Deep functional and phenotypic characterization* of expanded and cryopreserved NK cells establishes consistent product identity across GMP and clinical-scale manufacturing sites and FC21 and PM21 platforms as intended for clinical use.

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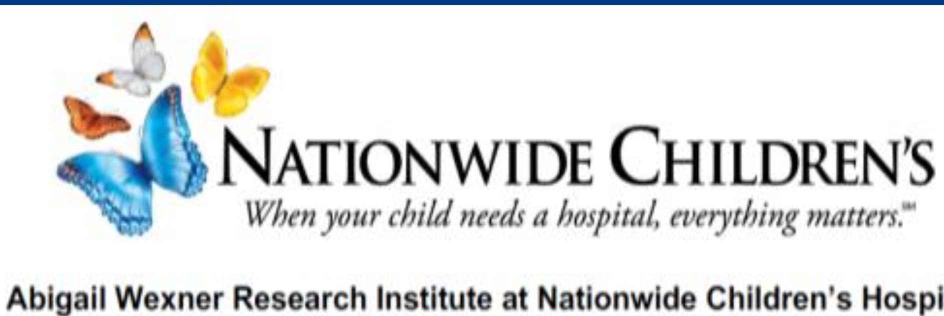

INTRODUCTION

We previously developed a feeder cell system (FC21) for *ex vivo* expansion of natural killer (NK) cells based on the K562 cell line genetically modified to express 4-1BBL and membrane-bound IL21 (mbIL21) . This system enabled us to robustly generate large numbers of highly active NK cells (FC21-NK) from various sources. We applied this approach in clinical trials assessing autologous and allogeneic FC21-NK cell products delivered with or without chemotherapy or allogeneic stem cell transplant. To date, over 300 doses of FC21-NK cells up to 10^8/kg/dose have been delivered to over 50 patients, without any major toxicities (see abstract S284 and EP585). The cell line was re-derived to enable broader academic access and the expansion process was refined through the development of a non-cellular membrane-particle preparation (PM21) to enhance safety, efficiency, scale and product uniformity for commercialization, while providing the same stimulus for expansion and activation as FC21. On a preclinical scale, these changes resulted in a highly similar final product, but deep characterization of the impact of these process variations in representative material intended clinical use had not yet been done.

OBJECTIVE

To perform deep characterization of *ex vivo* expanded, cryopreserved FC21-NK cells and PM21-NK cells, in order to assess uniformity of product identity and potency across production sites (GMP or large-scale process development) and platforms (FC21 (Clone9), FC21 (CSTX), and PM21) intended for use in clinical trials (PM21-NK, e.g. NCT04395092) for AML.

METHOD(S)

NK cell expansion: Up to 10 samples of each NK-cell type were obtained for comparison: (1) freshly isolated NK cells from peripheral blood, (2) cryopreserved FC21-NK cells expanded with the original Clone9.mbIL21, (3) cryopreserved FC21-NK cells expanded with the newly-derived CSTX002, and (4) cryopreserved PM21-NK cells expanded with PM21 particles from CSTX002. All cells were expanded at large scale in the process-development laboratory or in manufacturing facilities under good manufacturing practice (cGMP) guidelines and are representative for clinical materials. Cells were cryopreserved in media containing DMSO and FBS or human serum albumin. Samples were thawed and rested in media containing low-dose IL2 (100 IU/mL) for 48h prior to testing. Recovery was determined by total viable cells by trypan-blue staining.

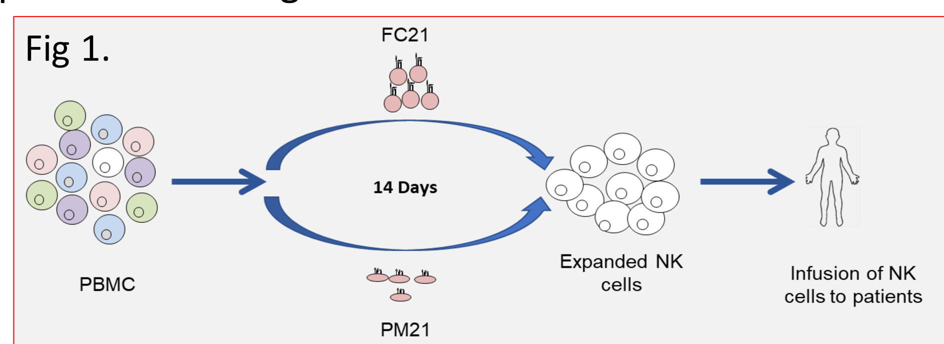

Figure 1. Peripheral blood mononuclear cells (PBMC) isolated from healthy donor leukopak expanded using feeder cells (FC21) or membrane particles (PM21) overexpressing the membrane bound interleukin 21 (IL21) and co-stimulatory molecule 4-IBBL for a period of two weeks.

Cytotoxicity Assay: NK cells were co-cultured for 4-hours with calcein-labeled K562 at 5:1 E:T ratio. Calcein release was determined and mean percent lysis calculated (1).
Mass Cytometry (CYTOF): NK cells were fixed in Prot-1 buffer (Smart tube Inc) for 10 mins at room temperature. Cells were labeled with a panel of 32 metal-conjugated antibodies against common NK cell surface markers, followed by Iridium for doublet exclusion, and acquired on a third-generation Helios mass cytometer instrument. The data was analyzed using clustering algorithms viSNE and SPADE (2,3).
Cytokine Assay: NK cells were stimulated with PHA. After 4-hours the levels of cytokines were measured in the supernatants using 37-plex bead-based array (MACS Plex cytokine kit - Miltenyi).

RESULTS

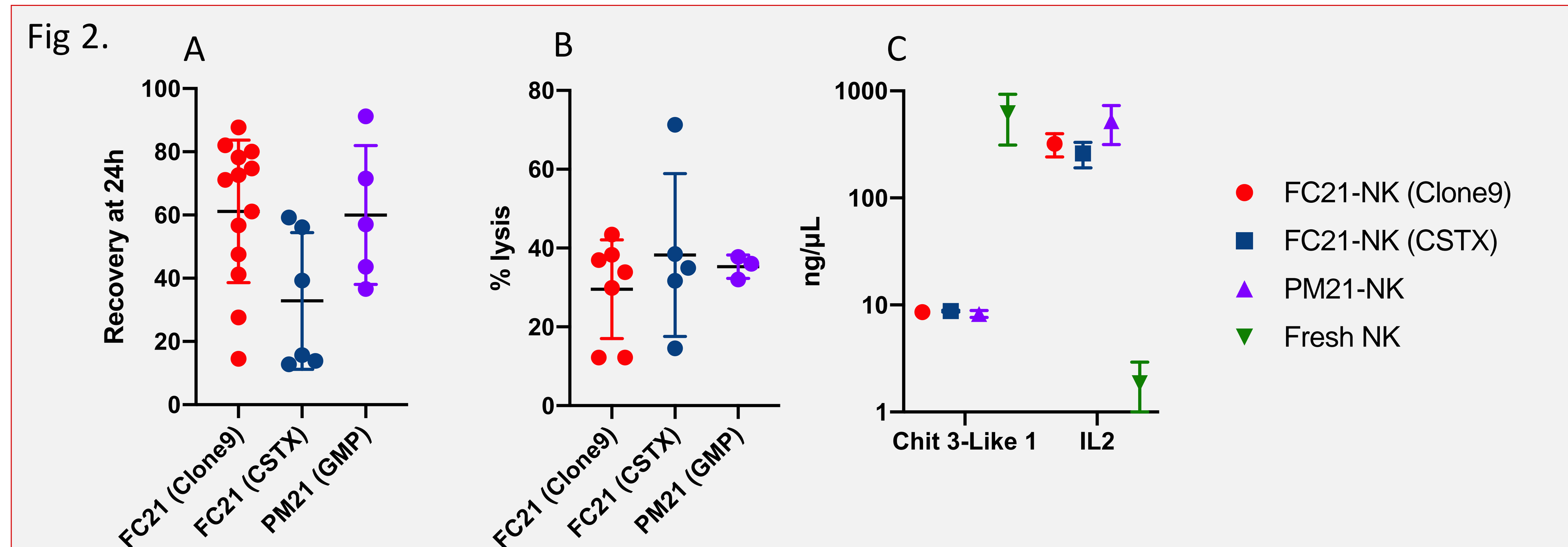

Figure 2. Recovery and function of expanded NK cells after cryopreservation. NK cells were generated with feeder cells (FC21, clone9 or CSTX002) or membrane particles (PM21 from CSTX002) for two weeks, then cryopreserved. Cells were thawed and rested 24h. A) Total viable recovery (indicated as % of product cryopreserved). B) Cytotoxicity by 4 hr calcein-release with K562 targets at 5:1 (E:T ratio. C) Cytokine production following PHA stimulation (10ng/ml) for 4hrs, assessed by bead array. Shown are cytokines previously demonstrated to be significantly different from Fresh NK cells. Mean ± SD or geometric mean ± SD.

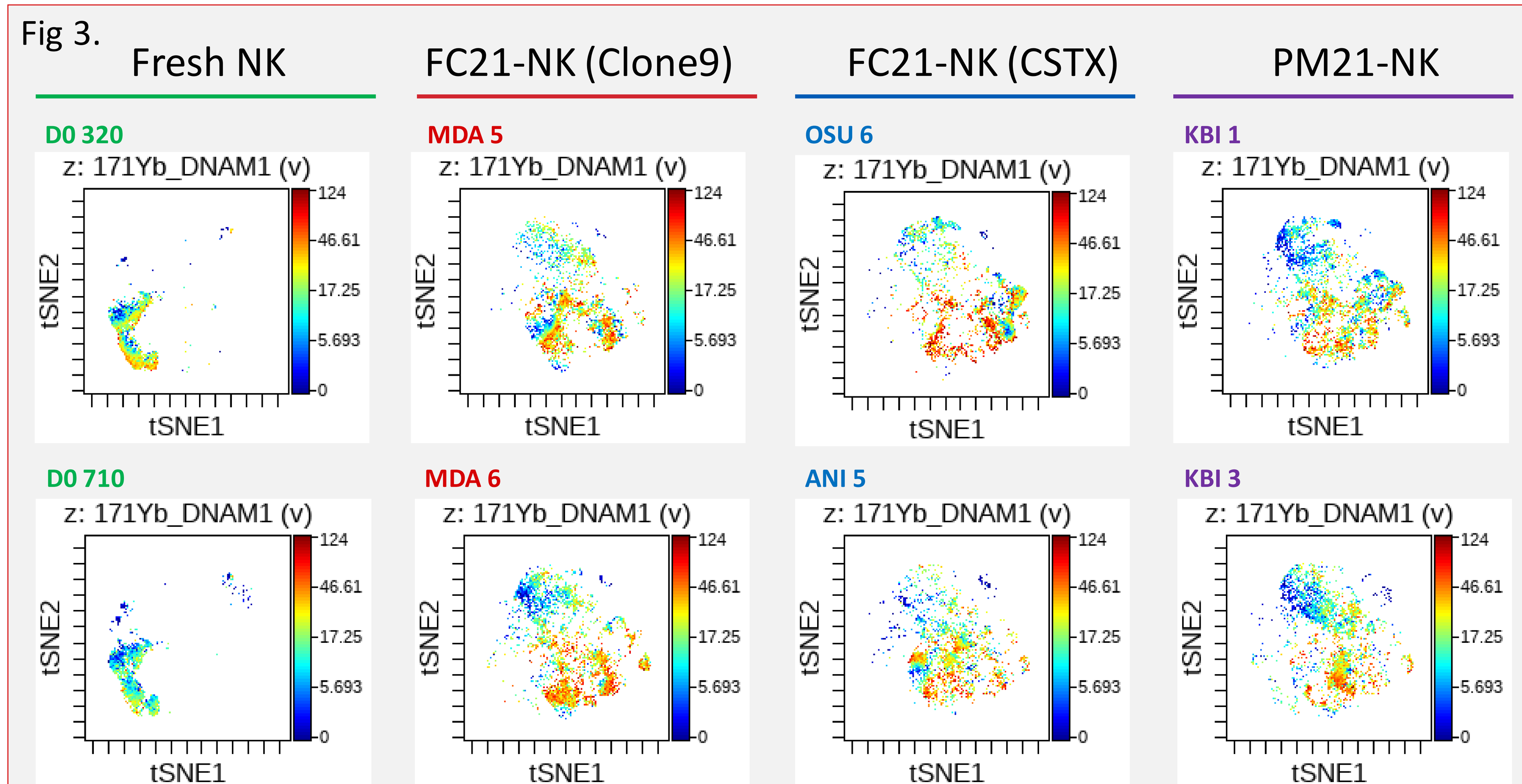

Figure 3: Broad assessment of NK cell phenotypes by 32-parameter time-of-flight mass cytometry (CyTOF). Expanded, cryopreserved NK cells were thawed and stained with metal-conjugated antibodies and acquired with a Helios cytometer. Peripheral blood NK cells were freshly isolated and stained in parallel for comparison. ViSNE analysis was applied to 16 surface markers with equal sampling on the Singlet, $CD3^{neg}$, $CD56^{pos}$, $cPARP^{neg}$ gate. DNAM-1 expression shown for reference. Two representative examples of each group are shown, demonstrating similar expression levels and clustering for expanded NK cells across platforms, and almost no overlap in phenotypes with freshly-isolated NK cells.

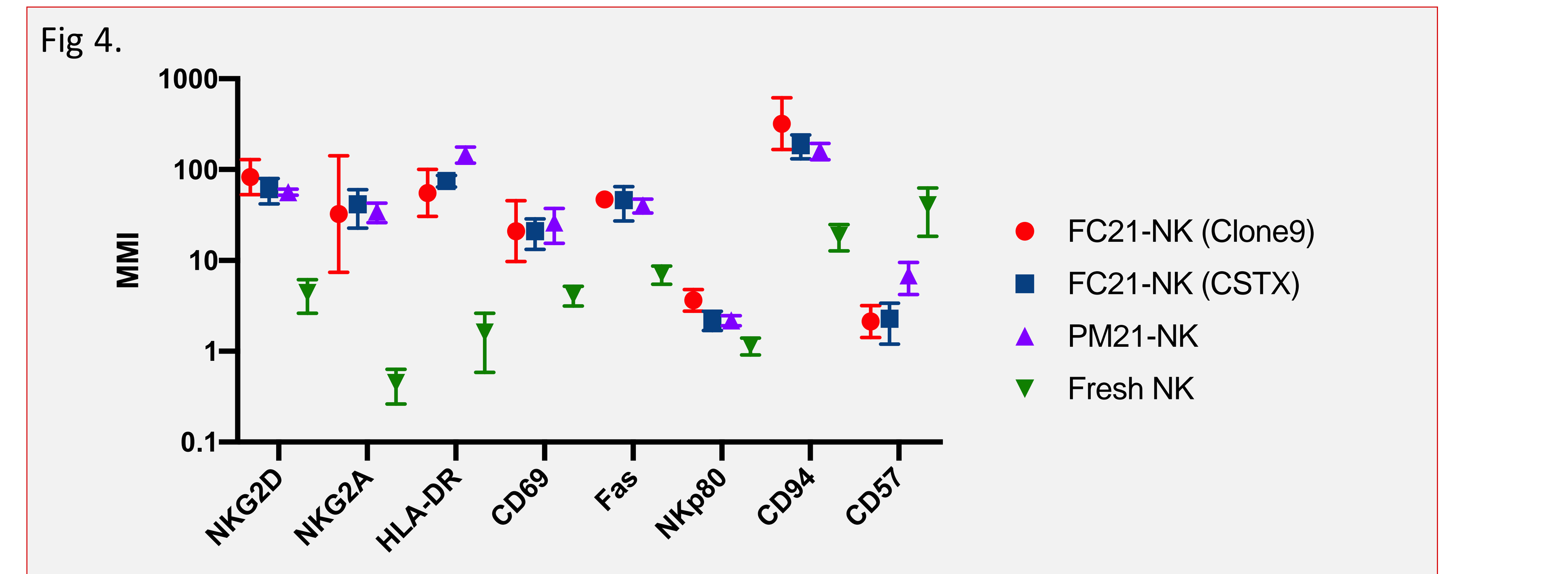

Figure 4: Phenotypic analysis of primary and expanded NK cells using mass cytometry (CyTOF). Mean metal intensity was determined on the population gated as for Fig. 3. Shown are all surface markers significantly different between fresh and expanded NK cells, wherein there are no significant differences observed between expansion platforms.

RESULTS

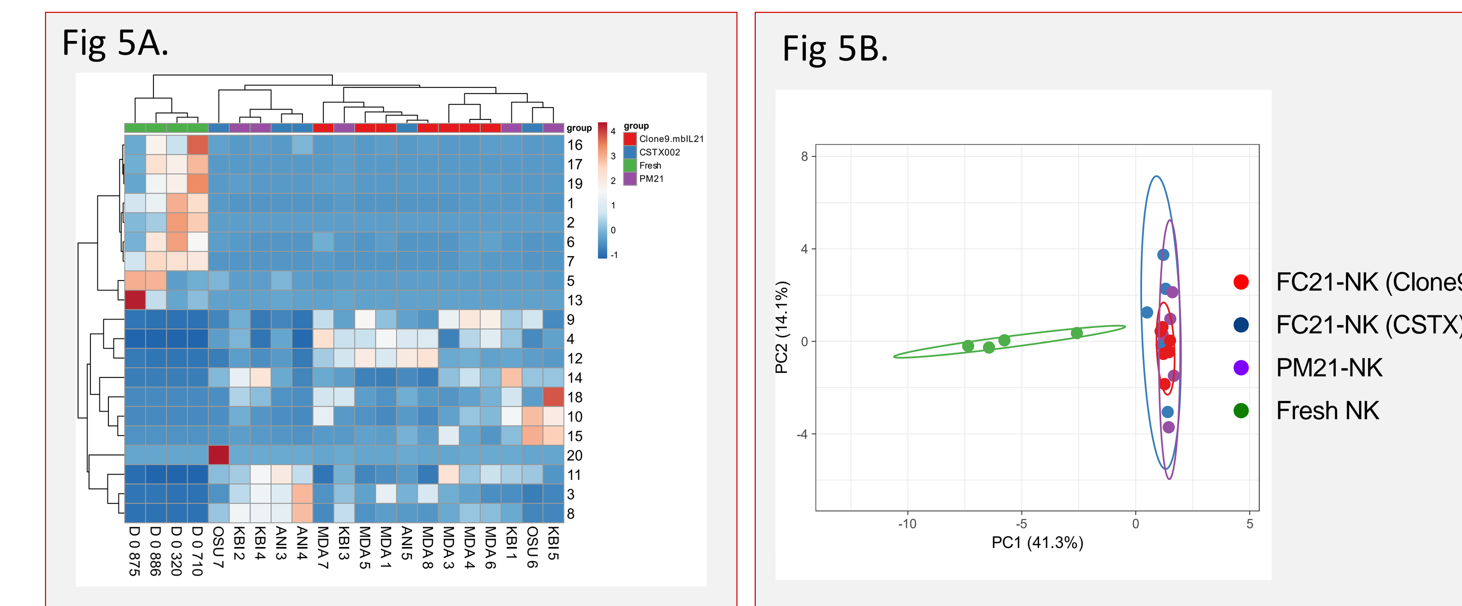

*Figure 5. Phenotypic identity of expanded NK cells is consistent across expansion methods but different from fresh NK cells. NK cells were phenotyped using multiparametric mass cytometry as shown in Figs 3, 4. 22 parameters were used for SPADE clustering into 20 nodes. There was no observable inter-method variation in expanded cells but moderate intra-method (donor-to-donor) variation was observed for all NK cell groups. **A)** Heatmap of node content (percent) for each primary and expanded NK cell sample. Both rows and columns are clustered using correlation distance and average linkage. **B)** Principle component analysis (PCA) analysis of SPADE nodal data. Unit variance scaling is applied to rows; SVD with imputation is used to calculate principal components. X and Y axis show principal component 1 and principal component 2 that explain 41.3% and 14.1% of the total variance, respectively. Prediction ellipses are such that with probability 0.95, a new observation from the same group will fall inside the ellipse.*

CONCLUSION(S)

- NK cells expanded with FC21 or PM21 are phenotypically distinct from non-expanded (primary) NK cells and posses a unique, yet highly consistent phenotypic and functional profile characterized by potent inflammatory cytokine production and cytotoxicity necessary for an effective cellular therapy.
- Expanded NK cells intended for clinical use using different manufacturing processes, different versions of the feeder cell, and different forms of the activator (feeder cells (FC21) or membrane particles (PM21)) are functionally and phenotypically indistinguishable.
- Intra-process variations account for the majority of differences observed, which may be attributed to donor-to-donor variation.
- The similarity between the cryopreserved NK cells produced under cGMP conditions for the past Phase 1/2 HSCT trial and for the recently FDA approved Phase 2 HSCT NK-REALM trial provides a bridge between materials used in these studies.

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KEY:

FC21-NK (Clone9)- NK cells expanded with feeder cell Clone9.mbIL21
FC21-NK (CSTX)- NK cells expanded with feeder cell CSTX002
PM21-NK- NK cells expanded with PM21 particles derived from CSTX002

***NOTE:** RNAseq comparisons are ongoing, but could not be completed for this poster as planned due to COVID-19.

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