Novel NK cell culture medium supports enhanced proliferation and cytotoxic activity of peripheral blood NK cells.

Authors: FORSBERG Matthew[1], SCHUERMAN Elizabeth[1], PICARD Lea[2], WINGERT Sabine[2], GILLIG Marc[3], OHRI Rachit[3 *], WATZL Carsten[2], CAPITINI Christian[1]

(*Corresponding Author, Email: rachit@enablelifesciences.com)

Affiliations: 1: Univ of Wisconsin, Madison. 2: Leibniz Research Centre, Germany. 3: Enable Life Sciences LLC, Worcester MA.

ABSTRACT

Natural killer (NK) cells have emerged as a promising cancer immunotherapy due to their innate abilities (e.g. antigen independent mechanisms) to recognize and kill tumor cells, as well as their allogeneic potential. However, NK cells are relatively more challenging to expand ex vivo; therefore, new cell culture media technologies are needed for ex vivo expansion of NK cells while retaining or enhancing their therapeutic functions. In this study, we investigated expansion, surface marker expression, and cytotoxic activity of primary human NK cell cultures using a novel 2-part media formulation, which combines several molecular ingredients and plant-derived extracts in an optimal media recipe. We achieved 10-fold expansion in the absence of feeder cells over 14 days, with 10% FBS in the presence of IL-2 and IL-15 – much higher compared to the primary NK cells from the same donor cultured in control media conditions i.e. RPMI with 10% FBS in the presence of IL-2 and IL-15. Cells cultured in the novel media had lower expression of CD56 after 14 days of expansion compared to the freshly isolated cells in control media, but lower expression of CD56 after 14 days of expansion in control media. Using the Incucyte real-time quantitative live-cell imaging platform, enhanced cytotoxicity was observed in novel media-expanded NK cells compared to freshly isolated NK cells against the human melanoma cell line M21 and the human pediatric neuroblastoma cell line CHLA-20. When the anti-GD2 monoclonal antibody Ch 14.18 was added to the cocultures, antibody dependent cellular cytotoxicity (ADCC) against the target cell lines M21 and CHLA-20 was also seen to be enhanced in novel media-expanded NK cells compared to freshly isolated NK cells from the same donor in control media. When the novel media were incorporated into feeder cell-based NK cell expansion protocols (e.g. using engineered K562 cells), the cultures using the novel media outperformed the feeder-based culture using control media in terms of both proliferation and cytotoxic activity. Finally, the cytotoxic activity of cryopreserved NK cells expanded in the novel media was superior compared to control media expanded NK cells. In summary, this novel 2-part culture medium performs well in expanding NK cells, both with and without feeder cells, and increases cytotoxic performance; the underlying biological mechanisms are manifested in a consistent expression pattern of cell surface biomarkers relevant to NK cell biology.

BACKGROUND and INTRODUCTION

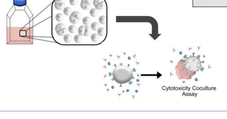
Enable-NK™ is a novel media formulation / media supplement for Natural Killer (NK) cells which incorporates plant-based extracts along with molecular ingredients - in a unique combination statistically optimized using Fractional-factorial Design of Experiment (DoE). Enable-NK™ was developed under the aegis of an NIH-SBIR grant, and is currently available as a Research Use Only (RUO) product.

Two-Phase Media Format

Phase 1: Adaptation and Growth

- The two phases (i.e. sub-formulations) can be used either independently, or sequentially
- Enable-NK can be added as a supplement to any basal medium; DMEM-F12 used for development
- IL-2 is the only required cytokine - all others are optional and customizable

Phase 2: Growth and Activation



Recommended Use:

Phase	NK Cell Lines	Primary NK Cells
1	3-5+ days	5-14 days
2	2-14 days	2-7 days

SIGNIFICANCE

Natural Killer (NK) cellular immunotherapies which offer broader cytotoxicity, better safety and improved allogeneic potential compared to Chimeric Antigen Receptor (CAR) T cellular immunotherapies. Yet, clinical response *rates* and consistency of clinical outcomes are disappointingly low with NK cellular immunotherapies. Biomanufacturing innovations - specifically, improved culture media composition(s) - represent the pathway to overcoming the entrenched hurdles of low patient response rates due to suboptimal NK cell activation, intratumoral NK cell "exhaustion", inconsistent batch-to-batch quality, and prohibitive cost. Potential benefits offered by next generation media formulations are:

[a] *enhanced cytotoxicity performance* of NK cellular immunotherapies against cancer cells, resulting in higher patient response-rates in ongoing clinical trials and emergent NK cellular immunotherapies

[b] *reduced batch-to-batch variability and cost*, by virtue of a serum-minimal formulation

[c] *increased proliferation and viability* of bioreactor expanded NK cells (both with and without feeder cells), resulting in improved dosage and therapy outcomes

[d] *mitigated "exhaustion" of NK cells* which will improve *in vivo* persistence and patient response rates,

[e] *improved transduction* of NK cells, leading to superior CAR-NK cellular immunotherapies

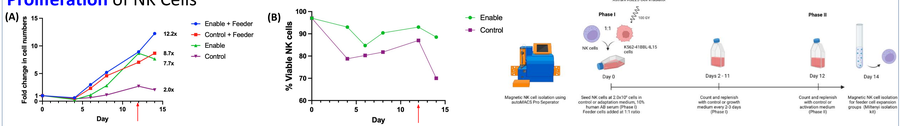
[f] *improved cryopreservation of NK cells* which can facilitate better logistics for effective clinical use

MATERIALS and METHODS

The control media used throughout the experiments was either RPMI-1640 supplemented with 10%FBS, 1% L-glutamine, 1% penicillin/streptomycin or RPMI-1640 supplemented with 10% human AB serum, 1% penicillin/streptomycin, 1% non-essential amino acids, 1% HEPES buffer. Additional media was added to the cultures every 2-3 days, to keep the cell concentration between $1x10^6$/ml and $2x10^6$/ml. 100 IU/ml IL-2 and 10 ng/ml IL-15 was also added to the cultures every 2-3 days. The feeder cells used were K562 cells engineered to express 41BBL and IL-15 (K562-41BBL-IL15). For all Incucyte assays, 10,000 target cells (either CHLA-20-GFP or M21-GFP) were plated in each well of a 96-well plate. The media for the Incucyte assays was DMEM supplemented with 10% FBS. 1ug of the anti-GD2 monoclonal antibody Ch14.18 was added to each ADCC well. Flow cytometry was performed on day 14 or day 18 using the Attune NxT flow cytometer, with 200,000 events collected for each sample. Antibodies were purchased from Biolegend (CD3-BV605, CD56-FITC or CD56-PE-Dazzle 594, CD16-AF700, LAG-3-BV711, NKG2A-AF700, TIM-3-BV510).

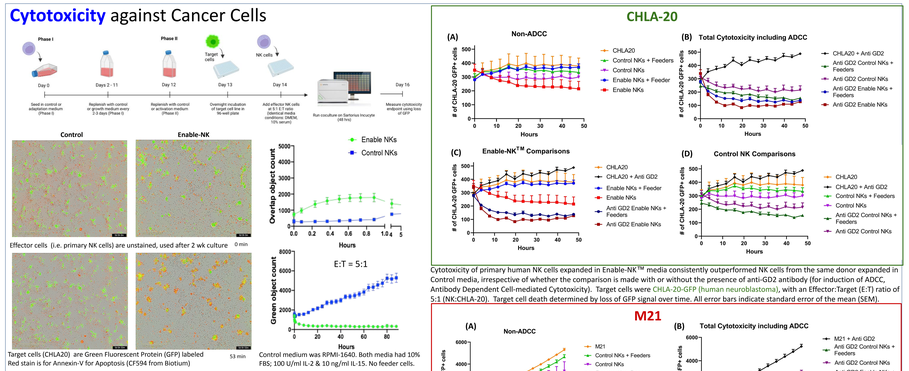
RESULTS organized by Performance Attribute of NK Cells

Proliferation of NK Cells



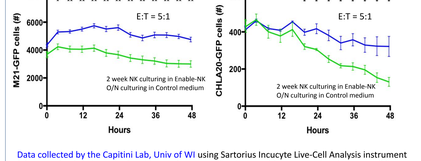
Proliferation **(A)** and viability **(B)** data of representative NK cell expansion. NK cells were isolated from Peripheral Blood Mononuclear Cells (PBMCs) using the Miltenyi NK cell isolation kit and separated with the autoMACS pro separator. 10% human AB serum was added to Enable-NK™ or Control media. Phase-I Enable-NK™ media was used for days 0-12; Phase-II Enable-NK™ media used for days 12-14 (switch indicted by red arrow). Cell numbers were determined using a Beckman Coulter Z1 Particle Counter for (Enable + Feeder) and (Control + Feeder) expansion conditions. Cell numbers for Enable and Control conditions (without feeders) were determined using a haemocytometer, with viability being determined by trypan blue exclusion.

Cytotoxicity against Cancer Cells



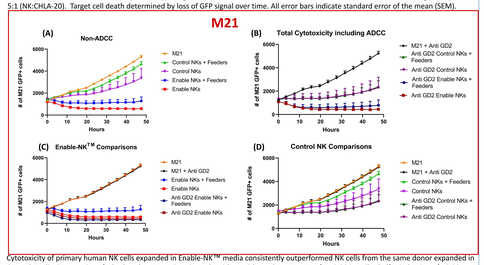
Target cells (CHLA20) are Green Fluorescent Protein (GFP) labeled
Red stain is for Annexin-V for Apoptosis (CF594 from Biotium)

Control medium was RPMI-1640. Both media had 10% FBS; 100 U/ml IL-2 & 10 ng/ml IL-15. No feeder cells.



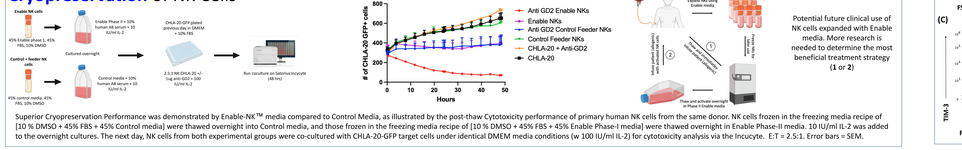
Data collected by the Capitini Lab, Univ of WI using Sartorius Incucyte Live-Cell Analysis instrument (above and right). CHLA-20 & M21 are a human pediatric Neuroblastoma and Melanoma cell line respectively. Primary NK cells were procured through fresh blood draws from healthy adult human donors. Bars indicate Standard Error of Mean (SEM).

Cytotoxicity of primary human NK cells expanded in Enable-NK™ media consistently outperformed NK cells from the same donor expanded in Control media, irrespective of whether the comparison is made with or without the presence of anti-GD2 antibody (for induction of ADCC, Antibody Dependent Cell-mediated Cytotoxicity). Target cells were CHLA-20-GFP (human neuroblastoma), with an Effector:Target (E:T) ratio of 5:1 (NK:CHLA-20). Target cell death determined by loss of GFP signal over time. All error bars indicate standard error of the mean (SEM).



Cytotoxicity of primary human NK cells expanded in Enable-NK™ media consistently outperformed NK cells from the same donor expanded in Control media, irrespective of whether the comparison is made with or without the presence of anti-GD2 antibody (for induction of ADCC, Antibody Dependent Cell-mediated Cytotoxicity). Target cells were M21 (human Melanoma), with an Effector:Target (E:T) ratio of 5:1 (NK:M21). Target cell death determined by loss of GFP signal over time. All error bars indicate standard error of the mean (SEM).

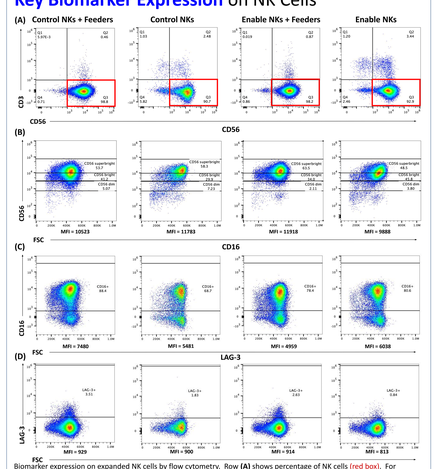
Cryopreservation of NK Cells



Cryopreservation Performance was demonstrated by Enable-NK™ media compared to Control Media, as illustrated by the post-thaw Cytotoxicity performance of primary human NK cells from the same donor. NK cells frozen in the freezing media recipe of [10 % DMSO + 45% FBS + 45% Control media] were thawed overnight into Control media, and those frozen in the freezing media recipe of [10 % DMSO + 45% FBS + 45% Enable Phase-I media] were thawed overnight in Enable Phase-II media. 10 IU/ml IL-2 was added to the overnight cultures. The next day, NK cells from both experimental groups were co-cultured with CHLA-20-GFP target cells under identical DMEM media conditions (w 100 IU/ml IL-2) for cytotoxicity analysis via the Incucyte. E:T = 2.5:1. Error bars = SEM.

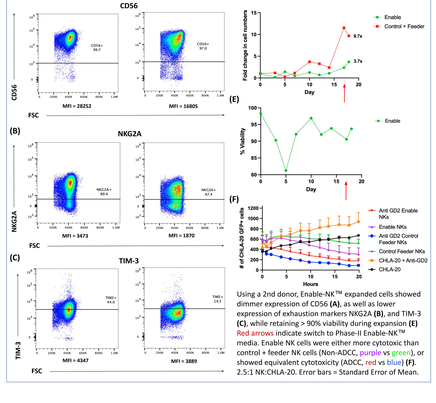
Potential future clinical use of NK cells expanded with Enable media. More research is needed to determine the most beneficial treatment strategy (**1** or **2**)

Key Biomarker Expression on NK Cells



Biomarker expression on expanded NK cells by flow cytometry. **Row (A)** shows percentage of NK cells (red box). For feeder-free expansion, Enable-NK™ had the dimmest expression of CD56 **(B)**, determined by MFI. Enable-NK™ cells had a higher percentage of cells expressing CD16 **(C)** compared to control NK cells, and showed the lowest expression of LAG-3 **(D)**.

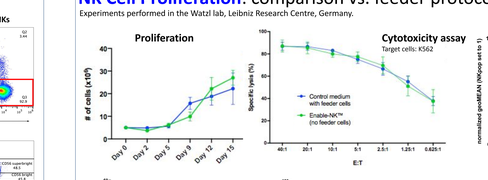
Additional NK expansion using different donor

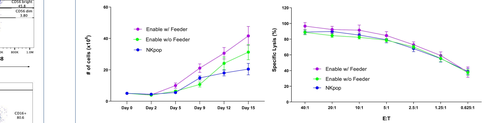


Using a 2nd donor, Enable-NK™ expanded cells showed dimmer expression of CD56 **(A)**, as well as lower expression of exhaustion markers NKG2A **(B)**, and TIM-3 **(C)**, while retaining > 90% viability during expansion **(E)** Red arrows indicate switch to Phase-II Enable-NK™ media. Enable NK cells were either more cytotoxic than control + feeder NK cells (Non-ADCC, purple vs green), or showed equivalent cytotoxicity (ADCC, red vs blue) **(F)**. 2.5:1 NK:CHLA-20. Error bars = Standard Error of Mean.

NK Cell Proliferation: comparison vs. feeder protocol

Experiments performed in the Watzl lab, Leibniz Research Centre, Germany.





Biomarker expression data was quantified for the n=4 human donors, with NK cells expanded either in Control conditions with feeder cells (IMDM medium w K562 feeders expressing membrane-bound IL-15 & 4-1BBL, with addition of soluble IL-21), or in Enable-NK™ with no feeder cells (and no soluble IL-21 added). Values are normalized to Control conditions ("NKpop") as the dotted Blue line at the value of 1.0 on the logarithmic y-axis scale. Relative biomarker quantification corresponding to Enable-NK™ is outlined in Green for the n=4 donors

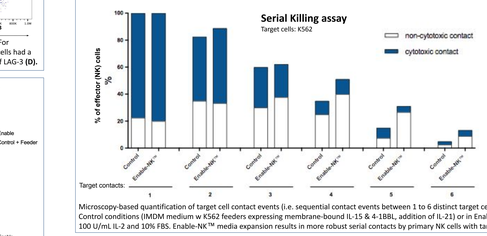
Control group: IMDM medium w K562 feeders expressing membrane-bound IL-15 & 4-1BBL, addition of IL-21.
Enable-NK™ without feeders: No feeder cells or IL-21 were used. All groups used 100 U/mL IL-2 and 10% FBS.
Enable-NK™ with feeders: Enable-NK™ media w K562 feeders (membrane-bound IL-15 & 4-1BBL), soluble IL-21.
Proliferation is average of data from 5 different human NK donors; Cytotoxicity is data from 6 donors.

The collected data establishes:

Opportunity for Synergy: between Enable-NK™ media and feeder cell based NK cell expansion protocols, and

Opportunity for feeder-free NK cell expansion with the use of Enable-NK™ media for small-fold expansions

Serial Killing by Primary NK Cells: comparison vs. feeder protocol



Microscopy-based quantification of target cell contact events (i.e. sequential contact events between 1 to 6 distinct target cells) in a co-culture assay with primary NK cells expanded either in Control conditions (IMDM medium w K562 feeders expressing membrane-bound IL-15 & 4-1BBL, addition of IL-21) or in Enable-NK™ media (no feeder cells or IL-21 were used). Both groups used 100 U/mL IL-2 and 10% FBS. Enable-NK™ media expansion results in more robust serial contacts by primary NK cells with target K562 cells, reflective of higher motility and lower exhaustion.

DISCUSSION: Our experiments illustrate that primary NK cells expanded in Enable-NK™ media demonstrate superior functionality in three key areas compared to NK cells expanded in Control media, with or without the presence of feeder cells. NK cells expanded feeder-free in Enable-NK™ have enhanced proliferation (7.7 fold over 14 days) compared to Control media (2 fold over 14 days), and this trend was also evident during NK cell expansion in the presence of K562-41BBL-IL-15 feeder cells, where use of Enable-NK™ media resulted in 12.2 fold expansion, while use of the Control media resulted in 8.7 fold expansion. The key finding in our studies is that primary NK cells expanded in Enable-NK™ media (rather than Control media) manifest significantly higher cytotoxicity against two separate human tumor cell lines, CHLA-20 (neuroblastoma) and M21 (melanoma). This superior cytotoxic capability was increased even further in the presence of the anti-GD2 monoclonal antibody Ch14.18, demonstrating that Enable-NK™ media expanded cells are especially potent killers via Antibody Dependent Cell-mediated Cytotoxicity (ADCC). Critically, these primary NK cells retained their superior cytotoxicity after cryopreservation, especially if the cryopreservation media and the post-thawing media also incorporate Enable-NK™. This functionality has been difficult to achieve in the past, as previous research has uncovered that in general, cryopreserved primary NK cells demonstrate much decreased migration and cytotoxicity compared to fresh (i.e. unfrozen) primary NK cells [1]. While not illustrated directly with primary NK cells cryopreserved in Enable-NK™ media, our studies do illustrate that fresh NK cells expanded in Enable-NK™ media did demonstrate enhanced migration compared to Control-media expanded NK cells, as quantified in the microscopy based serial-contact / serial-killing assay data on this poster. Biomarker data, assessed by flow cytometry, provides some explanation for the increased cytotoxic function, with primary NK cells expanded in Enable-NK™ demonstrating a larger $CD56^{dim}$ population (two separate donors). Previous research has shown that $CD56^{dim}$ NK cells show greater motility than $CD56^{bright}$ NK cells [2], which potentially explains why primary NK cells expanded in Enable-NK™ - with a larger $CD56^{dim}$ population - have enhanced migration and cryopreservation. Primary NK cells expanded in Enable-NK™ also have a smaller population of cells expressing the inhibitory marker NKG2A and the exhaustion marker TIM-3, touching upon the synergy of biomarker-centric mechanisms that the Enable-NK™ media is likely invoking. Finally, Enable-NK™ media offers practical, money-saving benefits by virtue of using less serum for NK cell expansions, as well as the potential of eliminating or reducing the need for feeder cells.

ACKNOWLEDGMENTS: Grant support for Enable Life Sciences LLC is acknowledged from the National Institute of Health (NIH) SBIR (Small Business Innovative Research) program Grant # 1R43GM137742-01. Support is gratefully acknowledged from: Massachusetts Biomedical Initiatives (MBI) Bioincubator (Worcester MA); Lifebridge Health Bioincubator (Sinai Hospital, Baltimore MD); Univ of MA (Amherst) Dept of Statistics; as well as the facilities (including Research Cores) at: Univ of MA, Univ of CT, Univ of MD, and Worcester Polytechnic Institute (WPI). Grant support for the Capitini laboratory is acknowledged from St. Baldrick's Stand up to Cancer (SU2C) Pediatric Dream Team Translational Research Grant SU2C-AACR-DT-27-17, NCI/NIH R01 CA215461, American Cancer Society Research Scholar Grant RSG- 18-104-01-LIB, and the Midwest Athletes Against Childhood Cancer (MACC) Fund. The authors thank the University of Wisconsin Carbone Cancer Center Flow Cytometry Laboratory, supported by P30 CA014520, for use of its facilities and services. Key contributions by Ms. Nicole Flegel to data graphing and are gratefully noted.

REFERENCES: 1. Mark, *C. et al.* Cryopreservation impairs 3-D migration and cytotoxicity of natural killer cells. *Nat. Commun.* **11**, 5224 (2020).2. Mace, E. M., Gunesch, J. T., Dixon, A. & Orange, J. S. Human NK cell development requires CD56-mediated motility and formation of the developmental synapse. *Nat. Commun.* **7**, 12171 (2016).