A Novel Medium for Increased Cytotoxicity and Serum-Minimal *ex vivo* Expansion of Natural Killer (NK) Cellular Immunotherapies

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ABSTRACT

Background: Despite significant clinical breakthroughs [1,2], cellular immunotherapy remains unacceptably variable in performance [3] and impractically expensive for mainstream adoption [4]. These hurdles are particularly true for new cancer immunotherapies with Natural Killer (NK) cells. Culturing NK cells *ex vivo* is challenging due to short half-lives, reduced functionality, and increased exhaustion [5]. Also, the use of serum introduces inconsistency and increases cost [6]. Here we introduce a novel prototype medium specifically formulated for NK cells, which uses a unique combination of plant extracts and molecular ingredients to increase cytotoxic performance & proliferation, and reduce NK cell exhaustion while allowing the reduction of serum by up to 90 percent.

Methods: The KHYG-1 NK cell line, or primary NK cells, were brought to 2% or 2.5% serum, respectively, in the prototype media. DMEM/F12 with 20% serum (for KHYG-1) or 10% human serum (for primary cells) was the control. All media was supplemented with 100 U/mL IL-2. Cell numbers were periodically assessed, using a cell counter or flow cytometry. For cytotoxicity assays, K562 target cells were co-cultured with KHYG-1 cells (Effector:Target, E:T ratio 20:1) for 5h, or with primary NK cells (E:T = 5:1) for 18h. Cells were stained with Annexin-V and Propidium Iodide to determine the levels of apoptosis and necrosis by flow cytometry. Levels of Biomarkers (e.g. CD56) were determined by Luminex (soluble biomarkers) and flow cytometry (cell-surface biomarkers).

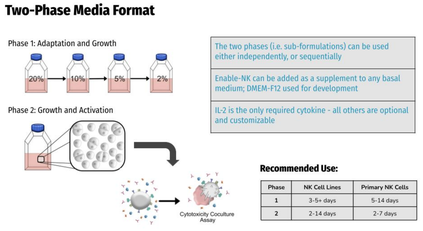
Results: Following adaptation to 2% serum, growth of KHYG-1 cells in the prototype media stayed nearly on pace with the control for over 3 weeks. Human primary NK cells grew 10-fold over 10 days in the prototype media with 2.5% serum, without feeder cells; in contrast, primary cells did not proliferate in control medium. Beta-testing with collaborating labs confirmed that the prototype media improved the proliferation of primary NK cells over a 12-day period, relative to prevalent gold standard media and methods. After culture in prototype media, both KHYG-1 cells and primary NK cells exhibited higher cytotoxic activity toward K562 and other cancer cells, compared with control medium. Secretion of interferon-γ, perforin, and granzyme A was increased in KHYG-1 cells cultured in prototype media. Additionally, NK cells are retained in a more CD56-dim phenotype, along with higher NKG2D, NKp46, CD69 and CD16: biomarker trends associated with increased cytotoxicity. Cell exhaustion biomarkers are reduced.

Conclusions: The prototype media, Enable-NK™, increases the cytotoxic activity of NK cells against cancer cells while mitigating NK cell exhaustion. It supports proliferation of primary NK cells, even at reduced serum content, and even without feeder cells.

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 at an advanced prototype testing stage.

Two-Phase Media Format



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

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:

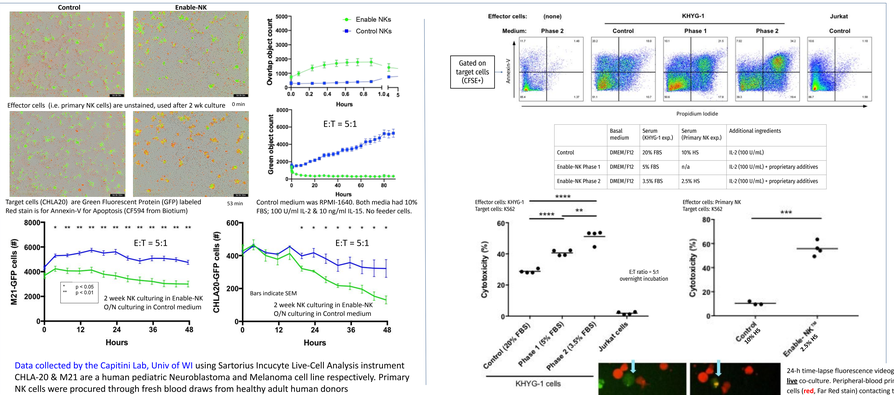
[a] *enhanced cytotoxicity performance* of NK cellular immunotherapies against cancer cells, resulting in higher patient response-rates in ongoing clinical trials and emergent NK cellular immunotherapies
[b] *reduced batch-to-batch variability and cost*, by virtue of a serum-minimal formulation
[c] *increased 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

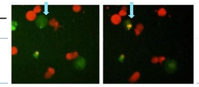
The co-culture studies involved the use of effector Natural Killer (NK) cells either as the KHYG-1 NK cell line or as primary NK cells (procured either from American Type Culture Collection (ATCC, Cat #PCS-800-019), or from fresh blood draws from healthy adult donors, subsequently purified from density-gradient isolated PBMC (Peripheral Blood Mononuclear Cells) using CD3+ cell depletion methods. Target cancer cells in the co-culture experiments were stained with CFSE (Carboxyfluorescein succinimidyl ester), which allowed them to be differentiated from unstained effector (NK) cells. Phycoerythrin (PE) labeled Annexin V and Propidium Iodide (PI) were incorporated after the co-culture incubation period to distinguish early apoptotic (Annexin V +, PI -), late apoptotic (Annexin V +, PI +), necrotic cells (Annexin V -, PI +) and healthy cells (Annexin V -, PI -) via flow cytometry. Various incubation periods and E:T (Effector : Target) ratios were employed in the experimental strategy against a range of target cancer cells.

RESULTS organized by Performance Attribute of NK Cells

Cytotoxicity against Cancer Cells



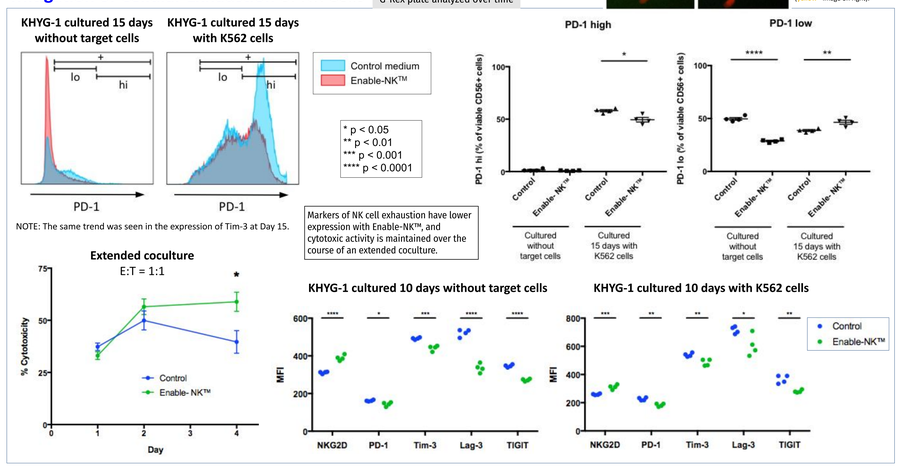
Data collected by the Capitini Lab, Univ of WI using Sartorius Incucyte Live-Cell Analysis instrument
CHLA-20 & M21 are a human pediatric Neuroblastoma and Melanoma cell line respectively. Primary NK cells were procured through fresh blood draws from healthy adult human donors



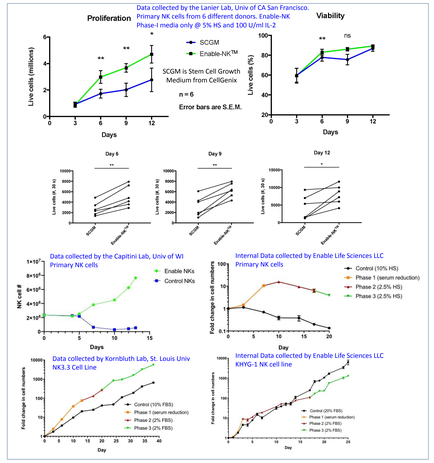
24-h time-lapse fluorescence videography of **five** co-culture. Peripheral-blood primary NK cells (**red**, Far Red stain) contacting target K562 leukemia cancer cells (**green**, CFSE stain - image on left), leading to direct killing evidenced by Propidium Iodide stain uptake (**yellow** - image on right).

Mitigation of Cellular Exhaustion of NK Cells

The *same* co-culture wells in a G-Rex plate analyzed over time

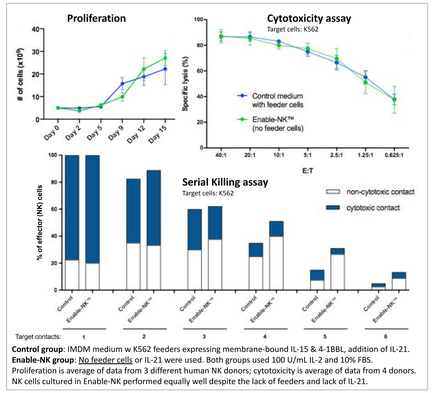


Feeder-free Proliferation of NK Cells



NK Cell Proliferation: comparison vs. feeder protocol

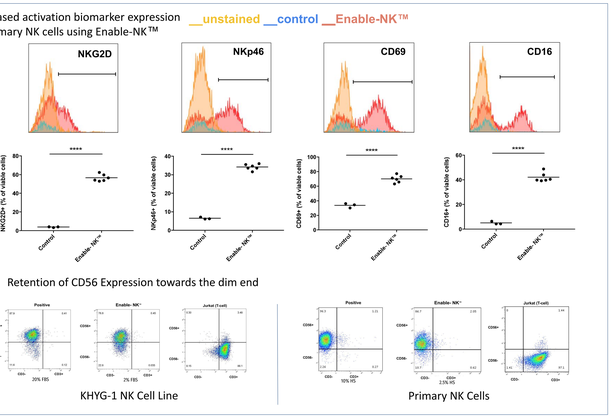
Experiments performed in the Watzl lab, Leibniz Research Centre, Germany.



Control group: IMDM medium w K562 feeders expressing membrane-bound IL-15 & 4-1BBL, addition of IL-21.
Enable-NK group: No feeder cells or IL-21 were used. Both groups used 100 U/mL IL-2 and 10% FBS.
Proliferation is average of data from 3 different human NK donors; cytotoxicity is average of data from 4 donors. NK cells cultured in Enable-NK performed equally well despite the lack of feeders and lack of IL-21.

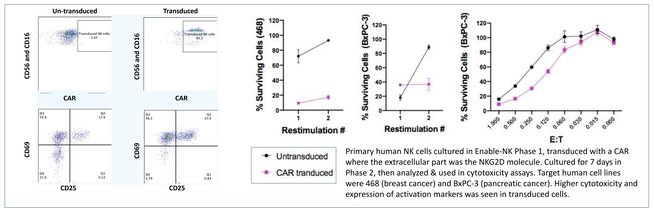
NK Cell Phenotype: surface marker expression

Increased activation biomarker expression in primary NK cells using Enable-NK™



Transduction of NK Cells

Experiments performed by Caroline Hull at Leucid Bio Ltd, London, UK.



Primary human NK cells cultured in Enable-NK Phase 1, transduced with a CAR where the extracellular part was the NKG2D molecule. Cultured for 7 days in Phase 2, then analyzed & used in cytotoxicity assays. Target human cell lines were 468 (breast cancer) and BxPC-3 (pancreatic cancer). Higher cytotoxicity and expression of activation markers was seen in transduced cells.

DISCUSSION: The cumulative datasets establish a consistent narrative, i.e. the prototype Enable-NK™ culture medium facilitates a significant increase (30-75% and higher) in the anti-cancer cytotoxicity performance of NK cells cultured in it, and supports NK cell proliferative capacity. Enhanced cytotoxicity, proliferation and viability was observed with 75-90% serum reduction, and also in the absence of serum reduction. The expanded NK cells retain their canonical CD3 /CD56⁺ phenotype, and their inherent capacity to create immunological synapse contact points with target cancer cells to induce their direct killing in a manner that keeps the cells less exhausted over time. Increased expression levels of several biomarkers pertaining to NK cell function and activation establish the mechanistic basis for the enhanced cytotoxicity, proliferation, viability and exhaustion performance of NK cells cultured in Enable-NK™. These results therefore offer a compelling basis to achieve development goals, by accomplishing a serum-minimal NK media formulation, and also impact *multiple* aspects of NK cellular therapies i.e. enhanced cytotoxicity, improved proliferation, increased viability and reduced cellular NK cellular exhaustion, *in addition to* eliminating serum-dependence, therefore leading to better long-term clinical outcomes for cancer patients.

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REFERENCES: (1) Hirayama AV, et al. Blood. 2019;134(7), pp.636-640. **(2)** Martinez VG et al. Philos Trans R Soc B Biol Sci. Royal Society; 2019 Aug 19;374(1779):20180214. **(3)** Burke C, et al. Regen Eng Transl Med. 2019;5:332–40. **(4)** Leech AA, et al. Balancing Value with Affordability: Cell Immunotherapy for Cancer Treatment in the U.S. Oncologist. John Wiley & Sons, Ltd; 2020 Jul 1;25(7):e1117–9. **(5)** Fang F, et al. Front Med. 2018;12(4):440–50. **(6)** Aijaz A, et al. Nat Biomed Eng. 2018;2(6):362–76.