IMPROVING CD34 + YIELDS LEADS TO A ROBUST AND REPRODUCIBLE FEEDER-FREE HUMAN iNK DIFFERENTIATION PROCESS

Poster # 130

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INTRODUCTION

Natural Killer (NK) cells are promising for cancer immunotherapy as they can target both hematological and solid tumors. However, current sources of NK cells such as umbilical cord (UCB) or Peripheral blood (PB) cannot easily expand to clinically relevant numbers and are heterogeneous in cell composition. Human iPSCs-derived-NKs (iNKs) may overcome these obstacles by provide a uniform and potentially unlimited source of NKs for allogeneic cell therapy. Current iNK differentiation protocols typically utilize xenogenic feeder cells, which could be problematic for patient safety, or they lack robustness and are highly variable in differentiation efficiency. Using animal free small molecules and recombinant proteins, we developed a feeder free differentiation process to reliably generate iNKs at scale by optimizing for CD34⁺ hematopoietic stem cells (iHSC) prior to downstream NK differentiation. These optimizations led to vastly improved iNK yields. Within 30 days, cells reached a purity of more than 95% CD56⁺, CD3⁻ with a single iPSC yielding up to 500 iNKs. Activation and expansion of iNKs via either irradiated K562 feeders or an antibody cocktail showed potent (>90%) killing of target cancer cells (K562) via an Incucyte killing assay. These results show that optimizing for CD34⁺ HSCs is a critical step for robustly differentiating iNK from iPSCs in a feeder-free manner. This protocol should greatly increase reproducibility and uniformity of clinically relevant iNK production for allogeneic cancer therapy.

METHODS

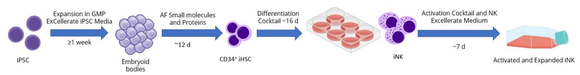

Figure 1. Overview of BT protocol: small molecule and animal free protein-based feeder-free iPSC to NK (Natural Killer) differentiation process

iPSCs were cultured on Cultrex™ UltiMatrix (Cat#: BME001-05) or Recombinant Animal-free Vitronectin with GMP iPSC ExCellerate™ Expansion media (Cat#: CCM036). Human iPSCs were differentiated into iNKs using the following animal-free proteins and small molecules:

Name	CHIR99021	SB 431542	BMP4	FGFb	VEGF	SCF	FLT3	TPO	IL3	IL7	IL15
Cat. #	4423	1614	AFL314E	3718-GMP	10318-VE	AFL255	AFL308E	288-TPE	AFL203	AFL207	247-GMP

iNKs and PBMC NKs were activated and expanded using ExCellerate Human NK Expansion Media, Animal Component Free (CCM037) and activated with irradiated K562 (mbIL21) feeders or an in house-developed antibody cocktail and the following cytokines:

Name	IL2	IL12	IL18	IL21
Cat. #	BT-002-AFL	219-IL	9124-IL	8879-IL

For flow cytometry, embryoid bodies (EBs) were dissociated via collagenase II (Worthington Biochemical), Triple E (Thermo Fisher) and filtered through a 20 µm filter to prepare single cells for flow cytometry. Dissociated EBs and NKs were assessed via the antibodies below or the FlowX Human NK Cell Phenotyping Flow Cytometry Kit (FMC033).

Name	CD56-A647	CD3-A405	CD34-PE	CD45-A647
Cat. #	FAB24086R	FAB100V	FAB7227P	FAB1430R

Cells were processed with BD LSRFortessa and data analyzed in FlowJo. Gates were set based on isotype controls. KG1α cells and PBMC NKs were used as positive controls.
For the killing assay K562s transfected with Red Lentivirus Reagent (Sartorius) were cultured in RPMI, 5% PBS and 1% PS. 5,000 cells/were plated per well in a 96 well plate with NKs added at the indicated effector: target ratio. Fluorescence was measured every hour by Incucyte and killing was measured as the loss of fluorescence normalized to timepoint zero and a no effector control. Data was analyzed via Graphpad.

RESULTS

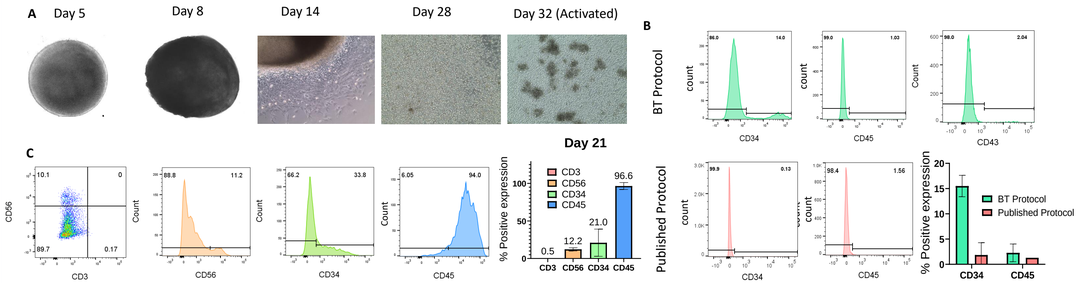

Figure 2. Feeder-free differentiation of iPSCs into HSCs (hematopoietic stem cells) and CLPs (common lymphoid progenitors) A) Bright field images of iPSC to iNK differentiation over time using the BT protocol: An embryoid body (EB) on day 5 and day 8. On day 14 an EB shows formation of a stromal like monolayer after plating on a TC-treated plate. iNKs on day 28 after differentiation and iNKs after activation via irradiated K562 feeders. B) On day 11, EBs show increased CD34⁺ population measured via flow cytometry compared to a published protocol, suggesting the development of hematopoietic stem cells (HSCs). CD45⁺ and CD43⁺ population are minimal; the latter suggesting the formation of definitive hematopoietic progenitors. C) On Day 21, common lymphoid progenitors (CLPs) have appeared as most cells are CD45⁺ leukocytes. A small subset of cells are CD56⁺, CD3⁻ suggesting the emergence of iNKs. All bar graphs show averages (N>=2) with error bars representing standard deviation.

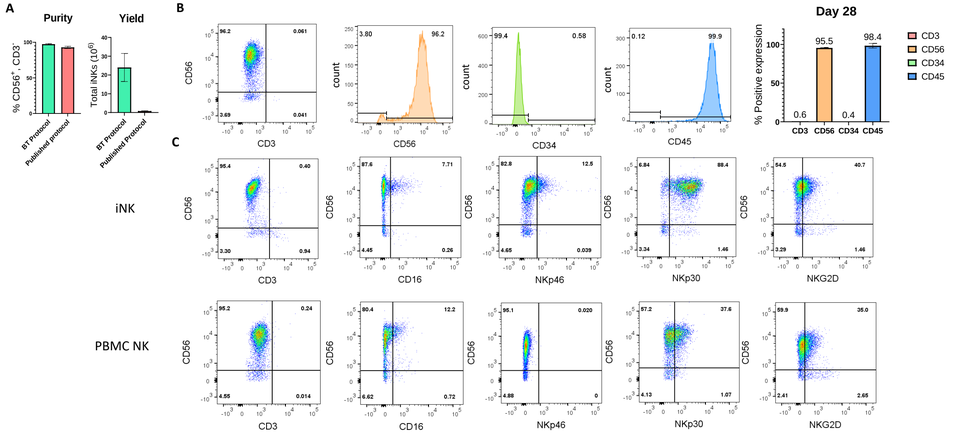

Figure 3. High purity feeder-free differentiation of iPSCs into iNKs A) iNKs derived via the BT protocol show similar purity (>95% CD56⁺, CD3⁻) and vastly improved yield (approximately 24-fold increase) compared to a published protocol. B) After 28 days, suspension cells are >95% CD56⁺, CD3⁻ via flow cytometry suggesting successful differentiation into iNKs. Cells remain CD45⁺ but have lost most CD34 expression as expected. Bar graph shows mean (N=4) with error bars representing standard deviation. C) These iNKs variably show other NK markers including CD16⁺, NKp46⁺, NKp30⁺ and NKG2D⁺ comparable to purified PBMC NKs.

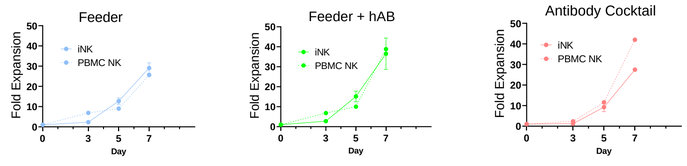

Figure 4. Using feeder and feeder-free systems, iNKs were activated and expanded comparably to PBMC NKs iNKs (N=3) expanded over 7 days in ExCellerate NK Expansion Media, Animal Free with irradiated K562 (mbIL21) feeders and human AB serum (left), irradiated K562 (mbIL21) feeders (middle) or an antibody cocktail (right). iNKs showed comparable expansion to PBMC NKs with expansions up to 36.5 (±7.9) fold after 7 days.

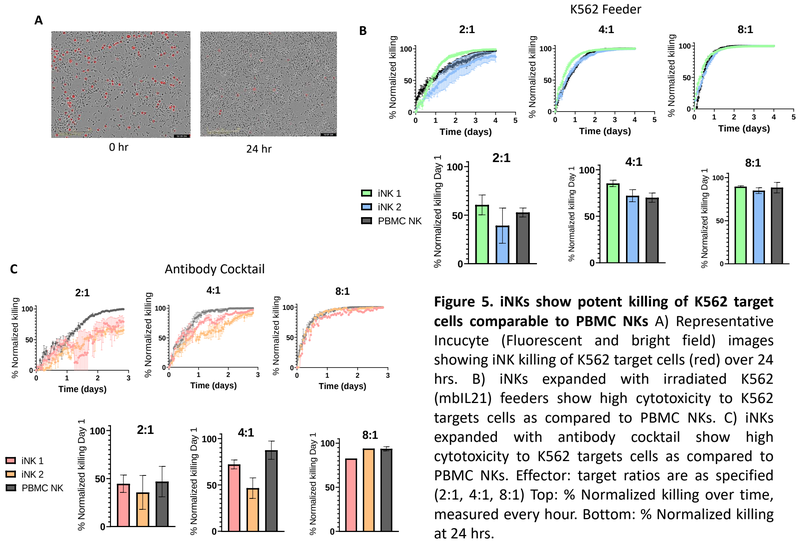

Figure 5. iNKs show potent killing of K562 target cells comparable to PBMC NKs A) Representative Incucyte (Fluorescent and bright field) images showing iNK killing of K562 target cells (red) over 24 hrs. B) iNKs expanded with irradiated K562 (mbIL21) feeders show high cytotoxicity to K562 targets cells as compared to PBMC NKs. C) iNKs expanded with antibody cocktail show high cytotoxicity to K562 targets cells as compared to PBMC NKs. Effector: target ratios are as specified (2:1, 4:1, 8:1) Top: % Normalized killing over time, measured every hour. Bottom: % Normalized killing at 24 hrs.

DISCUSSION

Optimizing CD34⁺ yield during differentiation of iPSCs to HSCs can significantly improve the yield of iNKs in a feeder-free differentiation process. Furthermore, the entire iNK workflow (i.e. from differentiation to activation and expansion) can be made entirely feeder-free by the substitution of antibody cocktail instead of irradiated K562 feeders during iNK expansion. Future work could include further improving the culture and expansion of CD34+ cells using small molecules or sorting for CD34⁺ cells. Additionally, non-viral gene editing techniques such as TC-Buster to engineer CAR-CD19 iPSCs can improve cytotoxicity towards B-cell lymphomas. These methods combined demonstrate a complete workflow to obtain highly cytotoxic NK cells derived from iPSCs appropriate for the clinic.

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