th1/Th2 Cytokine Kit (BD Biosciences) and analyzed by flow cytometry according to the manufacturer's instructions. The concentrations of IL-2, IL-4, IL-5, IL-10, IFN-γ, and TNF-α were based on the standard curve for each cytokine

Flow cytometry-based cytotoxicity and degranulation assays

Cytolytic activity of the expansion product was tested using a flow cytometry based method using CellTrace CFSE Cell Proliferation Kit (Invitrogen) (9). In short, 1×10^7 cells/mL target K562 cells were labeled with 0.5 mM carboxyfluorescein diacetate succinimidyl ester (CFSE) at 37°C for 5 min. The reaction was quenched by adding an equal volume of FBS. The cells were washed twice and resuspended in K562 media to a final cell concentration of 1×10^6 cells/mL. The expanded product was washed and co-cultured with labeled target cells at different ratios and time points. Effector cells and target cells were seeded alone as controls. The specific lysis was calculated as follows:
% Lysis = 100-{[absolute no. viable CFSE + target cells co-cultured with NK cells]/[absolute no. viable CFSE + target cells cultured in medium]} × 100%.

Degranulation of NK cells was also measured by co-culturing expanded PBMCs (5×10^6 cells/mL) with K562 target cells at ratios of 10:1, 5:1, and 1:1 (expanded effector cells:target K562 cells) in K562 media for 2 h at 37°C and in 5% CO_2. Two control samples consisting of NK cells cultured without K562 cells were also prepared. Samples, except one control, were incubated at room temperature with anti-CD107a-PE (H4A3; BD Biosciences), CD3-FITC, and CD56-PE-Cy7 (BD Biosciences) in the dark for 20 min and the samples were analyzed by flow cytometry for cell surface expression of CD107a on $CD56^+$ cells.

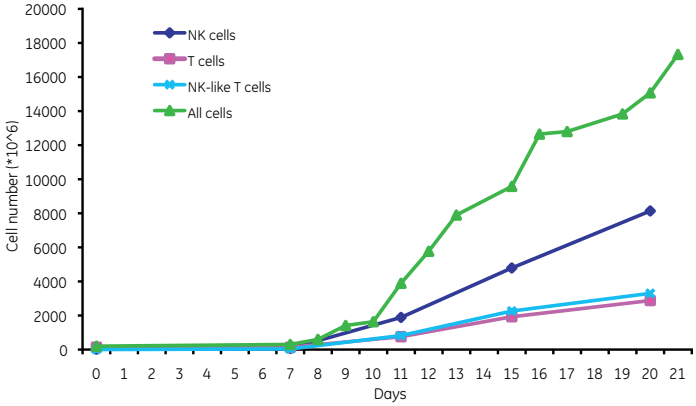
Results

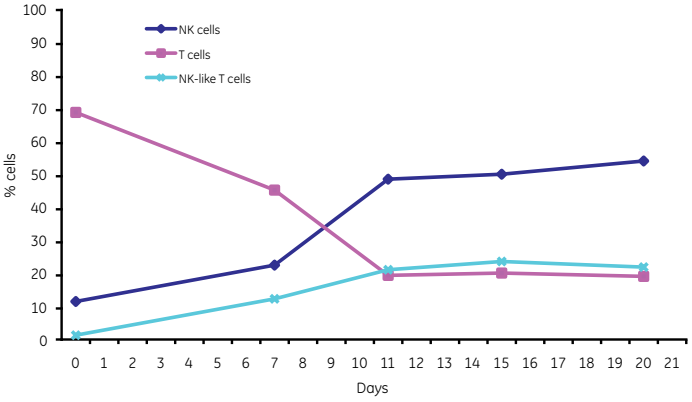
Various NK cell-activation and expansion protocols were evaluated in small-scale static cultures and the presently described method was then selected for large-scale cultures using the WAVE Bioreactor 2/10 system. Here we present results from three independent NK cell cultures using PBMCs from healthy donors (culture 1: 51-year-old female, culture 2: 51-year-old female, and culture 3: 29-year-old male). A culture from a fourth donor (33-year-old male) was also initiated but the cells failed to grow despite a high proportion of NK cells at the start of the experiment (17%) and the culture was therefore terminated on day 10 (results not shown). In fact, exceptional donor-to-donor variability, with respect to expansion potential, activity, and phenotype of the cells was observed, thus the results from each individual culture are presented separately.

In addition to cell counts and viability; pH, CO_2, lactate, glucose, ammonia, and glutamine levels were monitored every 1 to 2 days. More media was added and/or the perfusion rate increased to keep lactate levels to a minimum and the pH, glucose, and glutamine levels kept within an optimal range (pH 7, glucose > 1.5 g/L, and glutamine > 1 mM) throughout the entire culture period despite a dramatic increase in the cell densities (results not shown).

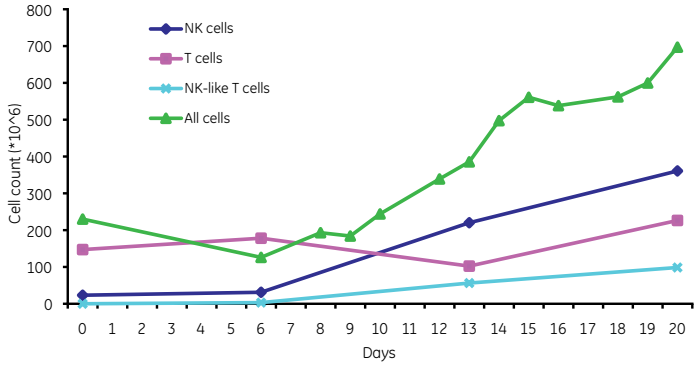
The cumulative cell growth from culture 1, 2, and 3 are presented in Figures 2 A, B, and C respectively. None of the cultures showed any significant increase in total cell number the first week of culture due to a delayed activation response and a selective loss of monocytes and B cells. The expansion rate differed considerably between the cultures after day 7, however. Whereas the cells from cultures 1 and 3 displayed

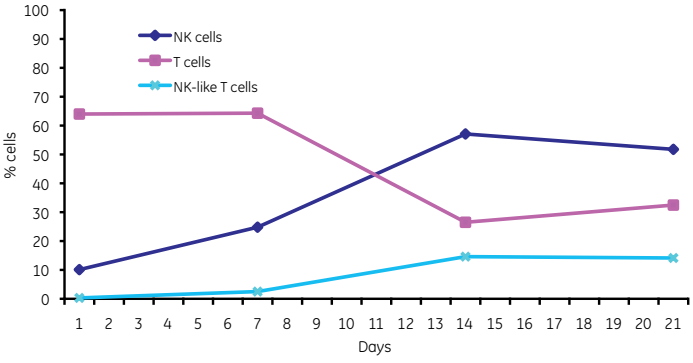
A. Culture 1.



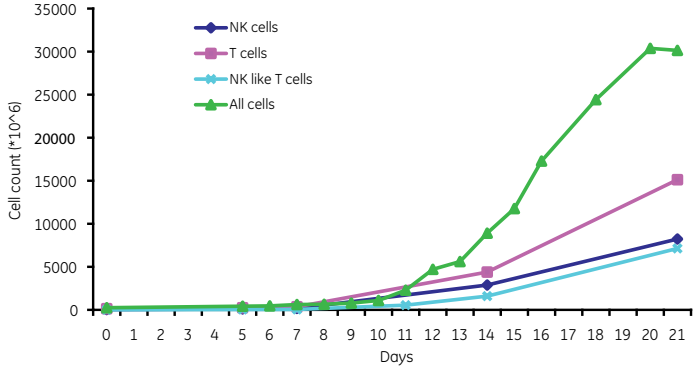


B. Culture 2.





C. Culture 3.



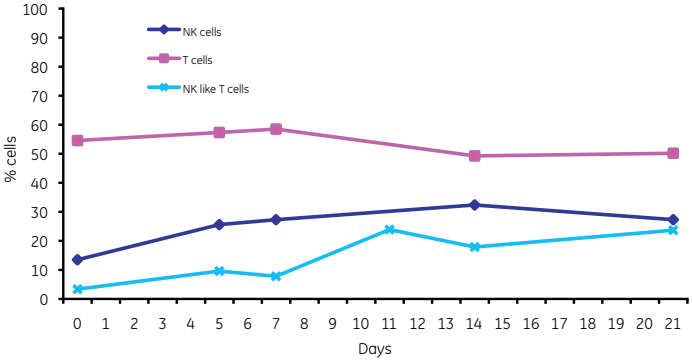
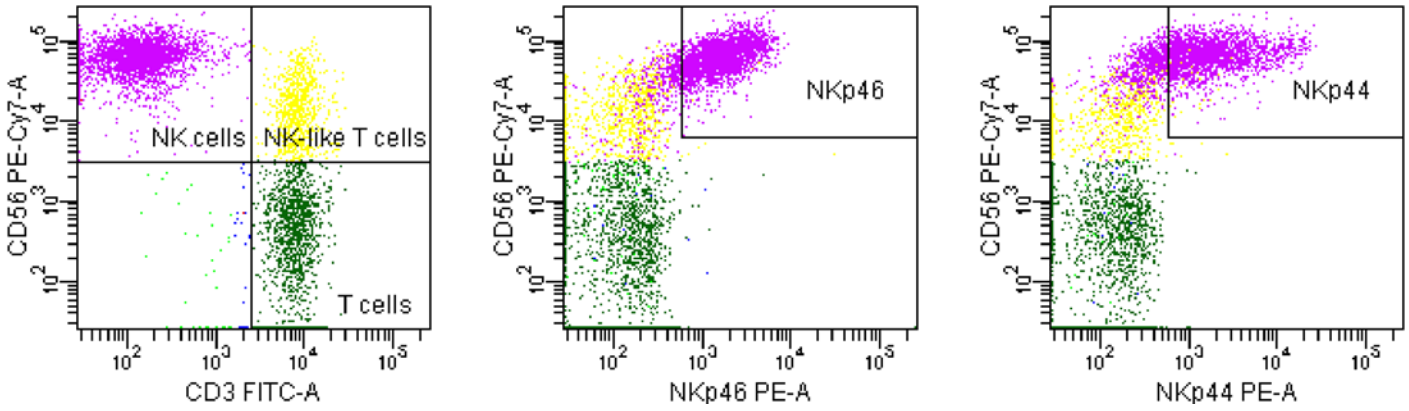


Fig 2. Cumulative growth (left) and proportion of NK cells, T cells, and NK-like T cells (right) for **(A)** Culture 1, **(B)** Culture 2, and **(C)** Culture 3.

A. Culture 2.



B. Culture 3.

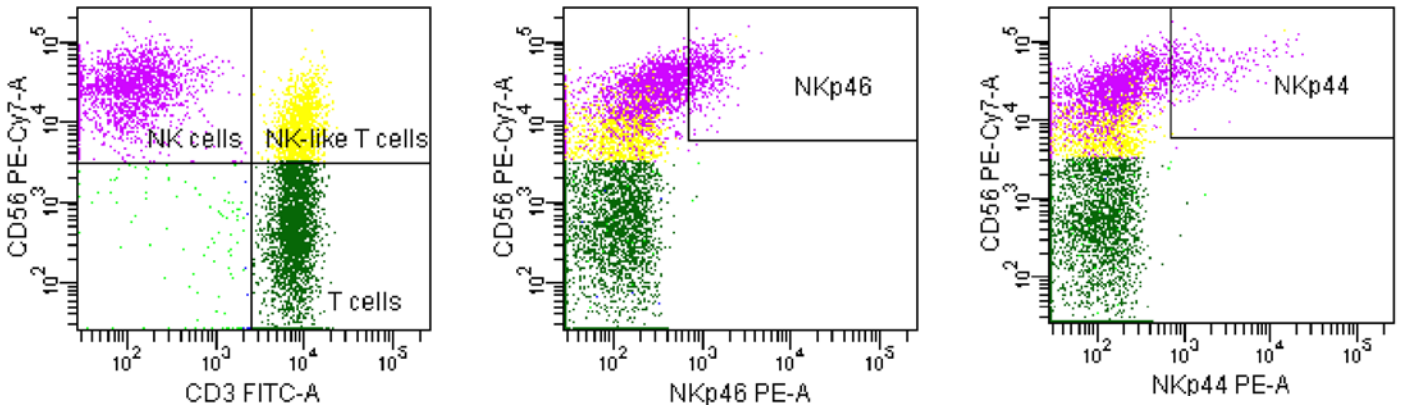


Fig 3. Phenotypic characterization of expansion product of **(A)** culture 2, and **(B)** culture 3 on day 21 using flow cytometry. Measurement of $CD56^+/CD3^-$ NK cells, $CD56^-/CD3^+$ T cells, and $CD56^+/CD3^+$ NK-like T cells (left); $CD56^+/NKp46^+$ NK cells (middle); and $CD56^+/NKp44^+$ NK cells (rightl).

good proliferation, the cells from culture 2 exhibited very slow growth throughout the culture period and only a 3-fold increase in total cell number and a 12-fold increase in NK cell number were achieved after a 3-week expansion period. The final yields for the three cultures were: 17×10^9, 0.7×10^9, and 30×10^9 cells respectively (Fig 2), with viabilities above 90% (results not shown).

The proportions of NK cells in the donor PBMCs at the start of the culture were 11%, 10%, and 14% in cultures 1, 2, and 3 respectively, of which most were $CD56^{dim}$ (results not shown). The total NK cell population increased in response to NK cell activation and expanded to 54%, 52%, and 27% of the total cell number giving a cumulative increase in NK cell number of 354-, 12-, and 250-fold in cultures 1, 2, and 3 respectively (Fig 2). The proportion of $CD3^+/CD56^+$ double positive NK-like T cells (also called cytokine induced T [CIT] cells) also increased considerably in the culture, from around 1% at the start of the culture to approximately 20% in the end product. The NK-like T cells have been shown to derive from $CD3^+CD56^-$ effector T cells and to possess greater cytolytic activity against various tumor cell targets, including K562 cells, than $CD3^+CD56^-$ T cells (10,11).

In contrast to cultures 1 and 2, the proportion of NK cells slightly decreased and the T cell population increased during the last week of culture 3. The reason for this is unclear, but might be due to the high levels of IFN-γ produced by the cell culture, which has the potential to activate T cells (2). The high final cell density (> 30×10^6 cells/mL) of this culture might also have a negative impact on NK cell growth. Support for the latter hypothesis comes from a parallel culture in which cells from the same donor were expanded in culture flasks for 10 days (compared to 5 days for culture 3) and subsequently 200×10^6 cells were transferred to a WAVE Bioreactor 2/10 system. This culture exhibited a similar proportion of NK cells to culture 3 on day 14 (~ 32%), but in contrast to culture 3; it exhibited 60% NK cells on day 21 although at a very low cell density of 1×10^6 cells/mL (results not shown).

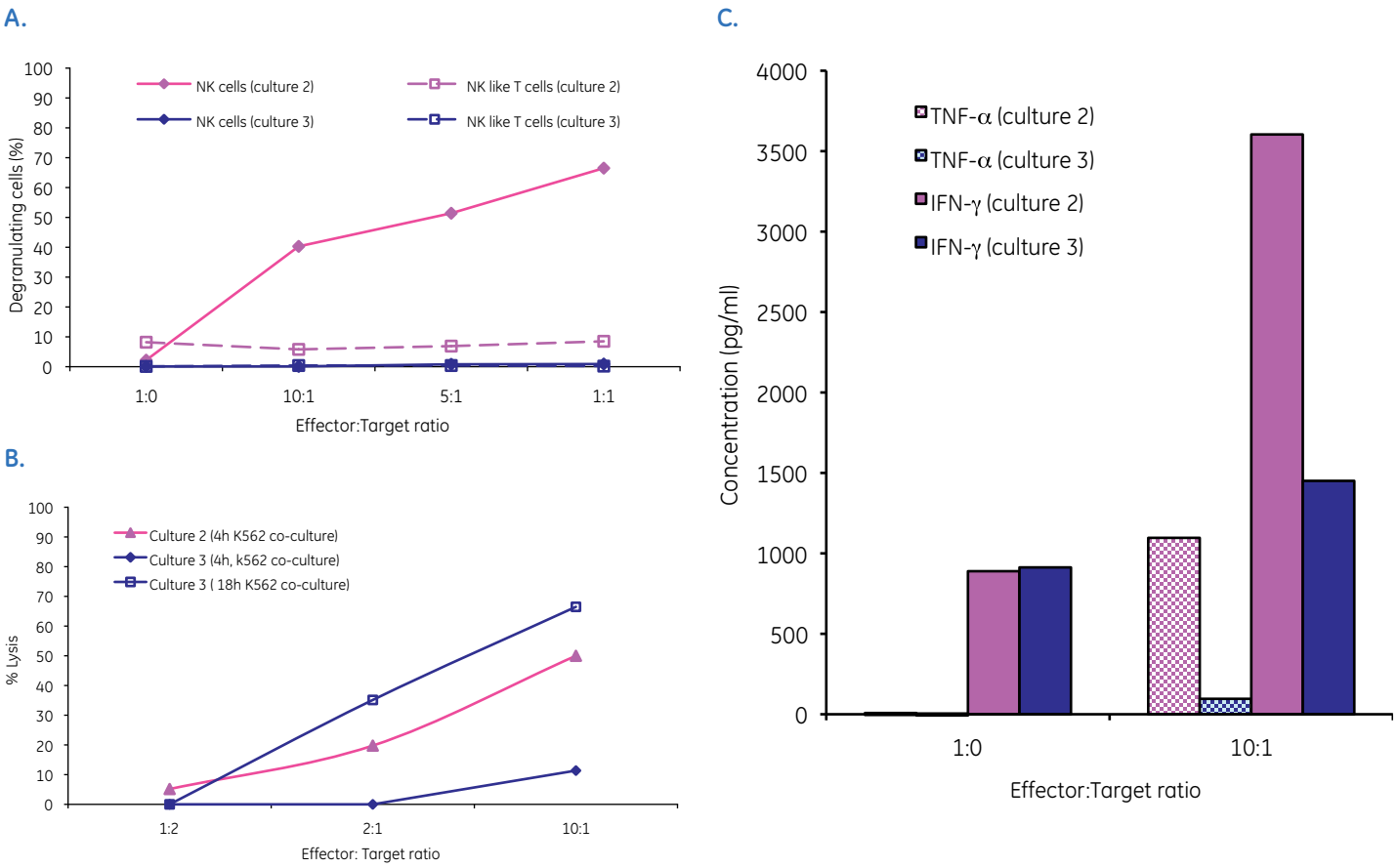


Fig 4. (A) Percentage of degranulating NK cells against K562 target cells as measured by CD107a expression after co-culture with K562 cells at different effector:target ratios. **(B)** Cytotoxic activity in response to K562 co-culture at different effector:target ratios. **(C)** Cytokine secretion of NK cells before and after co-culture with K562 cells at a ratio of 10:1 for 5 h. The results are mean values from triplicate wells. Note that TNF-α was undetectable in the unstimulated effector cell controls.

To evaluate the activation status of the cell products from cultures 2 and 3, the up-regulation of the activating receptors NKp44 and NKp46 was assessed and the cytolytic activity, degranulation, and cytokine production against the NK target cell line K562, were measured. There was a surprisingly large inter-individual variability between the two cultures (Figs 3 and 4). Interestingly, the cell product from culture 2, which displayed very slow NK cell growth, was highly active. Not only did ~ 70% to 85% of the NK cells express the activating receptors NKp46 and NKp44 (Fig 3A), but the cells degranulated, produced high amounts of IFN-γ and TNF-α, and displayed cytolytic activity against target cells in a dose-dependent manner upon co-culture with K562 cells (Fig 4). In contrast, the product from culture 3 was not as active. No CD107a surface expression or cytolytic activity was detected after a 4-h co-culture with K562 cells (Figs 4A and B). Some cytolytic activity was, however, detected after an 18-h co-culture with K562 cells at the two highest effector cell doses. Although the cells from culture 3 secreted high basal levels of IFN-γ, the cytokine production was only marginally increased after a 5-h incubation with target K562 cells (Fig 4C). Interestingly, only 30% of the NK cells from culture 3 were NKp46^{+} and 16% expressed NKp44. As mentioned in the introduction, NKp44 is expressed exclusively on activated NK cells. Although both activated NK cells and T cells secrete IFN-γ and TNF-α, neither culture 2 nor culture 3 expressed any detectable levels of IL-4, IL-5, or IL-10, which are generally produced by activated T cells (results not shown).

Conclusion

In this application note, we present data on the expansion of three independent NK cell populations using the WAVE Bioreactor 2/10 system. The results confirm the well known, but yet problematic, high inter-individual variations in NK cell expansion potential and activity. It is therefore recommended to monitor the cells carefully every day, provide media as required, perform frequent functional and phenotypic analyses, and to harvest the cells when the optimal cell concentration and cellular activity are reached. We recommend transfer of the cells to the WAVE Bioreactor instrument when the cells are activated and expanding and the target cell number of at least 200 × 10^6 cells has been reached.

We have observed that moving the cells into the WAVE Bioreactor instrument too early, when they are still being activated and are sensitive to stress, may halt proliferation for a few days before the cells have adapted to the new environment (results not shown). It should be noted, however, that it has been shown that NK cell cultures can be initiated directly in a WAVE Bioreactor instrument (7). Although WAVE Bioreactor instruments with perfusion supports very high cell concentrations, too high cell densities (> 20 × 10^6 cells/mL) might have a negative impact on NK cell growth and activity.

Major advantages of the WAVE Bioreactor system, compared to tissue culture flasks or gas-permeable bags, are that the cells are cultured in a single disposable bag and that continuous perfusion of fresh media is possible, which supports high cell concentrations and reduces the cost of goods. As an example, to produce 2 × 10^{10} cells at a density of 2 × 10^6 cells/mL in static culture requires multiple bags or flasks and at least 10 L of media. Expanding the same number of cells using the WAVE Bioreactor 2/10 system according to the protocol described herein requires only one Cellbag-2L, one transfer flask, and 5 to 6 L of media. The cost-saving on labor by using the WAVE Bioreactor system is one of the biggest advantages due to the handling of only one bag and the automated media-exchange, which dramatically reduces the hands-on-time in large-scale expansions. In addition, handling only one bag with high-density cell cultures reduces the risk of cross-contamination and the time and effort needed to concentrate and harvest cells at the end of culture.

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Ordering information

Product	Code no.
WAVE Bioreactor System 2/10 (BASE2/10 EH)	28-9377-86
System 2/10 Perfusion Controller (PERFCONT2E)	28-9884-64
Filter Heater (FLTHTR2)	28-4116-39
Protective White Opaque PVC Lid (LID2/10W, OPAQUE)	28-9376-33
CO2/Air Aeration Controller (CO2MIX20)	28-9377-95
Cellbag-2L (BioClear™ 10, Perfusion, DO)	28-9376-52
Cellbag-10L (BioClear 10, Perfusion, DO)	28-9376-62
M*BAG-20L	MB0020L10-01

Note: In the US, for research use and for use under IND or IDE cleared by the FDA.

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First published Apr. 2011

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28-9936-25 AA 04/2011