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Optimal Combinations of CellGenix® Reagents Boost Natural Killer Cell Expansion, Viability, and Purity

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Abstract

Cell and gene therapies, including immunotherapy with natural killer (NK) cells, have emerged as promising avenues for treating various diseases. However, successfully producing these therapies requires manufacturers to overcome operational challenges associated with yield and long-term cell culture. The selection of an optimal cell culture media and cytokine cocktail is an important step in generating high yields of functional cytotoxic NK cells.

This application note presents the performance of CellGenix® GMP Stem Cell Growth Medium (SCGM) and CellGenix® recombinant human cytokines for the expansion and cultivation of NK cells. Fold expansion, viability, phenotype, and cytotoxicity of NK cells cultured in CellGenix® GMP SCGM were compared to those cultured in other commercially available NK cell media. The results suggest that the chosen medium and cytokine cocktails deliver the highest expansion while maintaining cytotoxicity and purity.

Introduction

Cell and gene therapies continue to be the fastest-growing area of therapeutics, with several new therapies available to patients and hundreds more in development. One example of an emerging therapy is natural killer (NK) cell therapy, which utilizes NK cells to target and kill tumor cells more effectively.

Developing such innovative therapies involves overcoming many operational hurdles, particularly during manufacturing (whether scaling up or scaling out). A critical aspect is the optimization of fold expansion, yield, and phenotype. Achieving high fold expansion ensures a sufficient quantity of therapeutic cells, while maximizing yield increases the overall efficiency of the production process. Maintaining the desired phenotype and cytotoxicity is essential to ensure therapeutic efficacy. By focusing on these key factors, researchers can drive the development of more effective and reliable therapies, ultimately improving patient outcomes.

This study aimed to test the ability of CellGenix® GMP Stem Cell Growth Medium (SCGM) and CellGenix® recombinant human cytokines to generate functional NK cells with the highest yield while maintaining cytotoxic activity. CellGenix® GMP SCGM is a xeno-free medium that can be used for various NK cell applications when supplemented with human serum and CellGenix® IL-2, IL-12, IL-18, and IL-21. CellGenix® GMP SCGM is produced following applicable GMP guidelines and allows for the safe use in accordance with USP Chapter <1043> and ISO 20399:2022.

This application note outlines an NK cell therapy production process, covering expansion and functional characterization, including cytotoxicity, phenotype, and viability. A 24-well scale-down model was used to compare CellGenix® GMP SCGM with other commercially available NK cell media.

Key Materials

- CellGenix® GMP Stem Cell Growth Medium (SCGM)
- CellGenix® rh IL-2
- CellGenix® rh IL-12
- CellGenix® rh IL-15
- CellGenix® rh IL-18
- CellGenix® rh IL-21

Methods

NK Cell Expansion With Different Cytokine Cocktails

NK cells were purified by negative selection from leukocyte reduction systems of healthy donors using the EasySep™ Direct Human NK Cell Isolation Kit (STEMCELL Technologies). The cells were aliquoted and cryopreserved until further use. NK cells were thawed and seeded at 1×10^5 cells per well in a 24-well plate, which already contained 2×10^5 genetically modified K562 feeder cells. Prior to co-culture, feeder cells were treated with Streck Cell Preservative® (Streck) to prevent further proliferation while retaining the cells´ integrity. The cells were cultured in 1 mL CellGenix® GMP SCGM supplemented with 5% human serum and different cytokine combinations:

- Cocktail 1: 500 IU/mL IL-2
- Cocktail 2: 500 IU/mL IL-2 and 140 IU/mL IL-15
- Cocktail 3: 40 IU/mL IL-2 (increased to 200 IU/mL from day 3) and 10 ng/mL each of IL-12, IL-18, and IL-21

Three days after seeding, fresh cell culture medium with the respective cytokine cocktail was added to the wells to reach a final volume of 2 mL. On days 5 and 7, NK cells were counted and adjusted to 1×10^5 cells/mL. Cell count, viability, and phenotype were determined by flow cytometry using the Attune NxT (Thermo Fisher).

Expansion of NK Cells From Peripheral Blood Mononuclear Cell Cultures

Peripheral blood mononuclear cells (PBMCs) were isolated from leukocyte reduction systems of healthy donors through a Ficoll density gradient centrifugation. The cells were aliquoted and cryopreserved until further use. Thawed PBMCs were seeded at a density of 2×10^5 cells per well in a 24-well plate with 1 mL of either CellGenix® GMP SCGM or a competitor medium. All media were supplemented with 5% human serum, 40 IU/mL IL-2 (increased to 200 IU/mL from day 3), and 10 ng/mL each of IL-12, IL-18, and IL-21. Three days after seeding, fresh medium containing human serum and cytokines was added to the wells to reach a final volume of 2 mL. The cell number was adjusted as required. PBMC cell count and viability were determined by flow cytometry using the Attune NxT.

In Vitro Cytotoxicity Assay

K562 cells were stained with CellTrace™ Violet (Invitrogen) and co-cultivated with NK cells at different effector-to-target ratios for 6 hours in CellGenix® GMP SCGM supplemented with 2% human serum. Wells containing only labeled K562 cells served as control. NK-mediated cytotoxicity was determined by analyzing the residual viable CellTrace™ Violet labeled target cells at each effector-to-target ratio. Absolute cell counts were determined on an Attune NxT.

Immunophenotyping

The cell composition of the culture was determined by flow cytometry using fluorochrome-labeled antibodies targeting CD3 and CD56. The analysis was performed to determine the cell composition of either isolated NK cells or PBMCs from healthy donors before and after expansion. The cell types were categorized as follows:

- NK cells — identified as $CD3^{-}CD56^{+}$
- T cells — identified as $CD3^{+}CD56^{-}$
- Natural Killer T (NKT) cells — identified as $CD3^{+}CD56^{+}$
- Other cells — identified as $CD3^{-}CD56^{-}$.

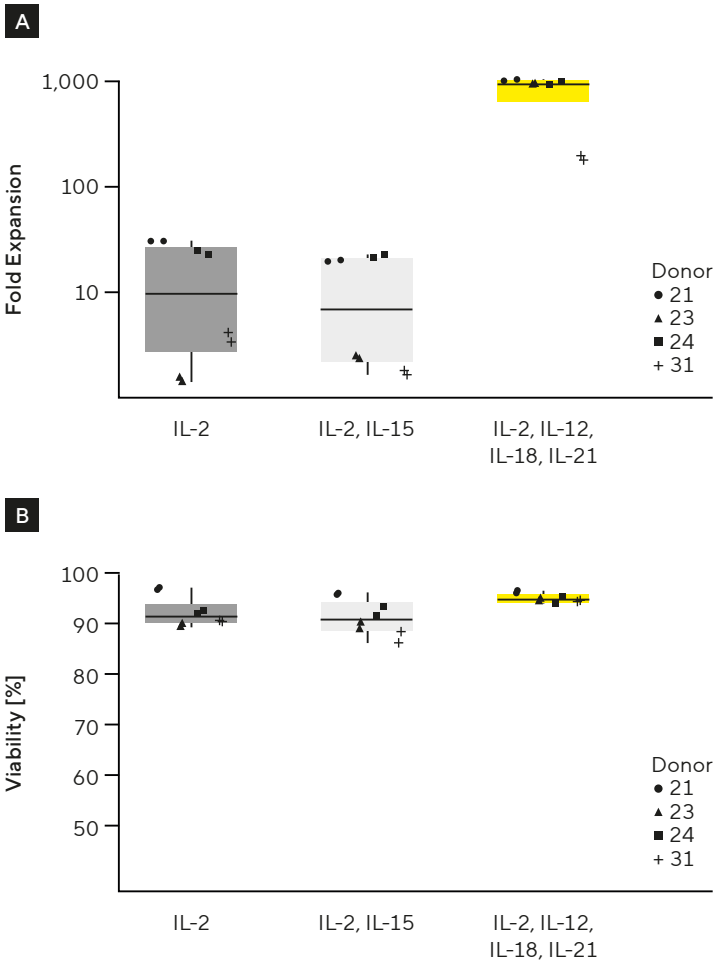
All samples were analyzed using the Attune NxT.

Results and Discussion

Choosing Optimal Cytokine Combinations Boosts NK Cell Expansion, Viability, and Purity

NK cell activation, viability, and expansion strongly depend on the effect of cytokine signaling, which makes the use of high-quality cytokines crucial in the process of ex vivo NK cell cultivation and expansion.[1] Various combinations and concentrations of cytokines have been previously described as effective for expanding NK cells for therapeutic purposes.[2-5] Our evaluation of the NK cell expansion and viability in CellGenix® GMP SCGM supplemented with three different combinations of well-described cytokine cocktails and feeder cell co-culture demonstrated that within a period of 10 days, the combination of IL-2, IL-12, IL-18, and IL-21 supported an up to 1,000-fold NK cell expansion, which was 50-fold greater than the expansion achieved with IL-2 alone or a combination of IL-2 and IL-15 (Figure 1A). While all tested conditions ensured viability of more than 90% after 10 days of culture, cells expanded with the 4-cytokine combination showed slightly improved viability for all donors (Figure 1B).

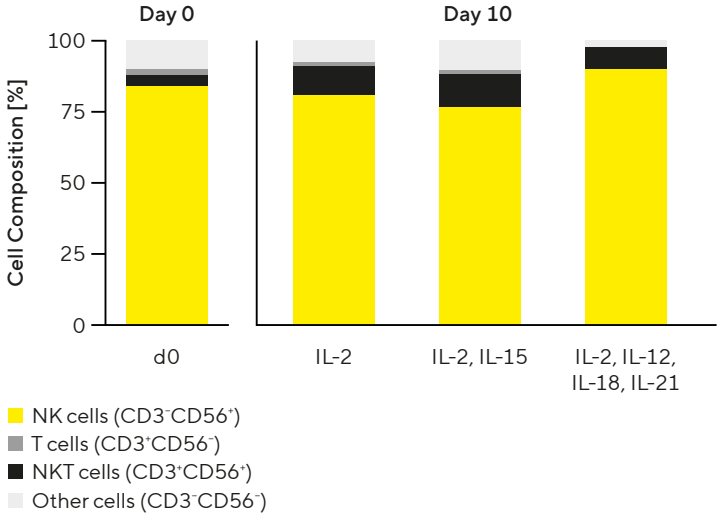
Figure 1: *NK Cell **(A)** Expansion and **(B)** Viability With Different Cytokine Combinations*



Note. NK cells from healthy donors were cultured in 24-well plates with CellGenix® GMP SCGM supplemented with IL-2, IL-2 + IL-15, or IL-2 + IL-12 + IL-18 + IL-21, and 5% human serum. Media addition was performed on day 3, and cells were passaged on day 5 and 7. Experiments were conducted with a sample size of n = 4. The data is presented as technical duplicates.

Another important aspect of the ex vivo NK expansion is the maintenance of a high NK cell purity to minimize contamination and potential side effects from other cell types. After 10 days, cells cultured in CellGenix® GMP SCGM supplemented with IL-2, IL-12, IL-18, and IL-21 showed an increase in $CD3^-CD56^+$ NK cells compared to the starting population on day 0 (Figure 2). The combination of IL-2 and IL-15, or IL-2 alone, led to a reduction in the NK cell proportion in culture, which coincided with an increase in $CD3^+CD56^+$ NKT cells and other cell types presenting a $CD3^-CD56^-$ phenotype. However, the $CD3^+CD56^-$ T cell population is marginal in all conditions and almost absent in cultures containing all four cytokines (Figure 2).

Figure 2: *Cell Composition Before and After Expansion of Purified NK Cells for 10 Days in CellGenix® GMP SCGM Supplemented With Different Cytokine Combinations*

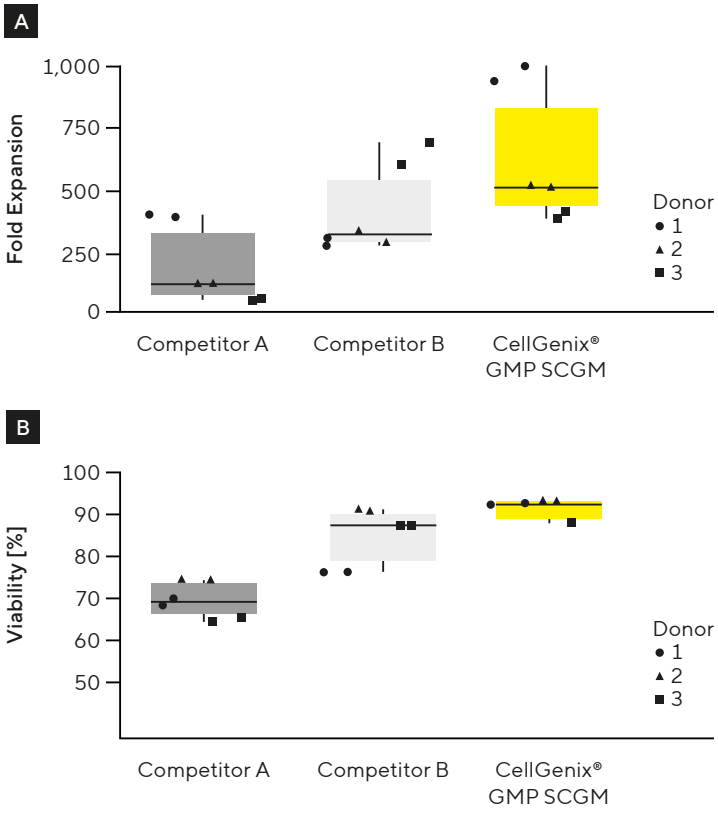


Note. Media were supplemented with IL-2 , IL-2 + IL-15, or IL-2 + IL-12 + IL-18 + IL-21, and 5% human serum. The average cell composition of NK cell cultures from four healthy donors is shown.

CellGenix® GMP SCGM Achieves the Highest NK Cell Expansion in Feeder-Free Conditions

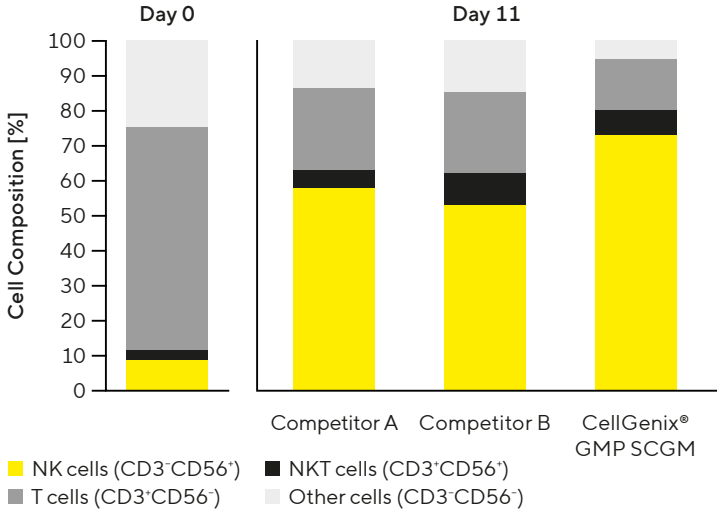
Of the three cytokine combinations tested, IL-2, IL-12, IL-18, and IL-21 facilitated the best NK cell expansion, viability, and purity throughout a 10-day ex vivo cultivation supported by a feeder cell co-culture, making it superior to the other cytokine cocktails tested. Further tests confirmed that the IL-2, IL-12, IL-18, and IL-21 cocktail sustains NK cell and PBMC expansion even in the absence of additional feeder cell stimulation (data not shown here). This was subsequently tested with different competitor media in comparison to CellGenix® GMP SCGM. The presented data demonstrate that PBMCs expanded in CellGenix® GMP SCGM showed the highest expansion among all media tested (Figure 3A) while maintaining over 90% cell viability after 11 days of cell culture (Figure 3B). Moreover, CellGenix® GMP SCGM increased the purity of NK cells in culture from 10% to over 70% in 11 days compared to 55% in competitor media (Figure 4).

Figure 3: *NK Cell **(A)** Expansion and **(B)** Viability in PBMC Cultures With Different Media in Feeder-Free Conditions*



Note. PBMCs from healthy donors were cultured in 24-well plates with CellGenix® GMP SCGM or competitor media supplemented with IL-2, IL-12, IL-18, IL-21, and 5% human serum. Media addition was performed on day 3, and cell number was adjusted as required. Experiments were conducted with a sample size of n = 3. The data is presented as technical duplicates.

Figure 4: *Cell Composition Before and After 11 Days of PBMC Expansion in CellGenix® GMP SCGM and Two Competitor Media*

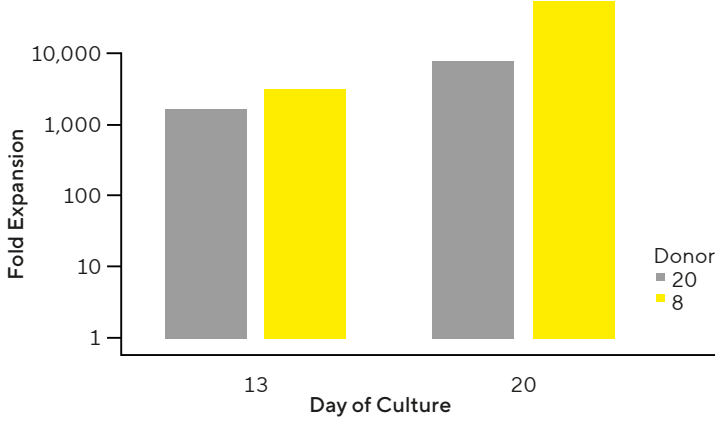


Note. Media were supplemented with IL-2, IL-12, IL-18, IL-21, and 5% human serum. The average cell composition of PBMCs from three healthy donors is shown.

CellGenix® GMP SCGM Supports Long-Term PBMC Expansion and Maintains NK Cell Cytotoxicity

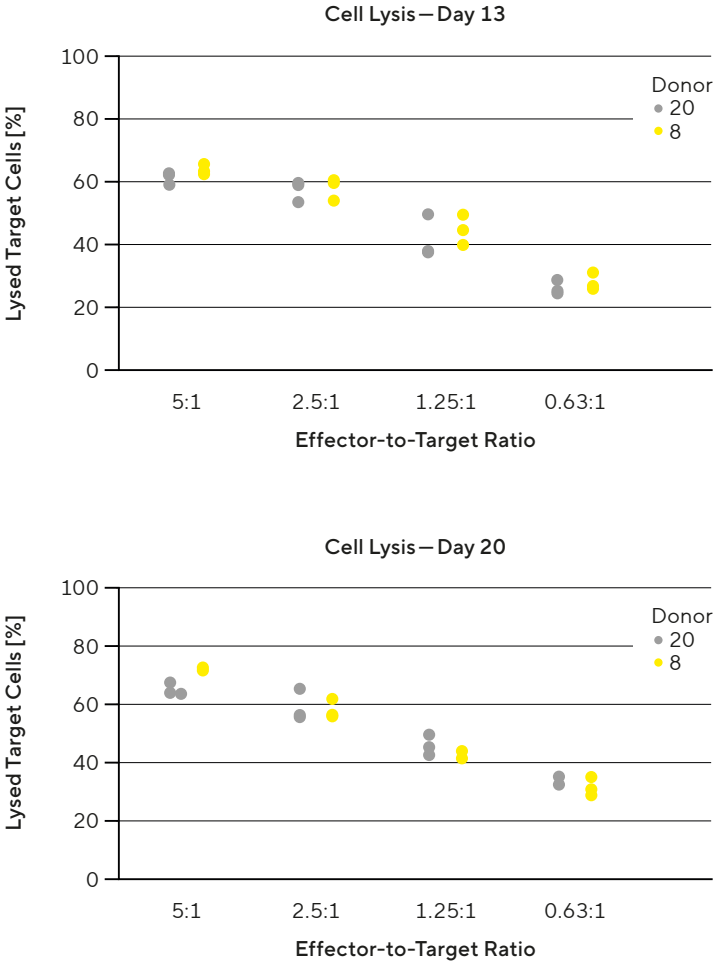
Clinical approaches may require a large quantity of allogeneic NK cells, necessitating a prolonged expansion period during which the cells' cytotoxicity must be maintained. Here, we demonstrate that CellGenix® GMP SCGM, supplemented with IL-2, IL-12, IL-18, and IL-21, supports robust long-term expansion for up to 20 days, achieving over a 10,000-fold increase in cell numbers (Figure 5).

Figure 5: *NK Cell Expansion Kinetics in Long-Term Culture With CellGenix® GMP SCGM*



Note. PBMCs from two healthy donors were cultured in 24-well plates with CellGenix® GMP SCGM supplemented with IL-2, IL-12, IL-18, IL-21, and 5% human serum. Media addition was performed on day 3, and cells were passaged as required.

Figure 6: *NK Cell-Mediated Cytotoxicity in Long-Term Culture With CellGenix® GMP SCGM*



Note. PBMCs from two healthy donors were cultured in 24-well plates with CellGenix® GMP SCGM supplemented with IL-2, IL-12, IL-18, IL-21, and 5% human serum. Cytotoxicity against K562 cells was measured at different effector-to-target ratios at day 13 and day 20. Three technical replicates are shown.

Additionally, the stable cytotoxic function of long-term expanded NK cells was confirmed by the elimination of co-cultured human immortalized myelogenous leukemia K562 cells. After 20 days of culture in CellGenix® GMP SCGM, NK cells continue to exhibit a strong cytotoxic effect, achieving around 70% elimination of K562 cells at a 5:1 effector-to-target ratio, which is comparable to the levels measured on day 13. This consistency suggests that there is no decrease in their cytotoxic capability over this culture period (Figure 6).

Conclusion

The use of CellGenix® GMP SCGM supplemented with IL-2, IL-12, IL-18, and IL-21 emerges as the most effective approach for ex vivo NK cell expansion, ensuring high viability and purity. This cytokine combination facilitates remarkable NK cell expansion, significantly outperforming other tested cytokine cocktails. The cells maintain over 90% viability and exhibit improved purity, increasing the proportion of $CD3^{-}CD56^{+}$ NK cells while minimizing contamination from other cell types. The use of IL-2, IL-12, IL-18, and IL-21 together sustains NK cell and PBMC expansion even without additional feeder cell stimulation, demonstrating that optimizing cytokine combinations is crucial for enhancing NK cell expansion and viability.

Additionally, the long-term expansion capabilities of NK cells cultured in CellGenix® GMP SCGM supplemented with IL-2, IL-12, IL-18, and IL-21 are evident, with NK cells retaining strong cytotoxic function even after 20 days of culture, achieving around 70% elimination of K562 cells at a 5:1 effector-to-target ratio. This consistency in cytotoxic capability underscores the potential of CellGenix® GMP SCGM with suitable cytokines for therapeutic applications, ensuring both efficacy and safety. Compared to competitor media, CellGenix® GMP SCGM not only supports the highest expansion rates but also maintains superior cell viability and purity, making it an optimal choice for clinical approaches requiring large numbers of allogeneic NK cells.

Ordering Information

Item	Cat. No.	Amount
CellGenix® SCGM | SCGM phenol red-free (GMP)	20802-0500 | 20806-0500 (bottles) 20902-0500 | 20906-0500 (bags)	500 mL 500 mL
CellGenix® rh IL-2 (Preclinical) CellGenix® rh IL-2 (GMP)	1420-050 1020-050	50 µg
CellGenix® rh IL-12 (Preclinical)	1428-050	50 µg
CellGenix® rh IL-15 (Preclinical) CellGenix® rh IL-15 (GMP)	1413-050 1013-050	50 µg
CellGenix® rh IL-18 (Preclinical)	1429-050	50 µg
CellGenix® rh IL-21 (Preclinical) CellGenix® rh IL-21 (GMP)	1019-050 1419-050	50 µg

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