Characterization of Natural Killer Cell Activation and Functionality for Cell Therapy Applications

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Dedication

To my parents, Jeff and Jackie,
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To all those that believed in me
And saw what I could be,
My deepest thanks and appreciation
For your boundless dedication.

Abstract

Natural killer (NK) cells are a promising emerging cell therapy due to their cytotoxic effector and cytokine producing functions, which play a role in targeting cancer cells. NK cells do not induce Graft-vs-Host disease and thus are suitable for allogeneic cell therapies targeting cancer cells. For allogeneic clinical applications, NK cells isolated from a single donor must be expanded through an efficient large-scale biomanufacturing process to treat many patients and produce an economical off-the-shelf therapy. Critically, the cultured NK cells must maintain functionality post-expansion to be an effective cellular therapy. Through multiple rounds of activation with K562 artificial antigen-presenting cells (aAPCs), the quantity of NK cells can expand by several orders of magnitude. However, the changes that chronic stimulation might induce in cell cycle status, metabolism, and *ex vivo* functionality of NK cells are not well understood. Even less is known regarding how changes in these cell characteristics may influence their *in vivo* functionality. In this study, we conducted a systematic evaluation of the activation and expansion process of NK cells through transcriptome analysis, dynamics of chromatin accessibility, metabolic characterization, and phenotypic analysis of exhaustion and senescence over time.

Transcriptomic methods were employed to explore activation-associated gene expression dynamics, and Assay for Transposase - Accessible Chromatin sequencing (ATAC-seq) was used to unveil the dynamic nature of epigenetic/transcriptional modifications during NK cell activation. Open chromatin regions and the genes associated with the peak signal from ATAC-seq were identified as enhancer genes for each sample. Furthermore, highly accessible regions or super-enhancers were evaluated to understand the changes in the epigenetic landscape. Differential accessibility of super-enhancers between pre-activation samples and K562-activated samples revealed highly expressed genes specific to each sample group. Furthermore, parallel analysis of transcription factor motifs identified highly accessible and active transcription factors that may contribute to the activated phenotype as master regulators. By understanding the transcriptional and epigenetic signature of the K562-activated NK cells, it is possible to find key genes or transcription factors that regulate K562 activation in order to develop bioprocesses that

phase out these feeder cells from the culture process. Eliminating K562 aAPCs will aide in optimizing biomanufacturing to reduce risk and variability.

Repeated stimulation and expansion of peripheral blood-derived NK (PB-NK) cells may lead to cellular senescence and exhaustion, both which are challenges in T-cell therapies due to how they can affect *in vivo* functionality. However, not much is known about potential senescence and exhaustion of NK cells through extensive stimulation with K562 aAPCs. In this thesis, repeated activation allowed for 10^6 - 10^8 fold expansion, although a reduced growth rate was observed from approximately day 21-26 onwards, which could be indicative of the presence of senescent cells in the later stage of culture. Efforts to characterize NK cell activity included utilizing flow cytometry to evaluate expression of various markers for exhaustion. Additionally, cell cytotoxicity assays were used to determine the presence of important secreted factors for NK cell functionality over multiple rounds of stimulation and to assess the killing kinetics during the different growth phases. Together, this work allowed exploration of the relationship between NK proliferative capacity and cytotoxic ability.

Surprisingly, characterization of NK cell receptor expression during expansion revealed fluctuations in many of the key activating and inhibiting receptors expressed on the surface of NK cells. Given that these receptors play a critical role in recognition of the target cells and NK cell effector functions, these changes in expression could impact efficacy in the clinic. The recycling and trafficking of NK cell receptors is not well understood; however, the development of therapeutics to control receptor surface expression to enhance NK cell functionality may be key in development of NK cell therapies.

Collectively, understanding the effects of activation and consequent proliferation of NK cells would remove a major roadblock in the biomanufacturing of NK cells, thus laying the groundwork for their potential use as an off-the-shelf allogeneic cellular therapy. Insights into mechanisms underlying activation and expansion provide a path to develop strategies to eliminate K562 aAPCs altogether, which would be desirable from a regulatory standpoint and further ease the transition from benchtop to biomanufacturing.

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List of Abbreviations

Abbreviation	Description
aAPC	Artificial antigen presenting cells
ADCC	Antibody dependent cell-mediated cytotoxicity
ATAC-seq	Assay for Transposase-Accessible Chromatin Sequencing
ECAR	Extracellular acidification rate
GO	Gene ontology
IFNγ	Interferon gamma
K562-mbIL-21	K562 cells expressing membrane bound IL-21
KIR	Killer immunoglobulin receptor
mbIL-21	Membrane bound IL-21
MHC	Major histocompatibility complex
MICB	MHC class I chain-related molecule B
NCR	Natural cytotoxicity receptor
NFκB	Nuclear factor kappa B
NK	Natural Killer
OCR	Oxygen consumption rate
OUR	Oxygen uptake rate
PB	Peripheral blood
PC	Principle components
PCA	Principle component analysis
PKC	Protein kinase C
RPKM	Reads per kilobase per million
TCA cycle	The citric acid cycle
TNFα	Tumor necrosis factor alpha
TPM	Transcripts per million

1 Introduction

Natural killer (NK) cells are part of the innate immune system, which initiates the first line of defense against infection and foreign pathogens. NK cells play a crucial role in immunosurveillance of malignancies due to their cytotoxic and cytokine-producing effector functions (Vivier et al. 2011; Terme et al. 2008). NK cells rely on a balance of activating and inhibiting receptors for activation and thus have the capacity to kill tumor cells without the requirement of antigen-dependent costimulatory signals for priming, unlike T cells (Vivier et al. 2011; Terme et al. 2008). Furthermore, this ability allows NK cells to recognize tumor cells in the absence of major histocompatibility (MHC) class I molecules and antigen, which are often downregulated on target cancer cells to prevent detection by T cells (Garrido, Ruiz-Cabello, and Aptsiauri 2017; Schreiber, Old, and Smyth 2011; Garrido et al. 2016). Regardless of MHC class I molecule mismatch between donor and recipient cells, various studies show no induction of Graft versus host disease upon adoptive transfer of NK cells (Terme et al. 2008; Farag et al. 2002), making NK cells an ideal allogeneic cell therapy.

One challenge for NK allogeneic cell therapies is that key activation signals have not been identified. Unlike T cells, which have specific signals for activation, NK cells are activated through a host of activating and inhibiting receptors (Rosenberg and Huang 2018). Various methods have been utilized to activate and expand NK cells, including the use of cytokine stimulation (Klingemann and Martinson 2004), monoclonal antibodies (Carlens et al. 2001), bead-tethered cytokines and receptors (X. Li et al. 2015), and various feeder cell lines (Berg et al. 2009; Denman et al. 2012; X. Wang et al. 2012; Kweon et al. 2019; Streltsova et al. 2019; Ojo et al. 2019; Fujisaki et al. 2009). The most common strategy is the use of artificially engineered antigen presenting cells (aAPCs), classically derived from the K562 leukemia cell line, which has been engineered to express various co-stimulatory ligands on the cell surface.

Studies have shown that the K562 aAPCs used to activate NK cells have significantly high expression levels of NKG2D ligands, and natural cytotoxicity receptor (NCR) ligands for NKp30, NKp44, and NKp46, which contribute to inducing signaling towards

preferential stimulation of NK degranulation as opposed to cytokine or chemokine secretion (Lisovsky et al. 2014; Long et al. 2013). However, those same ligands are also present on other less effective feeder cells utilized for NK cell activation (Tremblay-Mclean et al. 2019; Ojo et al. 2019) and thus are not considered to be master regulators of K562-mediated activation (Long et al. 2013). Identifying the key regulators mediating K562 aAPC activation is critical for developing alternative strategies that do not require the use of the feeder cells for biomanufacturing.

The other main challenge for developing a cost and time efficient cell therapy product is the ability to expand the primary cell source to a sufficient amount of cells. Clinical trials involving NK cells often call for higher doses, such as up to 10^9 cells/kg, for increased efficacy (Dahlberg et al. 2015; Knorr et al. 2014; Ojo et al. 2019). As such, the quantity of cells desired for a uniform product to treat multiple patients would be on the order of 10^{12} cells. Given that the number of cells taken from a donor is not enough to meet this demand, NK cells isolated from a single donor must be expanded through an efficient large-scale biomanufacturing process to treat many patients and produce an economical off-the-shelf therapy.

To obtain the desired fold expansion, outside stimulus for activation and expansion of NK cells is necessary. Current research is predisposed toward investigating optimization of the surface receptor expression to enhance expansion. *Denman el al.* found that K562 aAPCs expressing membrane-bound IL-21 (mbIL-21) allowed for 10^4 fold expansion in 3 weeks and claimed the NK cells did not exhibit evidence of senescence until 6 weeks of expansion (Denman et al. 2012), thus making the use of K562 cells expressing mbIL-21 an optimal feeder cell line to meet expansion potential requirements. While the fold change expansion capacity of NK cells using aAPCs, specifically the K562 leukemia cell line, holds the potential to meet biomanufacturing standards for an economical off-the-shelf product, rigorous and dynamic characterization to assess the functionality and metabolism of the resultant cells has not been previously performed. Chronic antigen stimulation has been found to induce genome-wide epigenetic programming and dysfunction in T cells, leading to the concern that this may also occur in NK cells (Bi and Tian 2017; Merino et al. 2019). Exhaustion and senescence of NK cells is not well understood. Therefore,

determining shifts in functionality and metabolism over time could be key in establishing whether cells generated under prolonged weekly expansion will meet targeted clinical and regulatory requirements.

1.1 Thesis organization

This work focused on understanding activation and expansion of peripheral blood-derived NK cells to remove major roadblocks in the biomanufacturing of NK cells. This goal was addressed on two fronts: understanding K562-mediated NK cell activation through transcriptional and epigenetic changes to phase out use of feeder cells in the bioprocess and characterizing the effect of repeated stimulation on NK cell growth kinetics, metabolism, transcriptional signature, and functionality to determine the best time to harvest NK cells during the expansion process. This thesis is arranged into 6 chapters. Chapter 2 details the methods used across all the studies completed for this work. Chapter 3 explores the transcriptional and epigenetic signature of K562-activated NK cells in order to identify key regulators of NK cell activation. Various super-enhancer genes and transcription factors are identified that potentially play a key role in K562-mediated activation. Chapter 4 investigates the effect of repeated stimulation with K562 aAPCs on NK cell growth, metabolism, transcriptome, and functionality in an effort to determine at what stage NK cells are potentially exhausted and should not be used in the clinic. Chapter 5 continues this study of NK cell phenotype and illustrates that key receptors have fluctuating expression over time, which has not been previously seen. Receptor turnover may play a more important role in upkeep of the functional NK cell phenotype than previously understood and thus controlling receptor expression may prove to be key in modulating the NK cell phenotype to have desired functionality. Finally, Chapter 6 provides a summary of key results and the future directions for this work.

2 Materials and Methods

2.1 Cell Culture and NK Cell Expansion

K562 artificial antigen-presenting cells (aAPCs) (ATCC) were engineered to express 4-1BBL and membrane bound IL-21 and obtained from FATE Therapeutics. K562 media formulation included RPMI 1640 Medium with L-glutamine (Corning), 1% penicillin/streptomycin (Gibco), and 10% heat inactivated fetal bovine serum (FBS, Hyclone). K562 aAPCs were irradiated at 10,000 rads and cryopreserved in 90% FBS and 10% DMSO (Sigma) prior to thawing and co-culture with NK cells as feeder cells. Peripheral blood mononuclear cells were obtained from Memorial Blood Center (Minneapolis, MN) using Ficoll-Paque Premium (GE Healthcare). NK cell isolation was performed using negative selection with the EasySep Human NK Cell Enrichment Kit (STEMCELL Technologies). NK cells were cultured overnight in B0 media (Cichocki and Miller 2010) supplemented with 100 U/mL IL2 (Proleukin) to recover from magnetic bead sorting. B0 media formulation included 2:1 (vol:vol) mix of Dulbecco's Modification of Eagle's Medium (DMEM) with 4.5 g/L glucose, L-glutamine, and sodium pyruvate and Ham's F12 Medium (Corning). Supplements included 20 μM 2-mercaptoethanol (Gibco), 50 μM ethanolamine (Sigma), 10 μg/mL ascorbic acid (Sigma), 1,6 ng/mL sodium selenite (Sigma), 1% penicillin/streptomycin (Gibco), and 20% heat-inactivated human AB serum (Valley Biomedical). A schematic for the expansion process is shown in Figure 4-1A. NK cells were collected the following day for functional and metabolic measurements prior to stimulation with 1:2 ratio of NK:irradiated K562 aAPCs in B0 media supplemented with 50 U/mL IL2. Half media changes occurred every 2-3 days throughout the continuous culture. Seven days after initial stimulation, and weekly thereafter, NK cells at 1.25E5 cells/mL were re-stimulated with K562 aAPCs at a 1:1 ratio. NK cell samples were removed for analysis immediately prior to each round of re-stimulation. During the period between each round of re-stimulation, the culture was diluted to 2.5E5 cells/mL every 2-3 days (Figure 4-1A).

2.2 Growth Kinetics and Cell Cycle Analysis

Cell counting was performed every 2-3 days using the Countess II Automated Cell Counter after adding a 1:1 ratio of Trypan Blue (Gibco) to the sample. To determine the cutoff between growth phases, cumulative total cell number data was log2 transformed to visualize the breakpoint between growth phases by a clear shift in slope. The doubling time for each growth phase was determine from the inverse of the slope of the log2-transformed data.

For cell cycle analysis, NK cells were collected weekly prior to re-stimulation. Cells were washed and stored in 100% Ethanol (Fisher Scientific) at -20°C. Once all samples were collected, cells were rehydrated in PBS for 15 minutes at room temperature and washed in FACS buffer (PBS supplemented with 2%FBS and 2 mM EDTA). Cells were stained with a 1:20 dilution of 1 mg/mL propidium iodide (Life Technologies) in FACS buffer for 15 minutes at room temperature in the dark. Samples were immediately analyzed on the LSR II H4710 instrument (BD Biosciences). ModFit LT 4.1.7 was utilized to analyze cell cycle distribution in the G0/G1, G2/M and S Phases.

2.3 Seahorse Metabolism and Oxygen Uptake Rate

Glycolysis and oxidative mitochondrial metabolism were concurrently analyzed using the Seahorse XF Cell Mito Stress Test Kit (Agilent Technologies) with modifications as described in a previous study (Cichocki et al. 2018). 10^6 cells per well were plated in at least triplicate for analysis in the XF^e 24 Extracellular Flux Analyzer (Agilent Technologies). Glucose, oligomycin, FCCP, rotenone, and antimycin A were sequentially injected to measure oxygen consumption rate (OCR) and extracellular acidification rate (ECAR). Basal respiration, maximal respiration, and ATP-linked respiration were calculated from the OCR values according to the manufacturer's instructions. Similarly, glycolysis, glycolytic reserve, and glycolytic capacity were measured from the ECAR values according to the manufacturer's instructions. Oxygen uptake rate (OUR) was calculated from the average basal OCR values for each donor at each timepoint.

2.4 Glucose Consumption and Lactate Production

Prior to each media change, the supernatant was removed and stored at -80 °C. For analysis, media samples were thawed and then centrifuged to remove any cell debris. 100 ul of cell-free supernatant was added to a 96-well plate (Corning) for analysis of glucose and lactate concentrations using the YSI 2950D-3 Biochemistry Analyzer (Xylem Inc.) according to manufacturer's instructions. Cumulative moles of glucose consumed, and moles of lactate produced were calculated according to the ratio of the volumes of new and old media at each media change timepoint. The ratio of lactate production to glucose consumption was calculated for each donor over time.

2.5 RNA Sequencing

Total RNA was purified form NK cells at day 0, 7, and 35 using the Qiagen™ RNeasy Mini Kit. The Illumina TruSeq® Stranded mRNA Sample Prep Kit protocol was used to purify poly-A containing mRNA molecules from the total RNA and create sequence libraries. Sequence libraries of each sample were pooled and sequenced using the Illumina HiSeq 2500 (High Output, 125 bp, Paired Reads, v4) instrument at the University of Minnesota Genomics Center. Low quality ends and adapter sequences were trimmed from the Illumina reads using trimmomatic. Processed reads were mapped and aligned with Spliced Transcripts Alignment to a Reference (STAR) to the Human reference genome (GRCh38.91) with default ENCODE parameter settings, except for '--runThreadN 16 --sjdbOverhang 76.' Reads Per Kilobase of transcript, per Million mapped reads (RPKM) values were computed using Cufflinks and then normalized to transcripts per million (TPM). Using R Studio, TPM was then log2 transformed before performing hierarchical clustering using the Euclidean distance and centroid linkage (UPGMC) method. Principal component analysis (PCA) was performed using the 'ggplot2' package in statistical software R (v 3.3.3).

Differential gene expression analysis was completed in R. Python software htseq-counts was utilized to extract the necessary raw read counts to upload into the 'EdgeR' package to determine differential gene expression. Lowly expressed genes (less than 6-7 counts) were filtered out and normalized with trimmed mean of M-values (TMM) to adjust for falsely positive downregulated genes. Negative binomial generalized linear models

were then fitted prior to either implementing the quasi-likelihood (QL) F-test or likelihood ratio test for verifying differential gene expression. The threshold criteria of P value and FDR ≤ 0.05 and a fold change of ≥ 2 in gene expression among the different pair-wise comparisons was used to identify the differentially expressed genes. Functional analysis or Gene ontology (GO) of the different differentially expressed genes was done utilizing DAVID v6.8, while pathway comparison analysis was performed in Ingenuity Pathway Analysis. Further visualizations of the data including the PCA plot and the comparative analysis of pair-wise comparisons to perform quadrant analysis was completed using the TIBCO Spotfire v7.6.1 OmicsOffice package

2.6 ATAC Sequencing

Samples were obtained from 5.0×10^4 cells with the OMNI-ATAC protocol (Corces et al. 2017). The MinElute PCR cleanup kit (Qiagen) was utilized to purify transposed DNA. Indexed sequence libraries were created for each sample sequenced using the Illumina NovaSeq 6000 (SP, 2x50 bp, paired-end reads) instrument at the University of Minnesota Genomics Center. Low quality ends and adapter sequences were trimmed from the Illumina reads using trimmomatic. Processed reads were mapped and aligned to the Human reference genome (GRCh38) using Bowtie2 with parameters -N 1 -L 20 -i S,1,0.50 -D 20 -R 3 -X 2000. For identifying super-enhancers, donor samples of the same time point were pooled, and peaks were called using MACS2 with parameters: -p 0.00001 --nomodel --shift -100 --extsize 200 --bdg. HOMER was utilized to exclude peaks -1kb to +100bp of transcription start sites. Bedtools merge stitched together neighboring peaks separated by less than 12.5kb. Read coverage in stitched peaks was computed and background coverage from NK cell input DNA sequencing (GEO accession number GSM2048316, Koues et al. 2016) in the same region was subtracted to give the ATAC signal. Peaks were ranked by strength of ATAC signal, in which the inflection point of the ATAC signal vs. rank curve was set as the cutoff between regular enhancers and super-enhancers (Whyte et al. 2013).

Transcription factor footprint analysis was performed as previously described (Brown et al. 2019). A universal peak list was generated using MACS2 with parameters: -p 0.01 --nomodel --shift -25 --extsize 50 --keep-dup 'auto' --bdg --SPMR --call-summits.

Peaks were also called for each sample with the same parameters. Irreproducible discovery rate (IDR) processing was applied to obtain reproducible peaks (at IDR < 0.05) between replicates using the universal peak list as the oracle peak set. The universal peak set was redefined as 150bp intervals centered around peak summits. ATAC-seq read counts in these peaks were calculated using Bedtools multicov, and differential accessibility analysis was performed using DEseq2 with a 2-fold-change cutoff and q-value < 0.01. Using HOMER, all available vertebrate transcription factor motifs that were present in peaks upregulated in day 7 samples were compared to the those present in peaks upregulated in day 0 samples using the following settings: -noweight -size given -p 16 -S 100 -N 1000000 -bits -cache 1000.

2.7 Flow Cytometry Characterization

Antibodies used for analysis of marker expression in NK cells are listed in Table 2-1. For weekly phenotypic analysis of surface markers (CD3, CD56, CD57, CD16, NKp30, NKp44, NKp46, CD94, KIR, NKG2A, CD96, and NKG2D), NK cells were stained with 1:1000 dilution of Fixable Viability Dye eFluor780 (Thermo Fischer Scientific) for 10 min at 4 °C in the dark. Cells were washed with FACS buffer (PBS supplemented with 2% FBS and 2 mM EDTA) prior to staining with corresponding surface markers for the different flow panels (see Table 2-1) for 20 min at 4°C in the dark. Cells were then washed with FACS buffer prior to fixation in 2% paraformaldehyde (PFA). Intracellular staining of perforin and granzyme was also performed weekly. For intracellular staining, NK cells were stained with identifying surface markers CD56 and CD3 following the surface staining protocol as described above. Upon fixation in 2% PFA, cells were incubated for 10 min at 37 °C. NK cells were washed in FACS buffer and incubated in 1X permeabilization buffer for 10 min at 4 °C (10X permeabilization buffer (eBioscience) diluted in distilled water according to the manufacturer's instructions). Cells were again washed with FACS buffer before incubating cells with intracellular antibodies for 30 min at 4 °C. Samples were then washed and stored in 2% PFA prior to analysis. Flow cytometry data was acquired using the LSR II H4710 instrument (BD Biosciences) and analyzed with FlowJo v10 software.

2.8 NK Cell Effector Function Assay

To determine NK cell degranulation, samples were cultured alone or with naïve K562 target cells at a 1:2 ratio of effector (NK) cells to target cells in 200 ul of B0 media in a 96-well round bottom plate. Cells were incubated with CD107a antibody (H4A3: BD Biosciences) for 1 hour at 37°C prior to the addition of 20 ul of 1:100 GolgiPlug and 1:150 GolgiStop (BD Biosciences). Cells were subsequently incubated for 3.5 hours at 37°C prior to being stained with Fixable Viability Dye eFluor780 (Thermo Fischer Scientific). Cells were stained for CD56 and CD3 and then fixed with 2% PFA. To stain for intracellular markers such as interferon gamma (IFNγ) and tumor necrosis factor alpha (TNFα), the steps for intracellular staining were followed as described earlier. Marker expression was analyzed with the LSR II H4710 instrument (BD Biosciences) and FlowJo v10 software.

2.9 Killing Kinetics Assay

A549, PANC-1, and SKOV-3 cells expressing NucLight Red (NLR) were utilized to assess the killing kinetics of the various donors over time. Each NLR target cell line was plated at a concentration of 5×10^3 cells per a well in a 96-well clear bottom, black plate (Corning). Cells were allowed to attach overnight in DMEM supplemented with 10% FBS, 1X penicillin/streptomycin, and glutamine. The IncuCyte® Zoom instrument (Essen BioScience) was used to quantify the amount of target NLR cells per well. After counting the target cells, NK cells were added at 1:1, 3:1, and 10:1 effector to target ratios in B0 media containing 50 U/mL IL2. Each condition was replicated in triplicate for each experiment. The number of viable target cells was tracked by imaging every 30 minutes over a period of 50 hours using the IncuCyte® Live Cell Analysis System. Quantification of live target cell number at each timepoint was performed using the IncuCyte® software and normalized to the number of live cells at the initial timepoint and the live cells remaining in the control group containing only target cells (Uppendahl et al. 2019; Felices et al. 2019).

2.10 Statistical Analysis

Data was calculated from independent donors for each assay and expressed as the mean ± SD. Differences between groups within each growth phase or timepoint was determined by paired one-tailed Student's *t* test unless otherwise specified in the figure legend. P values < 0.05 were considered statistically relevant.

Table 2-1. Antibodies used for flow cytometry Table. List of antibodies used in various flow cytometry panels and their corresponding vendor, clone/product number, and ul/Test or dilution.

Target antigen	Antibody species	Vendor	Clone or product number	ul/Test	Panel
eBioscience™ Fixable Viability Dye eFluor780		Thermo Fischer Sci.	65-0865-14; APC/Cy7	1:1000	All
CD3	Human	BioLegend	317330; BV785 Clone: OKT3	3ul	IC, DG
CD16	Human	BioLegend	302038; BV421 Clone: 3G8	3ul	P1, P2, P3
CD25	Human	BioLegend	302634; BV650 Clone: BC96	3ul	P3
CD56	Human	BD Cell Analysis	564058; BV786 Clone: NCAM16.2	3ul	P1, P2, P3
CD56	Human	BioLegend	318318; PE/Cy7 Clone: HDCD56	3ul	IC, DG
CD57	Human	BioLegend	393304; BV605 Clone: QA17A04	3ul	P3
CD94	Human	BioLegend	305516; PE/Cy7 Clone: DX22	3ul	P3
CD107a	Human	BioLegend	PE/Cy5.5 Clone:	3ul	DG
c-kit (CD117)	Human	BioLegend	323416; PerCP/Cy5.5 Clone: A3C6E2	3ul	P3
DNAM-1 (CD226)	Human	BioLegend	338312; APC; Clone: 11A8	5ul	P2
Granzyme B	Human/mouse	BioLegend	372206; FITC Clone: AQ16A02	5ul	IC
IFNγ	Human	BioLegend	BV650 Clone: B27	3ul	DG
IL-7Ra (CD127)	Human	BioLegend	351344; Alexa Fluor™ 700 Clone: A019D5	3ul	P3
KIR2DL1 (CD158)	Human	BioLegend	339510; APC Clone: HP-MA4	3ul	P3
KIR2DL2 (CD158b)	Human	BioLegend	312612; APC Clone: DX27	3ul	P3
KIR3DL1 (CD158e1)	Human	BioLegend	312716; APC Clone: DX9	3ul	P3
NKG2A (CD159a)	Human	Miltenyi Biotec	130-113-565; FITC Clone: REA110	2ul	P3

NKG2D (CD314)	Human	BioLegend	320806; PE Clone 1D11	5ul	P1
NKp30 (CD337)	Human	BioLegend	325210; APC Clone P30-15	3ul	P1
NKp44 (CD336)	Human	BioLegend	325108; PE Clone: P44-8	5ul, 3ul	P2, P3
NKp46 (CD335)	Human	BioLegend	331922; FITC Clone: 9E2	3ul	P1
PD-1 (CD279)	Human	Thermo Fisher Sci.	11-9969-42; FITC Clone: MIH4 eBioscience	5ul	P2
Perforin	Human	BioLegend	353304; PE Clone: B-D48	5ul	IC
TACTILE (CD96)	Human	BioLegend	338416; PE/Cy7 Clone: NK92.39	3ul	P2
TNFα	Human	BioLegend	502932; BV421 Clone: Mab11	3ul	DG

IC = intracellular, DG = degranulation assay, P1 = panel 1, P2 = panel 2, P3 = panel 3

3 Identifying Key Regulators of NK Cell Activation

3.1 Introduction

One of the main challenges facing NK cell therapy is the use of K562 aAPCs to activate and expand NK cells. The use of these cancerous feeder cells can potentially cause regulatory concerns when converting this culture process to meet industrial standards. K562 aAPCs are often used in conjunction with IL-2 stimulation to activate and expand NK cells. These cells have been engineered to express various receptors and ligands, specifically 4-1BBL and membrane bound IL21 (mbIL21), to enhance activation and expansion (Denman et al. 2012; X. Wang et al. 2012). However, when these ligands and receptors are expressed on Dynabeads™, the expansion potential associated with activation is significantly decreased (X. Li et al. 2015). As such, there are aspects of K562 cells beyond these ligands and receptors that are key for activation.

While K562 aAPCs have been known to aid in activation and expansion of NK cells, the exact relationship and mechanism of interaction is still unknown. In this chapter, we sought to understand the key biological processes and potential transcription factors that may regulate NK cell activation upon interaction with the K562 aAPCs. Identifying the mechanisms by which K562 aAPCs achieve such robust activation and proliferation of NK cells is key to developing alternative strategies that can eliminate these feeder cells from the culture.

3.2 Results

3.2.1 Initial activation results in slower growth

PB-NK cells were isolated from the blood through Ficoll separation and magnetic bead selection of CD56+ cells. NK cells were cultured overnight in IL-2 for the cells to recover from the stress of separation and prevent the resting phenotype from being dominated by the response to the stress that the separation caused. NK cells from three different donors were activated via K562 aAPC stimulation and cultured for one week in the presence of IL-2 stimulation. Activated NK cells were collected one week post-stimulation to ensure that all the K562 cells were killed in culture and would not confound the RNA-sequencing and ATAC-sequencing results. Flow cytometry results confirmed

that the culture had high purity of NK cells (average of 97.0 ± 0.1% expression of CD56+CD45+ live NK cells) at day 7 (Figure 3-1C).

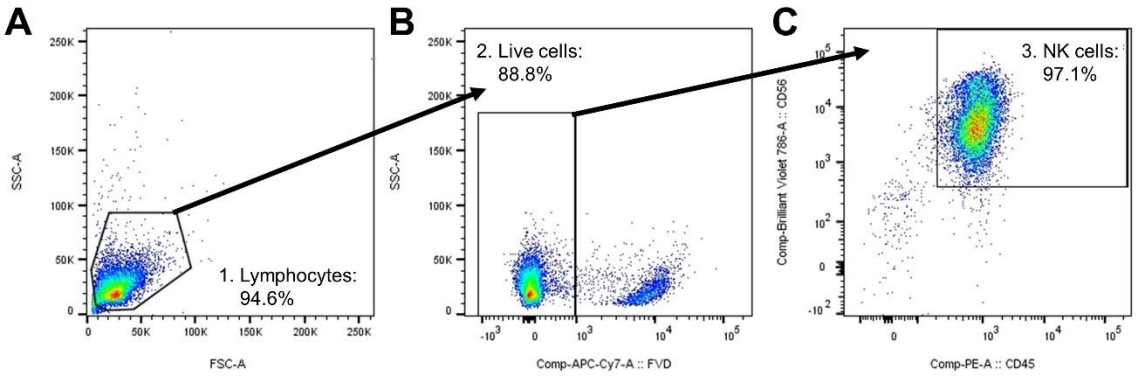

Figure 3-1. Flow cytometry of activated NK cells. Flow cytometry performed on K562-activated NK cells (Day 7) was first gated for lymphocytes (A), followed by live cells (B) and then NK cells using CD45+ and CD56+ markers (C). Representative figure of flow cytometry gating scheme for Donor A Day 7.

3.2.2 Transcriptomic comparison of activated and non-activated samples

RNA sequencing was performed on NK cells from three donors (Donors A-C) at Day 0 (pre-activation control; "d0"), Day 7 for the K562-activated samples ("d7"), and Day 7 for the sample receiving only IL-2 activation ("d7_unstim"). Hierarchical clustering (Figure 3-2A) and principal component analysis (PCA) (Figure 3-2B) were done to evaluate the difference in overall gene expression for each donor and activation stage. Both methods indicated that the samples clustered together by day and activation stage rather than by donor. The PCA analysis demonstrated a difference between the sample with IL2-only stimulation compared to the K562-activated samples.

A subset of genes related to NK cell activation, expansion, maturation, proliferation and/or cytotoxicity as detailed in Table 3-1 was mapped to the TPM gene expression data and was scaled by row to visualize the expression dynamics across samples (Figure 3-2C). Through hierarchical clustering of this subset of NK-related genes, we identified 4 clusters that related to enriched expression in Day 7 K562-activated samples (green bar), genes that are similarly expressed between timepoints (orange bar), enriched expression in Day 0 pre-activation samples (yellow bar), and enriched expression in Day 7 IL2-only activated sample (purple bar).

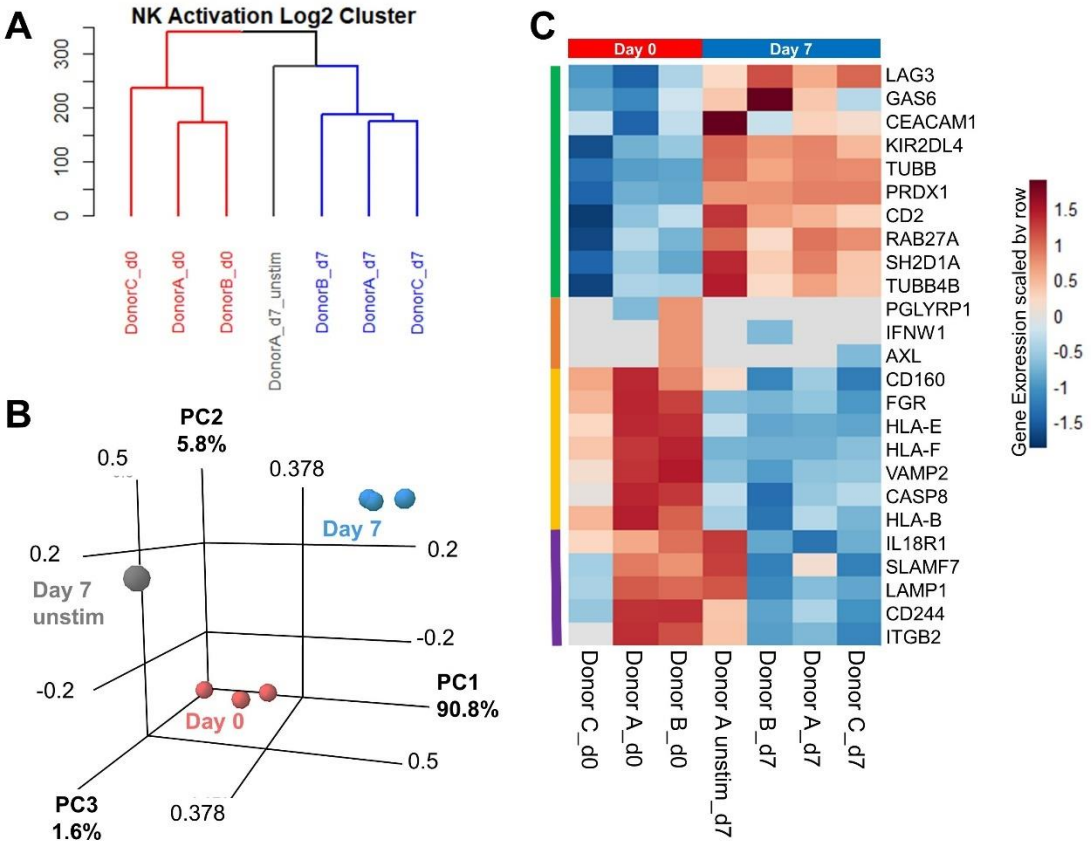

Figure 3-2. RNA-sequencing analysis of K562-activated NK cells. (A) Hierarchical clustering and (B) principal component analysis of the RNA sequencing analysis for Donors A-C, which included pre-activation samples (Day 0 - red), K562-activated samples (Day 7 - blue), and IL2-activated sample (D7 unstim - grey). (C) Subset of NK-related genes that play a role in activation, maturation, proliferation and/or cytotoxicity that are either preferentially expressed in Day 7 samples (green bar), not highly expressed in samples (orange bar), preferentially expressed in Day 0 samples (yellow bar), or preferentially expressed in the Day 7 IL-2 stimulated sample (purple bar). Gene expression is scaled by row from TPM values.

Table 3-1. Subset of NK-related functional genes. List of genes with specific functions related to activation, expansion, maturation, proliferation and/or cytotoxicity that were found to be differentially expressed in the RNA-sequencing data. Cutoff at log2 fold change =1, P value < 0.05, and FDR <0.05.

SYMBOL	GENE NAME	FUNCTION	NKd7vsNKd0 Log2FC	REFERENCE
GAS6	growth arrest specific 6	NK cell maturation	3.37	Park et al. 2009
PRDX1	peroxiredoxin 1	NK killing gene upregulated in expanded NK cells	2.22	Un Park et al. 2010
CASP8	caspase 8	Regulates the magnitude of NK cell activation	-1.88	Feng et al. 2019

SLAMF7	SLAM family member 7	Upregulated in IL-2 stimulated cells; CD2 related receptor for activation/cytotoxicity	-2.04	Giuliani et al. 2018
ITGB2	integrin subunit beta 2	Adhesion receptor; Role in NK cell maturation but downregulation results in a hyporesponsive phenotype in vitro	-2.29	Vivier et al. 2011; Crozat et al. 2011
CD160	CD160 molecule	Controls production of proinflammatory cytokines IFN-γ, TNF-α, IL-6, IL-8, and MIP1-β chemokine	-3.28	Tu et al. 2015; Le Bouteiller et al. 2011
AXL	AXL receptor tyrosine kinase	Essential role in the regulation of NK cell development as well as NK effector function	-4.01	Kim et al. 2017; Park et al. 2009
FGR	FGR proto-oncogene	Role in release of perforin and granzymes and transcription of cytokine and chemokine genes	-4.24	Paul and Lal 2017
CD244	CD244 molecule	Essential for optimal NK cell proliferation in response to IL-2; may play a role in the development of NK cell exhaustion	-4.29	Agresta, Hoebe, and Janssen 2018

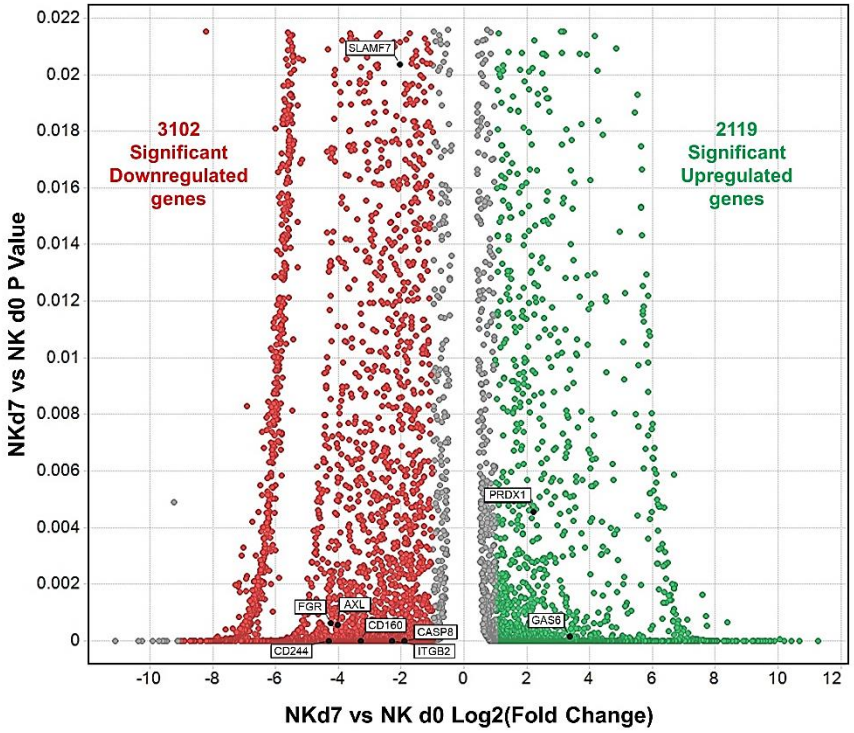

Figure 3-3. Differentially expressed gene analysis of K562-activated NK cells. Differentially expressed upregulated genes (green) and differentially expressed downregulated genes (red) in the Day 7 K562-activated samples compared to the Day 0 pre-activation samples. Genes highlighted in black were significant differentially expressed genes from the subset of NK-related genes. Criteria for significant differentially expressed genes were as follows: log2 fold change ≥ 1 or ≤ -1, p value < 0.05, and FDR < 0.05.

Analysis of significant differentially expressed genes between the pre-activation (Day 0) and K562-activated (Day 7) samples indicated that 3,102 genes were significantly downregulated (red) and 2,119 genes were significantly upregulated (green) (Figure 3-3). A few genes that were known to have NK-related functionality (black) were shown to also be differentially expressed. These results illustrated that there is a relatively large subset of genes that distinguish the cells in their differing activation states.

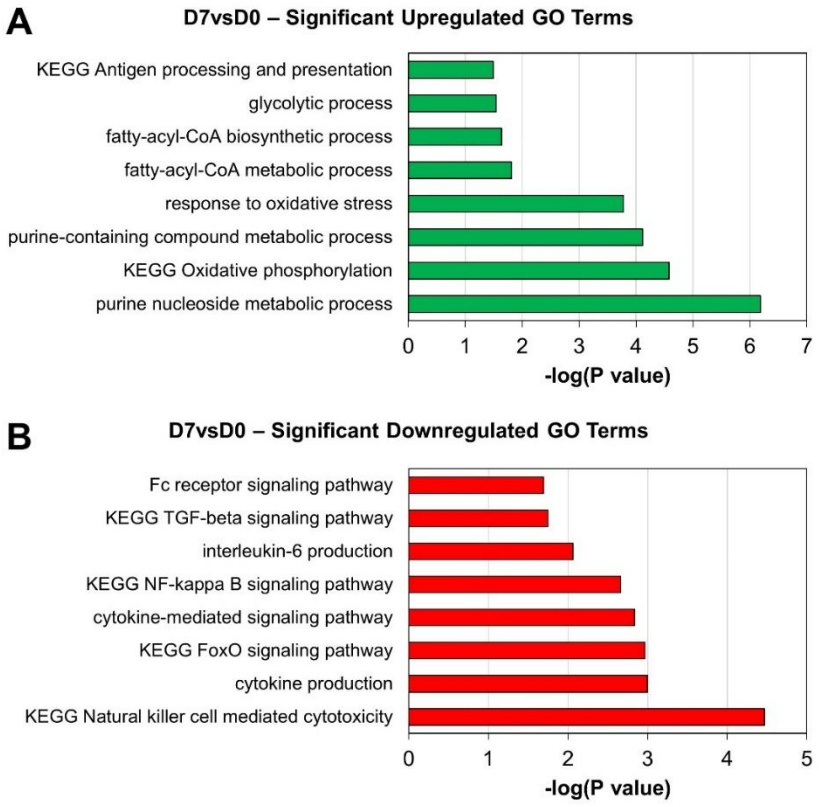

Figure 3-4. Enriched GO terms in pre-activation and K562-activated NK cells. Gene ontology analysis showing key significant upregulated (A) and downregulated (B) terms in the K562-activated Day 7 NK samples compared to the pre-activation Day 0 samples.

Gene ontology (GO) analysis of genes that were upregulated in K562-activated Day 7 samples included enrichment of terms related to metabolic processes such as glycolysis, oxidative phosphorylation, purine metabolism, and fatty acid metabolism (Figure 4-11A). Antigen processing and presentation was also represented in the significant upregulated biological processes, which was expected due to the interaction between K562 aAPCs and NK cells. At Day 7, pathways related to cytokine production/signaling and NK cytotoxicity were downregulated (Figure 4-11B), indicating the K562-activated NK cells

were moving away from a cytokine-producing phenotype and that the initial activation signals do not seem to be directly related to enhancing NK cytotoxicity.

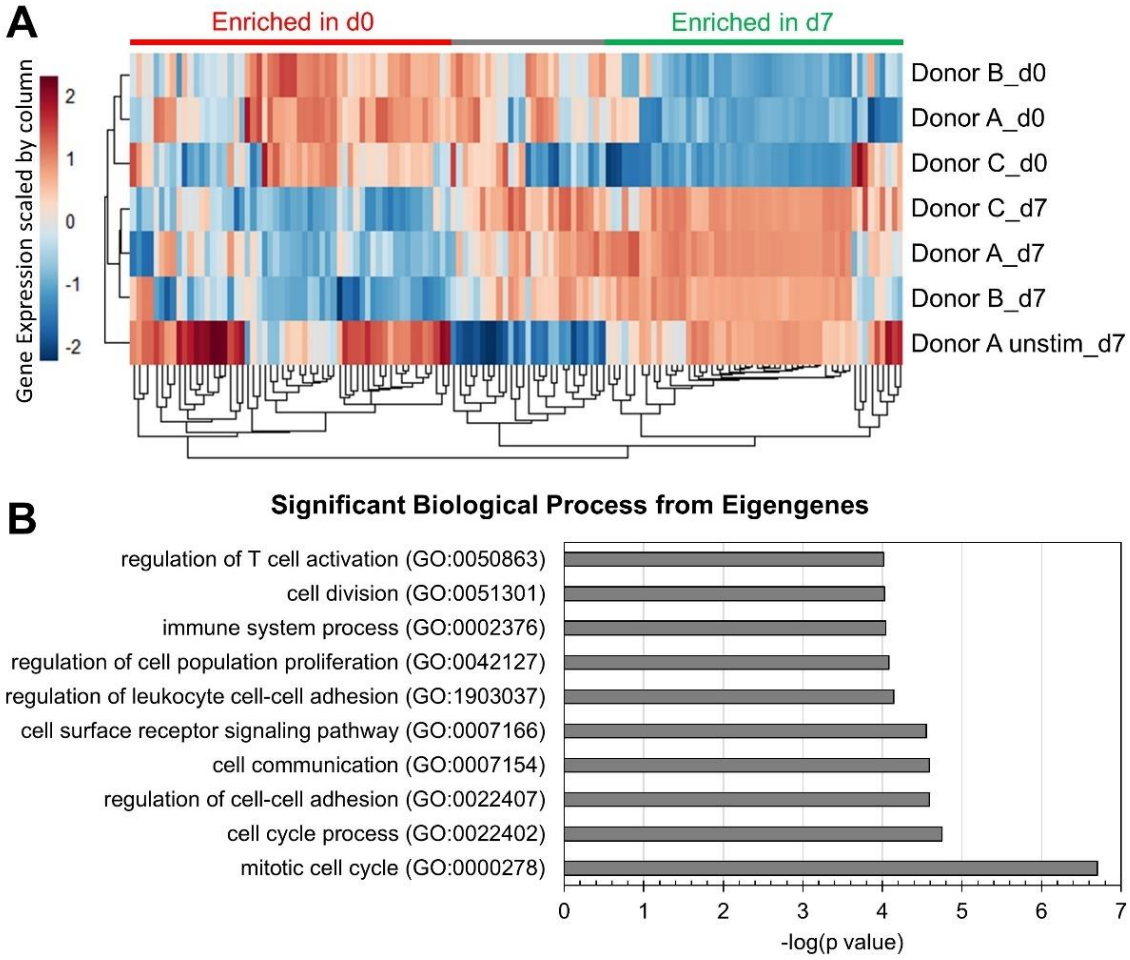

Figure 3-5. Eigengenes that contribute to variability between activation states. (A) Differential gene expression of the top 135 genes that contribute to PCA variability in the first 3 principal components (PC). Eigengenes were clustered and grouped according to genes that were specifically enriched at Day 0 (red bar), genes specifically enriched at Day 7 (green bar), and genes similarly enriched at both timepoints (grey bar). TPM values are scaled by column. (B) Significant GO biological processes that the PCA eigengenes influence.

To understand which genes and biological process contribute most to the variability of gene expression dynamics between the pre-activation Day 0 and K562-activated Day 7 samples, the top 50 genes that contributed to the variation between samples in the first 3 principal components (PC) were identified and termed eigengenes. Given that there was some overlap of genes that contributed to the variance in each individual PC, a total of 135 unique eigengenes were discovered. These eigengenes were then mapped to visualize the

differential expression of the TPM values across the different donors and their activation states (Figure 3-5A). Hierarchical clustering of the genes revealed three different clusters of eigengenes that were specifically enriched in Day 0 samples (red bar), eigengenes highly expressed at both timepoints but lowly expressed in the Day 7 IL2-only stimulation sample (grey bar), and eigengenes specifically enriched in Day 7 samples (green bar). GO analysis of these 135 eigengenes (Figure 3-5B) revealed that the significant biological processes that play a role in distinguishing the different activation states according to PCA mainly included terms related to cell division and the cell cycle. These GO terms were unsurprising given the cells are proliferating in culture. However, terms related to the immune system process and T cell activation were significant when GO analysis was performed on the eigengenes, indicating the difference between the activation states may be related to those specific genes in those terms. Interestingly, terms related to cell-cell interactions, specifically surface receptor signaling and cell-cell adhesion, were significant and seem to be key in initial activation.

To further elucidate which biological processes are enriched in the K562-activated Day 7 sample compared to the pre-activation Day 0 sample, GO analysis was performed on the eigengenes that clustered together to be specifically enriched at either Day 0 and Day 7 (Figure 3-6A,B). Eigengenes enriched at Day 0 indicated that the pre-activation samples had significant upregulated biological processes related to regulation of activation in response to cell surface signaling (Figure 3-6A). Eigengenes enriched at Day 7 showed the K562-activated samples had significant upregulated biological processes related to cell division or the cell cycle, as expected (Figure 3-6B).

Given that the two clusters used for the GO analysis were groups of specific eigengenes uniquely expressed in either the pre-activation Day 0 or K562-activated Day 7 samples and did not account for the second cluster of eigengenes that was expressed in both groups (grey bar in Figure 3-5A), we sought to add these eigengenes and perform GO analysis to see if other significant biological processes would be revealed. When the cluster of eigengenes that were enriched in both the pre-activation samples and K562-activated samples was grouped with the specific eigengenes more highly expressed in the Day 0 samples (red bar in Figure 3-5A), the primary biological processes were more focused on

the cell surface receptor interactions, especially as it relates to cell adhesion (Figure 3-6C). When the same analysis was repeated with eigengenes enriched in the K562-activated samples, there was no change in the top biological processes that were significantly upregulated at Day 7 (Figure 3-6D) in comparison to the GO terms obtained from eigengenes differentially enriched at Day 7 only (Figure 3-6B).

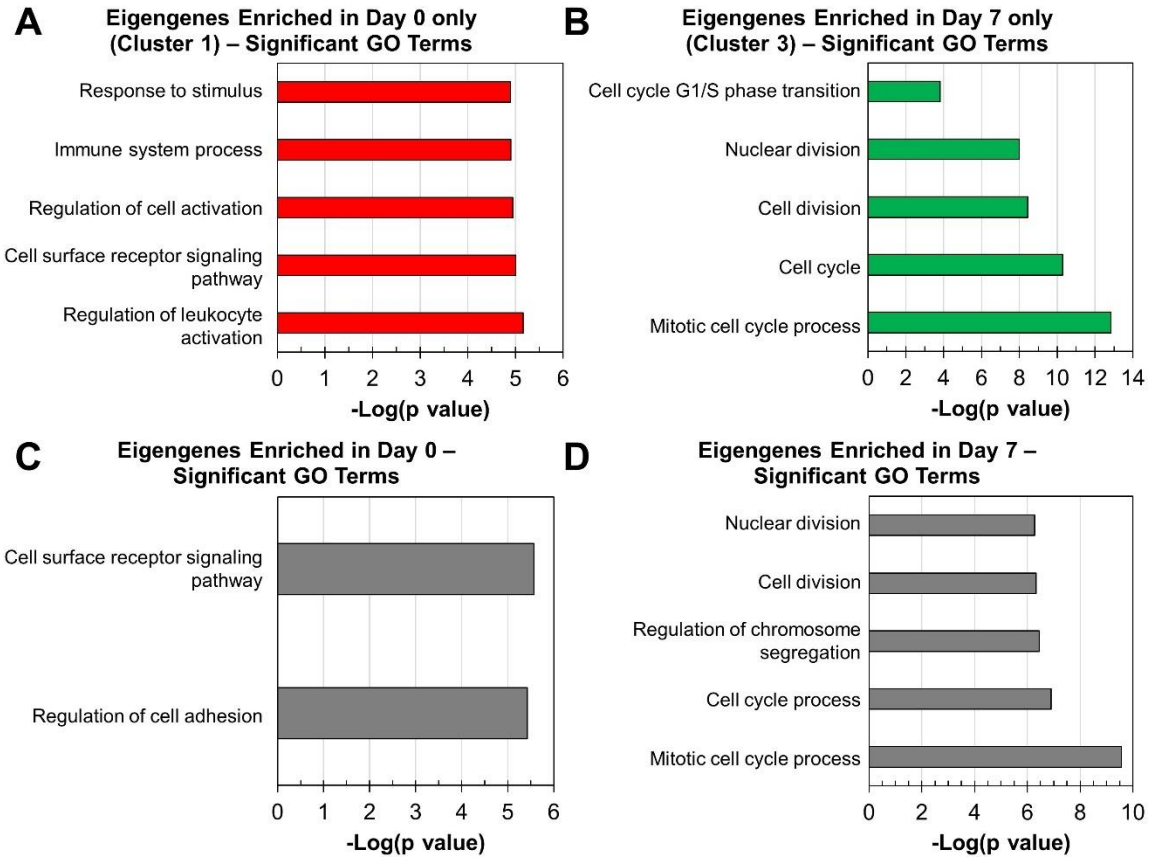

Figure 3-6. Enriched GO terms of PCA eigengene clusters. (A-B) GO analysis showing (A) enriched terms for pre-activation samples from the first cluster of highly enriched eigengenes specific to Day 0 (red bar in previous figure) and (B) enriched terms for K562-activated samples from the third cluster of highly enriched eigengenes specific to Day 7 (green bar in previous figure). (C-D) GO analysis was performed on the cluster of eigengenes that were enriched in both the pre-activation samples and K562-activated samples (grey bar previous figure) grouped with the specific eigengenes more highly expressed in the Day 0 samples (C) or Day 7 samples (D) to show upregulated terms for eigengenes highly expressed at differing activation states.

3.2.3 Chromatin accessibility landscape of K562-activated NK cells

To further understand the mechanism of activation via K562 stimulation, Assay of Transposase Accessible Chromatin sequencing (ATAC-seq) was utilized to find regions of increased accessibility and map transcription factor binding sites that are driving K562-

mediated activation of NK cells. Ranked peaks with accessible chromatin were identified through ATAC-seq signal, which was background-subtracted read coverage versus the peak rank (Figure 3-7A,B). The closest gene to those peaks was annotated to reveal the potential super-enhancers (red) that had high ATAC-seq signal compared to regular enhancers (black), which had low ATAC-seq signal but were still expressed. Of the 29,514 stitched peaks for Day 0, only 1,883 were identified to be potential super-enhancers. *CD247*, *JUND*, *PDE4C*, *BTG2*, and *FMOD* were identified to be top potential super-enhancers with related NK cell or immune system function for the pre-activation Day 0 sample (Figure 3-7A, Table 3-2). For Day 7, 2,352 super-enhancers were identified from a total of 35,000 accessible peaks. Of the top ranked super-enhancers, *BHLHE40*, *TRGs*, *ZBTB16*, and *FYN* were identified to have NK related function or immune system related function (Figure 3-7B, Table 3-2). These super-enhancers may be key in regulating the K562-activation phenotype and aide in the understanding of K562-mediated activation of NK cells.

Table 3-2. Top super-enhancer genes with NK-related function. List of genes that corresponded to highly accessible chromatin peaks in the ATAC-sequencing data.

SYMBOL	GENE NAME	FUNCTION	ENRICHED	REFERENCE
CD247	T-cell receptor zeta	Role in antigen recognition on T cells, hypo-responsiveness and differentiation of NK cells, linked to NKp30 expression	Day 0	Lau et al. 2018; Valés-Gómez et al. 2016
JUND	JunD proto-oncogene, AP-1 transcription factor subunit	Role in T cell activation, more accessible in memory NK and CD8+T cells	Day 0	Lau et al. 2018
PDE4C	phosphodiesterase 4C	Regulates cAMP which depresses NK cell cytotoxicity	Day 0	Fertig and Baillie 2018; Ullberg et al. 1983
ADGRE5/ CD97	adhesion G protein-coupled receptor E5/CD97	Related to proliferation (IL-2 stimulation) and results in decrease in cytolytic effector cells; Higher in memory T cells	Day 0	Kop et al. 2009
BTG2	BTG anti-proliferation factor 2	tumor suppressor gene, anti-proliferation protein	Day 0	Tsui et al. 2018
FMOD	fibromodulin	Inhibits NFκB pathway, which plays a role in release of perforin	Day 0	Lee and Schiemann 2011; Zhou, et al. 2002

BHLHE40	basic helix-loop-helix family member e40	Cofactor of T-bet in the regulation of IFN-γ production in *i*NKT cells	Day 7	Kanda et al. 2016
TRGs	T cell receptor gamma components	Role in T cell antigen recognition	Day 7	Rosenberg and Huang 2018
ZBTB16	zinc finger and BTB domain containing 16	Directs the effector function of NKT cells	Day 7	Savage et al. 2008
FYN	FYN proto-oncogene, Src family tyrosine kinase	Role in regulation of killer cell effector functions and differentially regulates cytotoxicity and cytokine production	Day 7	Gerbec, Thakar, and Malarkannan 2015

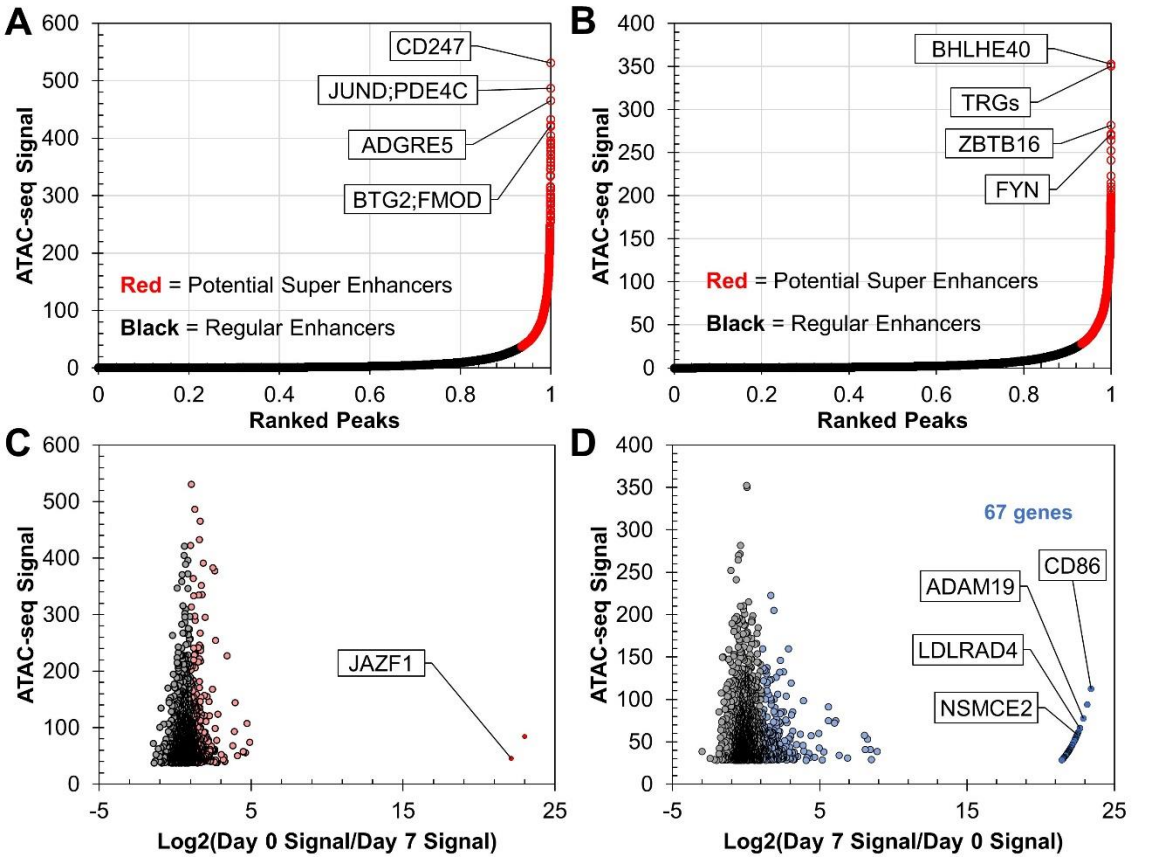

Figure 3-7. Chromatin accessibility of ATAC peaks in each activation state. (A-B) Peaks were ranked according to ATAC-seq signal, or chromatin accessibility, with the highly accessible chromatin regions indicating potential super-enhancers (red) while the rest are regular enhancers (black). Top super-enhancers and the corresponding closest gene in the pre-activation sample at Day 0 (A) and K562-activated samples at Day 7 (B) were identified and those related to NK cell function were labeled. (C-D) Levels of chromatin accessibility were compared between activation states to give unique genes that correspond to highly accessible super-enhancer peaks specific to the Day 0 (C) and Day 7 (D) samples. Grey = Log2 Fold change < 1; Pale red and blue = Log2 Fold change ≥ 1 and < 20 for Day 0 and Day 7, respectively; Red and blue = Log2 Fold change ≥ 20 for Day 0 and Day 7, respectively.

Table 3-3. Unique highly accessible super-enhancer genes with important related function. List of genes that corresponded to highly accessible chromatin peaks specific to each sample (Day 0 vs Day 7) in the ATAC-sequencing data.

SYMBOL	GENE NAME	Function	ENRICHED	REFERENCE
JAZF1	JAZF Zinc Finger 1	Role in glucose homeostasis, mediates metabolic stress via p53 stress pathways	Day 0	Kobiita et al. 2020
KRT71, KRT72, KRT74	Keratin	NK cells control tumor architecture through NKp46; potentially affect EMT genes such as keratins	Day 0	Dyck and Lynch 2018
CD86	CD86 molecule	NK activation receptor; Target molecule in CTLA4Ig-mediated enhancement of NK cytolytic activity	Day 7	Peng et al. 2013
ADAM19	ADAM Metallopeptidase Domain 19	Upregulated in cytokine-activated NK cells with IL12 and IL18; activation-independent TGFβ-responsive signature	Day 7	Sitrin et al. 2014
LDLRAD4	Low Density Lipoprotein Receptor Class A Domain Containing 4	Negative regulator of TGFβ signaling; TGFβ negatively affects NK cell function	Day 7	Ito et al. 2020; Regis et al. 2020
NSMCE2	NSE2 (MMS21) Homolog, SMC5-SMC6 Complex SUMO Ligase	Complex is required for telomere maintenance via recombination in ALT (alternative lengthening of telomeres) cell lines	Day 7	Jacome et al. 2015

Given that there were super-enhancers common to both timepoints (2,280 duplicate associated genes of the total 3,904 super-enhancers between both samples), levels of chromatin accessibility may be key in determining specific super-enhancers regulating each phenotype. To compare chromatin accessibility between super-enhancers, the log2 of the ratio between Day 0 and Day 7 ATAC-seq signal was computed. This analysis identified 2 differentially highly accessible genes in Day 0: *JAZF1* and keratin genes (*KRT71*, *KRT72*, *KRT74*). GO analysis of all genes with log2 ratio > 2 resulted in enriched terms for antigen processing and presentation, negative regulation of NK cell cytotoxicity, and Type I interferon signal pathway at Day 0 (data not shown). For the highly accessible peaks and associated genes at Day 7, there were 36 genes that had a log2 ratio > 20. Of those highly accessible genes, four had potentially key functions for K562-mediated activation: *CD86*, *ADAM19*, *LDLRD4*, and *NSMCE2* (Figure 3-7D). GO analysis of the log2 ratio > 2 genes for Day 7 resulted in enriched biological processes including positive regulation of IL-2 and IL-4 production, T cell co-stimulation, positive regulation of T cell

proliferation, and regulation of leukocyte differentiation (data not shown). This data suggests the super-enhancers with highly accessible chromatin play a major role in K562-mediated activation of NK cells (Table 3-3).

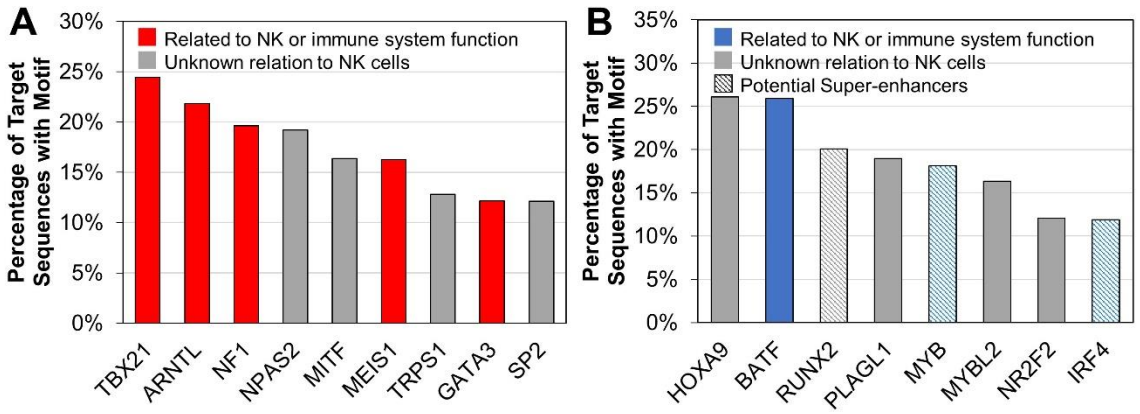

Figure 3-8. Transcription factors enriched in each activation state. (A-B) Highly expressed motifs from accessible peaks that correspond to transcription factors (TFs) enriched at Day 0 (A) and Day 7 (B). Transcription factors with related NK function at Day 0 (red) and Day 7 (blue). Pattern fill indicated that selected TFs to also be potential super-enhancers. Filtering criteria: % of Target Sequences with motif > 10%, P Value < 0.05, Preferential enrichment factor using average of RNA-sequencing TPM values for comparison > 1.5.

Table 3-4. Unique transcription factors with important related function. List of genes that corresponded to highly active transcription factors specific to each sample (Day 0 vs Day 7) in the ATAC-sequencing data.

SYMBOL	GENE NAME	Function	ENRICHED	REFERENCE
TBX21	T-Box TF 21	Role in IFNγ production and regulation of cytotoxicity in NK cells	Day 0	Marcenaro et al. 2016
ARNTL	Aryl Hydrocarbon Receptor NTL	Highly expressed in immature NK cells and enhances secretion of an array of cytokines including IFNγ	Day 0	Moreno-Nieves et al. 2018
NF1	Neurofibromin 1	Negative regulator of the Ras signal transduction pathway which affects NK cell-mediated cytotoxicity and IFNγ production; impairs NKT cytotoxicity	Day 0	Yuan et al. 2018; S. H. Lee et al. 2009
MEIS1	Meis Homeobox 1	Crucial regulator of early hematopoietic differentiation; immature marker	Day 0	Zeddies et al. 2014
TRPS1	Transcriptional Repressor GATA Binding 1	Represses GATA-regulated genes, which play a role in NK maturation	Day 0	Samson et al. 2003; Y. Wang et al. 2018
GATA3	GATA Binding Protein 3	Promotes maturation, IFN-gamma production, and liver-specific homing of NK cells	Day 0	Samson et al. 2003
BATF	Basic Leucine Zipper ATF-Like TF	Regulates activation of innate and adaptive immune systems	Day 7	Costanzo et al. 2018; Campbell et al. 2015

MYB	MYB Proto-Oncogene, TF	Role in NK cell activation, specifically the secretion of IFNγ and the cytotoxicity of NK cells.	Day 7	Shin, Lee, and Kim 2018
IRF4	Interferon Regulatory Factor 4	Regulates activation of innate and adaptive immune systems	Day 7	Costanzo et al. 2018; Campbell et al. 2015

ATAC-sequencing peaks were combined into reproducible peaks among the different activation states (Day 0 and Day 7, excluding the Day 7 unstim sample). There were 190,092 peaks in the Day 0 sample and 237,604 peaks in the Day 7 sample. From these reproducible peaks, differentially accessible peaks were identified with the following criteria: Fold-change cutoff =2, and q <0.01. At Day 7, there were 36,977 peaks upregulated, and 10,648 peaks were upregulated at Day 0. Motif finding on each peak set identified differentially active transcription factors from a list of 440 transcription factor motifs and generated p-value-ranked lists for differentially accessible peaks in Day 7 vs Day 0. To find the most significant transcription factors at each timepoint, the transcription factor list was filtered by p value < 0.05. Furthermore, if the target motif percentage was > 10% (highly expressed) and the ratio of average TPM values for Day 0/Day7 > 1.5 (preferentially expressed at Day 0) then the transcription factor was considered enriched at Day 0. The addition of the RNA-sequencing data as a filtering criterion helped eliminate potential false positives given than some of the motifs are very similar and the enrichment seen in one transcription factor could be attributed to a different one. The RNA-sequencing results validated that the identified transcription factors were turned on and enriched. This same method was applied to the Day 7 samples with the ratio of average TPM values for Day 7/Day0 > 1.5 (preferentially expressed at Day 7).

For the pre-activation Day 0 samples, 9 differentially activated transcription factors were identified (Figure 3-8A). Of interest, four of those transcription factors affected NK cell function or the immune system: *TBX21*, *ARNTL*, *MEIS1*, and *GATA3* (Table 3-4). For the K562-activated Day 7 samples, 8 differentially activated transcription factors were identified (Figure 3-8B). Three of those transcription factors affected NK cell function or the immune system and of those, two were also found to be related to super-enhancer peaks: *MYB*, *IRF4*. *RUNX2* was also found to be related to the super-enhancer peaks (Table 3-4). These transcription factors related to the super-enhancer peaks, especially those associated

with NK cell function (Table 3-4), may be potential master regulators of the K562-activated Day 7 phenotype. As such, these transcription factors are promising targets for small molecule regulation as a replacement for K562 aAPCs in the culture protocol.

3.3 Discussion

Activation is a key component in NK cell proliferation, maturation, and functionality. However, unlike T cells which have specific activation signals (Rosenberg and Huang 2018), NK cells are activated through a host of activating and inhibitory receptors (Vivier et al. 2011; Shimasaki, Jain, and Campana 2020; Paul and Lal 2017). Given the numerous receptor-ligand interactions between NK cells and target cancer cells, it is difficult to determine the most critical contributing receptors for activation. Furthermore, some activating receptors such as CD16, NKG2D, and NKp46 (Bryceson et al. 2006; Bryceson, Ljunggren, and Long 2009), do not active by themselves and often require co-stimulatory signals to synergistically activate NK cells (Long et al. 2013). As such identifying the key activation signals is challenging.

In attempt to gain a better understanding of NK activation upon stimulation with K562 aAPCs, whole transcriptome analysis and the changing epigenetic landscape were evaluated in pre-activation Day 0 samples and K562-activated Day 7 samples. This analysis revealed a shifting transcriptional and epigenetic signature. So far, there has been limited work performed to understand NK activation (Costanzo et al. 2018; Bezman et al. 2012; A. R. Campbell et al. 2015) and even less has been done to understand the epigenetic signature of activated NK cells (K. Li et al. 2019; Lau et al. 2018). Here, we attempted to expand on this limited knowledge and identified a shift in NK cell functionality upon K562 aAPC activation towards perhaps an intermediate stage between a cytokine-producing phenotype and cytotoxic phenotype. This intermediate stage seems to have similar characteristics to NKT cells, which are a subset of T cells with similar effector functions and characteristics as NK cells (Godfrey et al. 2000).

One of the differences between the pre-activation Day 0 samples and K562-activated Day 7 samples was the way activation was regulated. The Day 0 transcriptional and epigenetic landscape indicated enrichment of genes related to the regulation of activation, specifically in modulating the activation response through *CASP8*, *CD247*, and *TBX21*

(Feng et al. 2019; Valés-Gómez et al. 2016; Lau et al. 2018). Day 0 samples downregulated *ITGB2*, which can result in a hyporesponsive phenotype (Crozat et al. 2011), indicating the pre-activation Day 0 cells were at a resting state, which is expected. Unsurprisingly, genes related to IL-2 stimulation (*SLAMF7, CD244, CD97/ADGRE5*) were also enriched in the Day 0 signature given the cells recovered overnight in the presence of IL-2 (Costanzo et al. 2018; Giuliani et al. 2018; Kop et al. 2009; Agresta, Hoebe, and Janssen 2018). At Day 7, genes related to IL-12 or IL-18 stimulation (*ADAM19, BATF, IRF4*) were enriched (Sitrin et al. 2014; Costanzo et al. 2018; Lau et al. 2018; A. R. Campbell et al. 2015), which was unanticipated given that neither IL-12 nor IL-18 were added to the culture. However, it is possible that K562 cells activate NK cells through some similar pathways as IL-12 and IL-18 stimulation.

GO analysis revealed that metabolic pathways such as glycolysis and oxidative phosphorylation were upregulated at Day 7. However, terms related to purine metabolism were also upregulated, which was interesting given that studies have shown that NK proliferation can be regulated through purine receptors by adenine nucleotides. Purine receptor signaling can influence chemotaxis and NK-cell mediated killing in response to different chemokines (Jacob et al. 2013; Miller et al. 1999). Furthermore, GO terms related to the fatty acid metabolism were upregulated in the K562-activated Day 7 samples. While not much is known about the relation between fatty acid metabolism and NK cells, it is not known to be an essential fuel. However, fatty acid metabolism is downstream of *SREPB*, which is crucial for metabolic responses in cytokine-activated NK cells, indicating that the fatty acid metabolism may play a more central role in initial NK activation as a consequence of *SREPB* activation (O'Brien and Finlay 2019).

The translational and epigenetic signature for Day 0 was primarily a cytokine-producing phenotype. Differential gene expression analysis indicated *AXL* and *CD160*, which are key for cytokine signaling/production (Park et al. 2009; Kim et al. 2017; Le Bouteiller et al. 2011), were enriched at Day 0, while GO analysis indicated that cytokine production was upregulated in the pre-activation Day 0 samples. Highly accessible genes that are involved in cytokine production included *ARNTL*, an aryl hydrocarbon receptor that upon IL-2 stimulation can increase secretion of key cytokines including IFN-γ, TNF-

α, MIP-1β, LT-α and GM-CSF (Moreno-Nieves et al. 2018). K562-activated Day 7 samples did not have genes related to cytokine production among the top highly accessible genes.

Cytotoxic potential, which is an effect of activation, appeared to be upregulated in the pre-activation Day 0 samples compared to the K562-activated samples according to the GO analysis. However, IL-6 and TGFβ signaling, which decrease expression of activation markers such as NKp30 and NKG2D (Cifaldi et al. 2015; Wu et al. 2019) and induce NK cells to have a less cytolytic phenotype, respectively (Regis et al. 2020), were also upregulated at Day 0. Furthermore, the epigenetic signature of the pre-activation samples suggests that the highly accessible chromatin regions belong to genes that depress cytotoxicity (*PDE4C*, *CD97*, *FMOD*, *NF1*). *FMOD* is known to inhibit NFκB signaling (Y.-H. Lee and Schiemann 2011) which affects release of perforin (Zhou, Zhang, Lichtenheld, and Medows 2002), while *PDE4C* is known to induce cAMP signaling (Fertig and Baillie 2018), which inhibits NK cytotoxicity (Ullberg et al. 1983). Moreover, *CD97* and *FN1* are attributed to decrease in cytolytic effector function in NK cells and NKT cells (Kop et al. 2009; Yuan et al. 2018). Alternatively, at Day 7 one of the highly upregulated genes was *PRDX1* which is a cell killing gene upregulated in expanding NK cells (Un Park et al. 2010). *CD86*, *LDLRAD4*, and *MYB* were among the highly accessible genes in the Day 7 epigenetic signature. *CD86* and *MYB* is are activation markers that play a role in cytotoxicity (Peng et al. 2013; Shin, Lee, and Kim 2018), while *LDLRAD4* inhibits TGFβ signaling (Ito et al. 2020), thus enhancing NK cytotoxicity at Day 7.

Interestingly, what was common in both samples was upregulation of genes related to IFNγ production. IFNγ is secreted by NK cells and has functions relating to NK cytotoxicity and modulate anti-tumor immunity (Paul and Lal 2017), which may be why the NK cytotoxicity pathways were upregulated at Day 0 in the GO analysis but not so much in the epigenetic signature. For Day 0 samples, *ARNTL*, *CD160*, and *GATA3* were enriched to promote IFNγ production (Moreno-Nieves et al. 2018; Samson et al. 2003; Tu et al. 2015), while at Day 7, *BHLHE40*, *ZBTB16*, and *MYB* were enriched (Kanda et al. 2016; Savage et al. 2008; Shin, Lee, and Kim 2018).

The transcriptional and epigenetic signature that was unique to pre-activation Day 0 samples included genes that played a role in the NK cell memory phenotype (*JUND, CD97)* (Lau et al. 2018; Kop et al. 2009), cell adhesion (*ITGB2*) (Crozat et al. 2011), potential remodeling of the tumor microenvironment (keratin genes) (Dyck and Lynch 2018), exhaustion (*CD244*) (Agresta, Hoebe, and Janssen 2018), and anti-proliferation (*BTG2*) (Tsui et al. 2018). All these genes are potential targets to downregulate through small molecule inhibition in order to push NK cells towards the transcriptional and epigenetic phenotype seen in the Day 7 samples in the absence of K562 stimulation.

The K562-activated Day 7 signature seemed to be perhaps more closely related to NKT cell phenotype. Flow cytometry analysis also indicated a large percentage of NK cells were positive for CD7 (data not shown), which has been found to play a role in NK cell proliferation, regulation of adhesion, secretion of IFNγ, and enhancement of cytotoxicity against K562 targets (Rabinowich et al. 1994). Of the highly accessible genes at Day 7, *BHLHE40, TRGs, ZBTB16,* and *BATF* were also known to be upregulated in T cells or played a role in NKT effector functions (Kanda et al. 2016; Savage et al. 2008; A. R. Campbell et al. 2015; Lau et al. 2018; Costanzo et al. 2018). Day 0 samples also had a few highly accessible genes that related to functionality in the T cell phenotype (*CD247,JUND, NF1*) (Valés-Gómez et al. 2016; Lau et al. 2018; Yuan et al. 2018), but none were specific to a T cell phenotype like the Day 7 samples.

One uniquely highly accessible Day 7 gene was *NSMCE2*, which plays a role in telomere maintenance. This gene was of interest since K562 aAPCs express mbIL-21, which is known to prevent shortening of telomeres even after repeated stimulation (Denman et al. 2012). This indicates that telomere maintenance may play an important role in activation and should be maintained in order to facilitate multiple simulations without reaching an exhausted phenotype.

Of all the genes and transcription factors investigated in the transcriptional and epigenetic landscapes of the pre-activation and K562-activated samples, the most compelling ones were *RUNX1, MYB,* and *IRF4*. These transcription factors are of particular import because they correspond to super-enhancers enriched at Day 7. *MYB* and *IRF4* are interesting due to their known role in NK cell activation (Shin, Lee, and Kim

2018; A. R. Campbell et al. 2015). *BATF* in conjunction with *IRF4* are of particular interest given that these genes cooperatively interact with each other and were found to be upregulated in activated NK cells from previous transcriptional analysis data (Costanzo et al. 2018; A. R. Campbell et al. 2015).

3.4 Conclusions

Overall, the transcriptional and epigenetic landscapes of the pre-activation and K562-activated samples indicate a shift in regulation of activation from IL-2 stimulation to similar pathways of IL-12/18 stimulation, from a cytokine-producing phenotype with reduced cytotoxic capabilities to perhaps the beginning stages of a more cytotoxic phenotype, a switch away from a memory-like phenotype, and a switch towards an NKT phenotype. Some transcription factors (*RUNX1*, *MYB*, *IRF4*) seem to be more important than others for the regulation of the K562-activated phenotype given that they are also potential super-enhancers, which makes these factors ideal targets to activate in place of K562 stimulation in future studies.

4 Characterization of NK cells undergoing repeated K562 Stimulation

4.1 Introduction

Allogeneic adoptive NK cell transfer is an emerging cell therapy due to the progress made in recent clinical studies (Gill, Olson, and Negrin 2009; Terme et al. 2008; Shimasaki, Jain, and Campana 2020; Dahlberg et al. 2015). However, the limitations of this type of therapy revolve around the difficulty in obtaining the necessary number of cells to produce a viable off-the-shelf therapy (Klingemann and Martinson 2004). NK cells have been shown to be a promising candidate for allogenic therapy given NK cells have a reduced risk of Graft-vs-Host disease upon implantation compared to other cell-based therapies (Farag et al. 2002; Olson et al. 2010; Terme et al. 2008).

One challenge of allogeneic NK cell therapy is the ability to achieve sufficient quantity of NK cells to generate enough material for multiple patients to attain an economical "off-the-shelf" therapy. Current methods of expansion require continuous cytokine stimulation and multiple rounds of stimulation with feeder cells such as K562-mbIL-21 aAPCs to reach sufficient cell numbers (Denman et al. 2012; X. Wang et al. 2012). There is concern that chronic stimulation may result in NK cell exhaustion (Bi and Tian 2017), thus negatively impacting NK cell efficacy post expansion.

In this chapter, rigorous assessment of the expansion potential via K562 aAPCs was evaluated through growth kinetics and shifts in cell cycle status. Functionality of NK cells was investigated by testing killing potential through expression of CD107a, perforin, granzyme, and IFNγ. Expression of these markers aide in the killing process as well as tumor killing ability against different cancer cell lines including a lung carcinoma (A549 cells), pancreatic cancer (PANC1 cells), and ovarian cancer (SKOV3 cells). Furthermore, our data was supplemented by Seahorse metabolic assays to assess mitochondrial metabolism and glycolytic metabolism dynamics over time. RNA-sequencing was used to understand the transcriptional changes occurring during the prolonged culture and to enhance understanding of the signaling pathways active during the different growth phases of the culture.

4.2 Results

4.2.1 A shift in growth kinetics is observed during sustained NK cell expansion

NK cells from four donors were expanded via eight rounds of weekly stimulation with K562 aAPCs (Figure 4-1A,B). The K562 cell line used in all of these studies is a previously established line engineered to express CD137L (4-1BBL) in addition to membrane-bound IL-21 (Suhoski et al. 2007; Singh et al. 2011). This K562 variant was utilized because it has previously been shown to be able to achieve more extensive fold-expansion of K562 cells than other K562 variants and thus is of interest for clinical biomanufacturing (Denman et al. 2012; X. Wang et al. 2012). All four donors exhibited a shift to slower cell growth around Day 26 of expansion (Figure 4-2A). The average

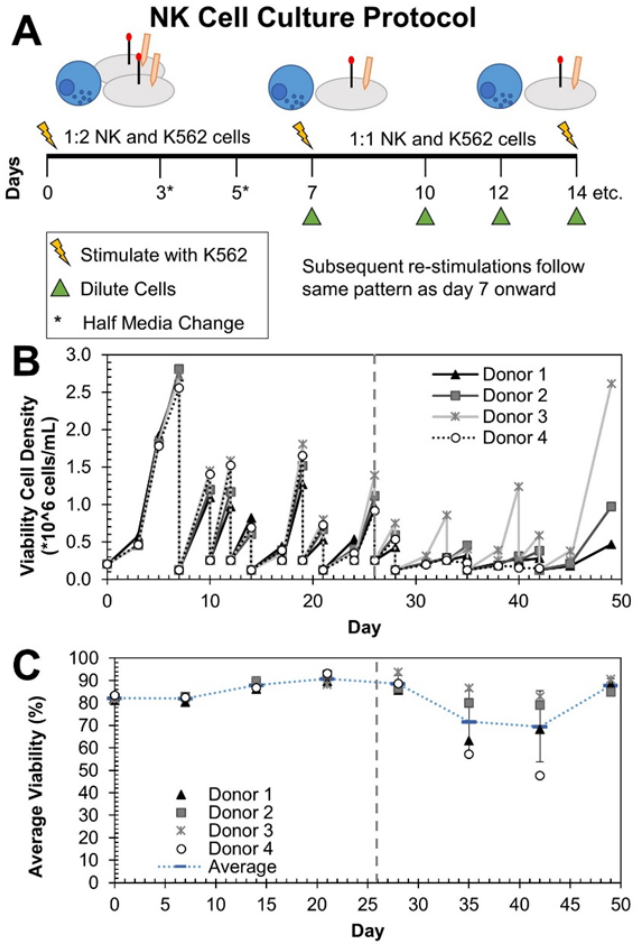

Figure 4-1. NK Cell Culture. (A) NK cell culture protocol. (B) Viable cell density of the NK cell culture over time for each donor. (C) Average percent viability (for each donor as well as the average across donors) every 7 days, immediately prior to addition of K562 aAPCs.

doubling time for the four donors during the first growth phase was 1.23 ± 0.04 days (median 1.23 days) (Figure 4-2B), which is the expected doubling time for healthy expanding NK cells in culture (Liu et al. 2013). During the slower growth phase, the average doubling time increased to 4.0 ± 2.33 days (median 3.86 days). Analysis of cell cycle distribution via flow cytometric analysis of propidium iodide staining showed a statistically significant decrease in the percentage of cells in S phase and increase in the percentage of cells in G0/G1 phase of the cell cycle after Day 26 (Figure 4-2C,D). Average NK cell viability remained at approximately 80-90% throughout the first growth phase, but decreased to 60-70% at Day 35 before recovering at the end of the culture (Figure 4-1C).

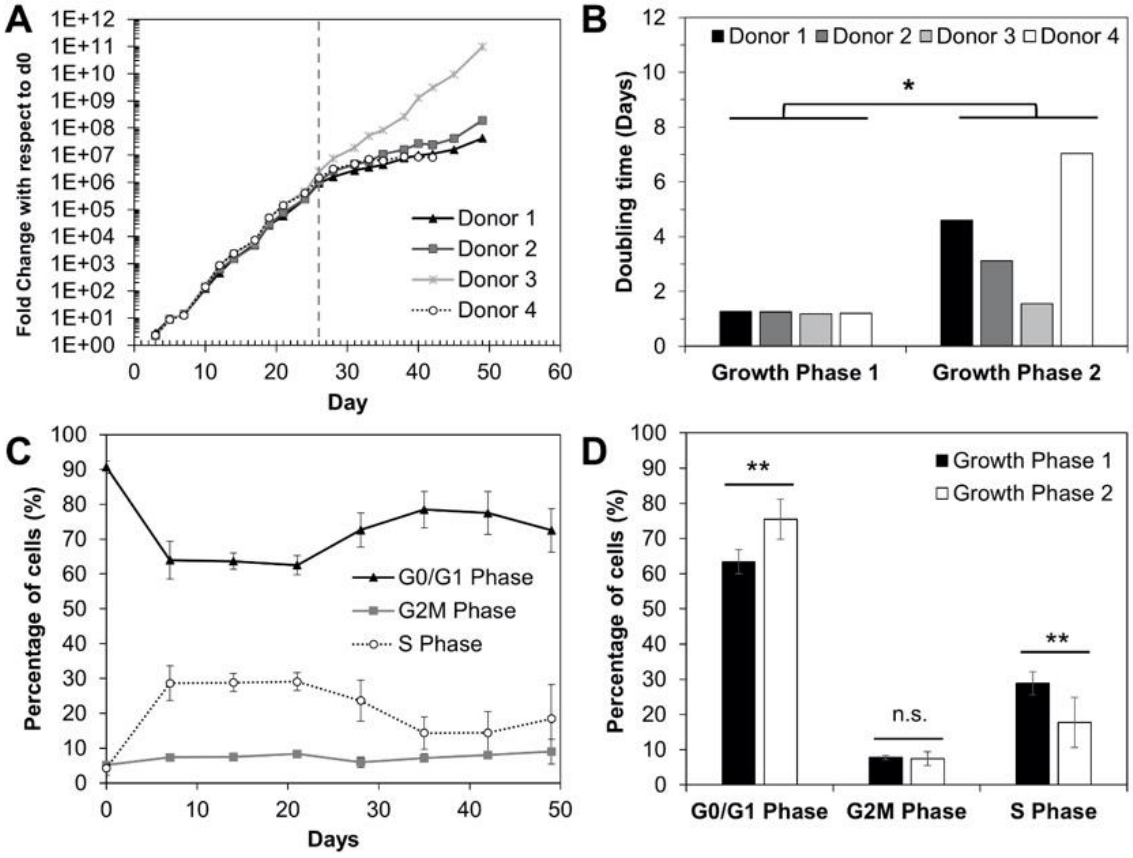

Figure 4-2. Characterization of growth kinetics during NK cell expansion. (A) Cumulative fold change over time for each donor (n=4); dashed line indicates the transition to a slower growth phase at day 26. (B) Doubling time in days for growth phase 1 (prior to day 26) and growth phase 2 (after day 26) for each donor. * $P < 0.05$ for growth phase 2 compared to growth phase 1. (C) Average percentage of cells in each cell cycle phase (G0/G1 phase, G2M phase, and S phase) from flow cytometric analysis of PI staining. (D) Average percentage of cells in each cell cycle phase for the different growth phases. Student's t tests were used to determine statistical significance in each cell cycle phase. * $P < 0.05$, ** $P < 1E-4$, n.s., not significant.

4.2.2 Metabolic shifts during NK cell expansion

NK cell metabolism was assessed by monitoring oxidative mitochondrial metabolism and glycolysis using Seahorse assays. Oxygen consumption rate (OCR) was measured at the basal state after the addition of an inhibitor of ATP synthesis (oligomycin), the maximal state after the addition of FCCP, which uncouples ATP synthesis from the electron transport chain, and the minimal state after addition of rotenone and antimycin A, which interfere with complexes I and III of the electron transport chain, respectively (Gerencser et al. 2009). NK cells exhibited lower basal, maximal, and ATP-linked respiration prior to stimulation (Day 0) as well as at the final timepoint (Day 49) (Figure 4-3A,B,C). However, there was no distinct shift in oxidative mitochondrial metabolism associated with the switch to slower growth kinetics. Oxygen uptake rate (OUR) results also indicated lower metabolic activity at Day 0 and Day 49 relative to the other timepoints (Figure 4-3D).

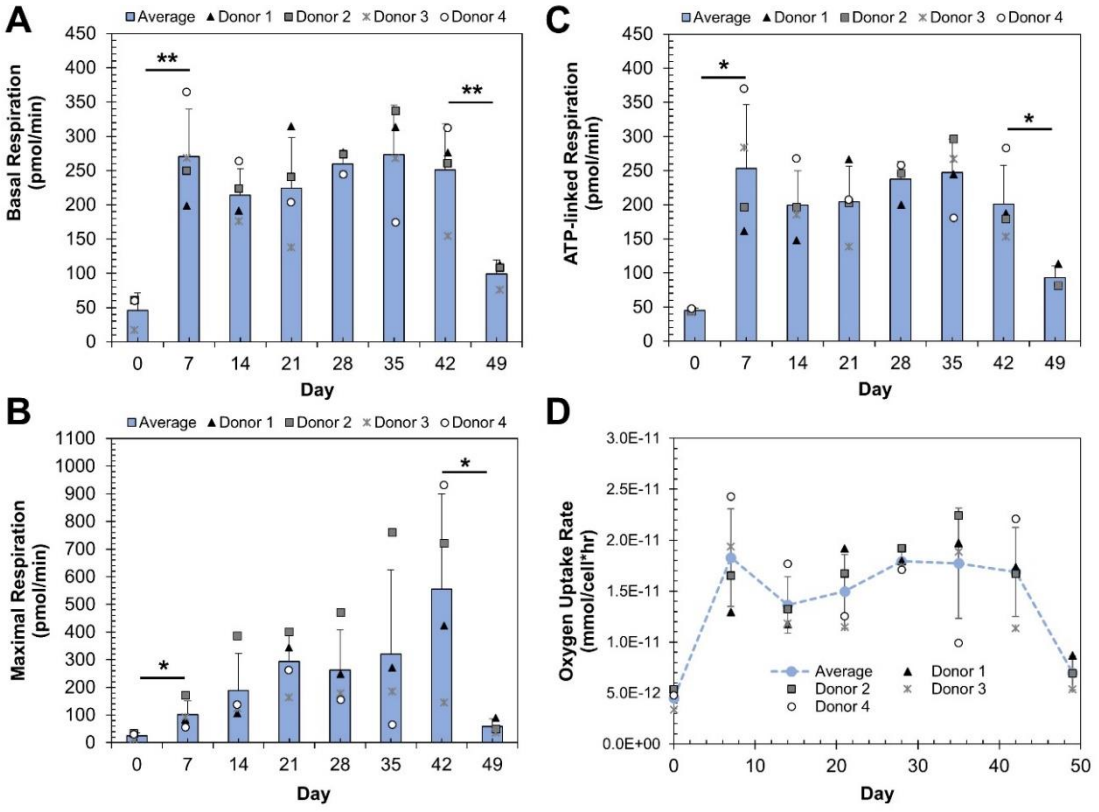

Figure 4-3. Characterization of mitochondrial metabolism during NK cell expansion. (A-C) Seahorse OCR profiles for each donor and average across all donors (blue bars) for basal respiration (A), maximal respiration (B), and ATP-linked respiration (C). (D) Oxygen uptake rate over time for each donor as well as the average across all donors. Student's t test was performed to determine if there was a change in OCR values from one day to the next.

To assess NK cell glycolytic metabolism, basal glycolysis, glycolytic capacity, and glycolytic reserve were calculated from the extracellular acidification rate (ECAR) (Figure 3A,B,C). ECAR values were measured at the basal level, maximal state after addition of glucose, and maximal state after the addition of oligomycin. Glycolytic metabolism showed a similar trend as oxidative mitochondrial metabolism, with the lower activity in prior to activation (Day 0) and at the final timepoint (Day 49) (Figure 4-4A,B,C). Furthermore, the basal glycolytic measurements showed a spike in the level of glycolysis at Day 7 after initial activation followed by lower yet steady levels through Day 42 (Figure 4-4 A), indicating a reduction in glycolysis after the first week of activation. To further assess metabolic flux, a ratio of lactate production to glucose consumption was measured (data not shown).

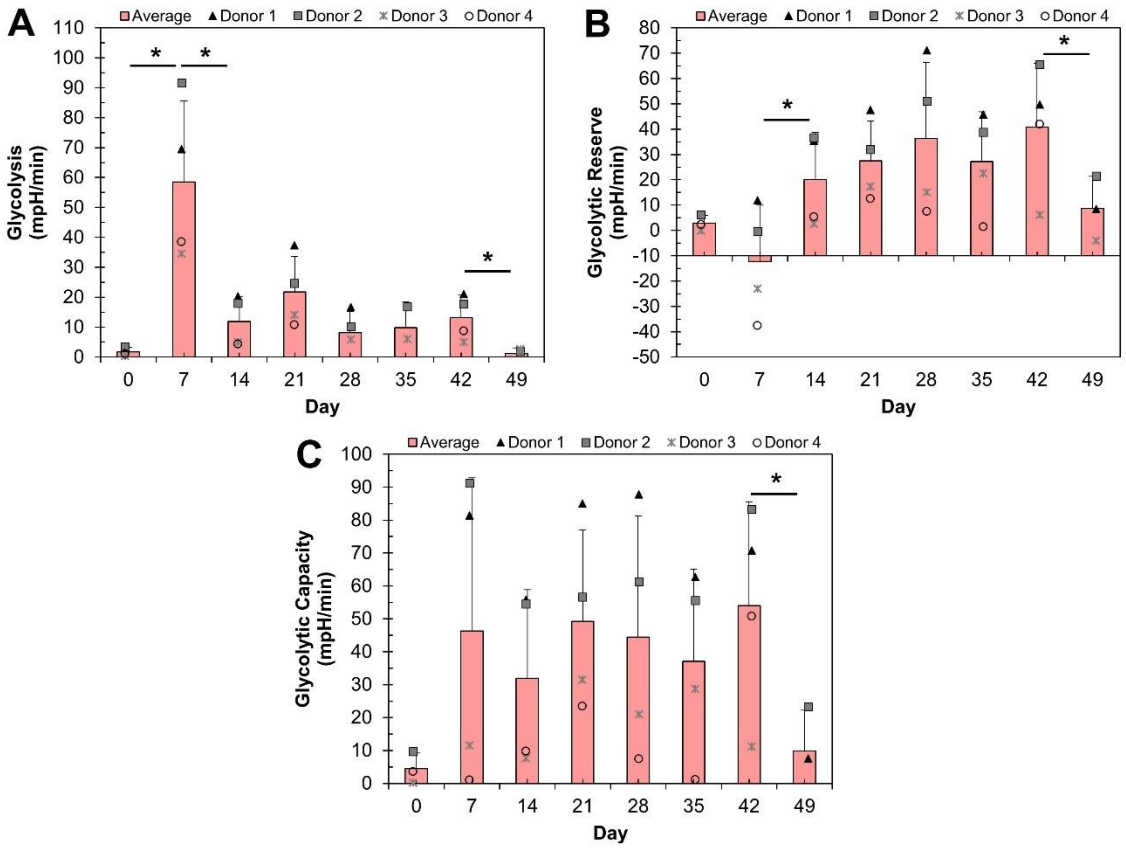

Figure 4-4. Characterization of glycolytic metabolism during NK cell expansion. (A-C) Seahorse ECAR profiles for each donor and average across all donors (red bars) for maximal glycolysis (A), glycolytic capacity (B), and glycolytic reserve (C). Error bars represent SD. Student's t test was performed to determine if there was a change in ECAR values from one day to the next. * $P < 0.05$.

4.2.3 Functional analysis of NK cells during expansion

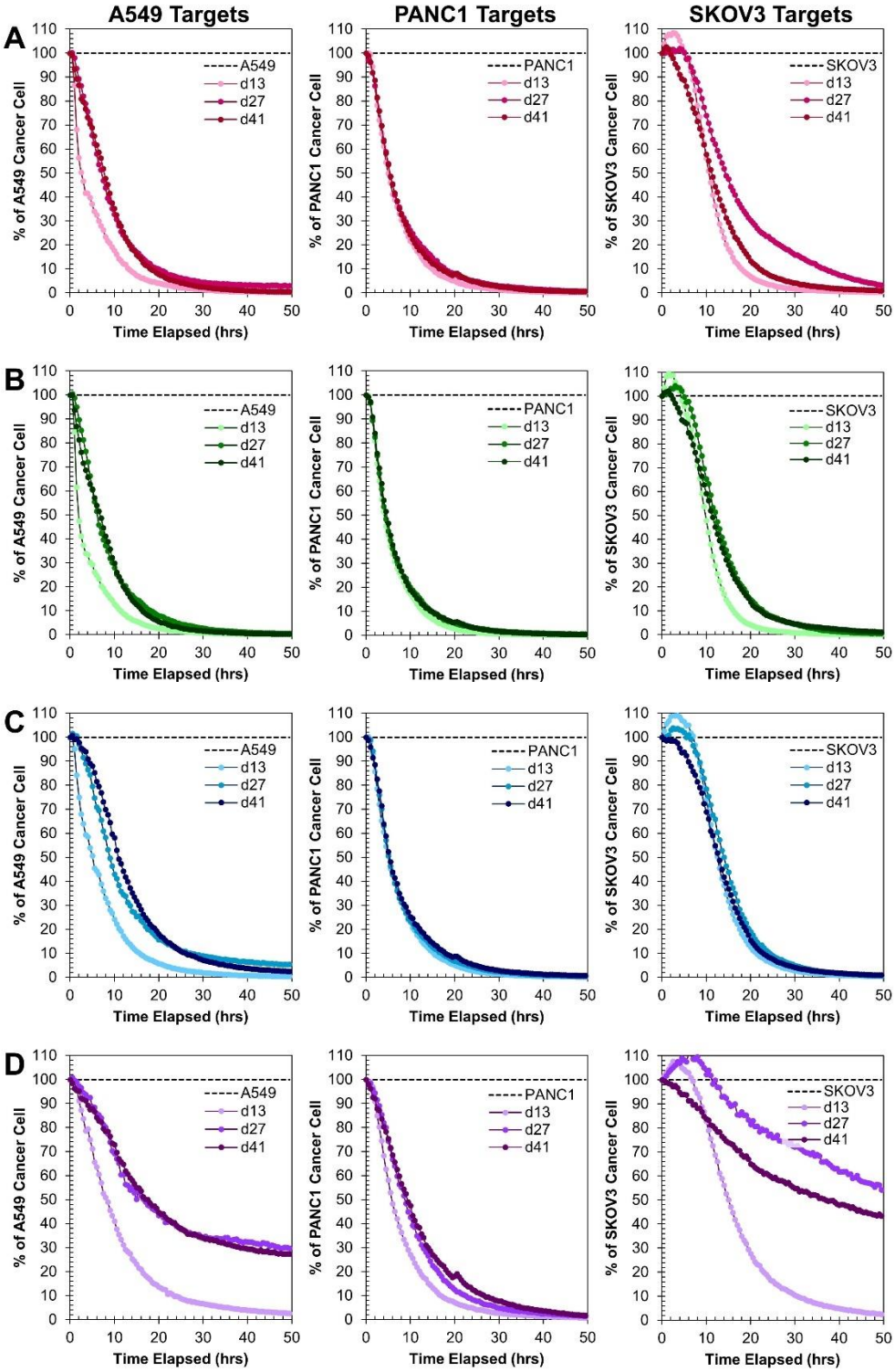

Figure 4-5. Characterization of killing kinetics of cancer cell lines by expanded NK cells. NK cells from Donors 1-4 (A-D) were incubated with A549 cells (left column), PANC-1 cells (center column), and SKOV-3 cells (right column) expressing NucLight Red fluorescent protein at a 10:1 ratio.

To evaluate NK cell functionality, their ability to kill cancer cells was evaluated using the IncuCyte® Live Cell Analysis System at three timepoints during the expansion process: Day 13 (normal growth phase), Day 27 (transition between normal and slowed growth), and Day 41 (slowed growth phase). The results demonstrated slower killing kinetics at Day 27 and Day 41 relative to Day 13 for many of the donors and target cancer cell lines, indicating that the slowed growth phase may be associated with changes in cytotoxic function (Figure 4-5).

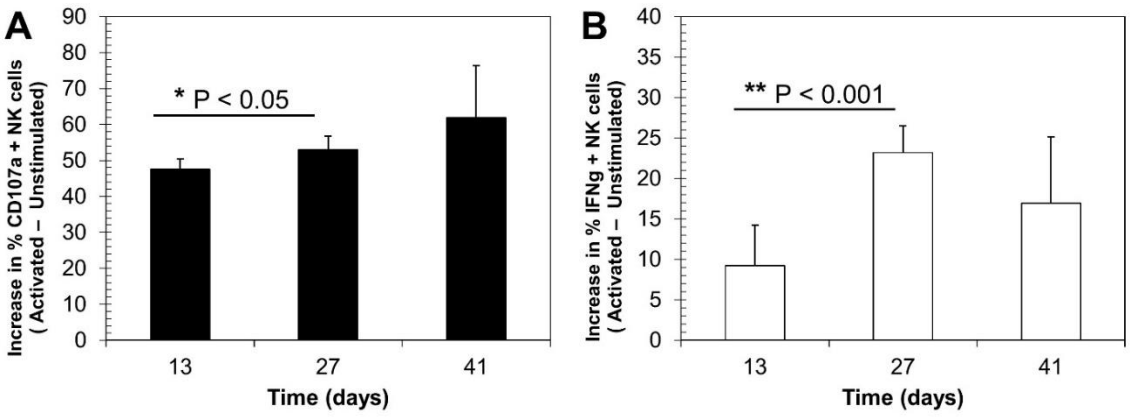

Figure 4-6. Degranulation assay to assess cytotoxicity potential of expanded NK cells. NK cells were incubated at a 1:1 ratio with and without naïve K562 cells for 3.5 hours. Flow cytometry was used to analyze the percent of live CD56+ NK cells expressing CD107a (A) and IFNγ (B) at Days 13, 27, and 41. Average results from Donors 1-4 are shown at each timepoint. Error represents SD. * $P < 0.05$, ** $P < 0.001$

A significant increase in CD107a and IFNγ expression upon stimulation was also observed at the later timepoints compared to Day 13, although the increase relative to Day 13 were only statistically significant at Day 27 (Figure 4-6). Weekly analysis of perforin B and granzyme expression immediately prior to re-stimulation showed that the percentage of NK cells expressing perforin and granzyme B decreased during the slowed growth phase (Day 28 – 49) (Figure 4-7). Collectively, these results demonstrate that the shift in growth kinetics may be associated with changes in NK cell cytotoxic functionality.

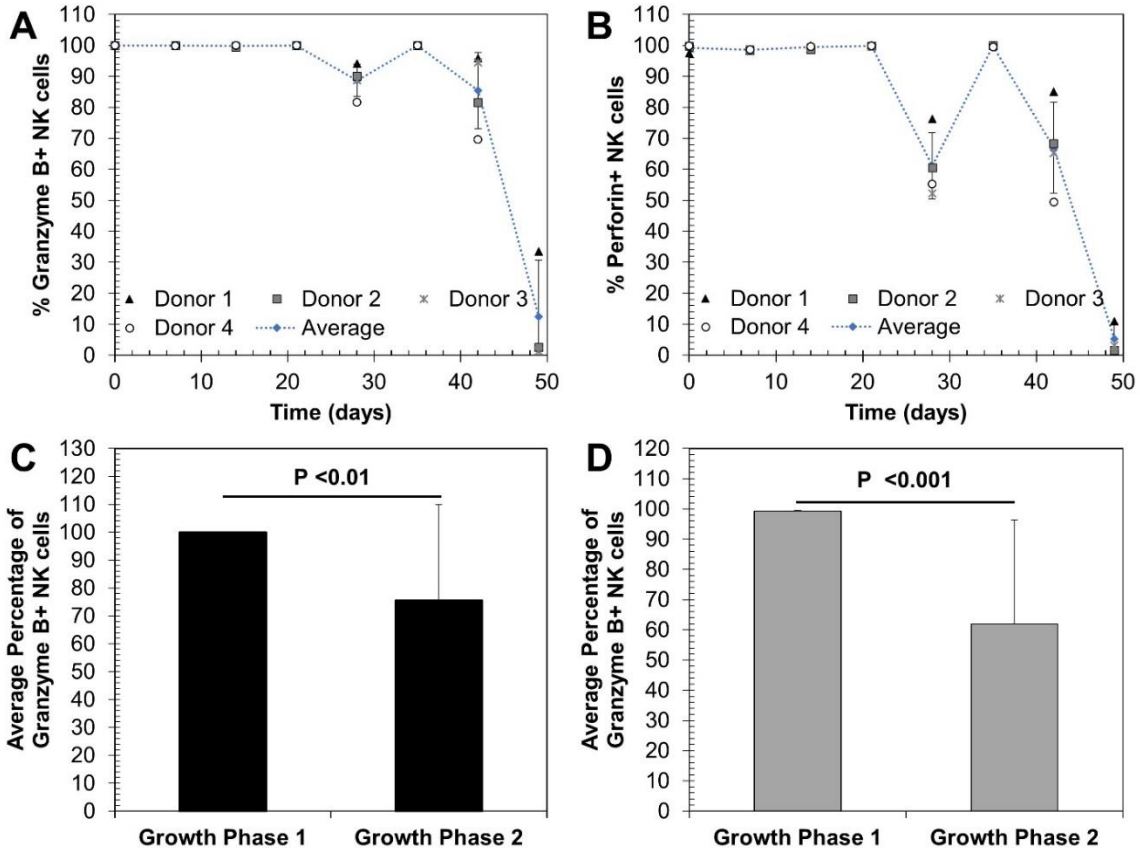

Figure 4-7. Assessment of cytotoxic functionality by flow cytometry. (A,B) Percentage of live CD56+ NK cells that express granzyme B (A) and perforin (B) prior to each re-stimulation. (C,D) Averaged results for granzyme B (C) and perforin (D) expression across all donors (1-4) for the first growth phase (Day 0 - Day 21) and second growth phase (Day 28 - Day 49). Error represents SD.

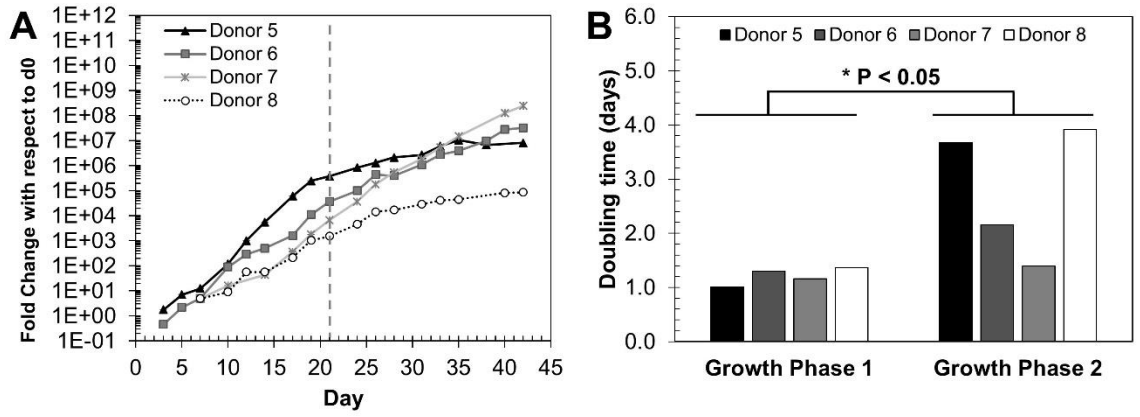

Figure 4-8. Growth kinetics of transcriptome analysis samples. (A) Characterization of growth kinetics during NK cell expansion for Donors 5-8. (A) Cumulative fold change over time for each donor; dashed line indicates the transition to the slower growth phase at day 21. (B) Doubling time in days for growth phase 1 (prior to day 21) and growth phase 2 (after day 21 for each donor. * $P < 0.05$.

4.2.4 Transcriptomic analysis of NK cells in early and late stage culture

RNA sequencing was performed on NK cells from four donors (Donors 5-8) at Day 0 (pre-activation control), Day 7 (normal growth phase), and Day 35 (slowed growth phase). Consistent with the first four donors, these donors exhibited a shift to slower cell growth during the culture, with the transition occurring at Day 21 (Figure 4-8). Hierarchical clustering (Figure 4-10A) and principal component analysis (Figure 4-10B) were performed to assess the similarity in overall gene expression for each donor and timepoint, with both methods indicating that samples clustered together by timepoint rather than by donor. Analysis of differentially regulated genes between timepoints indicated that 1,770 genes that differentially up- or down-regulated in both Day 7 and Day 35 samples compared to Day 0, while 149 genes and 101 genes were uniquely up- or down-regulated at Day 7 and Day 35, respectively (Figure 4-9). These results indicated that there is a relatively small subset of genes that distinguish the cells in normal vs. slowed growth phases.

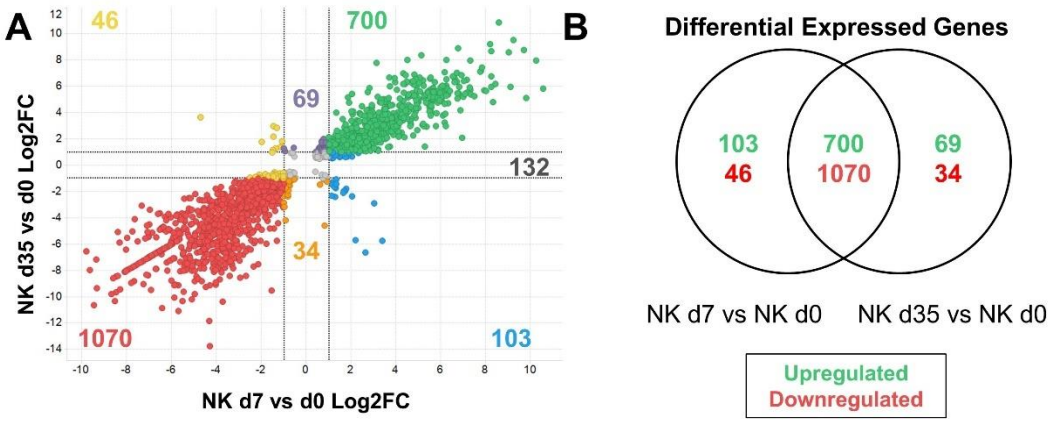

Figure 4-9. Differential gene expression analysis of expanded NK cells. (A) Quadrant analysis showing the pairwise comparison of activated NK cells at day 7 (growth phase 1) and day 35 (growth phase 2), both normalized to day 0. Plot shows genes that are commonly upregulated (green) and downregulated (red) between the pairwise comparison. Genes that are differentially expressed between the pairs are shown as follows: upregulated (blue) and downregulated (yellow) genes for day 7 vs. day 0, and upregulated (purple) and downregulated (orange) genes for day 35 vs. day 0. Grey indicates insignificantly expressed genes. (B) Venn diagram of the differentially expressed genes for the pairwise comparison. Pairwise comparison criteria for differentially expressed genes included: log2 fold change expression > 1 or < -1, p-value < 0.05, and FDR < 0.05.

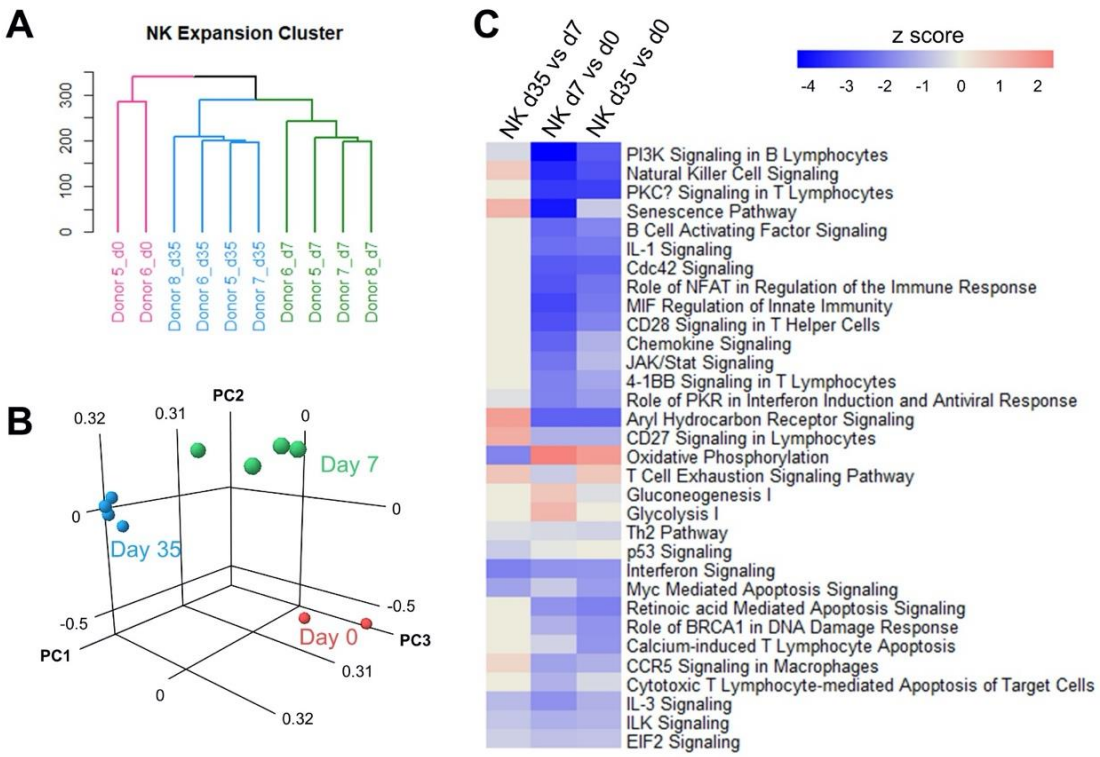

Figure 4-10. RNA sequencing analysis of NK cells in different growth phases. (A-B) Hierarchical clustering (A) and principal component analysis (B) of RNA sequencing analysis for Donors 5-8, which include non-activated samples (Day 0) and activated samples from the normal growth phase (Day 7) and slowed growth phase (day 35). (C) List of important canonical pathways that are activated (red) and inhibited (blue) according to the z score, which was computed through analysis of differential gene expression in Ingenuity Pathway Analysis.

Ingenuity pathway analysis was utilized to identify important signaling pathways that are differentially regulated between NK cells in the normal and slowed growth phases (Day 7 and Day 35, respectively) (Figure 4-10C). Of note, pathways associated with senescence and T cell exhaustion signaling were more active at Day 35 compared to Day 7, while metabolic pathways such as gluconeogenesis/glycolysis had higher activity at Day 7 than Day 35. Meanwhile, oxidative phosphorylation pathways were activated at both Day 7 and Day 35. Gene ontology (GO) analysis of genes that were downregulated at Day 7 compared to Day 0 resulted in enrichment of terms related to cytokine mediated pathways, cytokine production and response to stress/cytokine (Figure 4-11A). At Day 7, GO terms related to NK cell metabolism such as gluconeogenesis, the glycolytic process, and oxidative phosphorylation were upregulated (Figure 4-11B), supporting the previous

results that have shown increases in metabolic activity at Day 7 compared to Day 0 (Figure 4-3, Figure 4-4). Genes that were downregulated at Day 35 compared to Day 0 enriched for GO terms related to lymphocyte proliferation, cell mediated cytotoxicity, gluconeogenesis, and response to cytokine stimulation (Figure 4-11C). Upregulated GO terms at Day 35 compared to Day 0 included terms related to the mitochondrial metabolism (Figure 4-11D), supporting the Seahorse data showing that NK cell metabolism remains active even after the shift in growth kinetics.

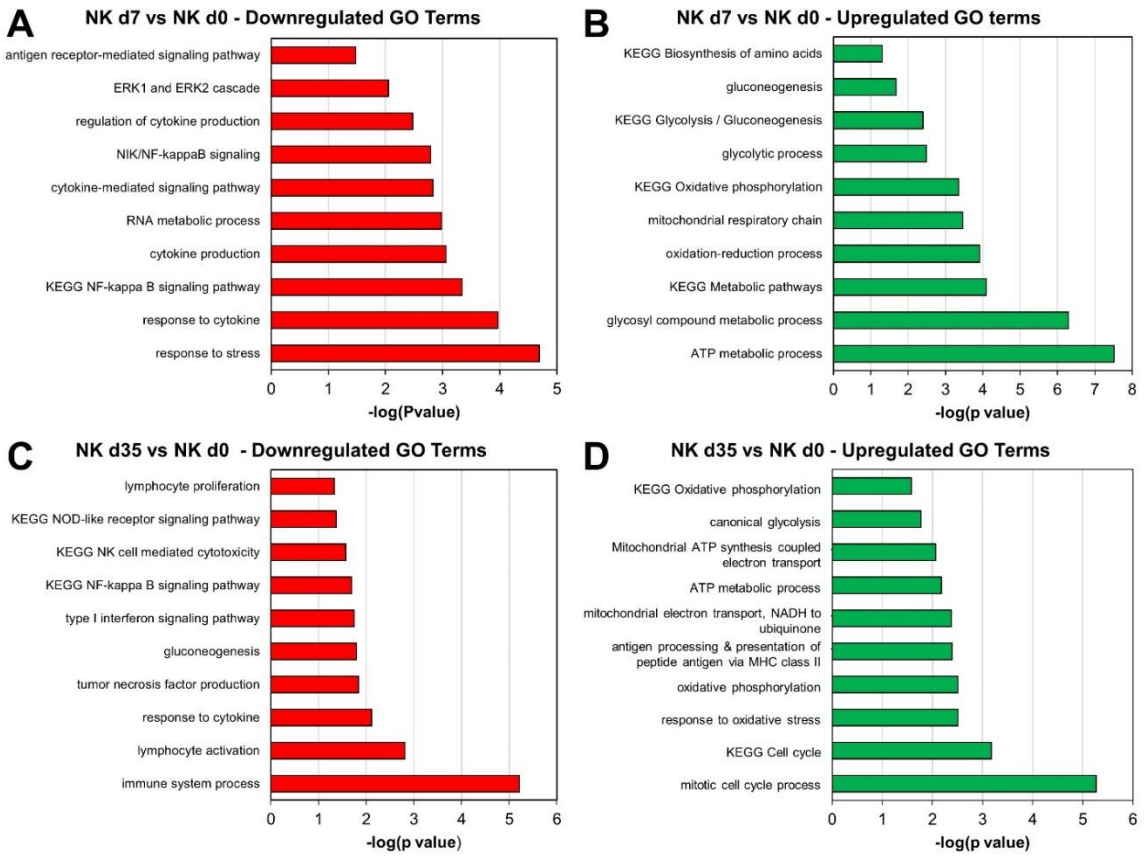

Figure 4-11. Enriched GO terms of early and late culture stage NK cells. (A-B) Gene ontology analysis showing key downregulated (A) terms and upregulated (B) terms in Day 7 RNA sequencing samples (compared to Day 0). (C-D) GO analysis showing key downregulated (C) and upregulated (D) terms in Day 35 RNA sequencing samples (compared to Day 0).

To more specifically assess changes in genes associated with key metabolic pathways in early and late-stage culture, the RNA sequencing results were mapped onto pathway diagrams for glycolysis, the tricarboxylic acid (TCA), the malate-aspartate shuttle, and part of the pentose phosphate pathway (Figure 4-12). While genes associated with the

glycolysis pathway generally increased at Day 7 and decreased at Day 35, expression of genes associated with the TCA or citric acid cycle maintained similar levels among both activated NK cell populations (Day 7 and Day 35) (Figure 4-12).

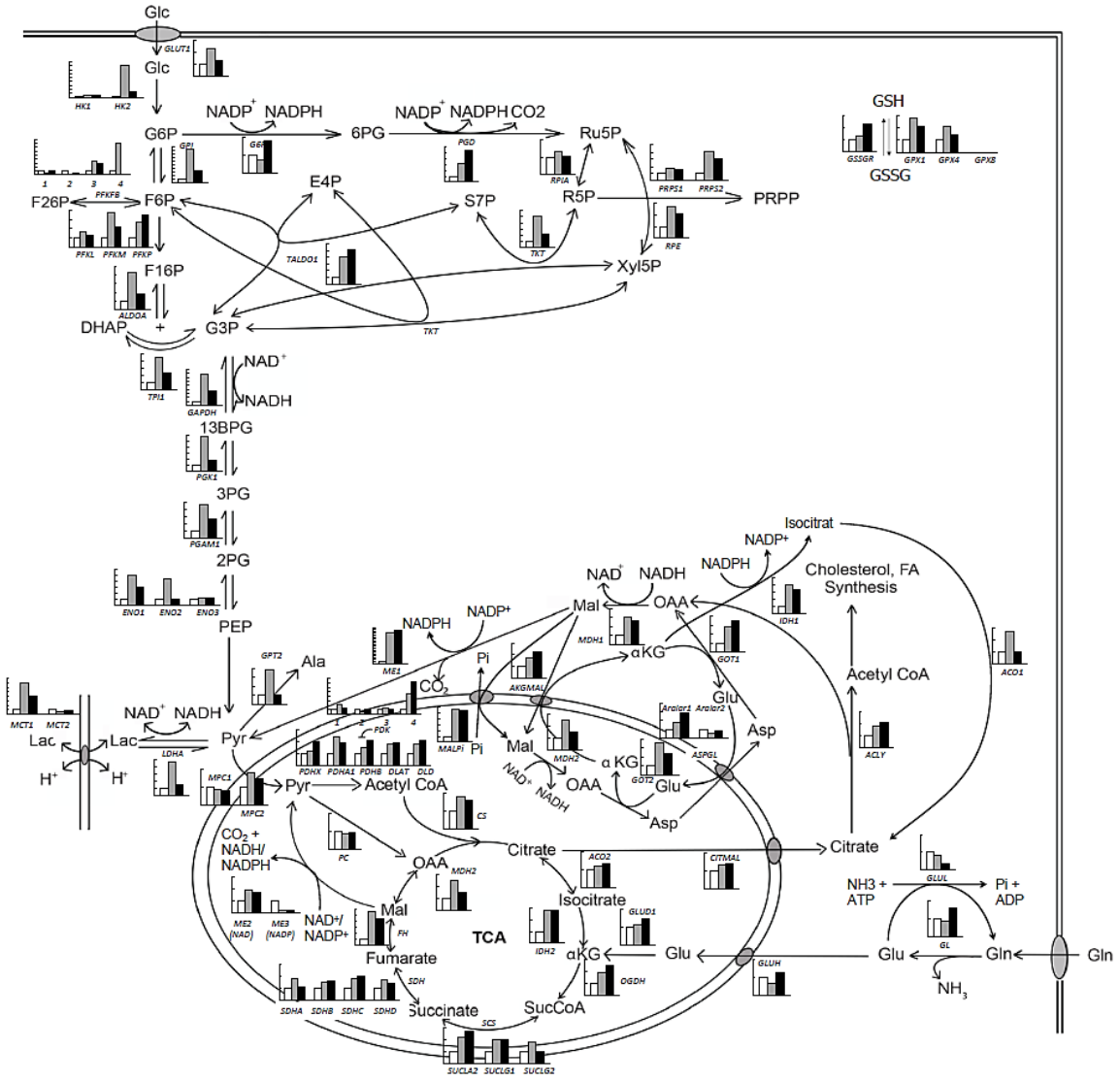

Figure 4-12. Mapping of RNA sequencing results onto key metabolic pathways. Bar graphs indicate relative TPM value expression compared to the control (Day 0). Each TPM value was the average of all the donors for that timepoint. Results were obtained from Donors 5-8 for Day 0 (white bars), Day 7 (grey bars), and Day 35 (black bars).

4.3 Discussion

From a biomanufacturing perspective for an allogeneic cell therapy, it is optimal to generate as many doses as possible from each donor's cells to minimize the cost and time

necessary for quality control for batches of cells from each different donor. The development of engineered K562 aAPCs expressing membrane-bound IL-21 (K562-mbIL-21) has enabled larger-scale expansion of NK cells derived from peripheral blood, with K562-mbIL-21 cells achieving 10^8 fold NK cell expansion by day 42 in culture, compared to 10^4 fold expansion when using aAPCs expressing membrane-bound IL-15 (K562-mbIL-15), as shown by *Denman et al* (Denman et al. 2012). Compared to NK cells expanded with K562-mbIL-15 cells, K562-mbIL-21-stimulated NK cells did not show signs of senescence and had longer telomeres, both indicative of greater expansion potential (Tran et al. 2008). Since K562-mbIL-21 aAPCs enable long-term NK cell expansion for bioprocess scale up, we sought to characterize the effects of repeated stimulation on NK cell growth kinetics, metabolism, and functionality, as these can ultimately impact the efficacy of the cell-based therapy.

While the initial report of K562-mbIL-21 aAPCs showed significantly more robust expansion potential than with K562-mbIL-15 cells, there may have been a subtle shift in growth kinetics after Day 21 of expansion, as prior to Day 21 approximately 10^4 fold expansion was achieved whereas the following 21 days exhibited 10^{3-4} fold expansion (Tran et al. 2008). Our results indicated a clear shift in growth kinetics around day 26 of expansion, as evidenced by a decrease in doubling time, and accumulation of cells in the G0/G1 cell cycle phase, and at later timepoints a slight decrease in viability. Transcriptomics analysis also confirmed the growth kinetics shift, with downregulation of biological processes of lymphocyte proliferation evident at Day 35. The more pronounced shift in growth kinetics in this study may be attributed to the difference in the K562-mbIL-21 aAPCs used, as *Denman et al*. utilized a cell line with additional transgenes including CD64, CD86, and truncated CD19. While our expansion process reached a similar level of 10^8 mean fold expansion by Day 42, our results indicated faster growth kinetics in the initial growth phase, given that a mean 10^5 fold expansion was achieved by Day 21, as compared to 10^4 fold expansion by Day 21 reported by *Denman et al*. with the K562-mbIL-21 aAPCs used in their study (Tran et al. 2008). Another study by *Wang et al*. using the same K562-mbIL-21 cell line utilized in our study did not show slowed growth over time, but a final population of 10^{13} cells was achieved over an 8 week period, reflecting 10^7 fold

expansion (X. Wang et al. 2012). This degree of expansion occurred in 4-5 weeks during our study, which was within the "normal" growth phase (doubling time ~1.23 days) (Liu et al. 2013), before slowed growth was observed. Given that NK cells must persist and proliferate within the patient to effectively kill targeted cancer cells, it is critical to determine the effect of the growth kinetics during expansion on NK cell functionality to ensure the efficacy of the expanded cells.

Cytotoxic functionality is often evaluated using a degranulation assay, which identifies NK cells that respond to outside stimulus by releasing cytotoxic molecules perforin and granzyme via measuring expression of CD107a, a lysosome membrane protein that is expressed on the NK cell surface upon release of cytotoxic granules (Alter, Malenfant, and Altfeld 2004). Upon exposing NK cells to target K562 cells, high levels of CD107a expression were detected in our study, indicating successful release of cytotoxic granules to kill the target cells. CD107a expression upon stimulation increased significantly from week 2 to week 4 of expansion, which is the border timepoint between the two growth phases, with no significant decrease observed in week 6. Furthermore, IFNγ expression was evaluated since it is known to be produced by primed NK cells and sensitizes K562 cells to perforin-dependent lysis (Berthou et al. 2000). Our data indicated that the level of IFNγ increased at week 4 before returning to normal levels (Scrivo et al. 2011). While *Wang et al.* used a different assay to evaluate cytotoxicity, their results indicated a similar trend, with NK cell killing activity peaking at 3-5 weeks before decreasing after 6 weeks.

Recent studies have shown that NK cell metabolism is intrinsically linked to effector function and IFNγ production (Donnelly et al. 2014; Keppel et al. 2015; Mah and Cooper 2016; Keating et al. 2016). Specifically, cytokine stimulation with IL-2 and IL-15 results in upregulation of NK cell metabolism, preferentially enhancing glycolysis after at least 3 days of high-dose stimulation (Mah and Cooper 2016). Our results showed that the glycolytic metabolism spiked at the end of week 1 and then returned to lowered levels for the remainder of the culture period. Oxidative metabolism was similarly enhanced after initial stimulation but maintained high levels of activity throughout the culture period. At week 7, metabolic activity decreased to similar levels of the unstimulated NK cells at day

0, indicating the cells may have returned to a non-proliferative phenotype after prolonged stimulation. This trend of initial increase at day 7 followed by maintenance of the metabolic rates and a decrease at the end stage culture, was also seen in the glycolytic capacity measurements. This phenomenon in conjunction with the changing dynamics of the basal glycolysis rates over time suggested that the NK cells have the capability of enhanced glycolytic metabolic rates but instead have maintained enhanced levels of oxidative phosphorylation over glycolysis post day 7. The transcriptomics data confirms the shift in activation of metabolic pathways between cells in the early stages of activation/growth (Day 7) and cells in the later "slowed" growth phase (Day 35). While there is decreased expression of metabolic genes in the glycolysis pathway at day 35 relative to day 7, genes associated with the citric acid cycle are similarly expressed at day 7 and day 35, indicating that oxidative phosphorylation metabolism is maintained for the duration of the culture.

This trend of enhanced glycolytic metabolism in response to stimulation, followed by a switch to more oxidative phosphorylation, and then a return to lowered levels of overall metabolism indicate that prolonged stimulation may follow trends seen in the *in vivo* effector T cell response as well as in NK cells during acute infection or an immune response (Poznanski and Ashkar 2019). *Chang et al*. found that glycolysis is key for T cell effector function but not necessary for cell proliferation and survival when supported by oxidative phosphorylation (Chang et al. 2013). Our data suggested that this may also be true for NK cells, given that the shift in cell growth kinetics does not coincide with the shift away from glycolytic metabolism. Enhanced glycolytic activity has been associated with short-lived $CD8^+$ T cells, whereas a decrease in glycolytic metabolism indicated a shift toward longer-lived memory T cells (Sukumar et al. 2013). Recent studies have also indicated that enhanced mitochondrial metabolism is a metabolic hallmark of memory NK cells (Cichocki et al. 2018; Poznanski and Ashkar 2019), which is reflective of our metabolic data for NK cells expanded through week 6. Thus, the NK cells in the slowed growth phase may represent a memory NK cell phenotype given that the lowered levels of glycolytic metabolism after a "contraction" phase of reduced proliferation and effector

functions is observed. This trend should be the subject of further investigation in future studies to determine if there is indeed a correlation to a memory NK phenotype.

To further understand the effect of prolonged stimulation on NK cell killing activity and whether exhaustion is occurring, we performed analysis of tumor cell killing via an IncuCyte® assay (Cichocki et al. 2017; Uppendahl et al. 2019; Felices et al. 2019) to quantify the percent lysis of target cancer cells normalized to the live cells remaining in the target cell-only control group. The killing curves for each individual donor revealed faster killing at day 13, during the first growth phase, compared to days 27 and 41, with day 27 being at the boundary between the two group phases. This shift at the boundary between the growth phases was most evident for the A549 lung carcinoma target cell line, followed by the SKOV3 ovarian cancer cells. No notable shift was observed in killing kinetics for the PANC1 pancreatic cancer cell line, indicating that certain cancer cell lines may be more sensitive to shifts in NK cell functionality. These studies used a high effector to target cell ratio (10:1), which could mask subtle differences in killing efficacy (Cerignoli et al. 2018), and thus lower effector to target ratios may be optimal for future work to fully characterize the link between growth kinetics and cytotoxic functionality.

The RNA-sequencing analysis also suggested that the NK cells seem to transition from a cytotoxic to a cytokine-producing phenotype. At day 35, which is during the slowed growth phase, there was downregulation of genes associated with lymphocyte activation, response to cytokine, and NK cell-mediated cytotoxicity. In particular, *BTN3A1/CD277* which is part of the lymphocyte proliferation GO term, was downregulated at day 35 compared to day 0 according to the gene expression data (data not shown). *BTN3A1/CD277* is known to play a role in IFNγ production and be downregulated in activated cells (Messal et al. 2011; Yamashiro et al. 2010). *PATZ1*, which was also downregulated at day 35, is known to play a role in depressing lymphocyte activation and proliferation (Keskin et al. 2015) and thus is one of the key genes contributing to the day 35 phenotype. The NFκB signaling pathway, which is correlated with IL2R signaling and control of perforin expression (Zhou, Zhang, Lichtenheld, and Meadows 2002), is also downregulated. Furthermore, downregulation of NOD-like receptor signaling, which is associated with regulating growth and immune response to stimuli (Platnich and Muruve

2019), may contribute to the observed shift in NK cell growth and killing kinetics. There was also increased activation of the senescence pathway and T cell exhaustion signaling pathway at day 35 when compared to day 7, indicating that cells in the slowed growth phase maybe shifting towards an exhausted or senescent phenotype. Furthermore, *CDKN2A*, which plays a role in senescence and aging (Baker, Jin, and van Deursen 2008), is highly expressed in the Day 35 samples compared to Day 7 and Day 0 samples and may play a key role in the NK cell exhaustion phenotype. While these phenotypes are not well-characterized in NK cells, decreases in IFNγ, CD107a, perforin and granzyme, and cytotoxicity are attributed to exhaustion, whereas loss of CD57 expression, increased NKG2A expression, and decreased proliferation are associated with senescence (Judge, Murphy, and Canter 2020; Streltsova et al. 2018). While we did not observe a decrease in IFNγ or CD107a expression, our data did show a decrease in perforin and granzyme expression during the slowed growth phase as well as decreased cytotoxicity against A549 and SKOV3 target cells, thus suggesting that prolonged stimulation may lead to senescence and an exhausted phenotype.

4.4 Conclusions

Understanding the link between the growth kinetics transition and functionality will be critical for determining the degree of expansion that should be utilized before harvesting NK cells for clinical use. In addition, this understanding could enable engineering of the expansion process to prevent these shifts in growth kinetics and prevent exhaustion or senescence from occurring. The timing of when NK cells are stimulated can potentially be used to tailor the biomanufacturing process. *Stretsolva et al*. showed that NK cells stimulated weekly with K562-mbIL-21 aAPCs could only maintain growth for 6-7 weeks, as seen in our study, but when stimulated with the K562 aAPCs only at week 1 and week 7, along with weekly IL-2 in between, the growth period extended to 8-14 weeks (Streltsova et al. 2019). However, there was a slower expansion rate and IFNγ production and CD16 expression decreased, which could negatively impact cytotoxicity (Streltsova et al. 2019). The continued rigorous characterization of how NK cell activation and repeated stimulation impacts cell growth and metabolism and the consequent link to functionality is critical for improving the bioprocess to ensure the efficacy of NK cell therapies.

5 Assessing changes in NK cell phenotype during repeated stimulation

5.1 Introduction

In the study of long-term NK cell expansion in Chapter 4, there was a transition away from a cytotoxic phenotype towards a cytokine-producing phenotype after multiple rounds of stimulation. Given that the characterization was focused on the overall functionality of the NK cells, in this chapter we sought to further understand the phenotype of NK cells in the slowed growth phase by assessing activating, inhibiting and cytotoxic receptor expression on the surface of the cultured NK cells. We hypothesized the cells were becoming less mature and more exhausted and sought to rigorously characterize the dynamics of NK cells over repeated stimulations using markers that are key in functional, mature, activated NK cells.

5.2 Results

5.2.1 Subtle growth kinetic shift observed during expansion

NK cells from three donors were expanded via eight rounds of weekly stimulation with K562 aAPCs (Figure 4-1A). The engineered K562 cell line used in all of these studies is a previously established line engineered to express CD137L (4-1BBL) and membrane-bound IL-21, which allowed NK cells to grow after repeated stimulation (Denman et al. 2012). All three donors exhibited a subtle yet statistically significant shift to slower cell growth around Day 24 of expansion (Figure 5-1A). This shift in growth kinetics was more subtle compared to previous studies (Figure 4-2A, Figure 4-8A), though the timing of the shift was similar. As expected for healthy expanding NK cells, the average doubling time for the three donors during the first growth phase was 1.20 ± 0.04 days (median 1.19 days) (Figure 5-1B) (Liu et al. 2013). During the slower growth phase, the average doubling time increased to 1.93 ± 0.17 days (median 1.91 days).

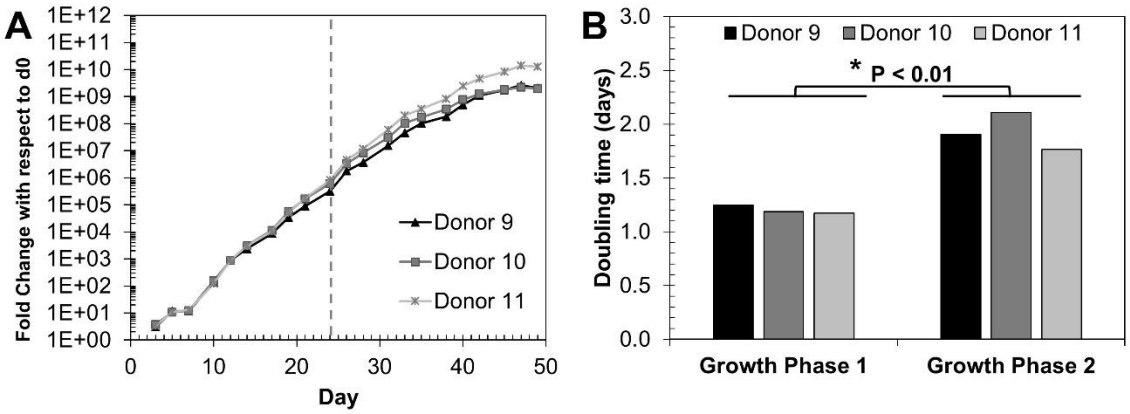

Figure 5-1. Characterization of growth kinetics during NK cell expansion. (A) Cumulative fold change over time for each donor (n=3); dashed line indicates the transition to a slower growth phase at day 24. (B) Doubling time in days for growth phase 1 (prior to day 24) and growth phase 2 (after day 24) for each donor. * $P < 0.01$ for growth phase 2 compared to growth phase 1.

5.2.2 Shifts in cytotoxic granule and cytokine expression during extended culture

Previously we assessed perforin and granzyme B expression weekly. Given that expression of these lytic granules decreased towards the end of the culture (Figure 4-7), we sought to characterize expression dynamics more thoroughly during the expansion process. As such, we evaluated perforin and granzyme B expression every 2-3 days when media was changed and the culture was diluted (Figure 5-2A,B). Expression of both perforin and granzyme B decreased immediately after K562 aAPC stimulation but recovered fully within the last 2 days before re-stimulation in the first growth phase. However, as cells entered the slower growth phase, it took longer for the NK cells to recover cytotoxic granule expression. In addition, at the Day 35 timepoint the expression or production of perforin and granzyme did not fully recover, with perforin expressed in 58.6 ± 2.48 % NK cells and granzyme B expressed in 83.8 ± 6.94 % NK cells. Another week was necessary to recover previous levels of perforin and granzyme expression in the NK cells.

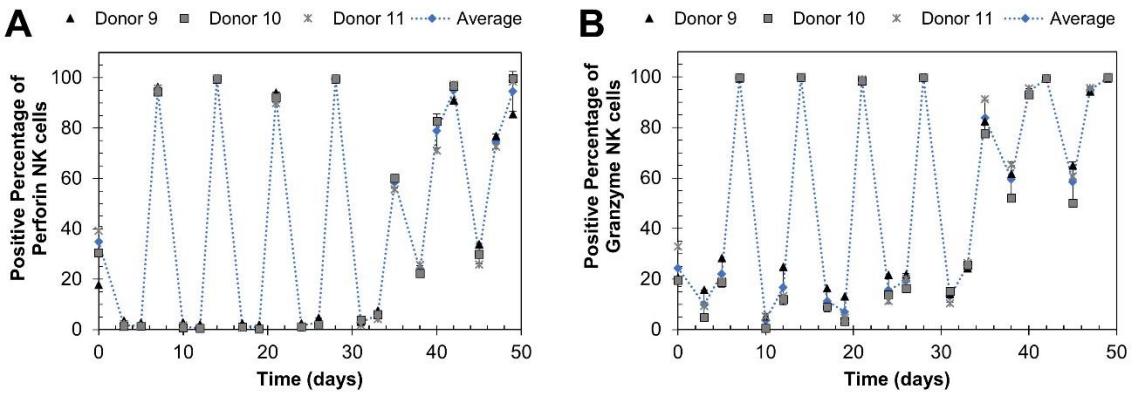

Figure 5-2. Assessment of cytotoxic granule production during NK cell expansion. Percentage of live CD56+ NK cells that express perforin (A) and granzyme (B). Data shown as the average from the three individual donors (error bars represent SD).

Stimulation with naïve K562 cells was used as an additional assay for cytotoxic functionality. An increase in CD107a expression was observed at each timepoint relative to NK cells not exposed to the naïve K562 cells, but the only timepoint that showed a statistically significant difference relative to the previous timepoint was Day 21 (Figure 5-3belowA). IFNγ expression significantly increased at Day 14 and subsequently decreased, but high IFNγ expression was also observed in the unstimulated NK cell population at that timepoint, which indicates the cells were producing a lot of IFNγ even without added stimulation of naïve K562 cells (Figure 5-3B). The percentage of cells producing IFNγ upon stimulation compared to the unstimulated cells significantly increased in the second growth phase (from an average of 4.98% to 12.02%). TNFα expression upon stimulation significantly increased compared to the previous timepoint at Day 28 and Day 35 (Figure 5-3C). Furthermore, while the average percentage of cells with CD107a and TNFα expression seemed to decrease in the final weeks of expansion, these differences were not statistically significant.

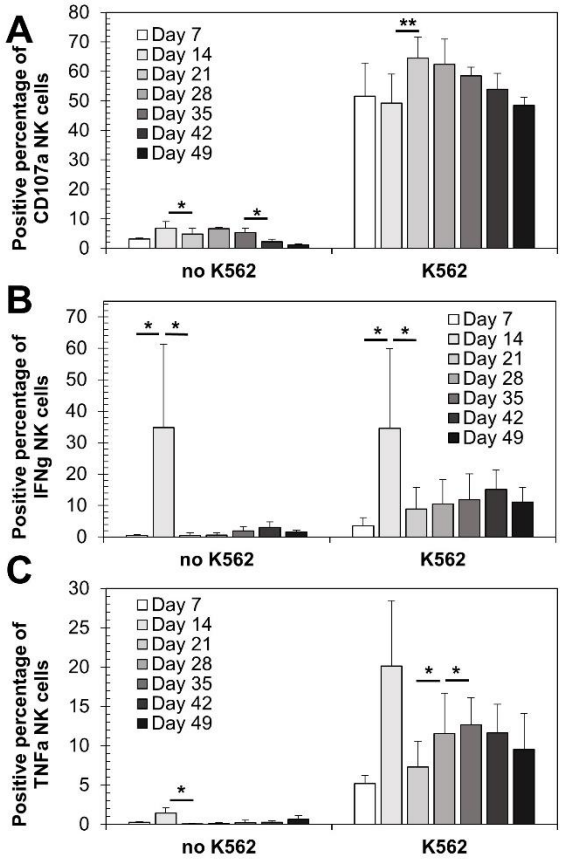

Figure 5-3. Assessment of cytotoxic functionality by flow cytometry during NK cell expansion. Percentage of live CD56+ NK cells that are positive for CD107a (A), IFNγ (B), and TNFα (C) with and without a 3.5 hour incubation with naïve K562 cells at a 1:1 ratio. Data shown as the average from the three individual donors (error bars represent SD). Student's t test was used to determine statistical significance from previous week. * $P < 0.05$, ** $P < 0.01$.

5.2.3 Changes in NK activating and inhibiting receptor expression during expansion

To investigate why there are changes in NK cell functionality as it relates to growth potential, activation and cytotoxicity, a host of receptors related to NK functionality were evaluated. Flow cytometry histograms where used to show the shifting mean intensities of the live CD56+ NK cells for each marker over time. A cocktail of killer immunoglobulin receptors (KIRs) was utilized to investigate changes in KIR or inhibitory receptor expression on NK cells (Pende et al. 2019; Purdy and Campbell 2009). KIR expression fluctuated for 14-day periods during expansion, reaching similar high expression levels as Day 0, 14, 28, 42, and 49. In the last week of expansion the cells recovered KIR expression much faster than previously shown. (Figure 5-4A,B). NKG2A was highly expressed

throughout the culture, however, in the later growth phase its expression decreased drastically at Day 31 before recovering (Figure 5-4A,C). CD94, an inhibitory receptor (Orr et al. 2010; Sáez-Borderías et al. 2009), was expressed highly throughout the culture (Figure 5-4A, Figure 5-5A), whereas NKG2D, an activating receptor (Wensveen, Jelenčić, and Polić 2018), fluctuated over 14 day periods, similar to the KIRs (Figure 5-4A, Figure 5-5B). CD96, an indicator of exhaustion and decrease in IFNγ and TNFα secretion (Sun and Sun 2019; Sun et al. 2019), was lowly expressed in the first growth period but increased with fluctuations at intermediate timepoints during the later growth phase (Figure 5-4A, Figure 5-5C).

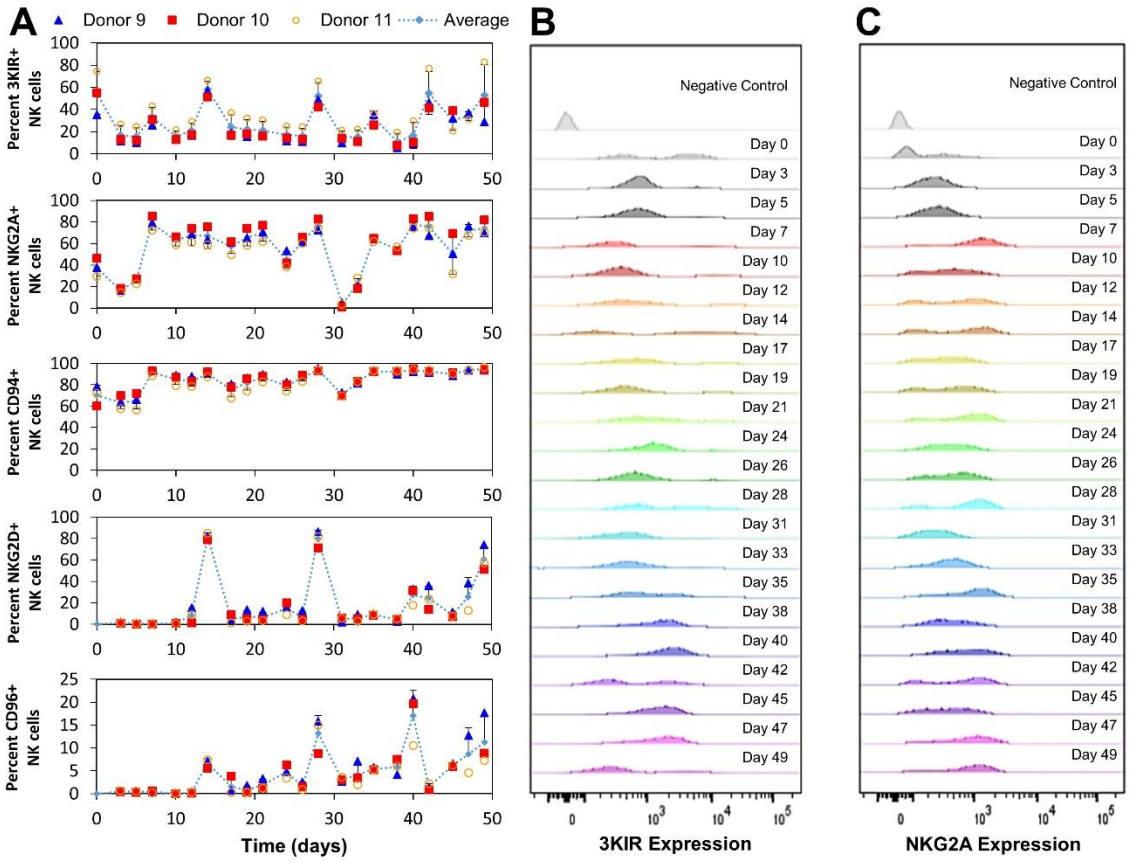

Figure 5-4. Flow cytometric analysis of changes in activating and inhibitory receptor expression during NK cell expansion. (A) Percentage of live CD56+ NK cells positive for 3KIR, NKG2A, CD94, NKG2D, and CD96. (B-C) Histograms showing expression of 3KIR (B) and NKG2A (C) at each timepoint for Donor 9.

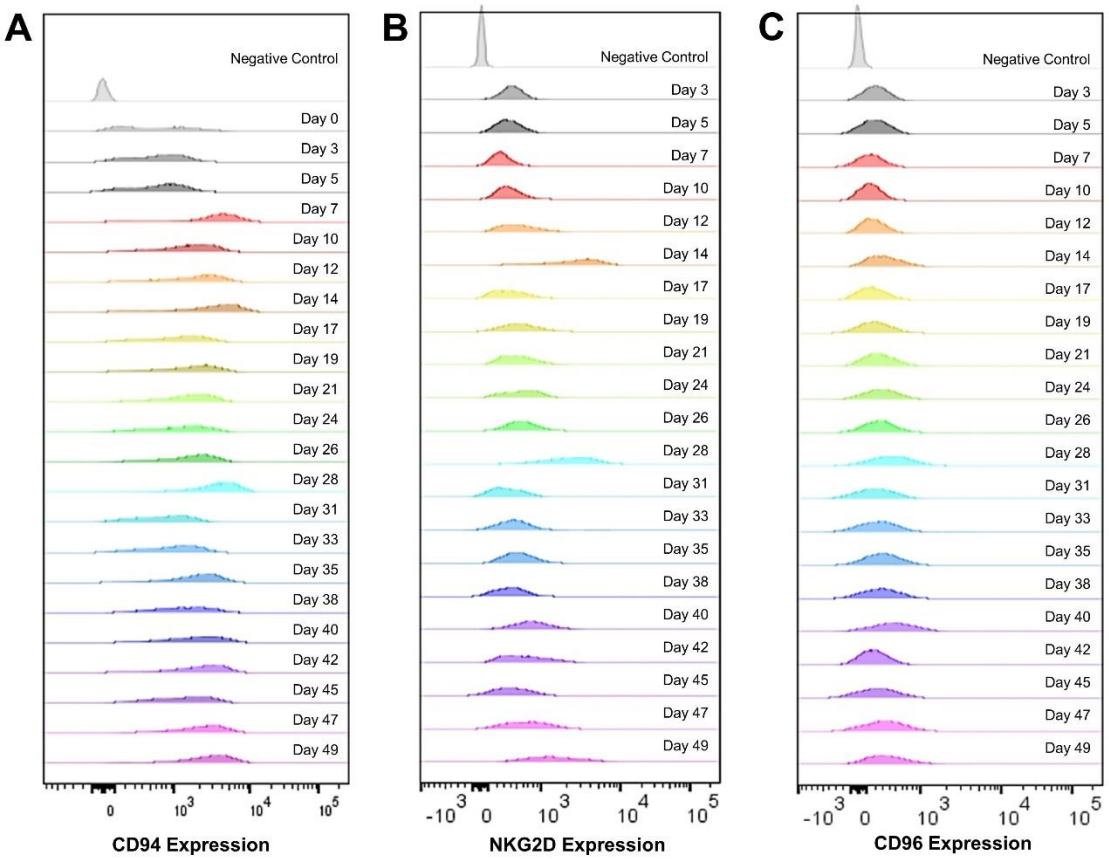

Figure 5-5. Flow cytometric histograms of intensity distribution in activating and inhibitory receptor expression during NK cell expansion. (A-C) Histograms showing expression of CD94 (A), NKG2D (B), and CD96 (C) at each timepoint for Donor 9 live CD56+ NK cells.

Natural cytotoxicity markers (NCRs), including NKp30, NKp44, and NKp46, are markers that play a role in non-MHC-restriction recognition and killing of target cells (Barrow, Martin, and Colonna 2019). NKp30, which is expressed on mature resting and activated NK cells and not on adaptive memory cells, reached a high expression level at Day 14 and then fluctuated over 7 day periods (Figure 5-6A, Figure 5-7A). NKp44 expression, which is generally enhanced by cytokine stimulation including IL-2, also fluctuated on a weekly basis, but expression remained relatively low throughout the culture. NKp46, which affects secretion of IFNγ and TNFα and plays a role in cytotoxicity, followed a similar trend of weekly fluctuations (Figure 5-6A,B). CD16, a marker for antibody-dependent cell mediated cytotoxicity (ADCC) (W. Wang et al. 2015), was highly

expressed at the beginning of the culture and recovered expression levels each week post-stimulation in the first growth phase. After entering the second growth phase, CD16 expression took much longer to recover to the high levels seen in the previous growth phase (2-3 weeks; Figure 5-6A,C). CD57, a marker of mature NK cells with higher cytotoxic potential and decreased responsiveness to cytokines (Nielsen et al. 2013), remained low throughout the culture (Figure 5-6A, Figure 5-7C).

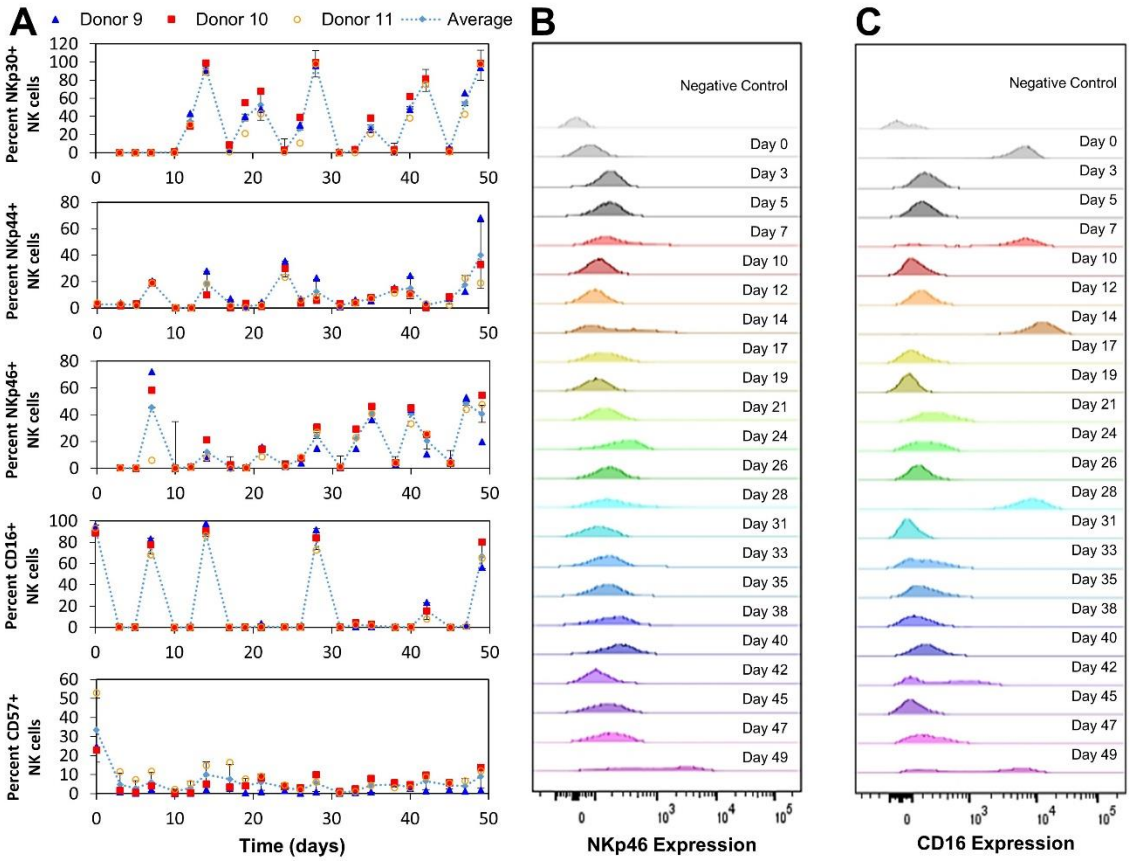

Figure 5-6. Flow cytometric analysis of changes in markers of natural cytotoxicity and maturation during NK cell expansion. (A) Percentage of live CD56+ NK cells positive for NKp30, NKp44, NKp46, CD16, and CD57. (B-C) Histograms showing expression of NKp46 and CD16 at each timepoint for Donor 9.

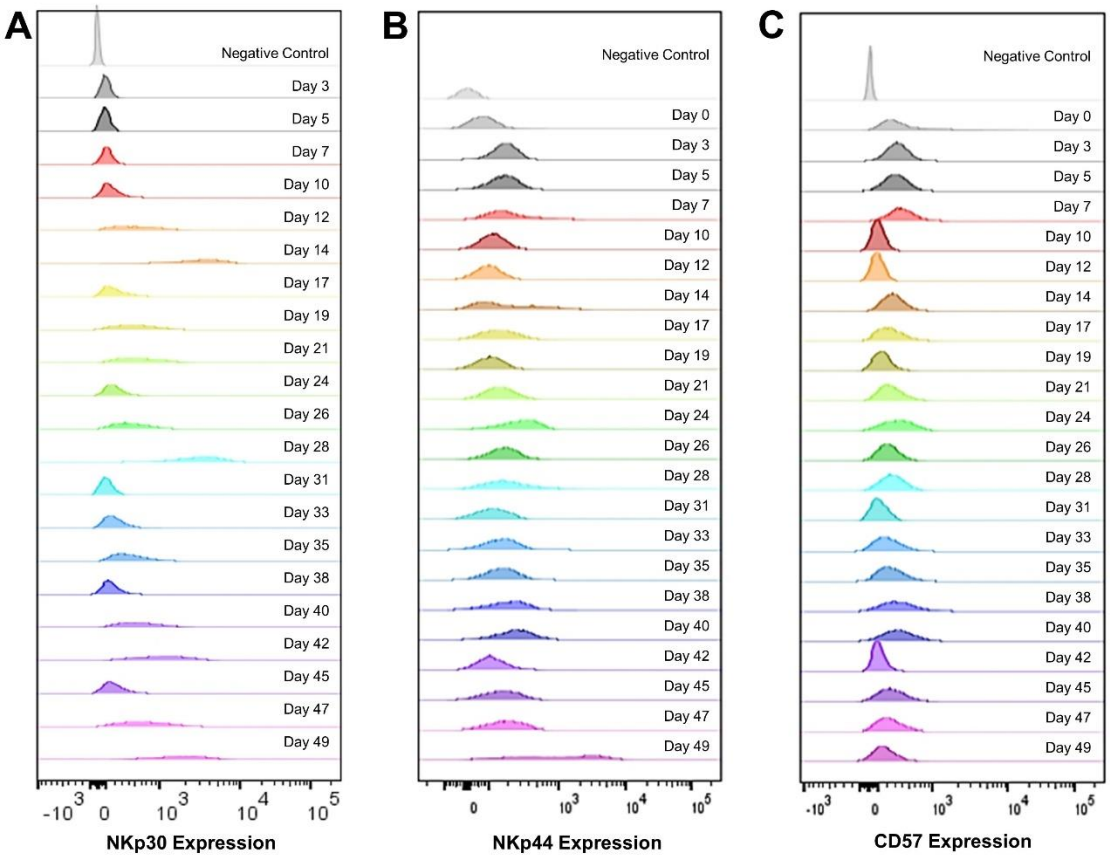

Figure 5-7. Flow cytometric histograms of intensity distribution of natural cytotoxicity and maturation receptor expression during NK cell expansion. (A-C) Histograms showing expression of NKp30 (A), NKp44 (B), and CD57 (C) at each timepoint for Donor 9 live CD56+ NK cells.

5.3 Discussion

Assessment of NK cell phenotype during expansion has not been investigated rigorously. Key NK cell markers have been evaluated at discrete timepoints such as week 1, 3, and 6 of expansion (X. Wang et al. 2012) rather than at intermediate timepoints to understand the changing dynamics and phenotype during multiple round of expansion. Activating and inhibiting receptor expression on the NK cell surface can influence the efficacy of the cell therapy and thus are important to track throughout the culture. As such, we sought to understand receptor expression and NK cell phenotype changes at more regular intervals. Given the results in Chapter 4, which indicated that the cells in the later growth phase were becoming less cytotoxic and shifting towards a cytokine-producing and potentially exhausted phenotype, we sought to rigorously characterize NK cell phenotype

over time. Various inhibiting receptors (NKG2A/CD94, 3KIRs, CD96) and activation receptors (NKG2D, NKp30, NKp44, NKp46, CD16) were evaluated to assess NK cell functionality and maturity.

Surprisingly, receptor expression of NKG2D, NKp44, NKp46, and CD16 decreased upon interaction with the K562 aAPCs and recovered in the 2 days before re-stimulation. To our knowledge, this phenomenon has not been seen before or previously investigated. Expression of the three KIRs fluctuated over 14-day periods, except for the final week in culture. KIR expression is associated with immune tolerance and KIRs are generally expressed on CD56dim cells, which are mature and exhibit strong cytolytic activity and rapid cytokine secretion capability upon activation (K. S. Campbell and Purdy 2011; Purdy and Campbell 2009; Pende et al. 2019). The exact mechanism of receptor recycling is not well studied, but serine and threonine phosphorylation events have been known to modulate KIR surface expression through receptor internalization and turnover (K. S. Campbell and Purdy 2011). Furthermore, protein kinase C (PKC) phosphorylation can act as a stabilizer for KIR expression on the cell surface (K. S. Campbell and Purdy 2011; Alvarez-Arias and Campbell 2007). Changes in these phosphorylation events may potentially be the cause of the fluctuating KIR expression throughout the culture.

CD96, a marker for decreased IFNγ and TNFα secretion and exhaustion (Sun et al. 2019; Sun and Sun 2019), increased over roughly 2 week periods for the first 40 days. Average high expression after each fluctuation increased with each cycle until Day 40, and after this timepoint CD96 expression recovered faster (within 1 week). Given that CD96 is a marker for NK exhaustion, it is interesting that expression fluctuated and gradually increased expression towards the end of the culture. The faster recovery and higher expression of CD96 may indicate the cells are less active and less sensitive to K562 stimulation over time, thus making the cells in the later growth phase not an ideal candidate for cell therapy applications. Furthermore, percentage of cells expressing perforin and granzyme B decreased upon interaction with K562 cells but recovered full expression in the 2 days before re-stimulation for the first 4 weeks. NK cells did not recover expression post week 4 and seemed to not release their entire payload of lytic granules in the subsequent stimulation at week 5. Rather, a small percentage of NK cells release their

granules and recovered expression in the following week 6 of stimulation. This could indicate that the NK cells were becoming de-sensitized to the K562 aAPCs, resulting in an active subpopulation to lyse the K562 cells. Collectively, it seems the NK cells are either slower in production of perforin and granzyme or there is a longer delay until the program for granule production is initiated. In either case, the NK cells show signs of exhaustion in the slower growth phase at Day 35.

NKG2D is an activating receptor that plays a role in stimulating cytokine and cytotoxic molecule production and promotes CD16 signaling. Expression of NKG2D is generally low but increases over time in mature NK cells. Furthermore, chronic engagement of this receptor can lead to a hypo-responsive phenotype to CD16, NKp30, and NKp46 (Jelenčić et al. 2018; Wensveen, Jelenčić, and Polić 2018). NKG2D expression fluctuated every 2 weeks up until Day 28. Expression was not fully recovered to previous high levels (as seen at Day 14 and Day 28), and it took 3 weeks to recover high levels of expression at the end of the culture. This shift in periodicity may be a consequence of the shift in growth kinetics, since it took longer to recover expression from week 4 to 7. However, it is interesting to note that in the first growth phase, during the 14 day cycling period once NKG2D expression started increasing it only required 2 days to reach maximum expression, and thus unknown mechanisms may be regulating the timing or delay of receptor cycling for 12 days. Recovery was delayed further (16 days) after Day 28, and at Day 28 NKG2D expression required at least 4 days to recover high levels once the receptor cycling began. NK cell activation has been known to increase expression of NKG2D at the cell surface and in the intracellular pool of receptors that recycles to the surface (Roda-Navarro and Reyburn 2009). However, MHC class I chain-related molecule B (MICB) can induce significant rapid endocytosis and degradation of NKG2D (Roda-Navarro and Reyburn 2009; Masilamani et al. 2009), which may be the reason for fluctuating expression of NKG2D on the NK cells.

Perhaps the most interesting shifts were in the expression of functional cytotoxic receptors (NCRs and CD16). The NCRs, which include NKp30, NKp44, and NKp46, play a role in NK cytotoxicity in a non-MHC dependent manner. NKp30 is expressed on mature resting and activated NK cells, but has decreased expression in adaptive memory NK cells.

NKp44 is enhanced by IL-2, IL-15 and IL1β and is often expressed by CD56-bright or immature NK cells. NKp46 plays a role in IFNγ and TNFα secretion, and calcium release, and it is expressed in NK cells irrespective of activation status (Barrow, Martin, and Colonna 2019). In our experiment, the NCRs fluctuated on a weekly basis. Upon stimulation with K562 cells, expression decreased in 3 days to almost no expression before recovering over the following 2-4 day period. NKp30 expression was high even in the later stage of the culture, while NKp46 seemed to gradually recover to elevated levels of expression seen in the early stage culture. NKp44 expression fluctuated at low levels before increasing to the highest level of expression in the last week of the culture. The cause of the fluctuation is unknown, however, heparin sulfate proteoglycans on the surface of NK cells have been known to impact trafficking of NCRs to intracellular degradation and recycling pathways upon endocytosis and thus may play a role in the fluctuations in NCR expression (Pazina et al. 2017; Brusilovsky et al. 2015; 2014). Treatment with a structurally related heparin has been suggested to improve NCR expression and ultimately NK cell function, which would improve NK cell efficacy (Pazina et al. 2017; Brusilovsky et al. 2015; 2014). CD16 expression, which is associated with ADCC (W. Wang et al. 2015), decreased immediately after K562 stimulation and recovered in the 2 days before re-stimulation until Day 14. After Day 14, CD16 expression took 2 weeks to recover at Day 28 and then 3 weeks to recover high levels of expression at Day 49. This unexpected shift indicates that the CD16 recovery or recycling process seems to be delayed upon extended stimulation. Ultimately, NK cells may rely on NCRs rather than CD16 receptor interactions to kill the target cells in the later stage of the culture.

NKG2A and CD94 are inhibitory co-receptors that are expressed on expanded cells that undergo intensive proliferation in response to IL-2/IL-15. These markers also indicate poor cytolytic activity and secretion of IFNγ and TNFα, which fits the profile of immature, cytokine-producing cells of the later growth phase (Purdy and Campbell 2009; Pende et al. 2019; Orr et al. 2010; Sáez-Borderías et al. 2009). While CD94 was highly expressed throughout the culture, NKG2A expression fluctuated slightly and was lower at the beginning of the culture and around Day 31 – 33, indicating the cells were probably in a mature state in the beginning of the culture and gradually began to become more immature,

the exception being at Day 31 when NKG2A expression was low. Lack of serious fluctuations in CD94 expression may be attributed to the continuous rapid recycling between the cell surface and cytoplasm (Borrego et al. 2002; Masilamani et al. 2009). Alternatively, CD57 expression, which is associated with mature, potent cytotoxic NK cells with decreased sensitivity to cytokine and decreased replicative potential (Nielsen et al. 2013), was expressed at a higher percentage at the beginning of the culture before reducing to steady low levels of expression after the first K562 stimulation.

5.4 Conclusions

Collectively, the data supports the conclusion that the NK cells in the later growth phase cells are becoming less mature and shift towards a cytokine-producing phenotype as opposed to a cytotoxic phenotype. Furthermore, the cells start exhibiting markers of exhaustion in the later stage of the culture. However, the data suggests that perhaps a subpopulation remains activated with high proliferative capability while the remaining cells exhibit an exhausted phenotype. While these changes in phenotype are somewhat expected, the drastic fluctuation of receptor expression over time was quite unexpected. Mechanisms underlying receptor recycling and degradation have not been well-studied in NK cells. However, limited knowledge suggests serine, threonine, and PKC phosphorylation affect KIR turnover, while heparin sulfate proteoglycans affect NCR recycling and degradation pathways. MICB plays a role in degradation of NKG2D receptor expression and could be a target for downregulation. Overall, there is much to be learned about receptor endocytosis and intracellular trafficking. A deeper understanding of the pathways regulating receptor recycling could aide in the development of therapeutics to control receptor surface expression and enhance efficacy in the clinic.

6 Summary and Concluding Remarks

In the recent years, cell therapy has gained momentum within the biopharmaceutical industry. Current FDA approved cell therapies are autologous and derived from the patient's own cells. This cell therapy platform is very costly and risky given that if something goes wrong during manufacture of the expanded cells, no product will be delivered. The future of cell therapy lies in using an allogeneic platform in which cells from one donor can be expanded to be given to multiple patients. This platform allows for more control over the bioprocess and can supply patients with cells as an "off-the-shelf" therapy. NK cells are unique since they do not induce Graft-vs-Host Disease upon transplantation, which makes primary peripheral blood-derived NK cells an attractive allogeneic cell therapy.

While NK cells have similar effector functions as T cells, the key mechanism of activation is not well understood. Engineered K562 aAPCs are used to activate and expand NK cells to reach desired fold expansion to have sufficient number of cells to be a viable economical "off-the-shelf" therapy. However, use of K562 aAPCs may cause regulatory concerns and increase cost, risk, and variability in the bioprocess. As such, understanding activation through changes in the transcriptional and epigenetic landscape is key to potentially phasing out the use of K562 feeder cells from the culture process. The transcriptional and epigenetic signature of K562-activated NK cells seems to be related to IL-12/IL-18 stimulation pathways, an intermediate cytokine-producing phenotype with reduced cytotoxic capabilities, a non-memory-like phenotype, and an NKT phenotype. Key super-enhancer genes and transcription factors were identified as potential targets to shift resting NK cells towards an activated phenotype. Altogether, investigation into NK cell activation is necessary to transition to an optimized bioprocess.

Besides NK cell activation, better understanding of the effect of repeated stimulation with K562 aAPCs is necessary to ensure efficacy of NK cells in the clinic. Changing dynamics of NK cell growth kinetics, metabolism, transcriptional landscape, and functionality through cytokine release and cytotoxicity have not been extensively researched. In this thesis, slowed growth after 3-4 rounds of stimulation was noted. Furthermore, changes in transcriptome as well as effector functions, indicated the cells

transition away from a cytotoxic, mature phenotype towards a cytokine-producing, immature phenotype. Changes in growth kinetics seemed to correlate with changes in cytotoxicity against cancer cells, but the effect depended on the target cell type. Fluctuations in expression of key activating and inhibitory receptors over time such as NKG2A, CD96, KIRs, NKG2D, CD16, and NCRs, indicated that receptor recycling may play a more important role in NK cell function than previously assumed. Better understanding of NK cell receptor turnover and use of therapeutics to control receptor expression on the surface may aide in improving NK cell functionality throughout the culture and prevent exhaustion in the later growth stages.

Overall, this work focused on the challenges that NK cell therapy faces in transitioning from the benchtop to a large-scale biomanufacturing process. The effects of activation and consequent proliferation of NK cells are necessary to understand in order to remove major roadblocks in the biomanufacturing of NK cells. Elucidating mechanisms underlying activation and expansion offer a pathway to developing strategies to eliminate K562 aAPCs from the cell culture process altogether, which is advantageous from a regulatory perspective, and further to improve the bioprocess to reduce risk and variability. Insights into the correlation between growth kinetics and cytotoxic potential as well as the role receptor cycling may have on functionality could prove to be key in improving efficacy and platform development of an allogeneic NK cell therapy.

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