Umbilical Cord Blood Stem Cells - A Comparative Analysis of Differential Stromal Cell Lines Used to Support Natural Killer Cell Development

A THESIS SUBMITTED TO THE FACULTY OF
THE COLLEGE OF BIOLOGICAL SCIENCES,
UNIVERSITY OF MINNESOTA, TWIN CITIES

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF
BACHELOR OF SCIENCE

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MARCH 2014

Acknowledgements

I would like to thank Dr. Michael Verneris for the research opportunity, laboratory training, wisdom, always supporting and encouraging me to finish this project. I truly respect him as a professor, his inspiration, and his enthusiasm for his research. I would like to acknowledge Matt Weeres for teaching me the crucial lab techniques, culturing each cell line, control conditions, machines control, understanding each step in the protocol, and detailed aspect of human NK cell development from bone marrow stem cells which allowed me to finish my research. I would also like to thank Dr. Hongbo Wang for providing me with procedures required in the lab, training, and precious advice with direction. I also appreciate the advice of Rachel Bergerson and David Knorr who provided me the direction and safety in my laboratory work.

This research opportunity was a very special experience. I learned about clinical research and this gave me a new vision to see academic research in my field. In addition, I have learned how to work with a wide variety of medical professionals and clinical scientists, produce accurate lab results in a fast paced environment. I have gained a deeper understanding of laboratory science as a whole and it was a memorable life experience with wonderful laboratory members.

Dedication

"My parents would give me an Asian name "Yuri," or "bright," as Barack Obama said, believing that in a tolerant America your name is no barrier to success. They imagined me going to the best schools in the land, even though they weren't rich, because in a generous America you don't have to be rich to achieve your potential."

I dedicate my research to my parents. They always believed in my potential and are always there to support me in achieving my dream.

All life demands struggle, my time at College of Biological Sciences School was wonderful. It has been the most competitive and challenging time in my life. This school gave me not only the full scholarship, but also taught me how to survive in the school, how to live like an energetic person, and how to see the big picture of my life.

Abstract

Based on murine models, the transfusion of natural killer (NK) cell that are derived from bone marrow stem cells can augment graft versus leukemia and prevent graft versus host disease; two major complications of allogeneic hematopoietic cell transplantation. Additionally, NK cell therapy has the benefit of potentially curing leukemia without the toxic side effects of chemotherapy. The NK development occurs in the bone marrow and then in the secondary lymphoid tissue (lymph nodes). NK cells may be used to target leukemic cells due to the ability to recognize and destroy them (Imai, 2005). One of the main obstacles to therapeutic use of NK cells is obtaining sufficient numbers to treat patients. We have devised a system to generate NK cells from hematopoietic stem cells (HSCs) that result in >2-3,000 fold expansion from a single stem cell. This developmental process is dependent on stromal cells and we have identified a number of different cell lines that support NK differentiation from HSCs. This research is a comparative analysis of differential stromal cell lines which support the NK cell generation from HSCs. The main purpose of the work is to compare the AFT, EL, HFWT, OP9, and UG cell lines in their ability to induce efficient NK differentiation HSCs. Our results show differences in five different cell lines in their ability to support NK development, NK cell counts and the ability to destroy leukemic cells. These findings will aid future clinical studies using these cells for leukemia treatment.

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(“unstim”) and after stimulation with IL-1β and IL-23 (“stim”). Listed on the x-axis are the stromal cell lines used to create the NK cells.)

Introduction

1.1 Natural Killer (NK) cells and subsets

Natural Killer (NK) cells are lymphoid cells that play vital role in innate immune responses. They represent up to ~15 % human peripheral blood mononuclear cells (PBMC) and are defined as $CD56^{+}$ $CD3^{-}$ lymphocytes. Peripheral blood NK cells can be separated into two major subtypes based on the staining intensity of CD56 and the presence/absence of CD16 (Figure 1). More specifically, $CD56^{bright}$ $CD16^{-}$ cells make up a minority of the PB NK cells (~15%) and $CD56^{dim}$ $CD16^{+}$ NK cells make up the majority (~85%) of the NK cells (Gharehbaghian, 2006).

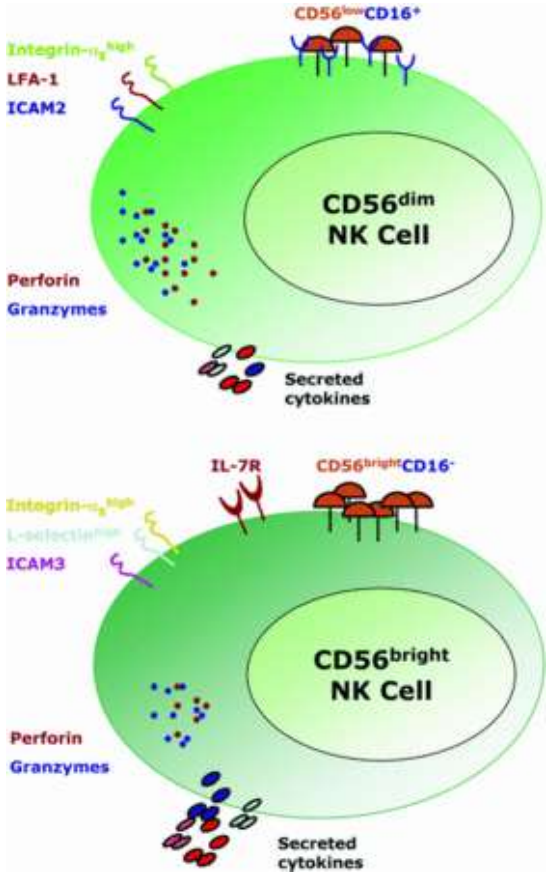

Figure 1. The major differences between human NK $CD56^{dim}$ and $CD56^{bright}$ subsets (Fairclough, 2008).

1.2 NK cell development

Human NK cells go through a series of development stages outlined in figures 2 and 3. NK cells are derived from HSCs and the initial steps of differentiation occur in the bone marrow (BM). The steps of differentiation have been broken into five developmental stages of NK cells. Stage I, pro-NK cell progenitors have a $CD34^{+}$, $CD117^{-}$, $CD94^{-}$, and $CD16^{-}$ phenotype. The stage I cells can be differentiated in to next stage in response to FLT3, IL-3, and IL-7. In stage II, pre-NK cells the cells express two key cytokines receptors CD117 and CD122 and have the functional capacity to respond to stem cell factor (SCF) and IL-2 or IL 15, which leads to development into stage III. Stage III NK cells are thought to be "committed" to the NK lineage and have referred to immature NK (iNK) cells. At this step, iNK cells lack all function (cytotoxicity or cytokine production). At stage IV, also known as $CD56^{bright}$ NK cells, they have the ability to make cytokines, but lack cytotoxicity. Stage V cells are released from the lymph node and enter the blood and are identified by the coexpression of $CD16^{+}$ and $CD94^{+}$ and are also called $CD56^{dim}$ NK cells These cells have robust cytokine production and cytotoxicity. The developmental system that I used closely resembles these steps (Grzywacz, 2011). .

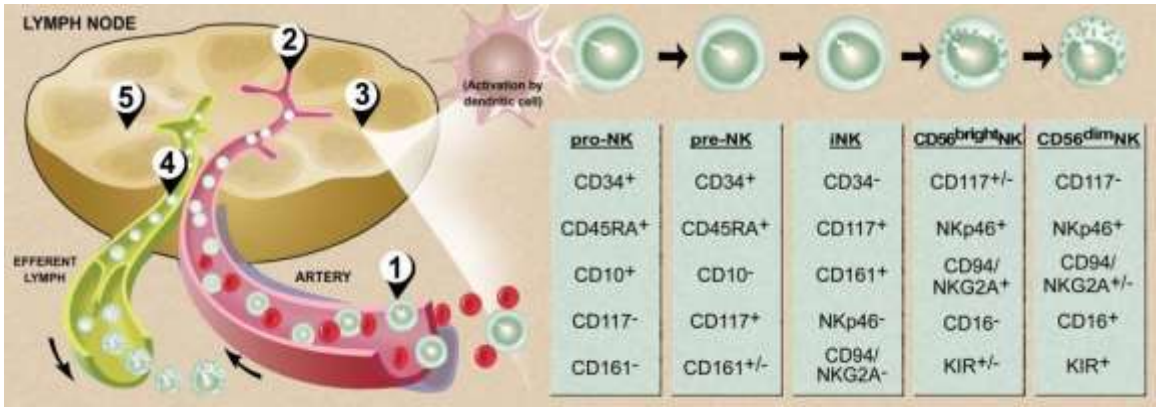

Figure 2. Model of human NK-cell development. Bone marrow – derived $CD34^{+}$ $HPCs^{+}CD45RA^{+}$ circulate (Caligiuri, 2008).

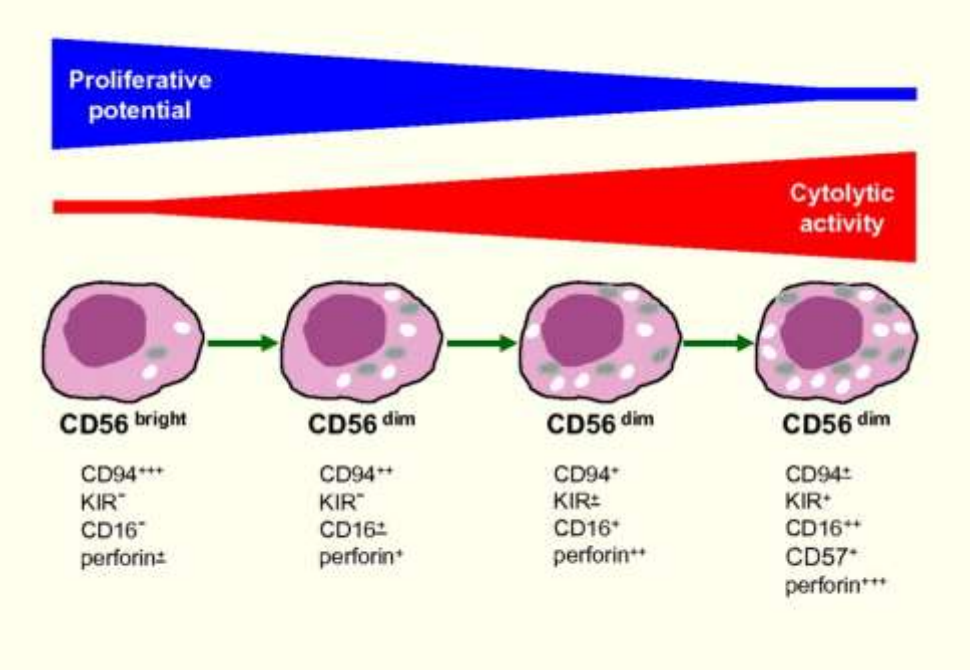

Figure 3. NK cell subsets, model of human CD56 NK-cell differentiation (Moretta, 2010).

1.3 NK Cell triggering and inhibitory receptors

NK cells can recognize aberrant cells and have the ability to kill them and also produce cytokines, such as interferon gamma (IFN-γ). In addition to recognizing tumor cells, NK cells also recognize virally infected cells (Mickel, 1988). NK cells recognize deranged cells due to the loss of MHC class I or the expression of "stress receptors" not normally expressed by healthy cells. NK cells recognize these cells using a variety of receptors that both activate and inhibit NK cell function, including Killer Ig receptors (KIR), NKG2D, DNAM-1, 2B4, NKp30 (Ahn, 2010; Vacca, 2008). Upon encounter with a potential target cells these receptors become engaged and it is the balance of signaling that determines whether the NK cells is activated. NK cells also become activated when cytokines are present including, IFN-γ and tumor necrosis factor-alpha

(TNF-α). As well, NK cells produce a number of cytokines including interleukin10 (IL-10), granulocyte macrophage-colony stimulating factor (GM-CSF), and tumor necrosis factor-beta (TNF-β) (Moretta A., Morretta L, 2004).

1.4 Goal of Research

One of the main limitations to the clinical use of NK cells is obtaining sufficient numbers to treat patients. We have previously found that a number of different cell lines support the development of HSCs into the NK lineage, in the presence of cytokines. The goal of this project was to compare these lines in their ability to give rise to NK cells. We also sought to compare the function of these NK cells developed under the influence of the different stromal lines.

2. Materials and Methods

2.1 Primary human CD 34^+ cells isolated from umbilical cord blood (UCB)

UCB was obtained after vaginal and cesarean deliveries and by drainage of blood into sterile collection tubes that have the anticoagulant citrate-phosphate-dextrose. All cord blood specimens were obtained from New York Blood center on IRB approved protocols. UCB samples were processed within 48 hours of collection. CB mononuclear cells (MNCs) include rare hematopoietic stem cells (~1%). $CD34^+$ cells were isolated from CB using positive immune-magnetic bead separation techniques (Miltenvi biotech). Selected cells were about 90% pure by FACS analysis.

2.2 Cell Culture of the Wilms' tumor cell line, HFWT

The human Wilms' tumor cell line, HFWT was taken from -80˚C liquid nitrogen tank. Ham's F12 media was used to maintain the HFWT cell line. The media was made before the start of HFWT cell culture, Ham's F12 with 15% fetal bovine serum (FBS) was used to make a supplement for HFWT cell culture. HFWT was cultured on gelatin coated 75ml flasks. HFWT cells are incubated at 37˚C with humidified atmosphere of 5% CO_2. HFWT was split into 24 well plate and incubated prior to use. When it was fully confluent, HFWT cells were irradiated at 3,000 rads (Harada, 2004).

2.3 Culture of the stromal cell line OP9

Murine OP9 bone marrow stromal cells, was taken from -80˚C liquid nitrogen tank and thawed using standard techniques. These cells were cultured alpha (α-MEM) media with L-glutamine, 2.2g of sodium bicarbonate, β-mercaptoethanol, and penicillin/streptomycin solution. OP9 was cultured at 37˚C and 5% CO_{2f} in incubator. When it was fully confluent, OP9 was split into 24 well plate and incubated prior to use and then irradiated at 3,000 rads (Saito, 2013).

2.4 Culture of the stromal cell line AFT-204

AFT-204 is the immortalized murine fetal liver stromal cell line. The media used to culture these cells was Dulbecco's modified Eagles's medium (DMEM) with 10 % heat-inactivated of fetal bovine serum (FBS), β -mercaptoethanol (50 μ M/L), L-glutamine, penicillin/streptomycin, streptomycin, and hydrocortisone solution were used to maintain the AFT-204 cells. As above, AFT-204 cells were grown to confluence and irradiated at doses of 3,000 rads before use.

2.5 Culture of the stromal cell line EL

The murine embryonic liver cell line, EL media used Alpha (α-MEM) media with 7.5% fetal bovine serum (FBS), 50% myelocult (M5300, glutamax (2mM), β-mercaptoethanol (50μM/L), hydrocortisone (10^{-6}), and penicillin/streptomycin solution were used to maintain the EL cell line. To co-culture with progenitor cells, EL cells were irradiated at doses of 3,000 rads.

2.6 Culture of the stromal cell line UG

UG is a murine embryonic liver cell line. UG media used Alpha (α-MEM) media with 7.5% fetal bovine serum (FBS), 50% myelocult (M5300, glutamax (2mM), β-mercaptoethanol (50μM/L), hydrocortisone (10^{-6}), and penicillin/streptomycin solution were used to maintain the EL cell line. To co-culture with progenitor cells, EL cells were irradiated at doses of 3,000 rads.

2.7 Fluorescence Activated Cell Sorting (FACS)

Fluorescence activated cell sorting (FACS) was used to determine the percentage of NK cells in culture. Cells were isolated from cultures and stained with CD56 antibodies and analyzed using a FACScanto. The percentage of NK cells was determined using Flowjo software.

2.8 Differentiation of CD34+ cells into NK cells

In lymphocyte development and homeostasis, cytokines play a vital role and various mixtures have been used to generate human NK cells. Cytokines play critical roles in regulating aspects of immune responses. $CD34^+$ HPC in vitro were cultured on irradiated stroma (above) in media containing IL-3, IL-7, IL-15, SCF, and FLT3. Cultures were refreshed by removing 50% of the

culture media and adding new cytokines twice weekly starting at D+14. Cells were analyzed on D28 of culture.

2.9 Cytotoxicity assay (Chromium-51 (^{51}Cr) Release Assay)

K562 erythroleukemia cell line targets were used in killing assays. K562 targets were labeled with ^{51}Cr by incubating 1 x 10^6 cells in 11.1 MBq, ^{51} Cr for 2 hours at 37˚C. 5% CO_2 (Schmidt, 2004). The cells were washed with PBS and resuspended in RPMI with 10% FBS. Plated in 96 well plates at 1 x 10^4 cells/well in triplicate. Incubated for 4 hours at 37˚C, 5% CO_2 (Grzywacz, 2011).

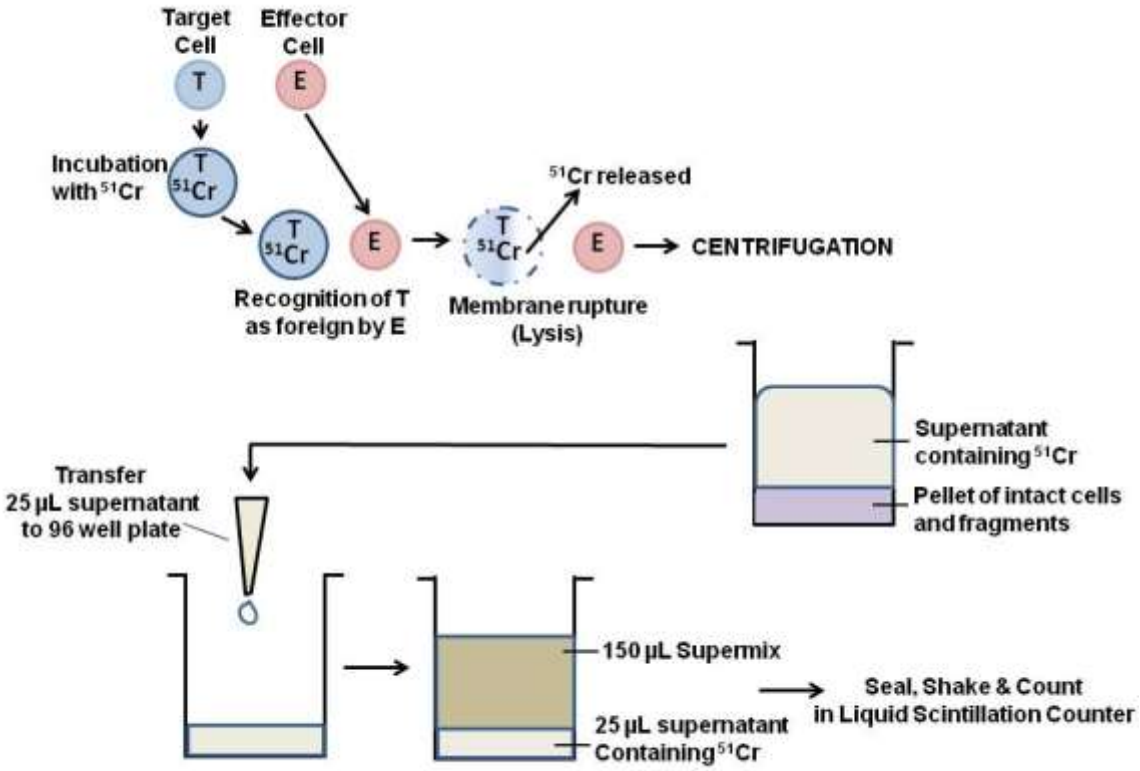

Figure 4. Principle of chromium release assay. 51Cr labeling the target cell, cytolysis the labeled 51Cr, and detection of the released 51Cr label, these are 3 main steps of the procedure (Schmidt, 2004).

$$\textbf{\% cytotoxicity} = \frac{CPM\ released\ from\ experimental\ sample - spontaneous\ release}{maximum\ release - spontaneous\ release}$$

3.0 Results

3.1 Comparison of the five different stromal cells and their ability to support NK development

CB $CD34^+$ cells were obtained from healthy human donors UCBs and were expanded from day 0 to day 28 of culture (as described in the methods section) using the following stromal cell lines: EL, AFT, UG, OP9 and HFWT. Controls included cultured in cytokines without stroma.

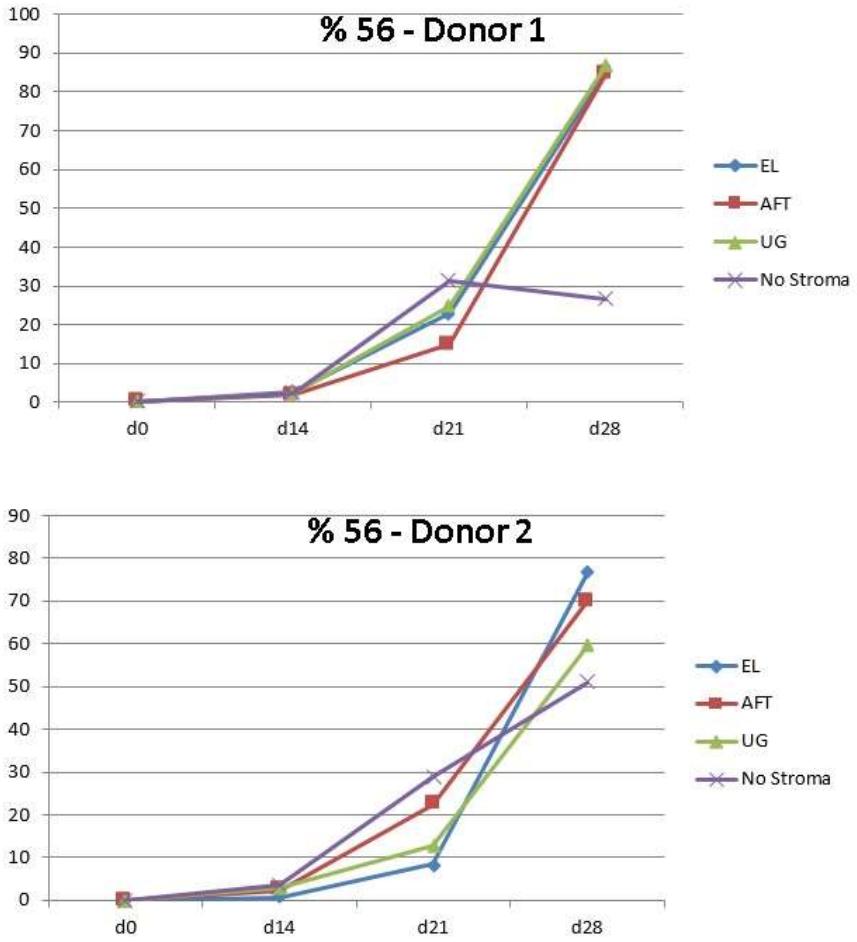

Figure 5. Impact of different stromal cells on the percentage of NK cells (defined as $CD56^+$) in culture over time. $CD34^+$ cells were cultured on the different irradiated stromal cells and the percentage of NK cells were assessed over time. Shown is the data from the donors.

The first experiment focused on exploring the NK cell yield from two separate donors using different feeder cell lines. I performed experiments comparing the AFT, EL, UG cell lines. As shown in figure 5, the percentage of $CD56^+$ cells starts to rise dramatically, starting at D14. By Day 21 the percentage of $CD56^+$ were relatively low for all conditions and seemed to not differ for the AFT, EL, UG cell lines or the no stroma control. Interestingly, HFWT and OP9 cell lines did not appear to support NK cells and are not shown. By day 28 both donors showed significant NK development, with the majority of the cells in culture being NK cells. For donor 1, there

appeared to be no differences between the conditions, while for donor 2 the results suggested more NK cell development in EL and AFT supported cultures.

3.2 Determination of the function of HSC-derived NK cells

Figure 6 shows the results of a killing assay where HSC-derived NK cells are used to kill ^{51}Cr labelled target cells. At day 21 the cytotoxicity was basically the same for each condition. On the other hand at day 28 the NK cells derived from either AFT or EL stromal lines retained their function, but those derived in the presence of UG (for donor 2) or no stroma lost cytotoxic activity.

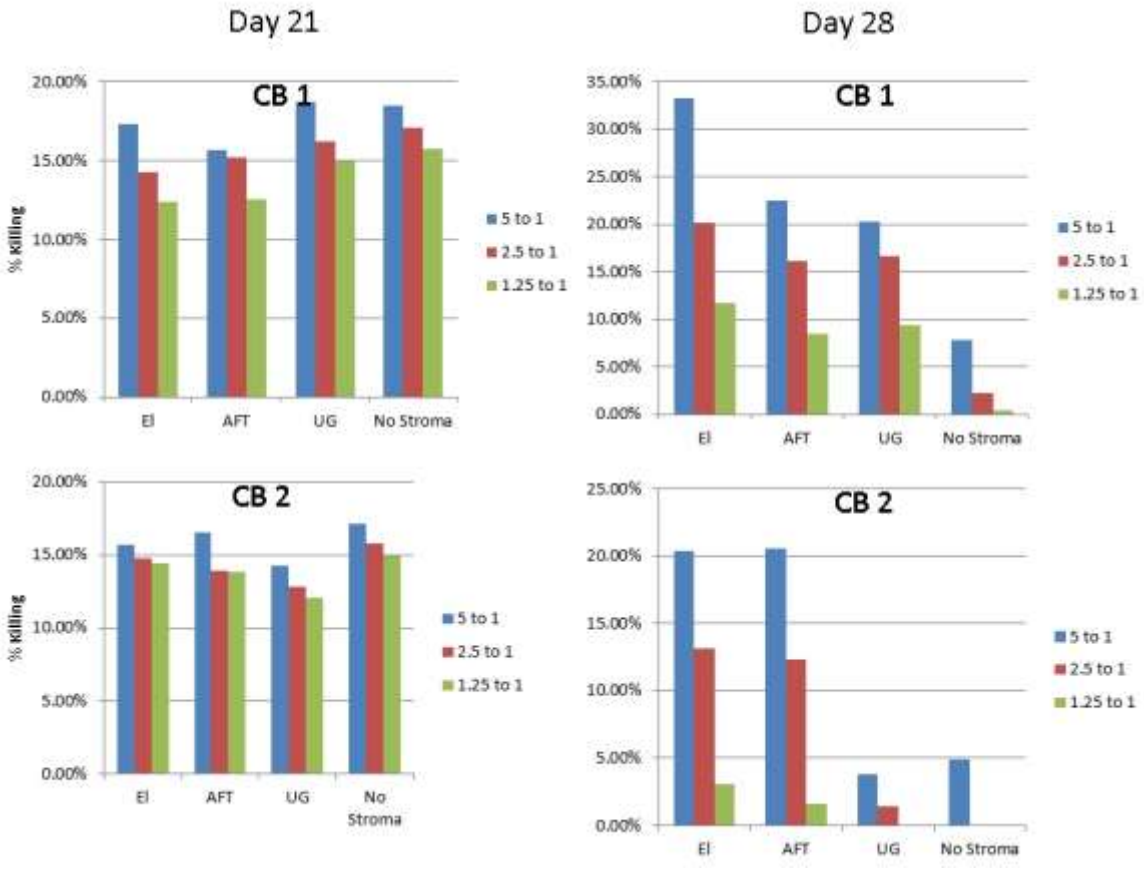

Figure 6. Cytotoxicity of NK cells generated on Different Stromal cell lines at Day 21 and Day 28 of culture. Shown are the results of cytotoxicity assays of HSC-derived NK cells generated on

the various stromal cell lines. Target cells were K562 and effector to targets ratios are show in the legend. The results for two donors at D21 and D28 are shown.

3.3 IL-22 Production at D21

Recently, our laboratory has shown that a unique population of NK cells differentiates in these cultures. These cells, which were originally called NK-22, have been more recently renamed as group 3 innate lymphoid cells. Unlike all other NK cells, these cells are characterized by high production of IL-22 upon stimulation with IL-1β and IL-23. To test whether these cells differentiate under the influence of various stromal cells the expanded cells were stimulated with the above cytokines and supernatant was assessed for IL-22 by ELISA. As shown in figure 7, IL-22 could be detected in both donors, but at differing concentrations depending upon the stromal cell line and the donor used. The concentrations varied between the donors, making it impossible to know whether the differences were due to the stromal cell lines or genetic differences in the donors.

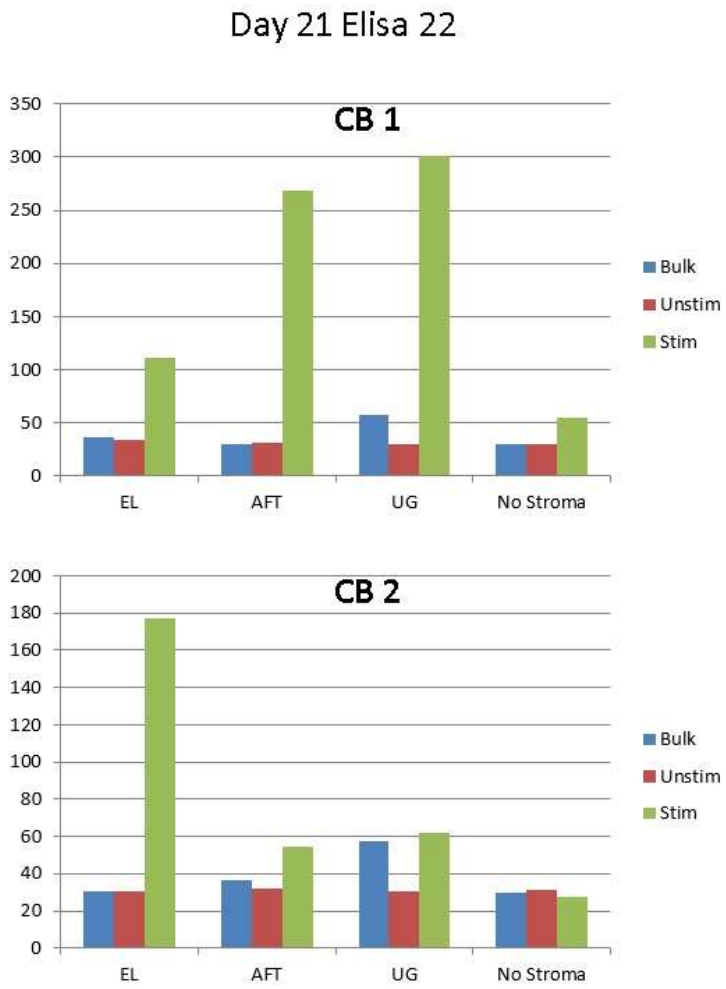

Figure 7. Day 21 ELISA for IL-22. $1x10^6$ cells were collected from cultures at D21 and stimulate with IL-1β and IL-23 and supernatant was collected 16 hours later. Shown are the supernatant of cells in culture ad D21 ("bulk"), after isolation but without stimulation ("unstim") and after stimulation with IL-1β and IL-23 ("stim"). Listed on the x-axis are the stromal cell lines used to create the NK cells.)

Discussion

This project examines the influence of five different stromal cell lines on the generation of NK cells from HSCs. From these studies, we observed that the cell lines HFWT and OP9 do not support the growth of NK cells from human CB $CD34^+$ cells. In contrast, AFT-024, EL, UG all support NK cell development and expansion. There were no clear differences noted in these latter stromal lines; with considerable donor to donor variation, which is common in human research.

In previous research, the Verneris' laboratory has demonstrated the necessity of stromal support in the differentiation of $CD34^+lin^-$ cells into NK cells. However the mechanism by which stromal cells mediate this activity has not been elucidated and is unknown. We speculate that the fetal stromal ells express surface receptors (or cytokines) that drive lymphopoiesis and NK differentiation, but the exact receptors involved in this process are unknown. Other investigators have used heparin in place of stroma to generate large numbers of NK cells. These studies show that large amounts of highly active NK cells can be produced in a closed system and under feeder free conditions, which is a benefit since regulatory approval will be much easier to obtain (Spanholtz, 2011). The mechanism of this approach is thought to be through the binding of heparin to cytokines and the presentation these cytokines to HSCs. As well, heparin may protect the cytokines from degradation. Our laboratory has recently compared the heparin-based method to the stromal-based method NK generation and found that both give rise to NK cells, but that the stromal-based method results in numerically more NK cells that are more mature (Dezell, 2012). While both methods generate clinical grade NK cells, the stromal method will require

significantly more regulatory oversight compared to the heparin method. Thus, it could be argued as to which approach is best for clinical translation.

Over the course of this research, I tested five different cell lines for their ability to support NK differentiation form HSC. While the AFT, EL, UG supported NK development, the OP9 and HFWT cell lines did not. Prior studies have shown that OP9 can support human NK differentiation (Beck, 2009), perhaps suggesting technical difficulties in our studies. The fact that HFWT is not a stromal cell line, but rather a cancer cell line makes it different from the other cells used in this study and may explain why it did not support NK development from CD34+ cells. Interestingly, HFWT does support the dramatic grown, expansion and activation of mature NK cells (Harada 2004). While this research did not identify a clearly superior stromal cell line for NK generation from HSC, these studies do further support the idea that HSCs can be differentiated into NK cells possibly for clinical use.

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