GENERATION OF HUMAN iPSC-DERIVED HEMATOPOIETIC PROGENITOR CELLS AND DIFFERENTIATION TO FUNCTIONAL iNK CELLS IN A FLEXIBLE AND FEEDER-FREE CULTURE SYSTEM

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Abstract

Having a reliable source of high-quality hematopoietic stem and progenitor cells (HSPCs) is critical for developing various autologous and allogeneic cell and gene therapies. iPSC-derived HSPCs (iHSPCs) can be a consistent source for generating a variety of clinically relevant cell types, including Natural Killer (NK) and T cells. However, the differentiation processes typically take weeks or months, making labor- and material-intensive testing costly during development.

Here we describe a fit-for-purpose feeder-free iHSPC differentiation system that uses all cell therapy grade and regulation-compliant reagents. This flexible protocol supports cost-effective, small-scale testing in multi-well plates and can be scaled up through shake flasks. By avoiding steps that can limit culture scale up, like spin embryoid body formation and complicated handling, this protocol facilitates the translation of process development learnings to large-scale manufacturing.

Using CTS™ StemPro™-34 serum-free xeno-free medium, we generated iHSPCs that were >65% CD34+CD43+ and >90% viable. To demonstrate functionality, the iHSPCs were differentiated into iNK cells in 100 mL cultures in shake flasks, followed by two weeks of expansion resulting in >10^9 CD56+ CD3- iNK cells, with >90% purity and >80% viability. NK cells have great potential as an "off the shelf" allogeneic therapeutic product, as they can target cancer cells in an antigen- and HLA-independent manner. We therefore tested the functional ability of the expanded iNK cells to mediate anti-tumor activity against the K562 erythroleukemia cell line and cancer organoids ("tumoroids") derived from human colorectal adenocarcinoma. The iNK cells displayed significant cytotoxic activity, killing >80% of target K562 cells and adenocarcinoma tumoroids in a dose-dependent manner, without requiring enrichment prior to use.

In conclusion, we demonstrate the use of a versatile differentiation workflow to generate clinically relevant cell types, including functional iHSPCs and iNK cells. Utilizing a cost-effective differentiation system during process development can aid in effectively characterizing both the therapeutic cells and production processes, to facilitate a smooth transition to scale-up or scale-out.

Introduction

Hematopoietic stem and progenitor cells (HSPCs) are critical for therapeutic applications due to their self-renewal and differentiation abilities. iPSC-derived hematopoietic and immune cells offer better consistency and scalability than primary cells. However, challenges include a lack of standardized protocols, reliance on proprietary iPSC lines and inefficient differentiation. In addition, characterizing materials and processes at scale is expensive, but small-scale testing may not fully translate to larger scales. Using a standardized protocol can streamline pre-clinical development efforts, allowing researchers to focus on efficacy, safety, and manufacturing.

Here we describe a simplified, xeno-free protocol for generating iHPSCs and differentiating them into mature, cytotoxic iNK cells. Using specific protein and small molecule cocktails, the protocol avoids embryoid body formation, scaffolds, and feeder cells. We demonstrate efficient differentiation in multi-well plates as well as shake flasks, and the capability of the iHSPC to differentiate into functional iNK cells.

Materials and methods

Cellular assessments: Cell counts and viability were determined using Countess™ 3 and Vi-Cell Blu automated cell counters. Assessment of surface markers and degranulation was performed using the Invitrogen™ Attune CytPix flow cytometer and analyzed by FlowJo software V10. Graphs generated using Excel, JMP V17 and GraphPad Prism software. Data is represented as Mean ±Standard Deviation.

iPSC to iHSPC Differentiation: Fibroblast-derived iPSCs were cultured in rhLaminin-521 coated plates with CTS™ StemFlex™ medium. For spheroid formation and mesoderm induction, iPSCs were dissociated using CTS™ TrypLE™ Select and seeded in CTS™ StemScale™ medium with Y-27632, BMP4, VEGF, and SCF, and kept on an orbital shaker for 2 days. For iHSPC differentiation, the medium was switched to CTS™ StemPro™-34 supplemented with SCF, BMP4, VEGF, FGFβ, MTG, ascorbic acid, and ITS-G. On Day 5, the medium was changed to either StemPro-34 SFM or CTS StemPro-34 SFM XF with SCF, FLT3, TPO, MTG, ascorbic acid, and ITS-G. Cell counts, viability, and surface marker expression were analyzed on Day 14. All cytokines and growth factors are PeproGMP grade.

iPSC to iNK Differentiation and iNK expansion: Fibroblast-derived iPSCs were differentiated into iHSPCs using CTS StemPro-34 medium until Day 8. The medium was then switched to iNK cell induction medium (CTS NK-Xpander™ medium, MEMα, Human AB Serum, BME, CTS GlutaMAX™, IL-3, SCF, FLT3, IL-7, and IL-15). From Day 15 to approximately Day 40, iNK cells were cultured in iNK maturation medium (CTS NK-Xpander™ medium, MEMα, Human AB Serum, BME, CTS GlutaMAX™, SCF, FLT3, IL-7, and IL-15). Cell counts, viability, and surface marker expression were analyzed weekly. Finally, iNK cells were expanded in CTS NK-Xpander™ medium with Human AB Serum and IL-15 for 2 weeks. All cytokines and growth factors are PeproGMP grade.

Scan for full protocol

K562 cytotoxicity assay: After expansion, iNK cells were co-incubated with CSFE-labeled K562 target cells at various ratios for 2 hours. Degranulation was assessed by CD107α expression on CD56+ iNK cells, and cytotoxicity was evaluated by measuring K562 cell death using the Attune CytPix flow cytometer.

Tumoroid killing assay: Human adenocarcinoma-derived tumoroids labeled with GFP were seeded in OncoPro™ Tumoroid medium. After 3 days, iNK cells were added at different ratios along with CellEvent Caspase-3/7 Red Detection Reagent. Control conditions included tumoroid-only, NK cells only, and medium only. iNK cell cytotoxicity was assessed over 3 days using live-cell imaging using the CellInsight™ CX7 LZR.

Science at a scan

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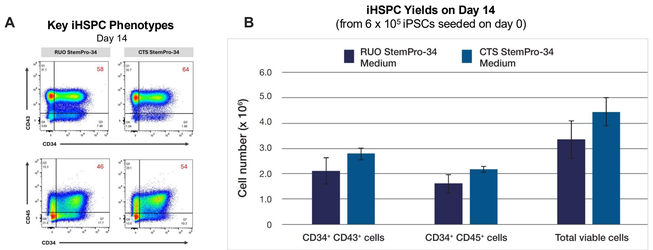
Results

DIFFERENTIATION TO iHSPC

Figure 1. Overview of the iHSPC differentiation method and key reagents.

D(0)	D(2)	D(6)	D(14)
CTS StemFlex	CTS StemScale	CTS StemPro–34 + Ascorbic acid, MTG, ITS	
rhLaminin-521 coated TC-treated	Non TC-treated	Non TC-treated	
Static adherent culture	Suspension culture on orbital shaker	Static suspension culture	
	Y-27632	BMP4	FLT3
	BMP4	bFGF	SCF
	VEGF	VEGF	TPO
	SCF	SCF	

Figure 2. iHSPC differentiation in CTS StemPro-34 produces superior yields and HSPC phenotypes compared to differentiation in StemPro-34.

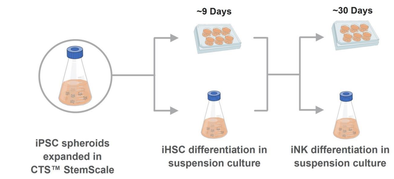


Differentiation was initiated from 6x10^5 iPSCs and carried out in 6-well plates. On Day 2, the medium was changed to CTS StemPro-34 SFM XF or StemPro-34, and iHSPC differentiation continued in static suspension culture. Representative phenotypic characterization (A), CD34+ CD43+ and CD34+ CD45+ iHSPC yields (B) were calculated by multiplying the number of total viable cells by the percentage of each phenotype. N=3.

The new CTS StemPro-34 SFM XF generated >2x10^6 iHSPCs from just 6x10^5 iPSCs in this small-scale testing. CTS StemPro-34 SFM XF is available in both 500 mL bottle and 2 L bag formats, with extensive product testing and regulatory support documentation, making it suitable for cell therapy development.

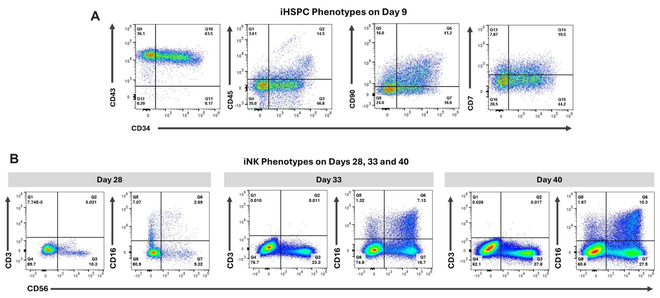
DIFFERENTIATION TO iNK CELLS

Figure 3. Overview of the iNK differentiation methods and key reagents.



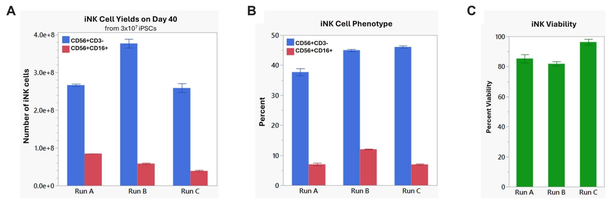
D(-5)	D(0)	D(2)	D(6)	D(8)	D(16)	D(58)
CTS StemScale	CTS StemScale	CTS StemPro–34 + Ascorbic acid, MTG, ITS		CTS NK-Xpander +MEMα, + 15%Human AB Serum		
Suspension (orbital shaker/bioreactor)				Suspension or static culture		
Y-27632	Y-27632	BMP4	FLT3	IL-15	IL-15	
DNAse I	DNAse I	bFGF	SCF	IL-7	IL-7	
	BMP4	VEGF	TPO	IL-3	FLT3	
	VEGF	SCF		FLT3	SCF	
	SCF			SCF		

Figure 4. Phenotypic characterization during iNK differentiation.



Surface marker expression on Differentiation Day 9 (A) shows a mixed progenitor population, positive for iHSPC markers CD34, CD43, CD45 and CD90, and the NK and T cell progenitor marker CD7. Between Days 28 and 40 (B) the percentage of CD56+CD3- and CD56+CD16+ cells increases as iNK differentiation continues.

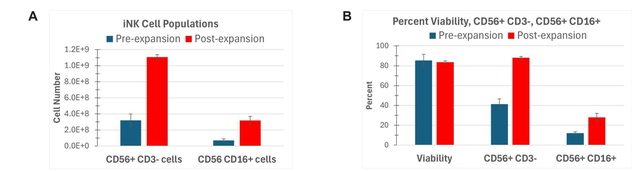
Figure 5. iNK cell yields, phenotype and viability on Differentiation Day 40.



Differentiation was initiated with 3x10^7 iPSCs in 100 mL culture volumes in 500 mL shake flasks. On Differentiation Day 40, CD56+ CD3- iNK cell yields exceeded 2.6x10^8 with 40-45% CD56+ CD3- purity and >80% viability. Data from 3 independent runs.

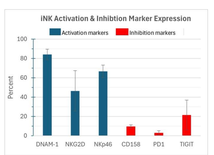
iNK CELL EXPANSION & FUNCTIONALITY

Figure 6. iNK cell enrichment following expansion in CTS NK-Xpander.



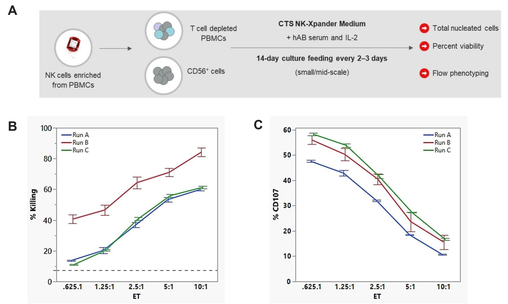
After differentiation, iNK cells were further expanded for 12 days in CTS NK-Xpander medium supplemented with 5% Human AB Serum and IL-2, in 40 mL GRex™ plates. CD56+CD3- and CD56+CD16+ iNK cell populations (A) and phenotype (B) before and after expansion in CTS NK-Xpander. N=2. Expanded iNK cells showed a significant increase in purity and were >80% CD56+ CD3- and >80% viable.

Figure 7. iNK cell activation and inhibition marker expression.



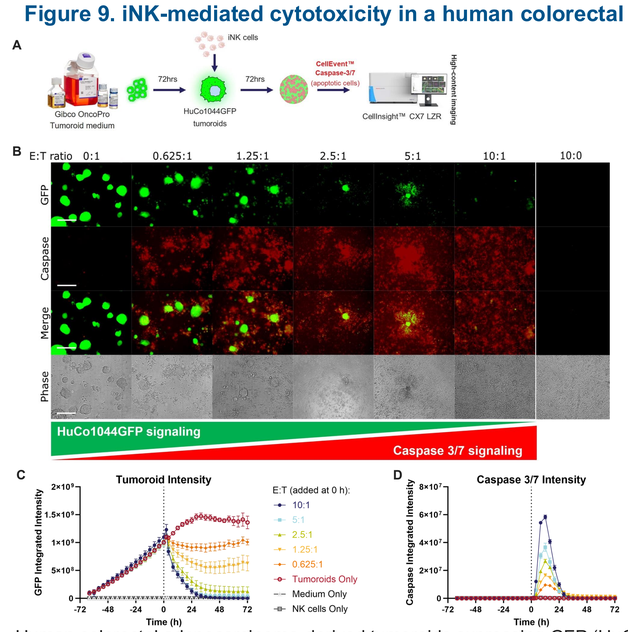
Expanded iNK cells express key activation markers (DNAM-1, NKG2D, NKp46) and show low expression of inhibition markers (CD158, PD-1 and TIGIT).

Figure 8. Expanded iNK cell cytotoxicity and degranulation.



Expanded iNK cells demonstrated cytolytic function as measured by K562 killing and degranulation (A). iNK cells were able to kill over 50% of K562 cells within 2 hours of co-incubation (B, % target cell death) and degranulate (C, % degranulation as measured by CD107a) and in a dose dependent manner. N=3.

Figure 9. iNK-mediated cytotoxicity in a human colorectal tumoroid model.



Human colorectal adenocarcinoma-derived tumoroids expressing GFP (HuCo1044GFP) were seeded in Gibco OncoPro Tumoroid medium. After 3 days, iNK cells were added to the tumoroids at different target ratios (E:T), along with CellEvent Caspase-3/7 Red Detection Reagent. iNK cell-mediated cytotoxicity was evaluated by live-cell imaging of Caspase 3/7 and GFP signals over 3 days, showing an increase in Caspase 3/7 signaling and concurrent decrease in tumoroid GFP intensity, in a dose dependent manner.

Conclusions

- This protocol is highly flexible and compatible with various culture formats from end-to-end.
- This method allows generation of clinically relevant cell types from iPSCs, including iHSPCs and iNK cells.
- The iNK cells can be expanded to increase yields and purity.
- The iNK cells were able to kill >50% of K562 cancer cells within 2 hours of co-incubation, and >50% of tumoroid cells within 12 hours of co-incubation.

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