Advancing Cancer Immunotherapy: Large-Scale Non-Viral Engineering and Feeder-Free Production of CAR-NK Cells

Deepak Kumar[&], Anna Liu[&], Erica L Heipertz[$], Namritha Ravinder[&]
[&]ThermoFisher Scientific, 5781 Van Allen Way, Carlsbad, CA 92008
[$]ThermoFisher Scientific, 6433 English Muffin Way, Frederick, MD 21703

Abstract

Purpose: Development of clinically relevant, efficient and safe, non-viral gene delivery methods for primary NK cells remain limited. To date, there is very limited published data demonstrating high-efficiency (>20%) non-viral gene knock-in for primary NK cells. In this study, we optimized a scalable, non-viral, closed-system gene delivery method for production of CAR-NK cells.

Methods: NK cells were enriched from PBMCs that were isolated from frozen leukopak using the Gibco™ CTS™ Rotea Counterflow Centrifugation system. The NK cells were cultured for 6 days in CTS™ NK-Xpander™ medium that was supplemented with IL-2. On day 6, NK cells were electroporated with Neon™ NxT or CTS™ Xenon™ electroporation system at a density of 50e06 cells / mL to deliver ss-DNA, CTS™ HiFi Cas9 protein and gRNA targeting Rab11a / AAVS1 loci. Alongside non-viral method we also tested viral based gene knock-in method. For this approach cells were electroporated with Cas9 RNP (HiFi Cas9 + AAVS1 sgRNA) followed by transduction using AAV6 virus for delivery of V5 tagged anti-meso3 CAR construct tailored for integration into the AAVS1 loci. The engineered CAR-NK cells were expanded and analyzed by flow cytometry for CAR expression. The cells were also further tested for cytotoxicity by co-culturing with GFP expressing SKOV3 cells for 6 hrs.

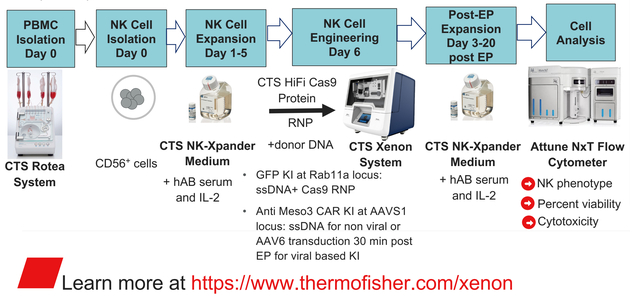
Results: The ss-DNA showed an average of 25% knock-in efficiency while anti meso-3 CAR knock-in efficiency was up to 35% over the time of culture. The edited NK cells maintained their phenotype and viability. Moreover, engineered NK cells expressing anti-meso-3 CAR demonstrated higher cytotoxicity against cancer cells as compared to non-engineered NK cells.

Introduction

Genetic engineering is a major driving force in advancing adoptive immunotherapy, a promising approach to treat cancers. Engineering of primary human natural killer (NK) cells in immuno-oncology holds great promise due to the potent antitumor properties of NK cells and their demonstrated safety in an allogeneic setting. In addition, unlike T cells, NK cells do not rely on a matching human leukocyte antigen to function, making allogeneic transfer safe from graft-versus-host disease. With these benefits engineered NK cells have the potential to be safer, less expensive, and more effective than current engineered T-cell therapies. One of the key challenges encountered by the cell and gene therapy industry is the ability to expand, engineer and process NK cells in large numbers using regulatory friendly ancillary reagents and closed, automated instruments. We have addressed these pain points by developing tools and protocols that facilitate manufacturing of engineered NK cells in a scalable, closed and automated manner. We developed CTS™ NK-Xpander™ Medium, a feeder-free NK cell culture media, that expands functional primary human NK cells to clinically relevant scales in two to three weeks. Additionally, the Gibco™ CTS™ Rotea™ system serves as a flexible closed automated cell processing and buffer exchange platform, while the Gibco™ CTS™ Xenon™ Electroporation System help ensures reliable delivery of a wide range of payloads, including DNA, RNA, and proteins, into NK cells for clinal applications. Together with the media system, editing tools and the instruments platforms described in this work we have developed a closed, modular, and semi-automated protocol that helps resolve cell therapy manufacturing challenges and ultimately get safer therapies to patients faster.

Materials and Methods

Human PBMCs were isolated from Leukopak by Rotea. Human NK cells were isolated from PBMC using negative isolation kit. Isolated NK cells were cultured with NK-Xpander media supplemented with human serum having IL-2. On day 6, cells were electroporated with Neon NxT/Xenon by targeting GFP or CAR construct to the Rab11a/AAVS1 locus using CRISPR/Cas9 system. EP conditions were 1st optimized in Neon NxT before scaling to Xenon. For CAR NK generation using viral based method, AAV6 carrying meso3 construct was added to Cas9 RNP transfected NK cells after 30 min of electroporation. Post-EP cells were cultured for 20 days and analyzed by Attune™ flow cytometry over time.



Results

Figure 1. Isolation of PBMC from Leukopak using CTS™ Rotea™ counterflow centrifugation system

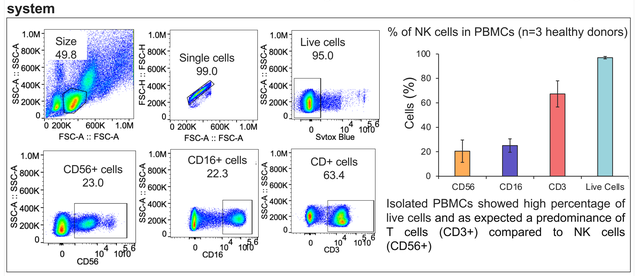


Figure 2. Enrichment of NK cells and immunophenotyping

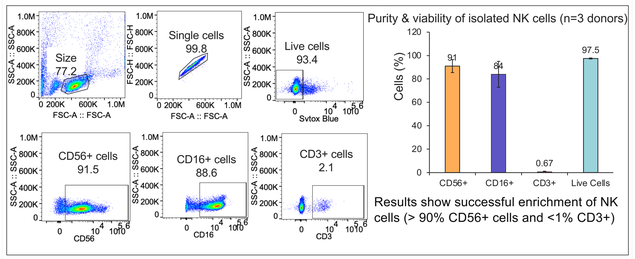


Figure 3. Non-viral transgene knock-in (KI) into NK cells (Rab11a GFP or V5 tagged meso-3 KI)

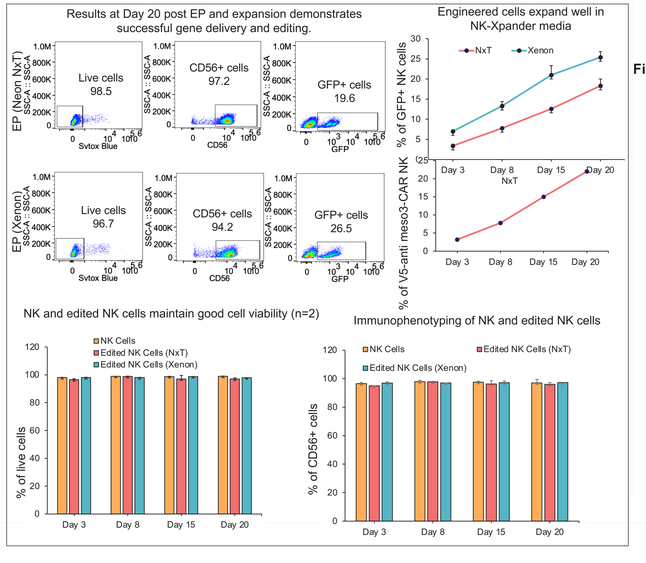


Figure 4. Generation of V5 tagged anti-meso3 expressing CAR NK cells using Xenon Electroporation for Cas9 RNP targeting AAVS1 loci followed by AAV6 transduction for anti meso3-CAR KI

a) Anti-meso3 CAR-NK (% V5) expression post Knock-in and expansion was calculated by Flow cytometry analysis at day 20 (100 uL: Neon, and 1 mL: Xenon Electroporation, 50M/ml of NK cell); **b)** Expression of anti-meso3 CAR NK over time; **c)** Cell good expansion up to 20 days post-EP; **d)** Edited NK cell immunophenotype over time; **e)** Viability of cells up to 20 days post-EP.

Results show 25-30% KI post EP and cells expand well in NK-Xpander media after EP while maintaining relevant NK cell markers and cell viability

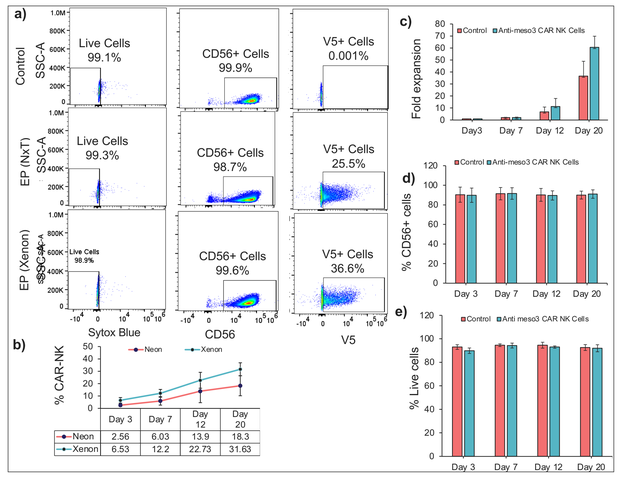
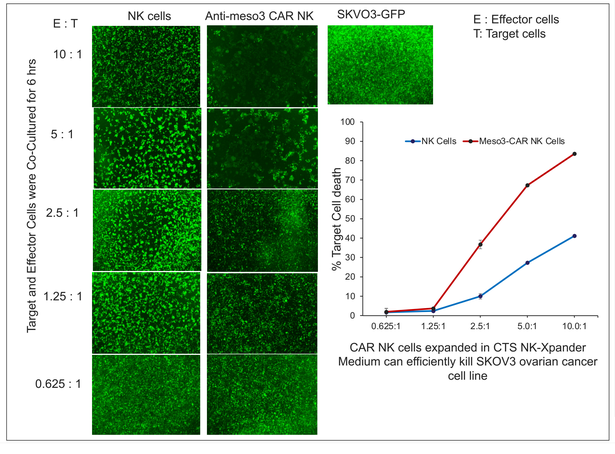


Figure 5. Killing efficiency of Anti-meso3 CAR NK cells against SKOV3 cells (Ovarian cancer)



Conclusions

- PBMCs were successfully isolated using the CTS Rotea system
- We successfully developed a feeder-free NK Cell Expansion medium, CTS™ NK-Xpander™ Medium, that expands human primary enriched NK cells to clinically relevant levels
- The CTS™ Xenon and/or NxT electroporation system showed around 20-25% KI efficiency with fully non viral protocol using either ss-DNA Rab11a GFP or ss-DNA meso3
- The CTS™ Xenon electroporation system together with CTS™ HiFi Cas9 and relevant sgRNA is able to edit clinically relevant levels of NK cells both for non viral and for EP + AAV6 based viral methods
- Engineered NK cells generated using methods described here expanded well in CTS NK-Xpander Medium and maintain their phenotype, viability and functionality
- CAR NK cells were able to kill around 80% of target cells (SKOV3) within 6 hours of co-incubation
- Data demonstrated successful use of modular automated platforms like Rotea and Xenon to manufacture clinically relevant CAR-NK cells

Acknowledgements

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