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Ex Vivo Expanded Cord Blood Natural Killer Cells as a Novel Therapeutic for Multiple Myeloma

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**EX VIVO EXPANDED CORD BLOOD NATURAL KILLER CELLS AS A NOVEL
THERAPEUTIC FOR MULTIPLE MYELOMA**

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THERAPEUTIC FOR MULTIPLE MYELOMA**

A

THESIS

Presented to the Faculty of

The University of Texas

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and

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by

Nina Shah, MD

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Dedication

This thesis is dedicated to my parents, Kumar and Salila Das, my sister, Mona Roy, my husband, Jay Shah, and my mentor Elizabeth J. Shpall – all of whom have pushed me to be better each day than I was the day before.

Acknowledgements

These studies were supported by the Paul Calabresi Clinical Oncology Award Program K12 CA088084.

EX VIVO EXPANDED CORD BLOOD NATURAL KILLER CELLS AS A NOVEL THERAPEUTIC FOR MULTIPLE MYELOMA

Nina Shah, MD

Advisory Professor: Scott Kopetz, MD, PhD

Multiple myeloma (MM) is the second most common hematologic malignancy in adults and, to date, is incurable. Allogeneic natural killer (NK) cells are active in various hematologic malignancies and may have a role against MM. Umbilical cord blood is a potential source for allogeneic NK cells and *ex vivo* expanded umbilical cord blood-derived NK (CB-NK) cells demonstrate activity comparable to that of peripheral blood-derived NK cells. However, large-scale expansion of these cells is required for clinical translation. Here we studied a potential method for *ex vivo* expansion of NK cells from fresh and cryopreserved CB. Using artificial antigen presenting cells (aAPCs), interleukin-2 (IL-2) and a gas permeable culture system we were able to expand CB-NK cells 1848-fold (fresh CB) and 2389-fold (cryopreserved CB). The resultant cells were >95% pure for NK cells and demonstrated an activated, unexhausted phenotype. Expanded CB-NK cells demonstrated formation of functional immune synapses with target MM cells and dose-dependent cytotoxicity against various MM cell lines. Finally, infusion of CB-NK cells to a murine MM model resulted in slower progression of disease and improved survival. Thus CB-NK cells can be expanded to clinically meaningful doses for cellular therapy and may be an important immunotherapy tool to treat MM.

TABLE OF CONTENTS

Dedication	iii
Acknowledgements	iv
Abstract	v
Table of Contents	vi
List of Figures	viii
List of Abbreviations	Xi
Chapter 1: Introduction	1
Multiple myeloma	
Multiple myeloma and the immune system	
NK cell alloreactivity	
NK cells and multiple myeloma	
Umbilical cord blood as a source of NK cells	
Summary	
Chapter 2: Materials and Methods	6
Ethics Statement	
Cells and cell lines	
Generation of eGFP-FFLuc-expressing ARP-1 cell line for <i>in vivo</i> experiments	
Isolation and expansion of umbilical cord blood-derived NK cells	
Original expansion techniques	

NK cell phenotyping via flow cytometry	
Immunofluorescence and confocal microscopy image acquisition	
NK cell ⁵¹Cr cytotoxicity assay	
ARP-1 myeloma murine model	
Chapter 3: Results	13
aAPC-mediated CB-NK expansion from fresh or cryopreserved CB units yields significantly greater fold expansion of NK cells than expansion of CD56⁺ cells with IL-2 alone	
aAPC-mediated expansion yields a pure population of NK cells with a mature phenotype	
CB-NK cells cultured with aAPCs demonstrate <i>in vitro</i> anti-myeloma activity	
Treatment with expanded CB-NK cells delays development of myeloma in a murine model	
Chapter 4: Discussion	22
Chapter 5: Strengths and Weaknesses	25
Chapter 6: Future Directions	27
References	28
Vita	36

LIST OF FIGURES

Figure	Description	Page
Figure 1	Culture method for expansion of CB-NK cells.	9
Figure 2	Co-culture of CB MNCs with IL-2 and aAPCs yields significantly greater expansion of NK cells than culture with IL-2 alone.	14
Figure 3	Phenotype of CB-NK cells cultured with aAPCs.	16
Figure 4	aAPC-expanded CB-NK cells form immunological synapses with and are cytotoxic against myeloma targets.	19
Figure 5	aAPC-expanded CB-NK cells delay development of myeloma in a NSG murine model.	21

LIST OF ABBREVIATIONS

MM	Multiple myeloma
NK	Natural killer
CB	cord blood
aAPCs	artificial antigen presenting cells
IL	Interleukin
HSCT	hematopoietic stem cell transplantation
GVM	graft versus myeloma
GVHD	graft versus host disease
MHC	Major histocompatibility complex
KIR	killer immunoglobulin receptors
HLA	Human leukocyte antigen
PB	peripheral blood
BM	Bone marrow
GMP	Good Manufacturing Practice
MDACC	MD Anderson
IRB	Institutional Review Board
IACUC	Institutional Animal Care and Use Committee
ATCC	American Type Culture Collection
⁵¹Cr	chromium-51
eGFP-FFLuc	green fluorescent protein- Firefly Luciferase
ELISA	enzyme-linked immunosorbent assay

MNCs	mononuclear cells
Eomes	Eomesodermin
MFI	mean fluorescence intensity
CMAC	7-amino-4-chloromethylcoumarin
NSG	NOD/SCID IL-2R γ ^{null}
IP	intrapertoneally
BLI	bioluminescence imaging
ROI	regions of interest
NCR	NK cytotoxicity receptors
NKIS	NK immune synapse
MM	Multiple myeloma

Chapter 1

INTRODUCTION

Multiple myeloma.

Multiple myeloma (MM) is a hematologic malignancy characterized by the clonal proliferation of plasma cells. It is the second most common adult hematologic malignancy and, to date is considered incurable. This disease is typically one of older individuals, with median age at diagnosis of 69 years [1], a pertinent statistic as a growing percentage of the population falls in this age category. Indeed approximately 24,000 new cases of MM were expected in 2014.[2] In addition to its chronic nature, MM also causes significant morbidity, including anemia, renal insufficiency, hypercalcemia, immune suppression and multiple bone fractures.

The landscape of MM has changed dramatically over the past 10 years, with numerous novel and effective agents, including immunomodulatory agents (thalidomide, lenalidomide, pomalidomide) and proteasome inhibitors (bortezomib, carfilzomib). These agents are usually given in an induction phase; thereafter eligible patients are consolidated with high dose chemotherapy and autologous hematopoietic stem cell transplantation (HSCT). Though the survival of MM pts has improved significantly because of these interventions[3], the 5-year survival is still only 46.6%.[1]

Multiple myeloma and the immune system.

MM is a disease characterized by immune dysregulation and exhaustion, whereby proliferation of malignant plasma cells is not checked by the native immune system.[4] In attempting to overcome this barrier, allogeneic (HSCT) has been studied for its graft versus myeloma (GVM) effect. Long term remissions of MM have been achieved with allogeneic HSCT, suggesting an immunological graft versus myeloma GVM effect.[5] However, treatment-related toxicity and graft versus host disease (GVHD) from donor alloreactive T cells limit the use of this modality. Natural killer (NK) cells are thus ideal candidates for this type of adoptive cellular therapy, as they exert anti-tumor effects without causing GVHD.[6]

NK cell alloreactivity.

NK cells are cytotoxic, non T/B-lymphocytes which are CD56⁺/CD3⁻ [7]. NK cells destroy cells lacking major histocompatibility complex (MHC)-class I molecules [8]. Normal cells are protected from NK cytotoxicity by interaction of inhibitory self-killer immunoglobulin receptors (KIRs) with self-MHC class I molecules. NK cells are thus well suited to kill tumor cells, which often down-regulate MHC class I to evade immune surveillance.[9]

Mismatch between the donor KIR and recipient MHC class I molecules is a proposed mechanism for NK-mediated allo-reactivity. Normally, KIR proteins interact only with specific (class I) human leukocyte antigen (HLA)-B and C molecules [7]. In an allogeneic HSCT with donor-recipient mismatch in the alleles of

HLA-B or C, the target tumor cells of the recipient lack the appropriate HLA alleles, thereby disinhibiting KIR signaling and promoting NK cell cytotoxicity. Alloreactive NK cell-mediated cytotoxicity in hematologic malignancies has been demonstrated *in vitro* and in murine models [10] [11]. Additionally, in patients with hematologic malignancies treated with allogeneic HSCT, NK cell alloreactivity appears to correlated with decreased relapse rate and protection against GVHD.[6] [12]

NK cells and multiple myeloma.

NK cells have demonstrated anti-MM activity *in vitro* and *in vivo*. [13, 14] Unfortunately, autologous PB-NK cells from MM patients appear to be hypofunctional, with a decrease in activating receptors [15] and increase in PD-1. [16] In addition, the variety of treatments for MM, including steroids and alkylating therapies can alter the predictability of obtaining sufficient numbers of activated NK cells. Thus *allogeneic* NK cells may be more a more optimal choice for this adoptive cellular immunotherapy. Several clinical trials of peripheral blood (PB)-derived allogeneic NK cell therapy have been performed. To date, these cells appear safe and, in the leukemia literature, potentially clinically active. [17, 18]

Umbilical cord blood as a source of NK cells.

While much of the work on NK cell adoptive therapy has been done with cells from peripheral blood, we have been interested in developing umbilical cord blood (CB) as a source of NK cells. CB has the benefit of being previously cryopreserved with HLA typing already performed. This allows for an “off-the-shelf” cellular therapy

that does not require manipulation of a live donor (as would be required in the case of PB).

CB is a well-established valuable source of hematopoietic stem cells. Multicenter clinical trials with CB HSCT for hematologic malignancies have shown efficacy comparable to that of unrelated or HLA-mismatched HSCT.[19-22] The lower rates of acute GVHD in some of these trials is attributed to the lower dose of T-cells in CB and proportionately more naïve T-cells compared with bone marrow (BM). Required matching for UCB at only 4/6 of the HLA-A, B and DR antigens allows more flexibility in graft selection than BM.

CB is also a promising source of NK cells; however, the quiescent state and limited baseline NK cell content (2×10^8 cells/ UCB unit) requires *ex vivo* activation and expansion before clinical use. Our laboratory has been able to demonstrate that, after culture with IL-2, CB-NK cells exhibit an activated phenotype comparable to that of PB-NK cells.[23] These CB-NK cells also demonstrate *in vivo* activity in a murine model of acute myelogenous leukemia.

Thus we have been interested in further developing this potential immunotherapy for eventual clinical translation, particularly in MM. To do this we would have to optimize a simple but powerful *ex vivo* expansion process for CB-NK cells that would eventually allow for Good Manufacturing Practice (GMP)-compliant

generation. In addition, the resultant NK cells would have to demonstrate anti-MM activity.

Summary.

In this study we utilized recently developed artificial antigen presenting cells (aAPCs)[24], IL-2 and a gas permeable flask culture system to grow NK cells from both fresh and frozen CB units. We compared this novel expansion procedure to the standard expansion of CB-NK cells from fresh CB, using IL-2 alone. We further examined these expanded NK cells to determine their phenotype, activation state and activity against MM. This is the first step in developing a novel cellular therapy for MM, with a goal of clinical translation in the near future.

Chapter 2

MATERIALS AND METHODS[25]

*This chapter is based on original work done by the student and published as: **Shah N**, Martin-Antonio B, Yang H, Ku S, Lee DA, Cooper LJ, Decker WK, Li S, Robinson SN, Sekine T, Parmar S, Gribben J, Wang M, Rezvani K, Yvon E, Najjar A, Burks J, Kaur I, Champlin RE, Bollard CM, Shpall EJ. “Antigen presenting cell-mediated expansion of human umbilical cord blood yields log-scale expansion of natural killer cells with anti-myeloma activity.” *PLoS One*. 2013 Oct 18;8(10):e76781. doi: 10.1371/journal.pone.0076781. eCollection 2013. *PLoS One* does not require permission for reuse/ reprint of content provided the original article is cited.*

Ethics Statement.

All research involving human materials was approved by the MD Anderson (MDACC) Institutional Review Board (IRB). Cord blood units were obtained from healthy donors who gave written informed consent. All animal work was performed under an MDACC Institutional Animal Care and Use Committee (IACUC)-approved protocol specific to this study.

Cells and cell lines.

K562-based aAPCs expressing membrane bound IL-21 “Clone 9.mblL21” were generously provided by Dr. Laurence Cooper (MDACC, Houston TX). Clone 9.mblL21 cells express membrane-bound IL-21, 41BB ligand, CD64 (FcγRI) and

CD86. This cell line has recently been shown to promote PB-NK cell expansion [24].and is GMP-grade for clinical use. Targets for NK cell functional assays consisted of K562 cells (American Type Culture Collection (ATCC), Rockville, MD) and MM cell lines RPMI 8226 (ATCC), ARP-1 (Multiple Myeloma Research Center, Little Rock AK), and U266 (ATCC). Autologous, unselected CB cells (from the same CB unit as the NK cells) were used as a negative control for 51 chromium (Cr) experiments.

Generation of eGFP-FFLuc-expressing ARP-1 cell line for *in vivo* experiments.

The generation of retrovirus vectors encoding green fluorescent protein (eGFP)-Firefly Luciferase (eGFP-FFLuc) and production of transient retroviral supernatant have been previously described [26, 27]. Briefly, the fusion protein eGFP-FFLuc was cloned into an SFG retroviral vector and retroviral supernatant was produced using 293-T cells co-transfected with the following retroviral vectors: eGFP-FFLuc SFG plasmid, the Peg-Pam-e plasmid containing the sequence for the MoMLV gag-pol and the RDF plasmid encoding for the RD114 envelope. Retroviral supernatant was collected at 48 and 72 hours after transfection and stored at -80°C for further use. For the generation of eGFP-FFLuc-expressing ARP-1 tumor cells, 50,000 cells were plated in presence of retroviral supernatant encoding eGFP-FFLuc in one well of a 24-well plate pre-coated with recombinant fibronectin fragment (CH-296; Takara Shuzo, Otsu, Japan). Transduced ARP-1 cells were expanded and eGFP expression evaluated by fluorescence-activated cell sorter (FACSCalibur; Becton-Dickinson (BD), San Jose, CA) analysis, whereas expression of FFLuc was

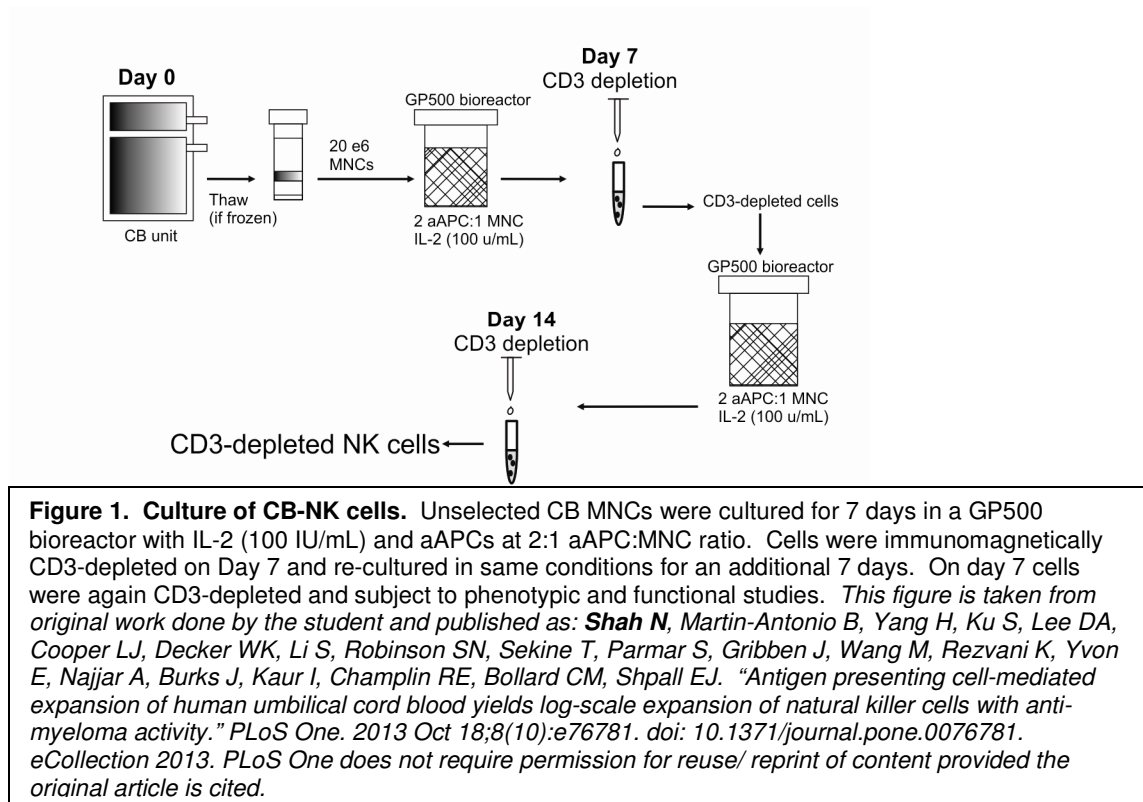
detected using D-luciferin (Promega, Madison, WI) and bioluminescence measured with a luminometer (Modulus; Turner BioSystems, Sunnyvale, CA). Because of the absence of selection gene in the eGFP-FFLuc retroviral construct, single cell cloning of the ARP-1-transduced cells was performed to isolate and expand an ARP-1 clone (clone # 24) with high level of eGFP and FFLuc expression. As ARP-1 expresses both CD138 and kappa light chain [28, 29], Clone 24 was further validated by flow cytometry analysis for CD138 and Kappa light chain expression and enzyme-linked immunosorbent assay (ELISA) for kappa light chain secretion.

Isolation and expansion of umbilical cord blood-derived NK cells.

CB units were obtained from healthy donors who gave informed consent under MDACC IRB-approved protocols. Culture media was comprised of 45% RPMI-1640 (Cellgro, Manassas, VA) and 45% Click's media (Irvine Scientific, Santa Ana, CA) supplemented with 10% AB human serum (Atlanta Biologicals, Lawrenceville, GA) and 100 IU/mL IL-2 (Proleukin; Chiron, Emeryville, CA).

CB mononuclear cells (MNCs) were isolated from fresh or frozen CB units by ficoll density gradient centrifugation. Twenty million MNCs were plated in 400 mL media in a GP500 gas permeable bioreactor (Wilson Wolf Corporation, New Brighton, MN) with irradiated (100 Gy) aAPC feeder cells (2:1 feeder cell:MNC ratio) at 37° C. IL-2 was replenished every 2-3 days. On day 7, cultured cells were CD3-depleted via immunomagnetic depletion according to manufacturer's instructions (Miltenyi Biotech, Auburn, CA). Remaining cells were then re-plated in the same

conditions, re-stimulated with aAPC feeder cells and cultured for an additional 7 days (Figure 1). Flow cytometric analysis was performed on Days 0, 7 and 14 during the expansion. NK cell number was determined by multiplying the live total nucleated cell count by the percentage of CD56⁺/CD3⁻ cells. Differences in cell growth were calculated using a 2-tailed student's t-test (Microsoft Excel 2010, Redmond, WA).



Original expansion techniques.

For comparison, CB-NK cells were also expanded by a method already known to be successful in our laboratory [30]. Fresh CB MNCs were isolated as above and then subjected to CD56⁺ immunomagnetic selection. These cells were then suspended at 1×10^6 cells/mL culture media with IL-2 at 500 IU/mL. The cells were cultured for 14 days at 37^o C; IL-2 was replenished every 2-3 days.

NK cell phenotyping via flow cytometry.

The following antibodies were used: FITC-conjugated CD45, CD158a, CD158b, CD94; PE-conjugated CD16, CD56, NKp30, NKp46, NKp44, NKG2C; PerCP-conjugated CD3; APC-conjugated CD56, NKG2A; Alexa Fluor 647-conjugated Eomesodermin (Eomes), T-bet (BD Biosciences); FITC-conjugated CD158e1 (BioLegend, San Diego, CA); aAPC-conjugated NKG2A (Beckman Coulter, Brea, CA). Intracellular staining for Eomes and T-bet was performed per manufacturer's guidelines (BD Cytotfix/ Cytoperm, BD Biosciences). Data were acquired by the BD FACSCalibur device using BD CellQuest-Pro software. Flow cytometry analysis was performed using CellQuest and FlowJo (Tree Star, Ashland, OR) software. Differences in mean fluorescence intensity (MFI) were calculated using a two-sided paired t-test (Microsoft Excel 2010).

Immunofluorescence and confocal microscopy image acquisition.

Immunofluorescent labeling was performed as previously described [31]. Target cells were labeled with CellTracker Blue CMAC (7-amino-4-chloromethylcoumarin, Molecular Probes, Eugene, OR). NK cell-target cell conjugates were formed by suspending equal volumes and cell numbers of NK effector cells and target cells (5×10^6 /mL) in culture media for 15 min at 37°C. Cells were then transferred onto microscope slides using a cell concentrator (Cytofuge 2, IRIS International, and Chatsworth, CA), fixed with 3% methanol-free formaldehyde and then permeabilized. NK effector cell F-actin was stained with rhodamine-

phalloidin (Molecular Probes, Invitrogen, Carlsbad, CA). Images were acquired using an Olympus IX81 microscope (Center Valley, PA).

NK cell ⁵¹Cr cytotoxicity assay.

Serial dilutions of NK cells were co-incubated in triplicate for 4 hours with 5000 ⁵¹Cr-labeled target cells (Amersham Pharmacia Biotech, Piscataway, NJ), in a total volume of 100 μ l in a V-bottom 96-well plate (Corning, Corning, NY).

Thereafter, supernatants (50 μ l) were harvested and transferred to a Luma-Plate-96 (Perkin-Elmer, Waltham, MA). After drying overnight, ⁵¹Cr release was measured on a TOPCount NXT microplate scintillation and luminescence counter (Perkin-Elmer).

Cytotoxicity was determined by the formula: cytotoxicity = (sample value - spontaneous lysis) / (max-lysis - spontaneous lysis) x 100%.

ARP-1 myeloma murine model

NOD/SCID IL-2R γ ^{null} (NSG) mice (Jackson Laboratories, Bar Harbor, ME) were irradiated with 300 cGy and inoculated with 1×10^6 eGFP-FFLuc -transduced ARP-1 cells (Clone 24) intravenously on day -1. Where indicated, 10×10^6 *ex vivo*, fresh, aAPC-expanded CB NK cells were given retro-orbitally on days 0, 12 and 19 with IL-2 (2000 IU intraperitoneally (IP) three times per week). Mice were subjected to twice weekly bioluminescence imaging (BLI) and weekly serum kappa light chain measurements. Prior to image acquisition mice were anesthetized with 2% isoflurane in 98% oxygen. BLI was performed using a Xenogen IVIS 200 system (Caliper, Waltham, MA) 10 minutes following a 100 μ L IP injection of D-luciferin (20

mg/mL phosphate buffered saline). BLI images were acquired at 5-minute exposures and superimposed on bright field photographs of the animals. Signal quantitation in photons/second (p/s) was performed by determining the photon flux rate within standardized regions of interest (ROI) using Living Image software (Caliper). Serum kappa levels were measured by a commercially available ELISA kit (Bethyl Laboratories, Montgomery, TX) according to manufacturer's instructions. Results reported are a representative experiment with 5 mice in each group. Differences in BLI and serum kappa levels were calculated using a 2-tailed student's t-test (Microsoft Excel 2010). Survival was calculated using the Kaplan-Meier method (SAS statistical software, version 9.2, Cary, NC).

Chapter 3

RESULTS[25]

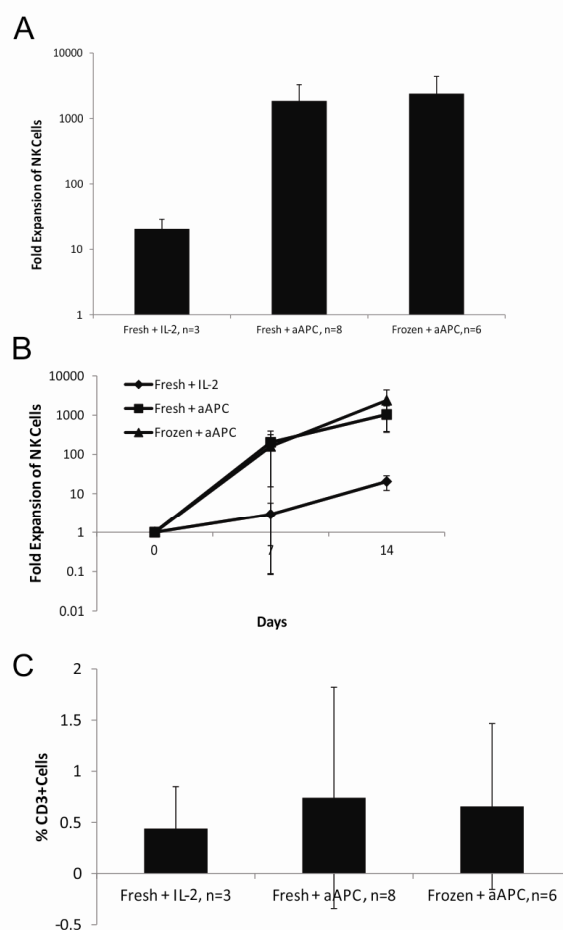
*This chapter is based on original work done by the student and published as: **Shah N, Martin-Antonio B, Yang H, Ku S, Lee DA, Cooper LJ, Decker WK, Li S, Robinson SN, Sekine T, Parmar S, Gribben J, Wang M, Rezvani K, Yvon E, Najjar A, Burks J, Kaur I, Champlin RE, Bollard CM, Shpall EJ.** “Antigen presenting cell-mediated expansion of human umbilical cord blood yields log-scale expansion of natural killer cells with anti-myeloma activity.” *PLoS One*. 2013 Oct 18;8(10):e76781. doi: 10.1371/journal.pone.0076781. eCollection 2013. *PLoS One does not require permission for reuse/ reprint of content provided the original article is cited.**

aAPC-mediated CB-NK expansion from fresh or cryopreserved CB units yields significantly greater fold expansion of NK cells than expansion of CD56⁺ cells with IL-2 alone.

In comparison with our original expansion approach of CD56-selected cells cultured with IL-2 alone, culture of either fresh or frozen CB MNCs with aAPC feeder cells resulted in greater expansion of NK cells after culture for 14 days ($p < 0.05$ for both fresh or frozen conditions, Figures 2A and 2B). Culturing of fresh CB MNCs ($n=8$) with aAPC feeder cells yielded a mean fold expansion of 1848 fold (609 fold – 4778 fold) while culturing of frozen CB MNCs ($n=6$) with feeder cells yielded a mean fold expansion of 2389 fold (103 fold – 4931 fold). This was in comparison to 20 fold (11 fold -27 fold) expansion from culture of fresh CD56⁺-selected cells with IL-2

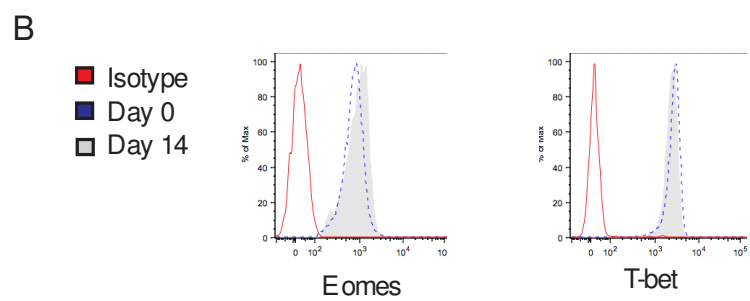
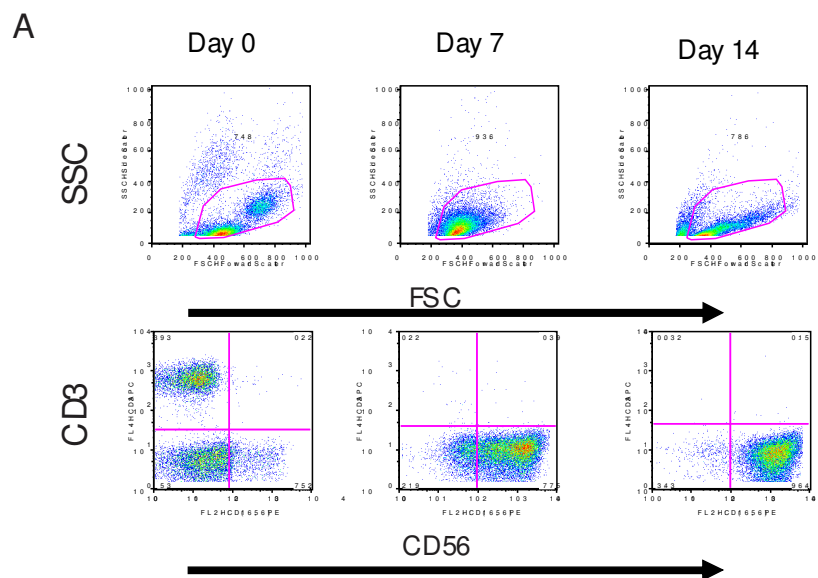
alone (n=3). The difference in NK cell yield was apparent by day 7 for the fresh CB culture with aAPC feeders ($p < 0.05$) but did not reach statistical significance for the frozen CB condition until day 14 ($p = 0.06$ at day 7). As seen in Figure 2C, the final culture contained very few ($\leq 1\%$) $CD3^+$ cells and this was not significantly different between the 3 culture conditions: mean value of 0.44% $CD3^+$ cells from the culture with IL-2 alone, 0.74% $CD3^+$ cells from fresh CB MNCs with aAPC feeders and 0.66% $CD3^+$ cells from frozen CB MNCs with aAPC feeders ($p > 0.5$ for all comparisons).

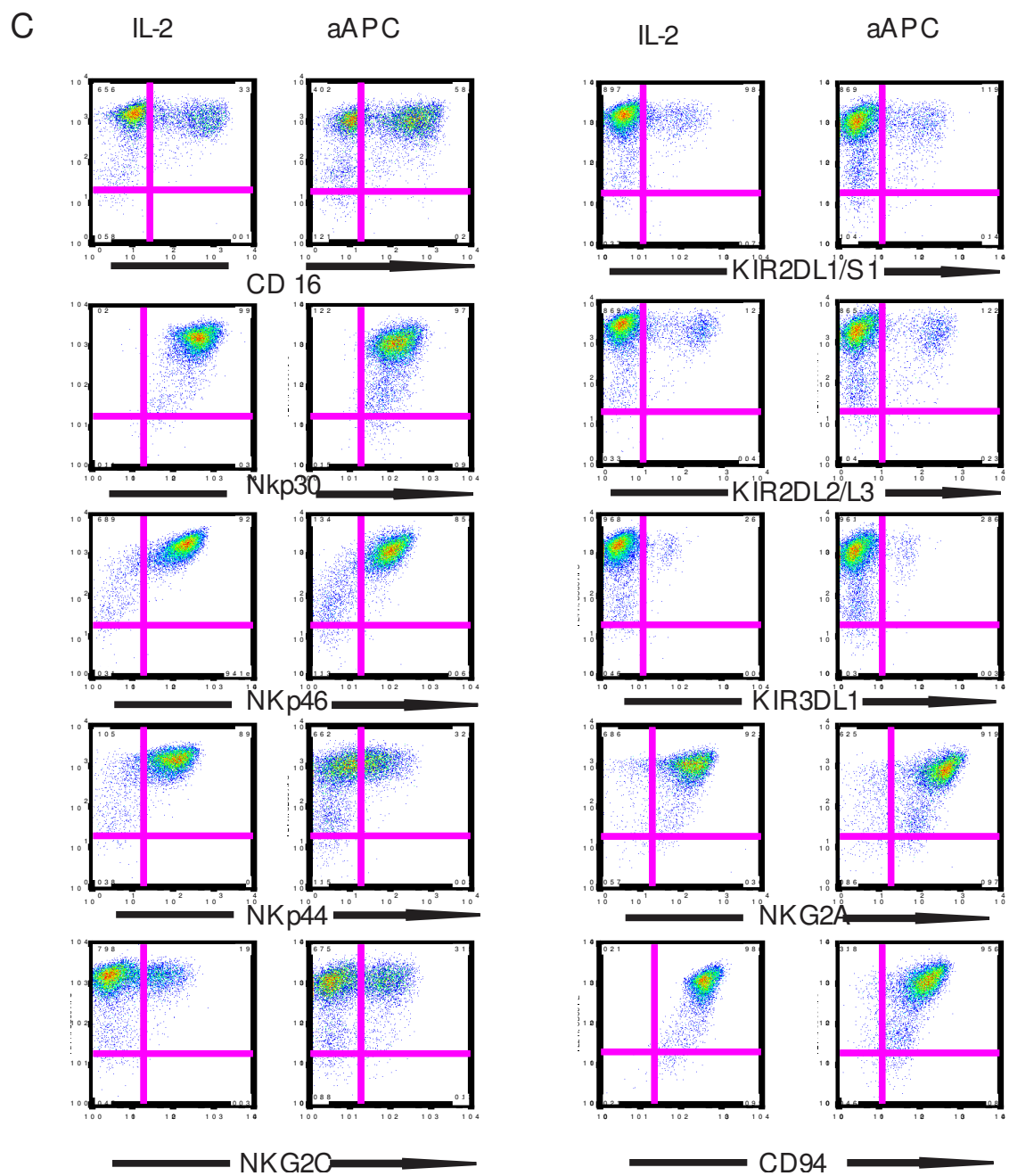
Figure 2. Co-culture of CB MNCs with IL-2 and aAPCs yields significantly greater expansion of NK cells than culture with IL-2 alone. A. Mean fold growth of $CD56^+/CD3^-$ NK cells from 8 fresh and 6 frozen cord blood expansions with aAPCs and IL-2 versus 3 expansions with IL-2 alone (14 day culture). B. Time course of NK cell growth over 14 day culture between all 3 conditions. By day 7, the fresh CB aAPC-containing culture demonstrated greater NK cell growth than culture with IL-2 alone ($p < 0.05$). The frozen CB showed a similar trend at day 7, which did not reach statistical significance ($p = 0.06$). C. All three culture conditions yielded comparable, low percentages of $CD3^+$ cells: 0.44%, 0.74% and 0.66% $CD3^+$ cells from the culture with IL-2 alone, fresh CB MNCs with aAPC feeders or frozen CB MNCs with aAPC feeders respectively ($p > 0.5$ for all comparisons). Mean \pm SD is shown for each figure. $P < 0.05$ where indicated (*). *This figure is taken from original work done by the student and published as: Shah N, Martin-Antonio B, Yang H, Ku S, Lee DA, Cooper LJ, Decker WK, Li S, Robinson SN, Sekine T, Parmar S, Gribben J, Wang M, Rezvani K, Yvon E, Najjar A, Burks J, Kaur I, Champlin RE, Bollard CM, Shpall EJ. "Antigen presenting cell-mediated expansion of human umbilical cord blood yields log-scale expansion of natural killer cells with anti-myeloma activity." PLoS One. 2013 Oct 18;8(10):e76781. doi: 10.1371/journal.pone.0076781. eCollection 2013. PLoS One does not require permission for reuse/ reprint of content provided the original article is cited*



aAPC-mediated expansion yields a pure population of NK cells with a mature phenotype.

As seen in Figure 3A, co-culture of CB MNCs with IL-2 and aAPC feeder cells yielded a population that was pure for NK cells at the end of the 2 week expansion period. After CD3-depletion, 96% of cells were CD56⁺/CD3⁻ and less than 1% were CD3⁺. CB-NK cells expanded with aAPCs demonstrated a CD56^{hi} phenotype similar to CB-NK cells expanded with IL-2 alone. Of note, culture of unselected CB MNCs with IL-2 and soluble IL-21 yielded a relatively pure CD56⁺/CD3⁻ NK cell population but with limited expansion of cells (mean expansion of 14 fold, data not shown). In addition, after log-fold expansion, aAPC-expanded CB-NK cells did not appear exhausted; rather, CB-NK cells continued to strongly express Eomes and T-bet, transcription factors recently recognized as necessary for NK cell maturation and activation [32, 33] (Figure 3B). Interestingly, the surface expression of NK cytotoxicity receptors (NCRs) NKp30, NKp46 and NKp44 was significantly lower for aAPC-expanded CB-NK cells versus IL-2-expanded CB-NK cells ($p \leq 0.05$ for all three NCRs). However, the expression of KIR antigens, NKG2A, co-receptor CD94 and the activating receptor NKG2C was similar between the two expansion methods (Figure 3C).





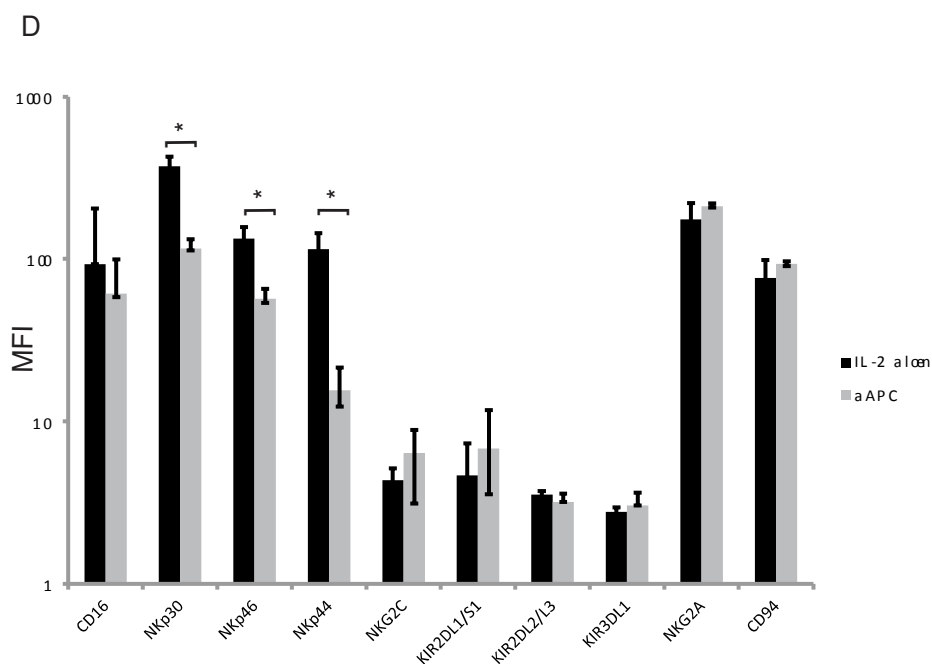


Figure 3. Phenotype of CB-NK cells cultured with aAPCs. A. Over the 14-day expansion, CB-NK cells cultured with aAPC feeder cells demonstrated a progressively pure, CD56⁺/CD3⁻ population, (representative dot plots of 17 expansions). B. aAPC-expanded CB-NK cells maintained Eomesodermin^{hi} and T-bet^{hi} phenotype after expansion. Representative histograms from 3 different CB-NK expansions; cells are gated on the live CD56⁺ population. C. CB MNCs from the same CB unit were expanded with aAPCs +IL-2 or IL-2 alone (n=3 separate CB units). Representative dot plots of NK cell surface receptor expression on day 14 are shown. D. By median fluorescence intensity (MFI), aAPC-expanded CB-NK demonstrated a decreased surface expression of the NCRs NKp30, NKp46 and NKp44. However there was a similar expression between the conditions of the KIR antigens, inhibitory receptor NKG2A, co-receptor CD94 and activating receptor NKG2C), (n= 3 paired expansions, mean +/- SD is shown, p ≤0.05 where indicated). *This figure is taken from original work done by the student and published as: **Shah N, Martin-Antonio B, Yang H, Ku S, Lee DA, Cooper LJ, Decker WK, Li S, Robinson SN, Sekine T, Parmar S, Gribben J, Wang M, Rezvani K, Yvon E, Najjar A, Burks J, Kaur I, Champlin RE, Bollard CM, Shpall EJ. "Antigen presenting cell-mediated expansion of human umbilical cord blood yields log-scale expansion of natural killer cells with anti-myeloma activity." PLoS One. 2013 Oct 18;8(10):e76781. doi: 10.1371/journal.pone.0076781. eCollection 2013. PLoS One does not require permission for reuse/ reprint of content provided the original article is cited.***

CB-NK cells cultured with aAPCs demonstrate *in vitro* anti-myeloma activity.

In order to kill targets, NK cells must directly contact the cell of interest and form the “NK immune synapse” (NKIS) [34, 35]. Our lab has previously demonstrated that expansion of CB-NK cells is necessary to repair the defective NKIS exhibited by naïve CB-NK cells [30]. To demonstrate that this synapse ability is maintained in CB-NK cells expanded with aAPC feeder cells, we performed a series of synapse assays with various MM targets. As shown in Figure 4A, NK cells

cultured with aAPC feeder cells formed a functional NKIS (demonstrated by F-actin polarization) with the classic NK cell target K562, MM cell lines RPMI 8226, aARP-1 and U266.

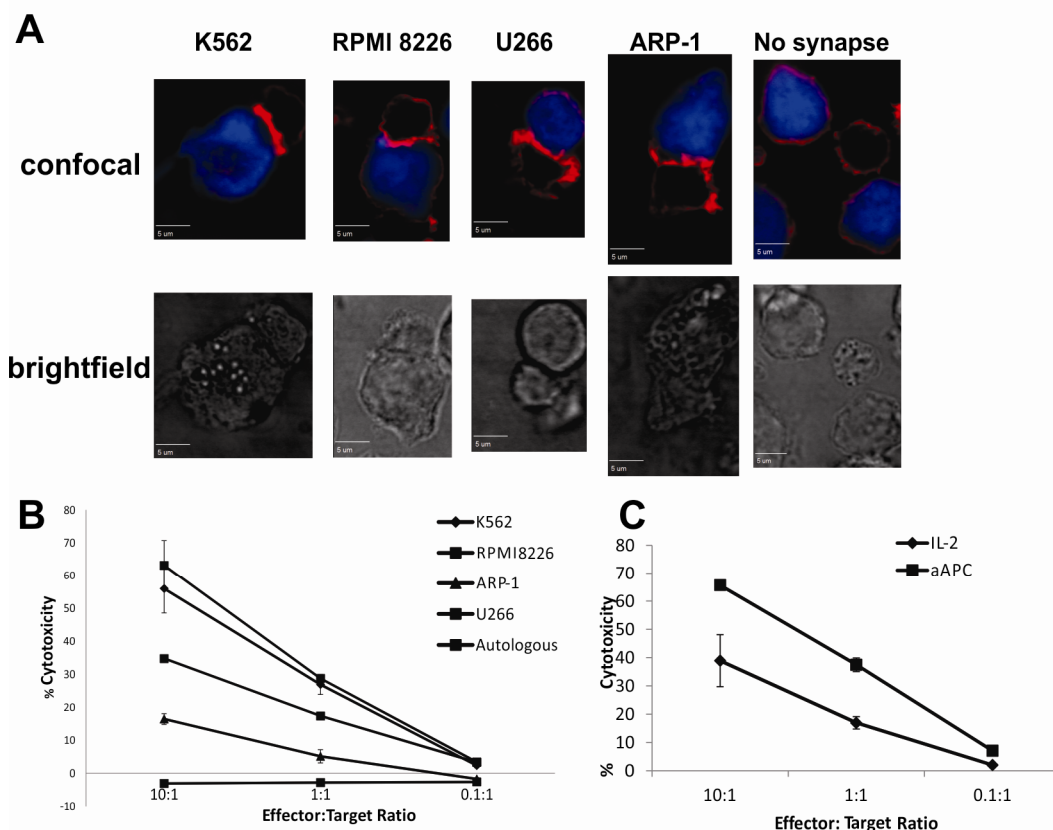


Figure 4. aAPC-expanded CB-NK cells form immunological synapses with and are cytotoxic against myeloma targets . A. CMAC-labeled tumor targets (blue) were incubated at a 1:1 ratio with aAPC-expanded CB-NK cells for 15 minutes. Conjugates were then fixed, permeabilized and stained for NK effector cell F-actin with rhodamine-phalloidin (red). Confocal and brightfield images were acquired; representative images from each slide are shown. aAPC-expanded CB-NK cells form immune synapses with the classic NK target K562 as well as a variety of MM cell lines. B. aAPC-expanded CB-NK cells were co-incubated in triplicate for 4 hours with ^{51}Cr -labeled target cells at ratios as shown. Supernatants were then harvested and analyzed the next day for ^{51}Cr content. % Cytotoxicity = (sample value-spontaneous lysis) / (max-lysis-spontaneous lysis) x 100%. CB-NK cells demonstrate dose-dependent cytotoxicity against K562 (classic NK cell target) and MM cells lines RPMI 8266, ARP-1 and U266 (representative of n>3 assays for each cell line). C. aAPC-Expanded CB-NK cells displayed equal or more cytotoxicity against K562 cells versus CB-NK cells expanded with IL-2 alone (representative from n=4 assays) . *This figure is taken from original work done by the student and published as: Shah N, Martin-Antonio B, Yang H, Ku S, Lee DA, Cooper LJ, Decker WK, Li S, Robinson SN, Sekine T, Parmar S, Gribben J, Wang M, Rezvani K, Yvon E, Najjar A, Burks J, Kaur I, Champlin RE, Bollard CM, Shpall EJ. "Antigen presenting cell-mediated expansion of human umbilical cord blood yields log-scale expansion of natural killer cells with anti-myeloma activity." PLoS One. 2013 Oct 18;8(10):e76781. doi: 10.1371/journal.pone.0076781. eCollection 2013. PLoS One does not require permission for reuse/ reprint of content provided the original article is cited.*

To demonstrate the functionality of CB-NK cells expanded with aAPC feeder stimulation, we performed a standard ^{51}Cr cytotoxicity assay. aAPC-expanded CB-NK cells were cytotoxic to all of the MM cell line targets (Figure 4B). Furthermore, despite the differences in phenotype with regard to the NCRs, in comparison with CB-NK cells expanded with IL-2 alone, the aAPC-mediated expanded CB-NK cells demonstrated equal or greater cytotoxicity against K562 (Figure 4C). This finding was consistent across the MM cell lines as well (data not shown). Neither of the CB-NK preparations demonstrated autologous cytotoxicity.

Treatment with expanded CB-NK cells delays development of myeloma in a murine model.

To investigate whether *ex vivo* expanded CB-NK cells can inhibit the growth of MM cells *in vivo*, we studied NSG mice treated with GFP firefly luciferase-transduced ARP-1 cells (Clone 24). Using the bioluminescent signal intensity as a surrogate for tumor cell density, serial images demonstrated that mice treated with CB-NK cells had a delay in the onset of MM (Figure 5A). After 1 week, the signal intensity (p/s) was significantly greater in those mice who received Clone 24 ARP-1 cells alone versus those who received Clone 24 ARP-1 cells and CB-NK cells (Figure 5B, $p < 0.05$ from Day 8-22) This was consistent with the ELISA analysis of serum kappa light chains; mice receiving Clone 24 ARP-1 cells alone had significantly more measurable serum kappa than mice who received Clone 24 ARP-1 cells and CB-NK cells, (Figure 5C, $p < 0.01$ at each time point). Finally, there was also a difference in survival between the 2 groups with a median survival of 31 days

in the mice who received Clone 24 ARP-1 cells alone versus 38 days for the mice who received Clone 24 ARP-1 cells and CB-NK cells, (Figure 5D, $p = 0.003$).

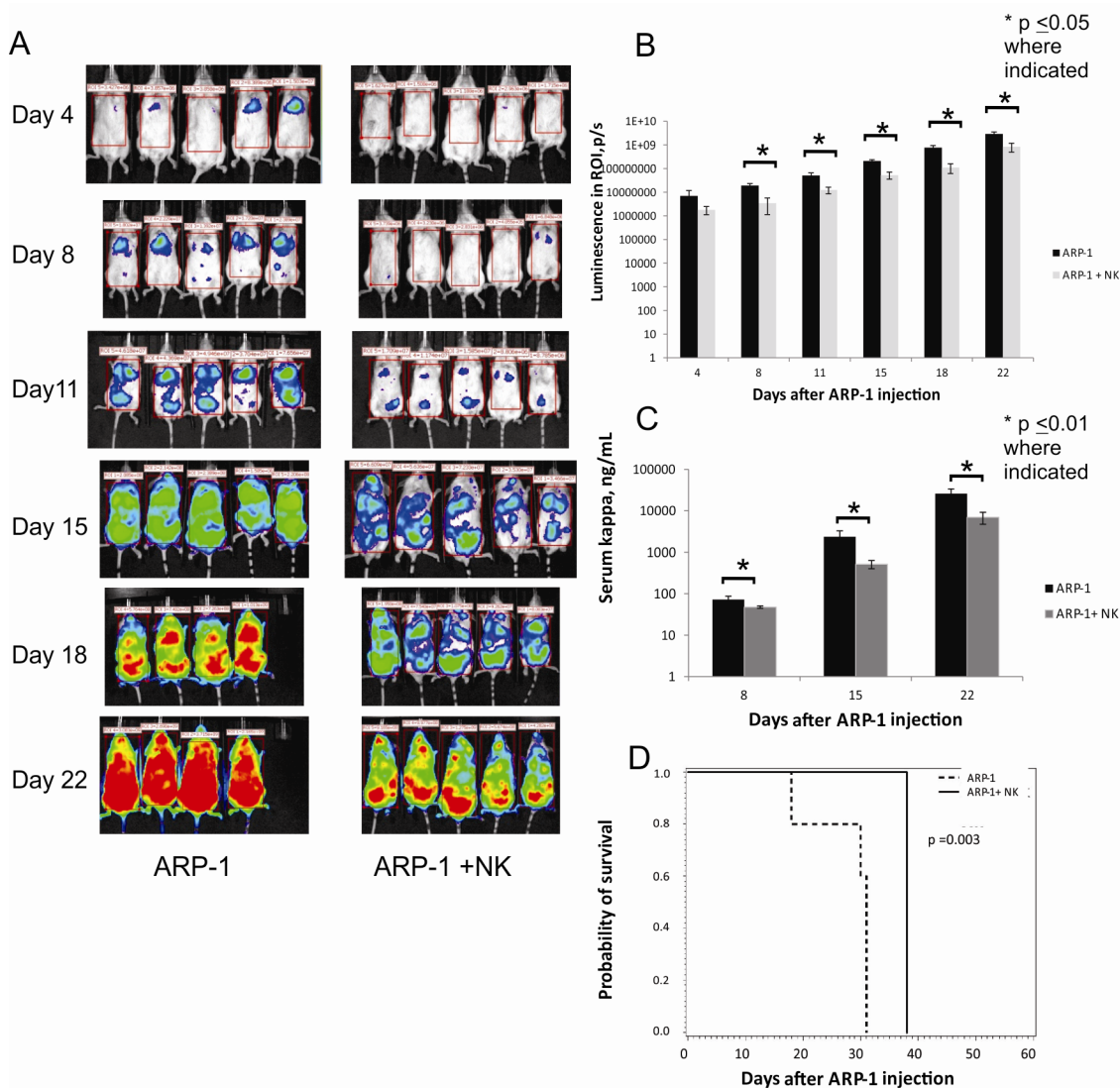


Figure 5. aAPC-expanded CB-NK cells delay development of myeloma in a NSG murine model. 1×10^6 GFP firefly luciferase-transduced ARP-1 cells (Clone 24) were given IV on day -1. In the CB-NK treated group, 10×10^6 *ex vivo*, aAPC-expanded CB NK cells were given retro-orbitally on days 0, 12 and 19 with IL-2, 2000 IU (IP) three times per week. Serial BLI and kappa ELISA measurements were acquired until day 18. Results represent mean values of $n=5$ mice in each group until day 18, by which time 1 mouse in the ARP-1 alone group had died. **A.** Serial BLI images demonstrate impaired myeloma development in mice receiving CB-NK cells. **B.** Signal intensity (p/s) was significantly greater in mice receiving Clone 24 ARP-1 cells alone versus those receiving both Clone 24 ARP-1 cells and CB-NK cells. Region of interest (ROI) is indicated by rectangles superimposed on each mouse from Figure 5A, $p \leq 0.05$ at days 8-22. **C.** Serum kappa levels (ng/mL) were significantly higher in mice treated with Clone 24 ARP-1 cells versus those treated with Clone 24 ARP-1 cells and CB-NK cells, $p \leq 0.01$ at each time point. **D.** By Kaplan-Meier method, there was a significant difference in survival of the mice, ($p=0.003$) in favor of the NK-treated group. The mice who received Clone 24 ARP-1 cells alone had a median survival of 31 days versus 38 days for the mice who received Clone 24 ARP-1 cells and CB-NK cells. *This figure is taken from original work done by the student and published as: Shah N, Martin-Antonio B, Yang H, Ku S, Lee DA, Cooper LJ, Decker WK, Li S, Robinson SN, Sekine T, Parmar S, Gribben J, Wang M, Rezvani K, Yvon E, Najjar A, Burks J, Kaur I, Champlin RE, Bollard CM, Shpall EJ. "Antigen presenting cell-mediated expansion of human umbilical cord blood yields log-scale expansion of natural killer cells with anti-myeloma activity." PLoS One. 2013 Oct 18;8(10):e76781. doi: 10.1371/journal.pone.0076781. eCollection 2013. PLoS One does not require permission for reuse/ reprint of content provided the original article is cited. Please note Dr. Beatriz Martin contributed significantly to this figure in taking images and performing the ELISA. Dr. Eric Yvon generated the ARP-1 cell line.*

Chapter 4

DISCUSSION

While there have been numerous advances in therapy options for myeloma, the disease remains incurable. As evidence accumulates to show an association between this disease and immune dysfunction [4, 36, 37] the urgency for immune therapies concurrently grows. These therapies currently include immunomodulatory agents and antibodies; experience with allogeneic HSCT and donor lymphocyte infusions [38] indicates that allogeneic cellular therapy is another approach for this modality.

We have been interested in developing NK cell therapy as a possible adjunct to traditional chemotherapy. NK cells have the benefit of having anti-MM activity without the risk of GVHD. Though PB-NK cells have been given for this patient population, PB requires a live, related, healthy donor to go through a separate procedure. Thus we have been working to develop CB-NK cell therapy, which would provide an “off the shelf” source of NK cells, obviating the need for an additional healthy donor procedure.

In order to bring CB-NK cell therapy to the clinic we had to overcome several barriers. CB-NK cells require robust, reliable *ex vivo* expansion. In addition, this expansion must be possible from a cryopreserved CB unit as this is how all banked

CB units are stored. CB-NK cells must exhibit an activated, unexhausted phenotype. Finally, these CB-NK cells must demonstrate anti-MM activity.

In this study, we were able to complete the first of many steps necessary to develop this novel immunotherapy, in preparation for clinical application. Our unique expansion process required fairly simple materials (gas-permeable cell culture bioreactors, IL-2 and aAPCs) and a relatively short expansion phase. Using this strategy we were able to expand NK cells 1000-2000 fold, a growth significantly greater than standard expansion with IL-2 alone. Importantly, this expansion was possible using fresh or cryopreserved CB units. Thus we may consider this method as a potential protocol for GMP-compliant CB-NK expansion.

In addition, the resultant CB-NK cells were a pure and active product. There was very little CD3 contamination (an important factor in avoiding GVHD) and the NK cells demonstrated activating receptors with no evidence of exhaustion. Furthermore, these cells were able to synapse with and kill MM cells and delay the development of MM *in vivo*. While there was a decrease in the MFI of NCRs (NKp30, NKp46 and NKp44) for the aAPC-mediated expansion, this did not translate to any decrease in cytotoxicity.

To put these results in perspective, the number of NK cells one could yield from a single 20% fraction of the CB unit could be as much as 1×10^9 cells or 1.5×10^7 /kg for a 75 kg person. Thus, this robust expansion protocol now also grants the potential for multiple NK infusions, depending on how the CB unit is frozen. This can

have significant implications when considering maintenance immunotherapy for a disease like MM, for which maintenance with the immunomodulatory drug lenalidomide is standard.[39]

With these important first steps made, the possibility of adoptive CB-NK therapy is closer in reach. This is even more so, considering the growth in public CB banks to greater than 160, with over 800,000 units banked worldwide. Indeed, the CB community has begun to expand its focus to include immunotherapy, including virus-specific T cells, regulatory T cells and NK cells.[40, 41] The results presented in this study are in line with this mission in the CB community.

Chapter 5

STRENGTHS AND WEAKNESSES

This study describes a novel, relatively simple expansion procedure for CB-NK cells. However, it is mainly a methods development study. Thus we are only able to make conclusions about the expansion capability and the potential for anti-MM effect. Less can be said about the specific mechanisms underlying NK cell activity or the reproducibility in the clinical setting.

Application of CB-NK cells.

Our results compare favorably with other NK expansion techniques and are among the first to report CB-NK activity against MM. Previous studies have shown efficient expansion of CB-NK cells; however, these techniques require several steps with upfront selection of CD34⁺ cells a culture period of 35-42 days.[42, 43] This could present a logistical problem when trying to plan for clinical use; in addition there would be more time for potential contamination, as well as cost of extended expansion. Finally, our method only requires a segment of the CB unit, which leaves the remainder for potential future cellular therapy use or for hematopoietic recovery in the case of allogeneic HSCT.

Role of CB-NK cells against MM.

Regarding the activity against MM, the novelty of this study rests in CB as the source for the NK cells, as others have demonstrated NK activity against MM.[14]

Unfortunately, due to the nature of CD138⁺ primary MM cells, we were unable to label these cells sufficiently with ⁵¹Cr, and thus unable to demonstrate dose dependent cytotoxicity against primary MM cells. In addition our *in vivo* model showed a delay of progression of MM but did not demonstrate eradication. This is not dissimilar to the clinical setting, in which immunotherapies are known to work best in the setting of concomitant chemotherapy. Finally, all of our functional data was acquired with infusion of freshly expanded CB-NK cells. Thus we are unable to make any statements about the activity of these expanded cells after freezing and thawing.

Other considerations not addressed in this study include the role of KIR in NK cell activity against MM, something that we were not able to study as we chose the CB units that were available to us and did not perform KIR or HLA typing on neither the CB-NK cells nor the MM cell lines. Additionally, though we were able to demonstrate an unexhausted phenotype of the expanded CB-NK cells with preservation of Eomes and Tbet, we were not able to examine this *in vivo*. This is known to be a potential limitation which has been highlighted by other adoptive transfer cell therapy studies.[32]

Chapter 6

FUTURE DIRECTIONS

Our next steps will be several. First, we will validate this protocol in a GMP-based setting to prove that the expansion process is reliable, in various technical hands. Second we intend to open a first-in-human phase I clinical trial of these cells in conjunction with high dose chemotherapy and autologous HSCT for patients with MM. If we can demonstrate safety then we will move on to an expanded trial to assess efficacy, particularly in high risk patients. Concurrently we will also continue *in vitro* studies to assess the viability and activity of frozen and then thawed CB-NK cells, which would have implications for the exportability of this technology.

Regarding the NK cells themselves, we are interested in further directing their cytotoxicity to the target MM cells that remain untouched by chemotherapy. Thus we are exploring the possibility of transducing these cells with a chimeric antigen receptor (CAR) construct specific for a MM antigen. We are also interested in applying the technique of fucosylation to these NK cells, as previous studies from our laboratory indicate that fucosylation can improve homing of cells to the bone marrow [44], the sanctuary site of MM.

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VITA

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