Cryopreservation of Natural Killer and anti-CD19 Chimeric Antigen Receptor Natural Killer Cells

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ABSTRACT

The therapeutic use of engineered natural killer (NK) cells in immuno-oncology holds great promise due to the potent anti-tumor properties of NK cells and their demonstrated safety in an allogeneic setting (Liu 2020 NEJM). Ideally, cell therapy products are cryopreserved to allow time for release testing and provide off-the-shelf accessibility to patients. However, NK cells recover poorly after cryopreservation with low viability and potency and require a "resting" culture period with cytokines (typically overnight) to regain function (Berg 2009 Cytotherapy). As a result, tightly coordinated laboratory resources are required to process fresh or rested cells in the clinic prior to dosing, significantly limiting the accessibility of NK cell drug products. Here, we screened GMP-grade cryopreservation formulations and developed processes that preserve the post-thaw viability of both non-engineered NK cells and engineered NK cells expressing an anti-CD19 chimeric antigen receptor (CAR). Importantly, we demonstrate potent cytotoxic activity of these cells post-thaw without requiring a rest period.

METHODS

Non-engineered human peripheral blood NK cells were expanded using irradiated feeder cells. Anti-CD19-CAR NK cells were engineered and expanded using irradiated feeder cells. The engineering efficiency was assessed by flow cytometry using biotinylated CD19 ligand and streptavidin. To identify suitable formulations for NK cells, non-engineered cells were cryopreserved using four GMP-grade formulations by controlled-rate freezing. Cell viability was quantified using acridine orange (AO) and propidium iodide (PI) staining. The cytotoxic activity of the NK or CAR-NK cells was assessed by incubating the cells with luciferase-expressing target tumor cell lines K562 or CD19⁺ Raji cells at an effector-to-target cell ratio (E:T) of 1:1 for either 4 hours or 24 hours and subsequently measuring the viability of the target tumor cells using the Steady-Glo® luciferase assay system (Promega® Corp.). Target cell killing was expressed as the percentage of target tumor cells killed by the non-engineered or engineered NK cells, normalized against the target cell-only controls.

Graphs and statistical analyses were generated using the GraphPad Prism® 9 software (GraphPad Software, Inc.)

References

Berg, Maria et al. *Cytotherapy* 2009;11(3):341-55.
Liu, Enli et al. *N Engl J Med.* 2020;382(6):545-553.

1 Process Flow Summary

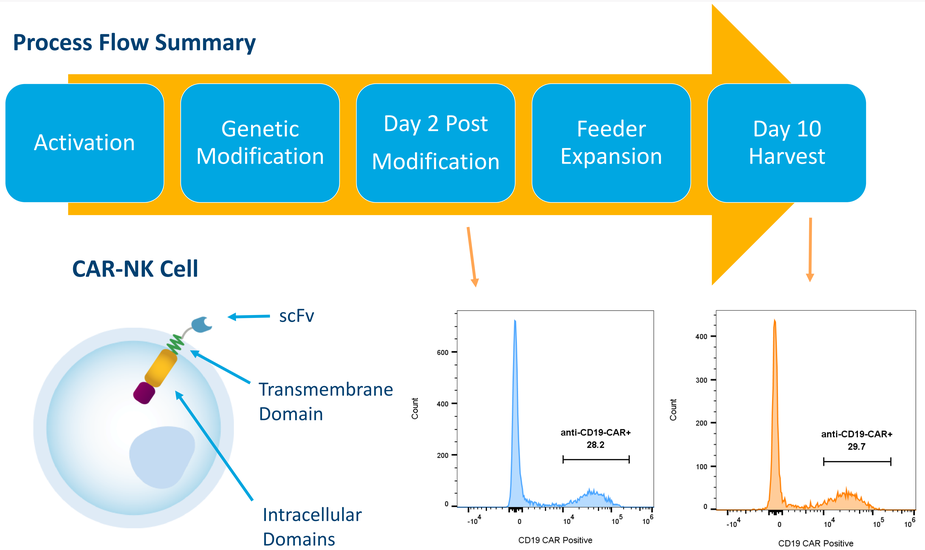

Figure 1. Human peripheral blood NK cells were activated, engineered to express anti-CD19-CAR, and expanded further to produce anti-CD19-CAR NK cell products. Engineering efficiency at day 2 post-modification was 28.2% analyzed by flow cytometry. The CAR-positive percentage was maintained (29.7%) after expansion (101-fold) prior to cell harvest and cryopreservation.

2 Identification of Two Suitable GMP Cryopreservation Formulations for NK Cells

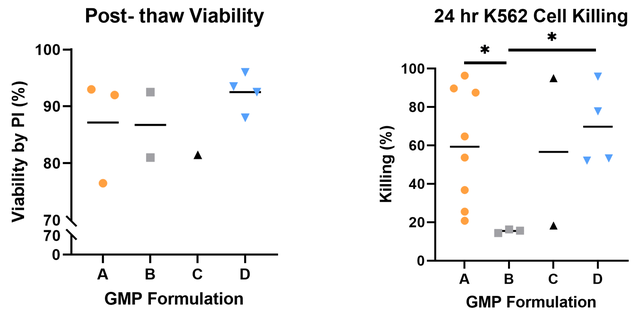

Figure 2. Cell viability after cryopreservation in 4 GMP formulations assessed by PI staining immediately post-thaw. NK cells cryopreserved in Formulation A (87 ± 8%, n = 3) and formulation D (93 ± 3%, n = 5) show the highest viability post-thaw. Cytotoxic activity of cells cryopreserved in GMP formulations was measured by a cell killing assay against K562 cells. The cytotoxicity of NK cells cryopreserved in Formulation A averaged 60 ± 28% (n = 8) killing of K562 cells while those cryopreserved in Formulation D averaged 70 ± 18% (n = 4), both significantly higher than NK cells cryopreserved in Formulation B (* = $p < 0.05$, A vs. B and D vs. B; t-test).

3 Cryopreservation Does Not Affect Cell Viability

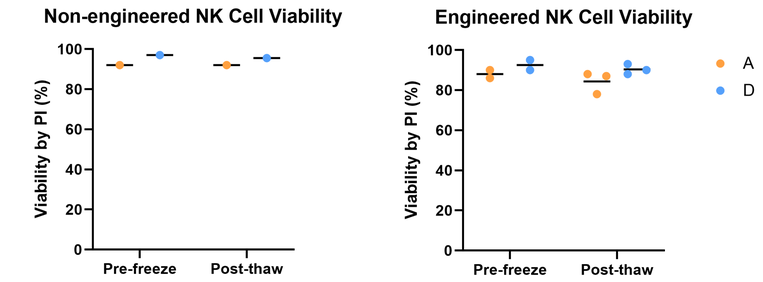

Figure 3. Cell viability of NK and CAR-NK cells before and after cryopreservation with formulations A and D. The viability of non-engineered NK cells post-thaw was not different from that prior to cryopreservation (p = 0.76). Similarly, anti-CD19-CAR NK cells are highly viable post-thaw (87 ± 5% viability, n = 6; compared to 90 ± 3%, n = 4, before cryopreservation; p = 0.35).

4 Anti-CD19-CAR Dependent Cytotoxicity Retained Post-thaw

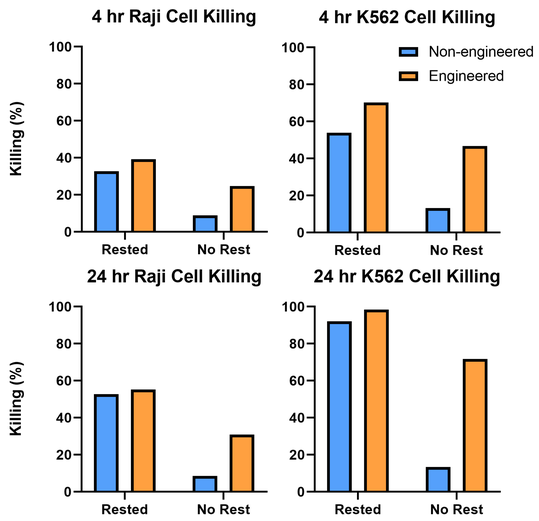

Figure 4. CAR-independent and CAR-dependent killing function was assessed using a cytotoxicity assay against K562 and CD19⁺ Raji cells, respectively. Non-engineered and anti-CD19-CAR NK cells (30% CAR-positive) were cryopreserved using formulation A. The cytotoxicity assays were performed using cells that were assayed immediately after thaw as well as cells that were allowed to rest overnight in culture medium with IL-2 (100 IU/mL) before the assay. CAR-NK cells that were not rested after thaw retain 50 to 65% of CAR-dependent cytotoxicity compared to rested cells.

CONCLUSIONS

We have developed a cryopreservation process and identified GMP formulations to cryopreserve non-engineered and engineered NK cells with high recovery that demonstrate high cytotoxic activity post-thaw. The ability to maintain potent tumor-killing function of CAR-NK cells after cryopreservation without requiring resting in culture is an important step toward the development of off-the-shelf CAR-NK cell therapies.