Comparative Analysis of Heparin Based Versus Stromal Cell Supported Methods for Natural Killer Cell Generation from Umbilical Cord Blood Stem Cells

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Dedication

I dedicate my research to my family, they have always been there for me, and they are my inspiration.

Abstract

The ongoing search for a cure for leukemia has many possible paths. One of which focuses on cell therapy and the transfusion of natural killer (NK) cells into patients. The innate cytotoxicity of NK cells allows them to target and destroy leukemic cells. NK cells have already helped some patients achieve remission after failure of traditional therapies. There are at least two possible sources of NK cells. First, peripheral blood NK cells can be obtained from a related donor and second, NK cells can be differentiated and expanded from unrelated donor umbilical cord blood (UCB) hematopoietic stem cells (HSCs). The later approach has the advantage of selecting donors with high cytotoxic capacity and cryopreservation of cells to be infused when needed. We previously described a method to generate NK cells from UCB-derived HSCs, using a murine fetal liver stromal cell line. However, use of murine cells is likely not allowed for human use. Thus, alternate clinical applications incorporating combinations of cytokines, additives, and procedural steps that would lead to highly cytotoxic NK cells are needed. The present research focuses on a comparison of a stromal cell supported method versus a liquid only based method for generating NK cells from UCB stem cells. Our comparative analysis focused on these two approaches in their ability to expand NK cells from stem cells and the resultant phenotypic functional capabilities of these HSC-derived NK cells. We demonstrate significant differences in absolute NK cell counts, nominal phenotypic differences, and similar functional capabilities between the two methods. Improvements in the techniques that result in NK generation will aid in the clinical use of these cells that have the potential to revolutionize leukemia treatment.

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Introduction

1. NK cells: general information.

NK cells play a vital role in innate immune responses with their ability to direct cytolytic activity against viral infected or malignant cells. They make up 10 to 15% of the peripheral blood (PB) lymphocytes [1]. NK cells also manufacture and release immunoregulatory cytokines including interferon-gamma (IFN-γ), tumor necrosis factor-alpha (TNF-α), tumor necrosis factor-beta (TNF-β), interleukin-10 (IL-10), and granulocyte macrophage-colony stimulating factor (GM-CSF) [2]. In addition to these capabilities, NK cells also mediate antibody-dependent cellular cytotoxicity (ADCC) through fragment crystallizable gamma region III receptors (FcγRIII), also known as CD16, that attach to the Fc portion of immunoglobin-gamma (IgG) [2].

1.1. NK cell subsets.

In the PB, there are two functionally distinct NK subsets which can be distinguished on a number of features, including the level of CD56 expression [3] (Figure 1). Around 90% of the NK cells display low levels of CD56, express high levels of CD16 ($CD16^{bright}$) and are called $CD56^{dim}$ cells. These cells have decreased proliferative capability ex vivo, are highly cytotoxic, demonstrate an abundance of granules and primarily deal in natural cytotoxicity and ADCC [4]. The other NK cell subset, known as $CD56^{bright}$ cells have higher levels of CD56 expression and make up approximately 10% of the PB. These NK cells have low levels of CD16 ($CD16^{dim}$) and have decreased cytotoxicity, but produce high quantities of immunoregulatory cytokines [5, 6]. The

$CD56^{bright}$ NK cells home to the lymph node and interact with dendritic cells (DC) during immune responses [7-9]. The CD56 cell surface marker on both subsets can participate in NCAM-to-NCAM binding [10, 11].

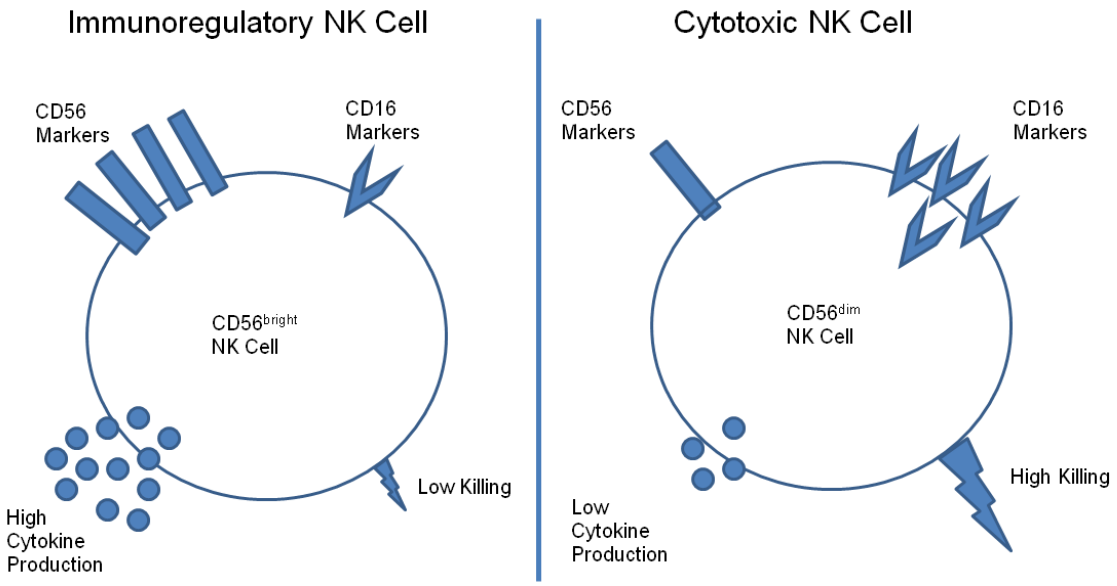

Figure 1. NK cells subsets adapted from Farag's 2006 article [12].

1.2. NK cell triggering receptors.

The overall interaction between NK cells and target cells is driven by a complex coupling of surface receptors and ligands that transmit activating or inhibitory signals into the cell. Many of these activating and inhibitory NK cell receptors have been identified, but the ligands on the tumor cells are still being investigated. The surface receptors are paired and categorized into three basic groups: killer Ig-like receptors (KIRs), heterodimeric C-type lectin receptors (CD94/NKG2), and non-MHC Class I-specific activating receptors or natural cytotoxicity receptors (NCRs) [12].

KIRs primarily distinguish between HLA-A, -B, and -C molecules. Structurally they are classified by the number of extracellular Ig-like domains and cytoplasmic tail length. Receptors with a long-tail mediate inhibitory signals and the short-tail receptors are associated with activating signals. Importantly, the KIR gene family is not linked to the genes of the MHC Class I molecules and various HLA-A and -B alleles have no "matching" KIRs [13]. The KIRs are encoded by a bundle of genes located on the leukocyte receptor complex (LRC) on chromosome 19q 13.4. They are situated in a continuous line and occupy 150 kb of the LRC. There are up to 14 expressed KIR genes and a few additional pseudogenes that are not expressed. There are two main groups of KIR haplotypes that are normally seen. The group A haplotype has six genes: four are inhibitory KIRs, one activating KIR, and a structurally divergent KIR. Group B has up to 12 genes with many combinations of activating KIR genes and at least one additional gene not in group A [14]. Individuals with KIR Group B genotypes have been found to be very effective in transplantation outcomes for acute myeloid leukemia (AML) patients displaying decreased relapse and increased disease-free survival [15, 16].

The C-type lectin receptors are not as diverse as the KIRs and are heterodimers that share an invariant subunit, CD94, which is non-covalently linked to a number of glycoproteins [13]. Only one receptor in this family, CD94/NKG2A, mediates inhibitory signals. NKG2A has a similar long tail structure that is seen with KIR. The other heterodimers, CD94/NKG2C and CD94/NKG2E, are activating receptors. The activating and inhibitory receptors in this family both recognize HLA-E molecules. HLA-E presents a small peptide fragment from the leader sequence of HLA-A, -B and -C and

therefore detect overall MHC class I expression on target cells [12, 17]. NKG2D is another activating C-type lectin receptor, but it is not associated with CD94 and instead exists on the cell surface as a homodimer [18]. NKG2D detects ligands that are usually displayed only on stressed and/or injured cells, including those experiencing DNA damage or viral infection. NKG2D ligands include MICA/B (MHC Class I Polypeptide-Related Chain A/B) and ULBP (Unique Long 16-Binding Protein) 1-4 which share similarity to MHC Class I structure.

NCRs make up a loosely defined group of receptors that all recognize non-MHC Class I-specific activating receptors on target cells. These Ig-like receptors are named by their molecular weight and include NKp30, NKp44, and NKp46. NKp30 and NKp46 are expressed on PB NK cells and NKp44 is only found on activated NK cells [19-21]. Also included in this group are activating receptors that are considered coactivators since they do not directly stimulate NK cell function [22]. The identified coreceptors are FcγRIII (CD16), CD2, 2B4 (CD244), NKp80, CD69, lymphocyte function-associated antigen-1 (LFA-1), and CD40 ligand. The numerous inhibitory and activating receptors in conjunction with their interaction with corresponding ligands play a vital role in the control of NK cytotoxicity and cytokine secretion.

1.3. NK cell killing of target cells.

NK cells play an important role in the innate immune response and interact with transformed cells such as AML cells or viral infected cells. As NK cells come into contact with various cells in the body, they make contact with these cells through the

above described activating and inhibitory receptors. It is the fine balance of signaling through these receptors that dictate whether NK cell activation and cytotoxicity occurs. As shown in figure 2 below, there are four basic scenarios which could occur. (a) NK interaction with normal cells displaying few, activating ligands and abundant amounts of MHC Class I (HLA-A, -B, -C molecules). In this scenario, engagement of inhibitory KIR or CD94/NKG2A with MHC class I results in a profound inhibition of NK cell triggering and tolerance to normal tissues. (b) Interaction with cells where the number of activating receptors outnumber the inhibitory receptors. The increased activating signal will lead to target cell killing. (c) Interaction with cells lacking self molecules. Self MHC molecules can be down-regulated due to viral infection or malignant transformation. To alert the immune system these abnormal cells decrease the expression of MHC class I (inhibitory receptor ligands) and display activating ligands that serve to activate NK cells. The end result is target cell killing. (d) Inhibition of lysis can also occur when inhibitory receptors outnumber the activating receptors. So despite an activating signal present, the inhibition signal is overpowering and no target cell killing occurs [12].

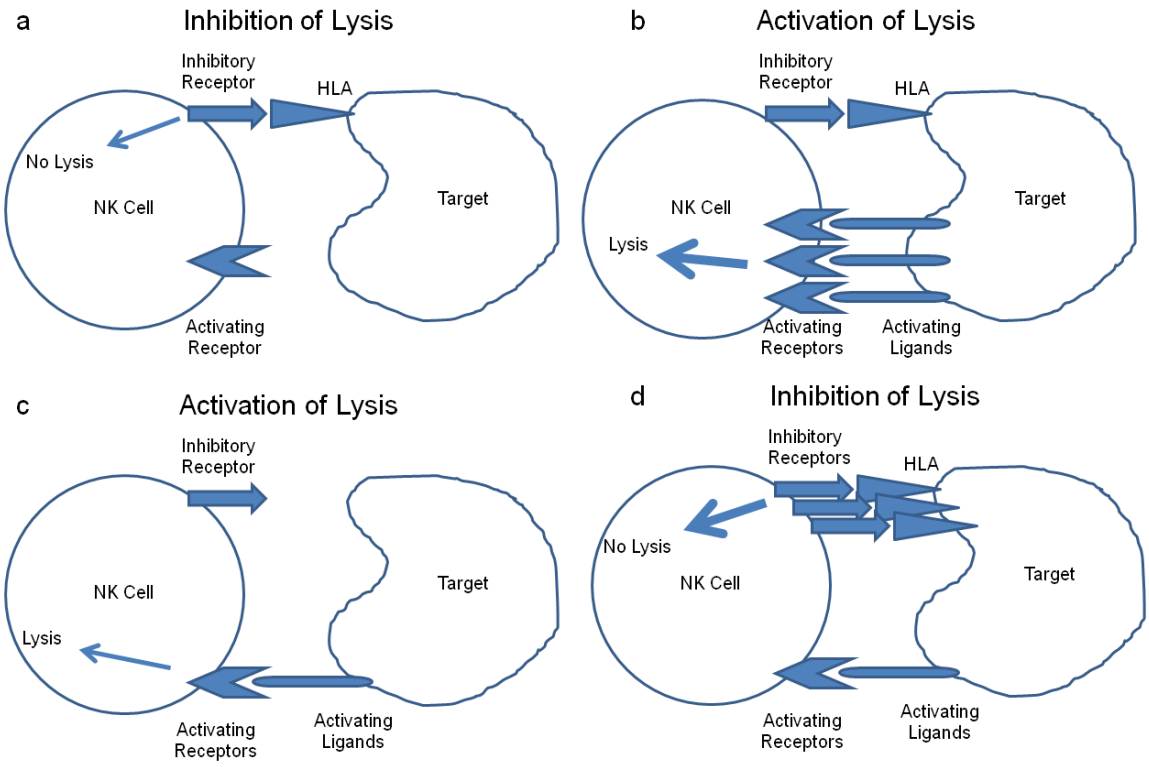

Figure 2. Basic interaction of inhibitory and activating receptors adapted from Farag's 2006 article [12].

When NK cell activation occurs, the following events culminate in target killing. NK cells release granules containing the cytotoxic proteins granzyme (serum proteases) and perforin. Perforin binds to the target cell and creates a pore, through which granzymes enter and induce apoptosis by cleaving specific intracellular substrates within the target cell and inducing apoptosis [23, 24]. As the target cell dies it releases antigenic cellular components that can be taken up by nearby DCs. Under the influence of inflammatory cytokines (IFN-γ and TNF-α) elaborated by NK cells, DCs process this material into peptide fragments and present them, complexed within MHC I and II molecules, to T cells. This interaction can incite an adaptive immune response [25, 26].

DCs also communicate with nearby NK cells through the release of additional cytokines including IL-12, IL-15, and IL-18. IL-15 promotes NK cell activation, expansion, and survival. IL-12 and IL-18 are inflammatory cytokines that promote IFN-γ production by NK cells, which affect the maturation and effector functions of DCs and the activation of other leukocytes including macrophages, granulocytes, and lymphocytes [27, 28]. IFN-α and IFN-β are type I interferons that are elaborated by activated DCs and are also able to activate NK cells. The NK and DC cell interaction is controlled by a feedback mechanism through the release of TNF-α and IFN-γ by activated NK cells [29]. TNF-α is able to trigger inflammation as well as induce apoptotic cell death, inhibit tumorigenesis, and hinder viral replication [30-32]. IFN-γ inhibits viral replication and plays a role in the immunostiumulatory and immunoregulatory process. The stimulation of NK cells also causes there proliferation and increased CD69 expression on their cell membrane. CD69 is one of the first surface markers expressed after activation and acts as a signal transmitting receptor [33].

1.4. NK cell development.

Initial NK cell development occurs in the bone marrow (BM) microenvironment. In the human BM, $CD34^+$ HSCs continually give rise to many different types of committed progenitor cells, some of which are NK cell precursors (NKp) [34, 35]. These findings in the BM do not preclude further NK cell development in other anatomical locations and research is underway to determine other possibilities. The $CD56^{bright}$ NK cells are mostly found in the lymph nodes, while the $CD56^{dim}$ NK cells predominate in

the blood. It is theorized that $CD34^{dim}$ $CD45RA^{+}\beta_7$ ^{bright} HPCs derived in the BM travel to the LN and then are driven to develop into $CD56^{bright}$ NK cells by local cytokines [36]. Researchers have also found that BM-derived $CD34^{+}$ cells cocultured with BM stroma produce cytolytic NK cells in vitro [37]. For research purposes, cytolytic NK cells can be differentiated and expanded from $CD34^{+}$ cells derived from fetal liver, BM, thymus, UCB, adult blood, or secondary lymphoid tissue (SLT) through exposure with IL-2 or IL15 [38].

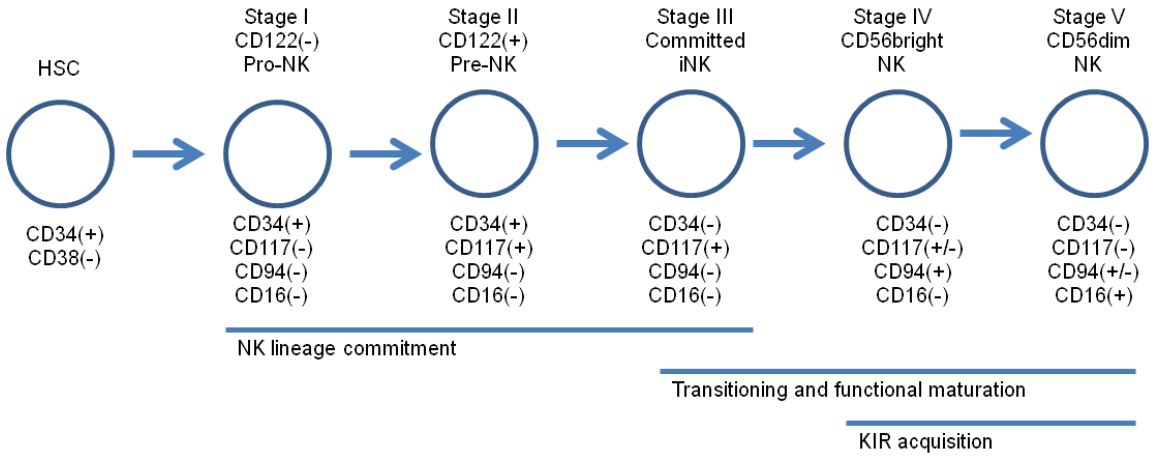

Figure 3. NK cell development and surface marker expression adapted from Freud's 2006 article [35].

As shown in figure 3 above, the combination of CD34, CD117, and CD94 discriminates five developmental stages of NK cells [39]. Stage I NK cell progenitors are marked by a $CD34^{+}CD117^{-}CD94^{-}$ phenotype. By utilizing FLT3, IL-3, and IL-7 cytokines, the stage I cells can be differentiated into stage II cells which express two key cytokine receptors CD117 and CD122. Accordingly, stage II pre-NK cells have the functional capacity to respond to stem cell factor (SCF) and IL-15 (or IL-2), respectively,

and can further differentiate into stage III cells. Stage III NK cells mark the functional maturation and transitioning process and represent immature NK (iNK) cells. Stage III cells differ from mature NK cells by the inability to produce IFN-γ or mediate perforin dependent cellular cytotoxicity [2, 39]. More recently a number of groups have demonstrated that some stage III NK cells have the ability to produce IL-22 [40]. These findings suggest the stage III progenitor fraction may be heterogenous. Stage IV NK progenitors, also known as $CD56^{bright}$ NK cells are $CD34^{-}CD117^{+/-}CD94^{+}$. Upon the acquisition of CD94/NKG2A, which confers MHC-I-dependent inhibition, these cells become functional [41, 42]. Many other phenotypic changes can be observed in stage IV cells including the downregulation of CD117, CD33, and CD127 and the acquisition of NKG2D, NKp46, CD122, intracellular perforin, and the ability for perforin and Fas-ligand-mediated killing and IFN-γ production [39, 42]. Terminally differentiated stage V NK cells are identified as $CD94^{+/-}CD16^{+}$ cells and are $CD56^{dim}$ NK cells. It is suggested that $CD56^{dim}$ NK cells are derived from $CD56^{bright}$ NK cells that are $CD94^{+}CD16^{-}$ [3, 43].

Cytokines play a vital role in lymphocyte development and homeostasis [44]. Various cytokine mixtures have been used to generate human NK cells from $CD34^{+}$ HPC in vitro, including: FLT3, SCF, IL-2, IL-3, IL-7, IL-12, IL-15, and/or IL-21. In addition, IL-2, IL-15, and IL-7 can support $CD3^{-}CD56^{bright}$ NK cells without additional cytokines or stroma support, but the cells are phenotypically and functionally immature when compared to PB NK cells [45-47]. IL-15 is considered the most important cytokine supporting NK cell development because of the pronounced NK cell deficiencies found

in mice lacking IL-15 [48, 49]. Solid evidence shows that trans-presentation of IL-15 by IL15Rα is necessary for mature NK cell survival [50-52].

BM stromal cells also play a critical part in maintaining lymphocyte development. The mechanisms that facilitate lymphocyte development are currently under investigation, but a few have been brought to light. One of the mechanisms involves growth arrest-specific gene-6 (GAS6) and its role as a ligand for several tyrosine kinase receptors. GAS6, in conjunction with protein S, forms the endogenous ligand for the Tyro 3/Axl/Mer (TAM) family of protein kinase receptors which are found on NK cell precursors. Experiments have shown that without the GAS6 genes BM stromal cells are unable to support HSCs [53]. Moreover, deficiencies in the Tyro 3 receptor family results in an arrest in NK cell development between stages II and III [54]. Another role of stromal cells involves the expression of Notch, which plays an important role in controlling progenitor cells that have the capacity to differentiate into multiple lineages [55]. Stimulation of $CD34^+$ cells with the Notch ligand (Delta4) results in the differentiation of functional NK cells [56]. Finally Wnt proteins play an important role in conjunction with stromal cell support in cultures. Wnt genes encode for a family of secreted glycoproteins that guide cell development activity. Experiments show that Wnt helps control stromal cell function [57].

Human and mouse experiments provide insight into possible sites of NK cell development, but a clear pathway has not been endorsed fully. It is proven that $CD34^+$ progenitors with NK cell differentiation capacity normally exists in the thymus, but a majority of the cells in this environment develop into T-cells and the importance of the

thymus in NK cell development has not yet been established [58-60]. Within the human BM all NK cell stages are present, but a distinct band of maturation does not appear to exist [36, 39]. More recent murine and human studies suggest that SLT may play an important role in terminal NK cell maturation by providing important cell-cell interactions with DCs, subsequent cytokine stimulation and interaction with mesenchymal cells may provide important surface bound protein stimulation [51, 61, 62].

2. Glycostem

Glycostem Basal Growth Medium or GBGM® (http://www.glycostem.nl/node/2) is a new growth medium designed for the expansion and differentiation of HSCs and progenitor cells. GBGM consists of "animal-component-free" media with good manufacturing practice (GMP)-compliant cytokines including SCF, IL-7, Flt3, IL-15, G-CSF, GM-CSF, TPO, IL-2, IL-6, and low molecular weight heparin (LMWH). GBGM is a commercialized product that follows GMP controls and thus, ensures consistent quality control measures in production, training, and documentation of all these areas.

The media is separated into Expansion Medium I, Expansion Medium II, and Differentiation Medium, each containing a different combination of cytokines and dosage. The mixture of various cytokines are claimed to be optimal for HSC and progenitor cell differentiation into NK cells. Research has shown the media's capacity for improved progenitor cell survival and NK differentiation and expansion, as compared with other serum free systems [63]. GBGM is the first clinical grade option for

generating defined HSC-derived NK cell products A schematized version of the culture technique is shown in figure 4 below.

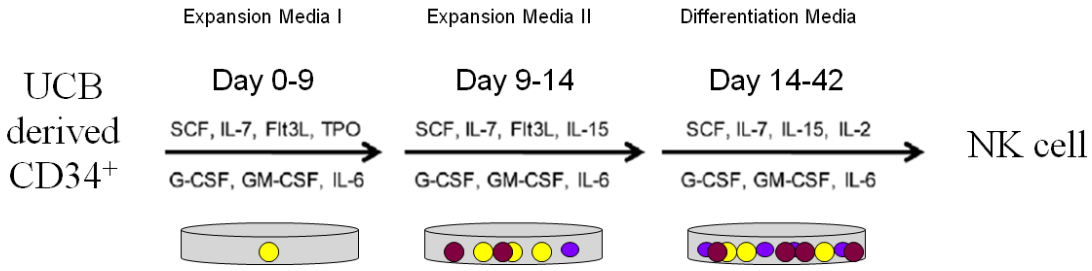

Figure 4. GBGM staged media protocol adapted from Spanholtz's 2010 article [63].

Certain forms of LMWH have been found to bind some cytokines that drive NK cell development and therefore may play a role in GBGM's success. For instance, SCF is able to bind with chemically modified heparins which resulted in increased stability and elongated activity of SCF [64]. SCF plays a vital role in hematopoiesis regulation by promoting proliferation and early differentiation of multipotential stem cells. It prevents apoptosis when it co-stimulates with other cytokines, playing an important role in NK cell development [64]. Another important NK cell cytokine that can interact with heparin and heparin sulfate (HS) is IL-7, a lymphoid growth factor that mediates progenitor development. Heparin may act as an IL-7 carrier by blocking its interaction with target cells and preventing degradation during transport [65]. IL-2 has also been found to bind with heparin and HS and maintain its capacity to bind with IL-2 receptors. The data suggests that heparin binding may be a mechanism for conserving cytokine activity closer to the area it was released in [66]. IL-2 is well known to play an important role in NK

cell proliferation and activation. As well, IL-12 binds to and dissociates from heparin/HS glycosaminoglycan molecules, potentially simulating cross presentation. The interaction between IL-12 and heparin allows for a localization of high concentrations of IL-12 to be delivered to cells. IL-12 plays a role in controlling NK cell cytokine production [67]. Thus, inclusion of LMWH in GBGM is hypothesized to work in a similar way as described above in the various cytokine/heparin experiments, specifically, it is hypothesized that LMWH concentrates and trans-presents the cytokines to the developing NK cells. Figure 5 below demonstrates the general differences between the "gold standard" stromal supported method and the GBGM heparin supported method for generating NK cells.

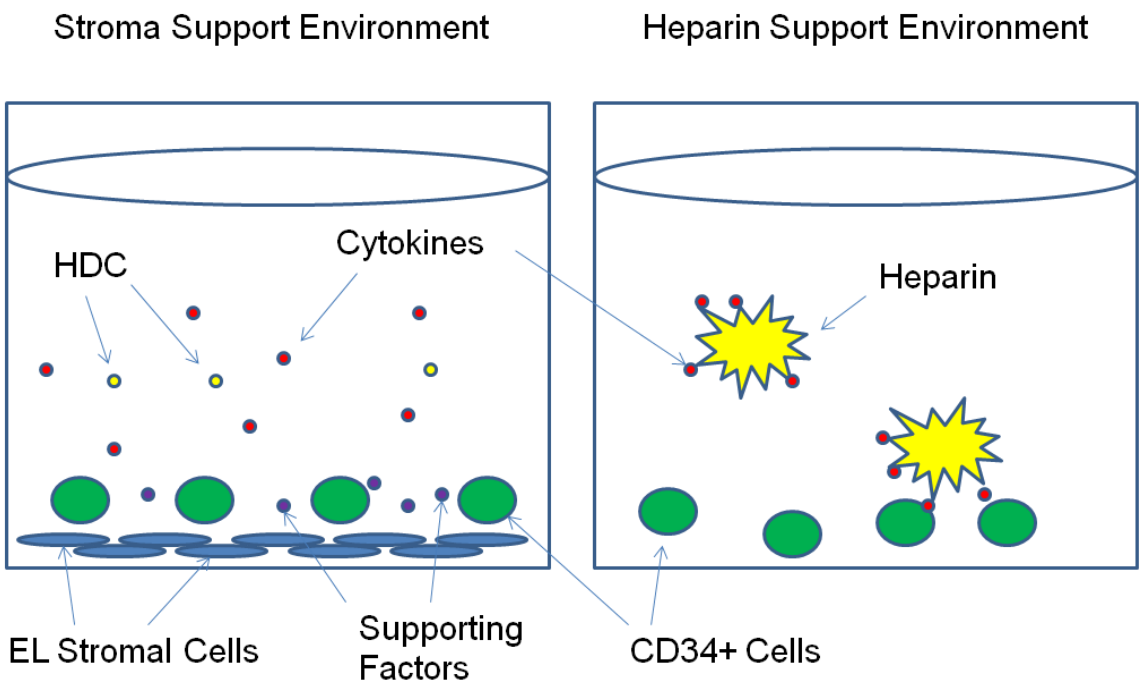

Figure 5. Stromal support environment compared to GBGM heparin environment.

3. Clinical Applications of NK cells: Successes and Barriers.

High-dose chemo- radiotherapy followed by the infusion of allogeneic HSCs (i.e., HSC transplantation (HSCT)) is uniquely curative for leukemia that is resistant to traditional chemotherapy. Donor lymphocytes (NK and T cells) recognize the recipient leukemia as being foreign and mount a cytotoxic attack, perhaps independent of chemotherapy resistance. Based on the above described biology of MHC-class I recognizing receptors, this may be most optimal when the donor and recipient differ at MHC class I loci and some studies have supported this concept [68, 69]. In this circumstance, the donor NK cells are considered "alloreactive" toward the patient. Alloreactivity is the basis for post-transplant graft vs. leukemia (GVL) reactions and while GVL can be curative, it is relatively weak and most optimal when patients have a low level of disease burden and are in remission.

In some patients, achieving remission can be difficult if not impossible with standard chemotherapy. At the University of Minnesota; we have used high dose chemotherapy and haploidentical NK cell infusion to treat such patients [70]. In this setting it is hypothesized that the high dose chemotherapy reduces the leukemia burden, kills the patient's own lymphocytes and allows the donor NK cells to proliferate and kill the residual disease. Post-infusional IL-2 stimulation is thought to stimulate NK cell expansion and effector function. At present only 30% of the individuals treated with this therapy enter remission [70]. As mentioned earlier, our collaborators have demonstrated that BM donors with KIR Group B genotypes are associated with reduced AML relapse

and increased disease-free survival after transplantation [15]. More recently, these same investigators further defined the region within the KIR loci that is most protective against relapse. They show that haplotype B donors that have KIR genes within the centromeric region of the KIR B loci were most protective against relapse [16]. These findings provide solid rationale for selecting NK donors based on genetic features, such as KIR Cen B/B genotypes. Unfortunately, only about 10% of individuals are KIR Cen B/B [16]. Considering this, most patients will not have a Cen B/B related donor. One possibility to overcome this barrier might be to identify UCB units that are Cen B/B and generate NK cells from isolated HSCs. Taking this approach it might be possible to create an "off the shelf" product that can be thawed and used to treat chemotherapy refractory AML patients. While stromal cell supported procedures are efficient in NK generation, there are potential regulatory issues regarding the use of xenogenic (murine), transformed fetal stromal cells. These concerns necessitate the evaluation of other methodologies, such as liquid-only based support media. In these studies we compare a stroma supported procedure to a liquid-only, heparin-based procedure (GBGM) for generating functional NK cells for possible clinical applications in leukemia patients. We hypothesized that the stroma method will provide the best expansion and differentiation of UCB stem cells into NK cells. We additionally hypothesized that stromal-based cultures would result in more mature and functional NK cells. To address this we directly compared optimized protocols for each method and determined NK cell differentiation, expansion, developmental progression and functional capability (cytotoxicity and cytokine production) of the NK cells generated from UCB stem cells.

We show that stromal supported cultures result in more robust expansion, but that heparin based cultures give rise to an equally functional population of cells that could be used therapeutically.

Materials and Methods

Isolation of $CD34^+$ cells from UCB and $CD56^+$ cells from PB

$CD34^+$ progenitor cells were isolated from UCB using magnetic bead selection (Miltenyi Biotech, Auburn, CA). Selected cells were routinely more than 90% pure. Where specified, enriched $CD34^+$ cells were sorted using fluorescence activated cell sorting (FACS) into $CD34^+lin^-$ cells (lineage markers were CD7, CD45RA, CD161, CD122, and Integrin β7). $CD56^+$ cells were isolated from PB using the Rosette separation method and LSM procedure.

Culture of the stromal cell line EL08.1D2

The embryonic liver cell line EL08.1D2 was cultured on gelatinized plates at 32°C in 40.5% α–minimum essential medium (α-MEM; Invitrogen, Carlsbad, CA), 50% myelocult (M5300; Stem Cell Technologies, Vancouver, BC, Canada), 7.5% fetal bovine serum (FBS), with β-mercaptoethanol (50 μM/L), glutamax (2 mM), penicillin (100 U/mL)/streptomycin (100 U/mL), and hydrocortisone (10^{-6} M). Prior to coculture with progenitor cells, EL08.1D2 cells were irradiated (3000 rads).

NK-cell differentiation cultures

Two different techniques were used. In the stromal-based cultures, $CD34^+$ selected cells (500 per well) were plated in a 24-well plate on an irradiated confluent monolayer of EL08.1D2 cells in Ham F12 plus Dulbecco modified Eagle medium (DMEM; 1:2 ratio) with 20% male, human AB^- sera (Sera Care Life Sciences,

Oceanside, CA), ethanolamine (50 μM), ascorbic acid (20 mg/L), 5 μg/L sodium selenite (Na_2SeO_3), β-mercaptoethanol (24 μM), and penicillin (100 U/mL)/streptomycin (100 U/mL). At the start of cultures, IL-3 (5 ng/mL), IL-7 (20 ng/mL), IL-15 (10 ng/mL), SCF (20 ng/mL), and FLT-3L (10 ng/mL) were added. Cultures were refreshed by demi-depletion (50% volume change) supplemented with the previously mentioned cytokines except IL-3. In the heparin-based cultures, CD34+ selected cells (50,000 per well) were plated in a 24-well plate. Expansion media I was utilized from days 0 to 8, expansion media II was utilized from days 9 to 13, and differentiation media was used from days 14 to 35. Media changes were performed through complete cell extraction and centrifugation with media evacuation with suction and the appropriate new media added. The cells were then plated into a new wells. Both cell culture conditions were refreshed at the same time and transferred to appropriate well sizes to accommodate increased cell numbers. Cell numbers below 200,000 were maintained in 24-well plates and those above were transferred to 6-well plates.

NK-cell precursor experiments

$CD34^+$ HPC or specified subsets were plated at 100 cells/well, 2 replicates/dilution [42]. Stroma supported conditions contained previously described above media and cytokines with stroma (EL08.1D2) and with HDC (10^{-6}M). Control conditions contained previously described above media with cytokines only and without stroma and HDC. Heparin conditions contained the GBGM media only without stroma and HDC. After 5 weeks NK

fold expansion was measured and flow cytometry performed. Anova statistical analysis was performed to determine significant relationships.

FACS staining and monoclonal antibodies

The following antibodies were used: CD7-FITC, CD16 (FITC or PE), CD34 (PercpCy5.5, PE, and APC), CD56 (PercpCy5.5 or Cy7), CD94-FITC, CD117 (PE or PercpCy5.5), CD161 (FITC or PE), CD158a-PE, and CD158b-PE (all from BD Biosciences, San Jose, CA). Additional antibodies included NKG2D-PE (R&D Systems, Minneapolis, MN), NKp30-PE, NKp44-PE, and NKp46-PE (Beckman Coulter, Hialeah, FL). Intracellular staining for IL-22–PE and IFN-γ–PE was performed using cytofix/cytoperm (BD Biosciences). IFN-γ staining was performed after 16 hours of stimulation with IL-12 (10 ng/mL) and IL-18 (100 ng/mL). IL-22 staining was performed after 16 hours of stimulation with IL-1β (10 ng/mL) and IL-23 (10 ng/mL). Brefeldin A was added for the last 4 hours.

FACS analysis and FACS sorting

Three to 5-color FACS was performed on a FACS Canto (BD Biosciences). Data was analyzed using Flowjo Version 7.6.1. FACS sorting was performed on either a FACS Vantage or FACS Aria (BD Biosciences).

IL-22 and IFN γ Production ELISA Assays

IL-22 or IFN γ production was measured using the DuoSet ELISA Development System (R&D Systems, Minneapolis, MN). 0.1 to 0.2 x 10^6 cells/mL were seeded in a 96-well plate with 100 to 200 uL per well of the appropriate conditions and IL 22 stimulatory cytokines IL-1β and IL-23 or IFN γ stimulatory cytokines IL-12 and IL-18. Wells were labeled based on donor and condition number. Three replicates with stimulatory cytokines and three without for control purposes. The plate was incubated overnight and analyzed the next day.

^{51}Cr release assay

K562, Daudi and Raji targets were labeled with ^{51}Cr (Dupont-NEN, Boston, MA) by incubating 1 × 10^6 cells in 11.1 MBq (300 μCi) ^{51}Cr for 2 hours at 37°C, 5% CO_2. The cells were washed with phosphate-buffered saline (PBS), resuspended in RPMI with 10% FBS, and plated in 96-well plates at 1 × 10^4 cells/well in triplicate. Effector cells were added at specified ratios (from 10:1 to 2.5:1) and incubated for 4 hours at 37°C, 5% CO_2. Supernatants were collected and counted (Wizard 1470; Perkin-Elmer, Shelton, CT). Specific ^{51}Cr lysis was calculated using the equation: % specific lysis = 100 × (test release–spontaneous release)/(maximal release–spontaneous release).

Statistical Analysis

Statistical analysis comparing the differences between heparin-derived NK cells and stromal supported cells were performed using student's t-test for the following parameters: number of mononuclear cells, number of NK cells, surface phenotype,

cytotoxicity, and cytokine production (IL-22 and IFN-γ). Timed analysis was calculated using a mixed model from PROC MIXED for repeated measures and used to look at the affect of heparin versus stroma on the absolute numbers of $CD56^+$ cells over 4 weeks [71]. This model incorporated all data and took into affect time, the matched pairs and the primary comparison as well as the interaction of the two. An F-test was used to calculate the p-values. A p-value of <0.05 was considered statistically significant for all tests.

Results

1. Determination of optimal conditions for comparing the stromal cell supported method versus the heparin supported method.

Our initial experiments focused on exploring the NK cell yield when $CD34^+$ cells were cultured with stromal cells and HDC versus a heparin-based media. Figure 6a summarizes the fold expansion for $CD34^+$ cells cultured in standard basal growth media and shows that the inclusion of both HDC and stroma cells provides the most optimum expansion, consistent with our prior results [42]. Figure 6b summarizes the results for the heparin-based media and shows that the condition with stroma cells and *no HDC* provides the best fold expansion. Interestingly the second best performance for the heparin-based media involved no stroma cells and no HDC, which did not perform as well in the corresponding standard basal growth media condition. Taking into consideration the regulatory issues of a stromal feeder line in clinical trials, we performed further experiments comparing the standard basal growth media with stroma cell support and HDC (stroma method) to the heparin-based media with no stroma cell support or HDC (heparin method). All subsequent experiments concentrated on these two methods. Analyzing the total cell fold expansion, we found that the stroma method yielded higher total cell fold expansion compared to the heparin method. Figure 6c shows that a 64 fold greater expansion was obtained using the stromal method compared to the heparin method ($p=0.001$, $n=22$ paired donors).

The UCB $CD34^+$ cell population is heterogeneous and our laboratory has previously shown that there is a small population of $CD34^+$ cells that express receptors

which indicate further progression along the lymphoid or NK lineage [42]. These receptors include: CD7, CD45RA, CD161, CD122, and Integrin β7. Some $CD34^+$ cells that express these markers can develop into NK cells without stromal support. This characteristic led us to name them $CD34^+NKlin^+$ cells. In contrast, the majority of the $CD34^+$ cell population (~90%) do not express these receptors and require stroma cell support to develop into NK cells; subsequently we called these $CD34^+NKlin^-$ [42]. At present, it is not known whether heparin supported culture systems can induce $CD34^+NKlin^-$ cell populations to become NK cells in the absence of stroma. To test this we purified $CD34^+NKlin^+$ and $CD34^+NKlin^-$ cells and cultured them in the presence of the stroma supported or heparin supported methods. Figure 6d shows that the heparin method is not as capable as the stroma method towards inducing expansion of $CD34^+NKlin^-$ cells into NK cells.

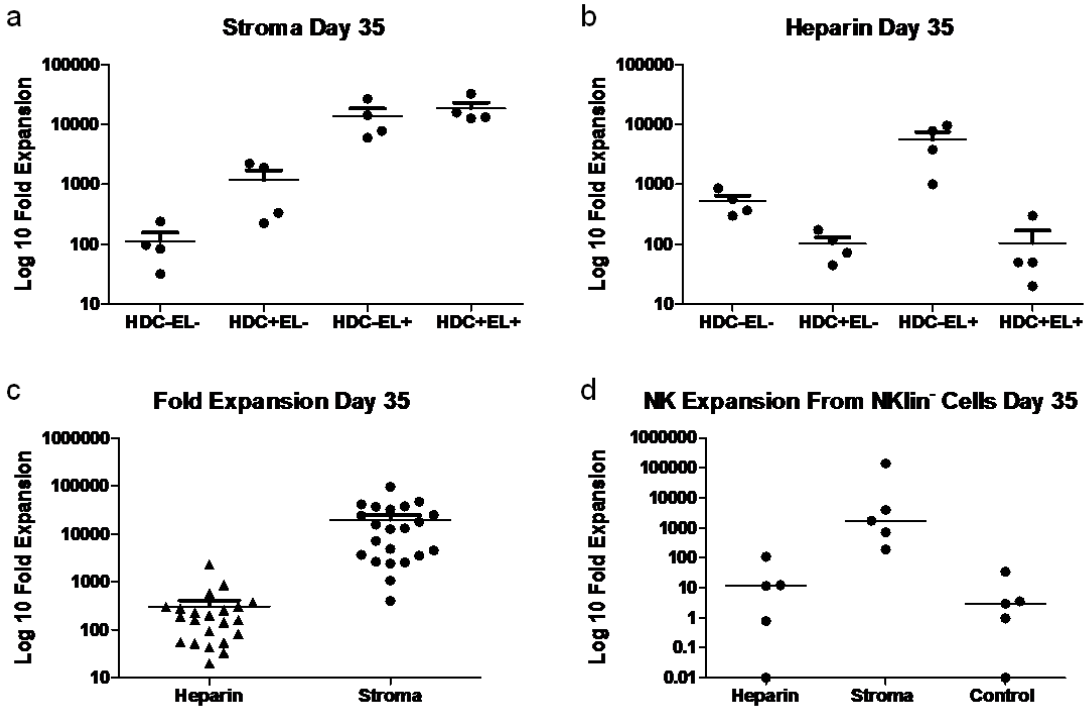

Figure 6. Stroma method provides best fold expansion and is able to induce expansion of NK lin$^-$ progenitors. **(a)** This experiment shows the effect of stroma cell support and HDC on total cell fold expansion utilizing the standard basal growth media. The average p-value for all experiments was <0.03, n=4. **(b)** This experiment compares the effect of stroma cell support and HDC on total cell fold expansion utilizing the heparin-based media. Average p-value for all experiments was <0.06, n=4. **(c)** Stroma method provides greater total cell fold expansion compared to heparin method. The stroma method increases total cell fold expansion by 64 times compared to the heparin method. p=0.001, n=22 paired donors. **(d)** Stroma method on average increases NK fold expansion from NK lin$^-$ stem cells by 6.92 units compared to the heparin method. The stroma method also shows an average increase of NK fold expansion from NK lin$^-$ stem cells of 7.64 units compared to a corresponding control. Anova statistic was used with 95% CI; respectively p=0.005, n=5, range=1.77-12.08; p=0.010, n=5, range=2.48-12.79.

2. The kinetics of NK cell development and the absolute numbers of NK cells generated by the stroma method and heparin method.

NK cells are marked by a $CD3^{-}CD56^{+}$ phenotype within the lymphocyte population [2, 35]. In >500 cultures, the Verneris laboratory has not been able to generate T cells using the stroma method (not shown), so for the purposes of this analysis CD56 was used to mark NK cells. Here we determined the kinetics of NK cell development and the absolute numbers of NK cells in these two culture conditions. Using flow cytometric analysis, we determined the percentage of $CD56^{+}$ cells at various times in culture. Figure 7a shows the gating strategy used to identify these cells including, the lymphocyte gate (left graph), the $CD56^{+}$ fraction (middle graph), and appropriate negative controls (right graph). Figure 7b displays the progressive acquisition of $CD56^{+}$ expression over 14-35 days in culture for a single donor. As shown in figures 7c and 7d, there was a sharp decrease in $CD34^{+}$ expression over the first 2 weeks in culture and a subsequent increase in $CD56^{+}$ expression beginning at day 14 (D14). At D14 there were equal percentages of NK cells in both conditions, but over the subsequent times in culture (D21-D35) a progressive increase in NK cells were noted in both conditions. However, higher percentage of $CD56^{+}$ cells were consistently observed in the stroma method compared to heparin method (figure 7d, D14-D35, $p=0.001$, $n=22$ paired donors). Comparing the number of NK cells generated in the two conditions, the stroma method resulted in significantly more NK cells at D35. In prior studies, we have shown that the stroma method increases $CD56^{+}$ expression and absolute counts starting on D21 [42]. Focusing on D21 for this experiment we also see similar results. In this

case the stroma method increased the percentage of $CD56^+$ by two fold relative to the heparin based cultures (figure 7d, D21, p=0.001, n=15 paired donors). Focusing on D35, the absolute numbers of NK cells for the two different methods were 3-fold in the stroma method compared to the heparin method (figure 7e, p=0.002, n=22 paired donors). These studies show that the two methods differ in rate of $CD56^+$ cell development, but that both are capable of expanding a substantial number of NK cells by D35.

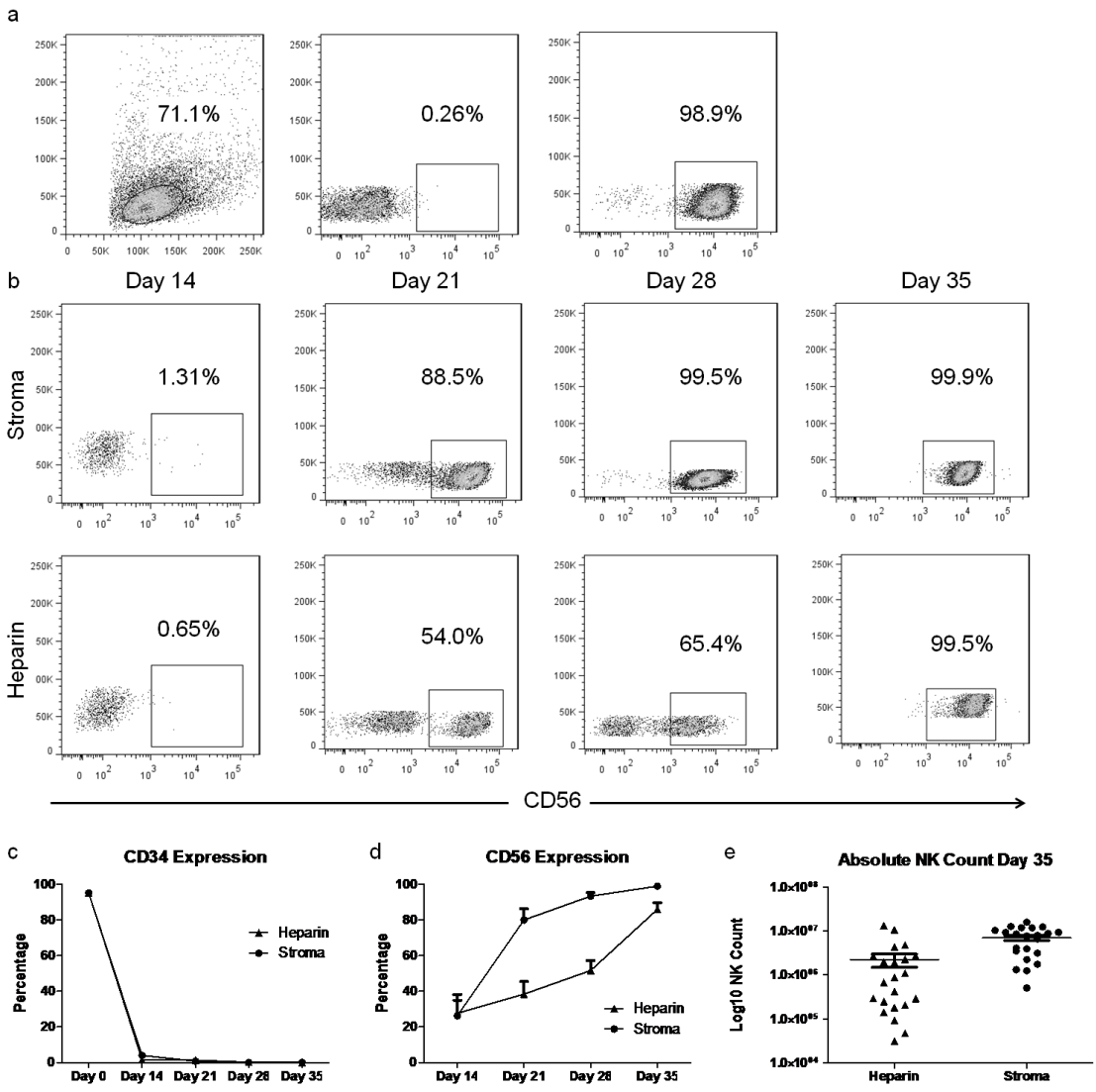

Figure 7. Stroma method provides greater absolute NK cell counts compared to the heparin method. (a) Shows the gating steps used with the initial lymphocyte population encircled in the first graph, the negative isotype control with negative cells right of the gate in the second graph, and the $CD56^+$ cells gated in a square in the third graph. **(b)** Displays sample raw data of the progressive acquisition of $CD56^+$ expression over 14-35 days in culture for stroma method on top and heparin method on bottom, paired donors for each day. **(c and d)** Shows the average decrease in $CD34^+$ expression and corresponding $CD56^+$ expression from day 14-35 for the stroma method and heparin method. p=0.001, n=22 paired donors. Focusing on D21 the stroma method increases $CD56^+$ expression two fold versus the heparin method. p=0.001, n=15 paired donors. **(e)** Shows the D35 absolute NK cell count for each method. The data shows that the stroma method develops 3 times as much NK cells compared to the heparin method. p=0.002, n=22 paired donors.

3. NK maturational status: a comparison of the Stage III and Stage IV NK progenitor populations generated under the stroma and heparin methods.

The differing percentages of $CD56^+$ cells seen at D21 in Figure 8d suggests that the two culture conditions generate NK cells at differing rates. NK cells progress through a series of orderly developmental steps, described as stages I through V (Figure 3). We and other researchers have used CD117 and CD94 expression to distinguish stage III and IV NK progenitors [35, 72]. To address the kinetics of NK cell maturation in the two methods, we performed FACS using antibodies directed against these antigens. Figure 8a and 8b are representative $CD56^+$ gated flow samples of $CD117^+$ and $CD94^+$ co-expression for a single donor cultured with either the stroma or heparin methods, respectively, over a 35-day period. For both conditions the CD117 expression progressively decreases as CD94 expression is acquired over time. As shown in figure 8c, cultures of HSCs developed with the heparin method tended to maintain high levels of CD117 expression for longer periods and the corresponding CD94 acquisition was slower. Comparing the heparin method to the stroma method, there were more stage III NK precursors ($CD56^+CD117^{high}CD94^-$). In contrast, the stroma method appeared to advance the cells to the stage IV NK cell more readily. These differences are most apparent on D28 ($p=0.001$, $n=13$ paired donors). Interestingly, at D35 both methods demonstrated similar proportions of stage IV (and V) NK cells ($p=0.3$, $n=22$ paired donors). Because the expansion was greater in the stroma method, the absolute numbers of stage IV cells were greater in the stroma method ($p=0.001$, $n=22$ paired donors).

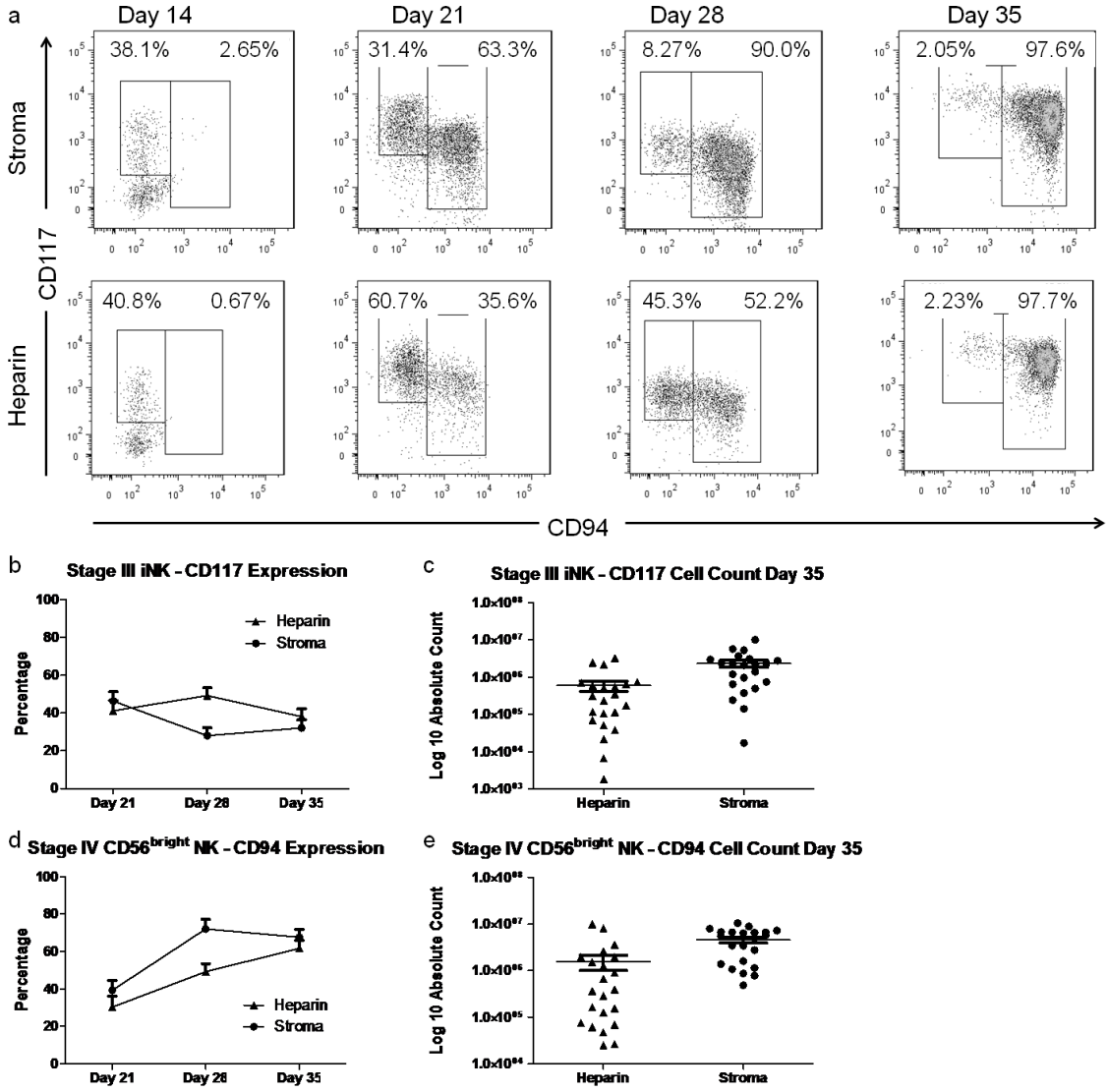

Figure 8. Stroma supported and heparin supported methods display differences in CD117 and CD94 expression on D28. (a and b) Sample flow cytometry data for CD117 and CD94 expression over a 35-day period for the stroma and heparin method respectively, paired donors for each day. **(c)** CD117 expression on D35 is very similar for both methods. D28 shows that the heparin method maintains 1.7 times more CD117 compared to the corresponding stroma method. D35, p=0.5, n=22 paired donors; D28, p=0.001, n=13 paired donors. **(d)** Data shows that the stroma method produces 3.9 times more NK cells that express CD117 on D35. p=0.002, n=22 paired donors. **(e)** CD94 expression on D35 is very similar for both methods. D28 shows that the stroma method maintains 1.5 times more CD94 compared to the corresponding heparin method. D35, p=0.3, n=22 paired donors; D28, p=0.001, n=13 paired donors. **(f)** Data shows that the stroma method produces 2.9 times more NK cells that express CD94 on D35. p=0.001, n=22 donors.

4. Examination of activating and inhibitory receptor acquisition for HSC-derived NK cells generated in stroma supported and heparin supported methods.

NK cytotoxicity is dictated by the expression of both inhibitory and activating receptors that engage ligands on the surface of potential target cells. The expression of these receptors is, in part, developmentally controlled [35]. Given the different kinetics of NK cell development between the two techniques, we interrogated the expression of key NK cell receptors. Figure 9a shows FACS histograms for NKG2D, 2B4, CD161, NKp30, NKp44, and NKp46 cultured in the stroma or heparin methods. In both conditions, these receptors were detected on nearly 100% of cells and were statistically similar (data not shown, p range 0.04 - 0.8, n=8 - 18 paired donors). A slight difference in mean fluorescence intensity is seen with NKG2D and CD161 where respectively the stroma method on average expressed 1.1 times more than heparin method; and the heparin method expressed 1.5 times more than the stroma method (p=0.04, n=18; p=0.0001, n=14).

While the above receptors were expressed on nearly 100% of the NK cells, other receptors are differentially expressed on developing NK cells. The two receptors where differences in expression were noted on the percentage of NK cells include KIR and CD16. At D35, the stroma method led to 3.4 times more KIR expressing NK cells on average than the heparin method (p=0.001, n=19 paired donors). Interestingly, the opposite was true for CD16 since the NK cells generated on stroma showed 10 times fewer NK cells expressing CD16 than the heparin method (p=0.001, n=14 paired donors).

These differing results are surprising considering that both receptors are considered to be associated with terminal NK cell maturation.

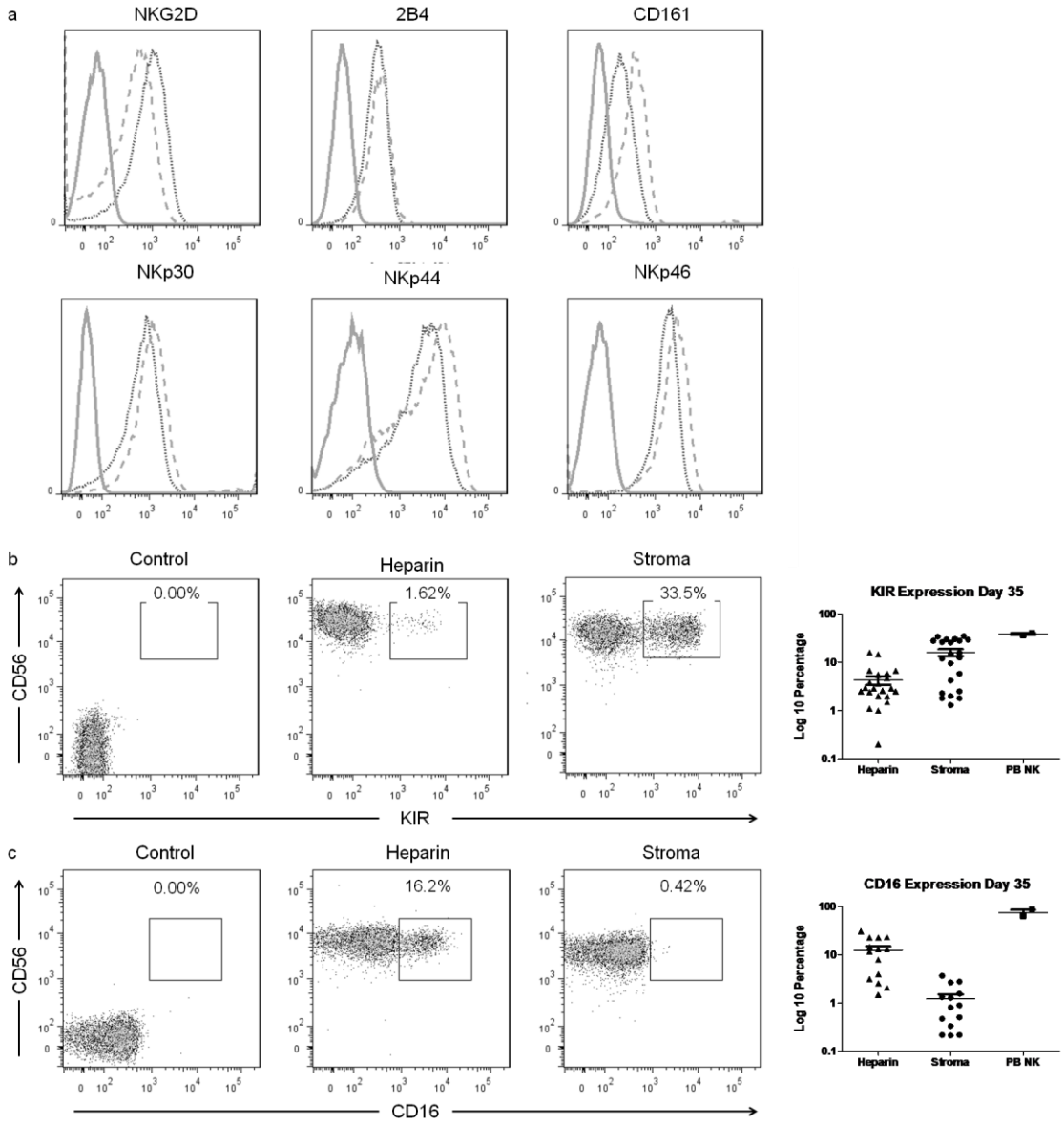

Figure 9. Activating and inhibitory receptor expression was very similar for both stroma and heparin methods with a few exceptions. (a) A representative sample is shown for a control (solid line), the heparin method (dashed line), and the stroma method (dotted line) for various receptors. p=0.04-0.8, n=8-18 paired donors. **(b)** Displays representative sample FACS data for KIR followed by summary analysis. Data shows that the stroma method leads to 3.4 times on average more KIR expression than the heparin method. p=0.001, n=19 paired donors. **(c)** Displays representative sample FACS data for CD16 followed by summary analysis. Data shows that the heparin method leads to 10 times on average more CD16 expression than the stroma method. p=0.001, n=14 paired donors.

5. Functional Properties of HSC-derived NK cells

IL-22 plays an important role in immune defense, inflammation, and tissue repair [73]. Our laboratory has recently shown that some stage III $CD56^{+}CD117^{+}CD94^{-}$ cells produce significant amounts of IL-22 after stimulation with IL1β and IL-23 [40]. Given the higher percentage of stage III NK cells in heparin based cultures (figure 8c) we hypothesized that there would be more IL-22 producing cells in this method. Our previous studies showed that IL-22 production peaks at D21 of culture and is more prevalent in cultures that do not contain hydrocortisone (unpublished). To investigate the IL-22 production in the two developmental techniques, HSC-derived NK cells generated with either the stroma or heparin methods were analyzed. At D21 cells were stimulated with IL-1β and IL-23 and supernatant was assayed for IL-22 using ELISA. As shown in figure 10a, the stroma supported cells provide 3.5 times more IL-22 production compared to the heparin supported cells (D21, $p=0.004$, $n=4$ paired donors).

IFN γ is a key immune regulator and activates other cells within the immune system, including antigen presenting cells and T cells. IFN γ production provides an indication of the developmental and functional capabilities of the $CD56^{+}$ NK cells, as this cytokine is produced by stage IV and V NK cells, but not stage III [72]. To date, it is not known whether these methods of generating NK cells from HSCs yields cells with differing abilities to produce this cytokine. To address this, NK cells at D35 were stimulated with IL-12 and IL-18 to provoke IFN γ production. As shown in Figure 10b, at D35 of culture, no differences are noted in the IFN γ production when examining the whole population of $CD56^{+}$ cells ($p=0.7$, $n=7$ paired donors). Similar results were

obtained when gating upon the more mature $CD94^+$ cells, which define the stage IV and V cell fractions ($p=0.5$, $n=7$ paired donors, data not shown). When examining the quantity of IFN γ produced, the heparin based method produced cells that made more IFN γ compared to the stroma derived NK cells. While these numbers were significant with heparin averaging 2751 pg/mL versus stroma averaging 2051 pg/mL ($p=0.03$, $n=6$), NK cells from these cell sources made IFN γ at D35, supporting the concept that the two methods are producing similarly functional cells.

Cytotoxicity is another hallmark measure of NK functionality and maturational status. At D35, cells from the two culture techniques were used for killing assays. To do this the cells were co-cultured with ^{51}Cr loaded target cells (K562, Raji, and Daudi leukemic cell lines) for 4 hours. As a control, IL-15 activated peripheral blood NK cells were also included. The experiments showed similar killing capacity for HSC-derived NK cells generated on stroma (circles) and heparin (triangles) cultures. Thus, both methods resulted in a population of equally cytotoxic NK cells.

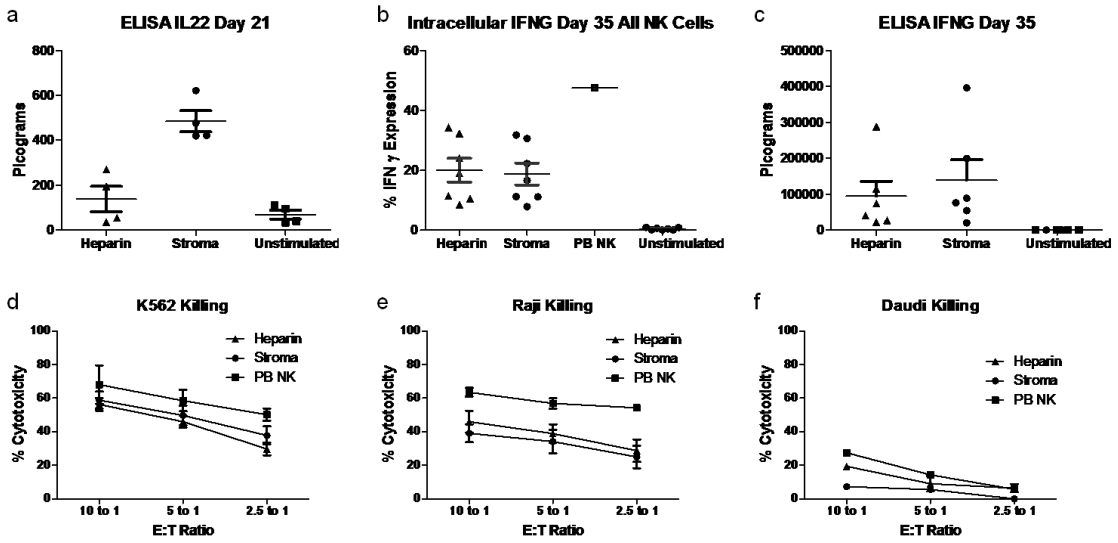

Figure 10. Stroma method provides greater IL-22 production while heparin method provides greater IFN γ production, but killing experiments are very similar. (a) The stroma protocol provides 3.5 times more IL-22 production than the heparin method for D21 NK cells derived from UCB HSCs. Intracellular IL-22 analysis was also performed, but none was detected (data not shown). p=0.004, n=4 paired donors. **(b)** Both methods provide similar IFN γ production by intracellular staining gated on $CD56^+$ cells. IFN γ production gated on $CD94^+$ cells (data not shown) was also similar. Respectively p=0.7 and 0.5, n=7 paired donors. **(c)** Analysis of the supernatant revealed there was 1.5 times more IFN γ production for the stroma method. p=0.07, n=6 paired donors. **(d-f)** Both methods provide similar killing ability when co-cultured with K562, Raji, and Daudi cell lines. Respectively p=0.7, 0.4, 0.1 and n=8, 5, 2 paired donors.

Discussion

Here, we compared two methods of generating NK cells from HSCs in vitro. In our studies we address the efficiency of a heparin supported approach and compare it to what might be considered the "gold standard," which is a stroma cell supported method. We show that the total cell expansion and NK cell generation was more robust for the stroma method, but that the heparin method could also generate large quantities of NK cells. Despite the significant differences in the total cell numbers, both approaches generate similar percentages of NK cells by D35. HSC-derived NK cells from both techniques displayed similar levels of activating and inhibitory receptors, with differences only in CD161, NKG2D, KIR, and CD16, favoring a slightly more mature product with stroma. Likewise, IL-22 production was significantly higher in stromal supported cultures, but IFN-γ production was higher in the heparin conditions. Lastly, cytotoxicity was similar between the two methods.

In previous work, our laboratory has demonstrated the necessity of stromal support for the differentiation of $CD34^{+}NKlin^{-}$ cells into NK cells [42]. The exact mechanism for this is not known, but possibilities include stromal cell expression of certain fate determining receptors (i.e., notch and/or gas-6) and/or elaboration of soluble factors including Wnt, cytokines, or extracellular basement membrane (such as heparin or periostin) [53, 55-57, 74, 75]. As part of these studies, we recently questioned whether the heparin supported method could also support the differentiation of $CD34^{+}NKlin^{-}$ cells into NK cells. We found that the heparin supported method is not as capable as the stroma method towards differentiating $CD34^{+}NKlin^{-}$ cells into $CD56^{+}CD117^{-}CD94^{+}$ NK

cells. This observation may explain the greater NK cell numbers observed with the stroma, since both $CD34^{+}NKlin^{-}$ and $CD34^{+}NKlin^{+}$ cells are being differentiated. Related to this, our laboratory recently observed that IL-22 producing NK cells from stromal-derived cultures are mainly derived from $CD34^{+}$ cells that are present after 5 days of culture. We speculate that these $CD34^{+}$ cells are derived from $CD34^{+}NKlin^{-}$ uncommitted progenitors (Yong-Oon Ahn and Verneris, unpublished). Thus, these two observations might suggest that the stroma acts on both "NK committed" and "uncommitted" progenitors, while heparin based media acts mainly upon the later population and that this accounts both for the differences in expansion as well as cell phenotype and function.

The differential expression of the receptor for SCF (CD117) and the iNK cell receptor (CD94) defines the stage III and stage IV NK cell developmental precursors [35]. Using this approach, we observed a significant difference between the two methods for NK cell maturation. At D14-D28 the heparin supported cultures showed a significant proportion of the cells were stage III NK progenitors, while in the stroma supported cells most were stage IV NK cells. Interestingly by D35, there were non-significant differences in the percentages of these two population, with the majority being stage IV cells for both conditions. These finding show that the two approaches differ in the kinetics of NK generation, but not in the final cell phenotype or function. Importantly, both methods generate stage IV NK cells, while the majority of NK cells in the peripheral blood are stage V cells, suggesting that additional signals, likely through cytokines or surface receptors, are not provided in the above culture methods.

The expression of inhibitory and activating receptors correlates with the maturational status and functionality of the NK progenitors. In these studies we evaluated the expression of NK activating receptors including the C-type lectin receptors (CD94/NKG2 heterodimers), CD161, and the NCRs (NKp30, NKp44, and NKp46) [12]. We also looked at one of the co-activating receptors CD244 (2B4). Both approaches yielded similar levels of expression for all these, except CD161 which was expressed at slightly higher levels of expression in the heparin cultured cells, and NKG2D which was slightly higher in the stroma cultured cells. Perhaps, the most interesting observations involved KIR and CD16 expression—both of which correlate with terminal differentiation. The stromal method provided significantly higher amounts of KIR, while in contrast, the heparin method had greater amounts of CD16. The percent expression, for either protein, were not as high as seen in PB NK cells (stage V NK cells), but it does question what mechanisms are causing these differences. The higher levels of CD16 on the heparin supported method may be attributed to the additional cytokines, such as G-CSF which leads to CD16 acquisition on neutrophils [76]. Alternatively, our laboratory has previously shown that upon activation, CD16 is shed from the surface in a matrix metaloprotease-dependent process and perhaps such proteases are expressed by stroma cells, explaining the lower CD16 expression in these conditions [77]. However, such a hypothesis is difficult to reconcile since the irradiated stroma cells are not morphologically detectable after ~2 weeks from the start of culture (unpublished). Related to this point, our studies show that the number and percentage of KIR expressing cells at D35 is higher in the stromal cultures. Thus, it appears that the early events (D0-

D14) provided by stroma appear to have later influence on maturation and KIR acquisition, in particular. The greater KIR expression in the stroma method could be associated with the early interactions between the Gas6/Protein S receptor on stromal cells and the Tyro3 ligand on the NK cell precursors, which modulate NK cell maturation [54].

Recent studies have shown that cells within the stage III NK progenitor fraction, present in secondary lymphoid tissues are capable of producing IL-22 [40, 73]. IL-22 acts on epithelial cells to induce proliferation, protection from apoptosis and the production of antibacterial compounds [73]. Due to these attributes, this cytokine is critically important in mucosal immunity. Our laboratory has previously shown that some stage III NK progenitors generated from HSCs are also able to produce IL-22 [40]. Given that the heparin based cultures had significantly more stage III NK cells, we hypothesized that there would be more IL-22 cells. Surprisingly, the stroma method produced significantly more IL-22 than the heparin method. As discussed above, these findings may point to certain cell surface or soluble proteins provided by the stroma cells that are not present in the heparin method. Alternatively the lymphoid tissue inducer-like cells (LTi-like) cells in the HSCs that give rise to IL-22 producing cells may not be supported in the heparin method and subsequently less to no IL-22 is produced [40]. Also recent experiments support the importance of Notch signaling and NK cytokine production [78]. Researchers have shown that stroma cells can provide this Notch signaling [55]. IL-22 has not been specifically tested in this correlation, but perhaps this experiment suggest that there may be a correlation between IL-22 cytokine production

and Notch signaling from stromal cells which may explain increased IL-22 levels in the stroma method.

IFN γ production is a critical regulator of immune responses and rapid production and release of NK-derived IFN γ which alerts components of the adaptive immune system. Classically, the stage IV NK cell subset ($CD56^{bright}$ cells) have been considered to provide the highest production of IFN γ [39], but this has been called into question recently with their alloreactive capabilities in HSCT [79]. In our experiments we considered that IFN γ production is most optimal at stage IV and therefore expect the stroma method will produce the most IFN γ, especially since they were more phenotypically stage IV NK cells. This was not the case and both methods produced similar percentages of IFN γ producing cells using intracellular analysis, but stroma showed slightly increased quantities of IFN γ production by ELISA. A possible explanation for the stroma method's IFN γ production may be once again associated with Notch signaling and NK cytokine production [78]. In addition, IFN γ production seen in the heparin media may be related to the IL-6 present. There has been research that shows IL-6 stimulated T cells show increased IFN γ production [80] and this may also be the case for NK cells.

HSC-derived NK cells have promise in the treatment of chemotherapy refractory malignancies. However, the methodology to generate these cells under GMP conditions is not trivial. In this study we compared stroma cell supported techniques to heparin supported methods. While the stroma supported cultures provide significantly higher numbers of NK cells, both methods produce remarkably similar cells with respect to

phenotype and function. The stroma supported method may have major obstacles with regard to FDA approval and integration into clinical trials. In contrast, the heparin method is a GMP compliant system that can be easily adapted into clinical trials, such as those that we have previously demonstrated to be effective in AML patients who have exhausted all traditional treatment [70]. Current Glycostem research has taken us closer to trials with the development of a NK growth protocol for a closed system utilizing a bioreactor method for generating pure NK cells from cryogenically frozen stem cells [81]. This protocol will allow for the development of transfusable NK products that can be more easily quality controlled. More recently, data showing superior transplant outcomes with donors displaying KIR Cen B/B haplotypes provides an interesting opportunity to apply this technology [15]. We envision a clinical trial where UCB donors are screened for favorable haplotypes (Cen B/B) and the creation of a frozen cell bank that can be utilized in conjunction with the heparin supported media to differentiate $CD34^+$ cells into NK cells for therapeutic purposes.

References

1. Robertson, M.J. and J. Ritz, *Biology and clinical relevance of human natural killer cells.* Blood, 1990. **76**(12): p. 2421-38.
2. Trinchieri, G., *Biology of natural killer cells.* Adv Immunol, 1989. **47**: p. 187-376.
3. Cooper, M.A., T.A. Fehniger, and M.A. Caligiuri, *The biology of human natural killer-cell subsets.* Trends Immunol, 2001. **22**(11): p. 633-40.
4. Robertson, M.J., et al., *Costimulatory signals are required for optimal proliferation of human natural killer cells.* J Immunol, 1993. **150**(5): p. 1705-14.
5. Cooper, M.A., et al., *Human natural killer cells: a unique innate immunoregulatory role for the CD56(bright) subset.* Blood, 2001. **97**(10): p. 3146-51.
6. Jacobs, R., et al., *CD56bright cells differ in their KIR repertoire and cytotoxic features from CD56dim NK cells.* Eur J Immunol, 2001. **31**(10): p. 3121-7.
7. Frey, M., et al., *Differential expression and function of L-selectin on CD56bright and CD56dim natural killer cell subsets.* J Immunol, 1998. **161**(1): p. 400-8.
8. Borg, C., et al., *NK cell activation by dendritic cells (DCs) requires the formation of a synapse leading to IL-12 polarization in DCs.* Blood, 2004. **104**(10): p. 3267-75.
9. Munz, C., R.M. Steinman, and S. Fujii, *Dendritic cell maturation by innate lymphocytes: coordinated stimulation of innate and adaptive immunity.* J Exp Med, 2005. **202**(2): p. 203-7.
10. Cunningham, B.A., et al., *Neural cell adhesion molecule: structure, immunoglobulin-like domains, cell surface modulation, and alternative RNA splicing.* Science, 1987. **236**(4803): p. 799-806.
11. Rutishauser, U. and C. Goridis, *Ncam - the Molecule and Its Genetics.* Trends in Genetics, 1986. **2**(3): p. 72-76.
12. Farag, S.S. and M.A. Caligiuri, *Human natural killer cell development and biology.* Blood Rev, 2006. **20**(3): p. 123-37.
13. Lanier, L.L., *NK cell receptors.* Annu Rev Immunol, 1998. **16**: p. 359-93.
14. Moretta, L. and A. Moretta, *Killer immunoglobulin-like receptors.* Curr Opin Immunol, 2004. **16**(5): p. 626-33.
15. Cooley, S., et al., *Donors with group B KIR haplotypes improve relapse-free survival after unrelated hematopoietic cell transplantation for acute myelogenous leukemia.* Blood, 2009. **113**(3): p. 726-32.
16. Cooley, S., et al., *Donor selection for natural killer cell receptor genes leads to superior survival after unrelated transplantation for acute myelogenous leukemia.* Blood, 2010. **116**(14): p. 2411-9.
17. Perez-Villar, J.J., et al., *Biochemical and serologic evidence for the existence of functionally distinct forms of the CD94 NK cell receptor.* J Immunol, 1996. **157**(12): p. 5367-74.
18. Wu, J., et al., *An activating immunoreceptor complex formed by NKG2D and DAP10.* Science, 1999. **285**(5428): p. 730-2.

19. Pende, D., et al., *Identification and molecular characterization of NKp30, a novel triggering receptor involved in natural cytotoxicity mediated by human natural killer cells.* J Exp Med, 1999. **190**(10): p. 1505-16.
20. Sivori, S., et al., *p46, a novel natural killer cell-specific surface molecule that mediates cell activation.* J Exp Med, 1997. **186**(7): p. 1129-36.
21. Vitale, M., et al., *NKp44, a novel triggering surface molecule specifically expressed by activated natural killer cells, is involved in non-major histocompatibility complex-restricted tumor cell lysis.* J Exp Med, 1998. **187**(12): p. 2065-72.
22. Moretta, A., et al., *Activating receptors and coreceptors involved in human natural killer cell-mediated cytolysis.* Annu Rev Immunol, 2001. **19**: p. 197-223.
23. Christensen, M.E. and N.J. Waterhouse, *Mechanisms of CTL cytotoxicity. A reactive response to granzyme B.* Immunol Cell Biol, 2010. **88**(5): p. 500-1.
24. Pardo, J., et al., *Granzyme B-induced cell death exerted by ex vivo CTL: discriminating requirements for cell death and some of its signs.* Cell Death Differ, 2008. **15**(3): p. 567-79.
25. Janeway, C., *Immunobiology : the immune system in health and disease*. 6th ed. 2005, New York: Garland Science. xxiii, 823 p.
26. Kindt, T.J., et al., *Kuby immunology*. 6th ed. 2007, New York: W.H. Freeman. xxii, 574, A-31, G-12, AN-27, I-27 p.
27. Akira, S., *The role of IL-18 in innate immunity.* Curr Opin Immunol, 2000. **12**(1): p. 59-63.
28. Andrews, D.M., et al., *Functional interactions between dendritic cells and NK cells during viral infection.* Nat Immunol, 2003. **4**(2): p. 175-81.
29. Schroder, K., et al., *Interferon-gamma: an overview of signals, mechanisms and functions.* J Leukoc Biol, 2004. **75**(2): p. 163-89.
30. Puro, R. and R.J. Schneider, *Tumor necrosis factor activates a conserved innate antiviral response to hepatitis B virus that destabilizes nucleocapsids and reduces nuclear viral DNA.* J Virol, 2007. **81**(14): p. 7351-62.
31. Biermer, M., R. Puro, and R.J. Schneider, *Tumor necrosis factor alpha inhibition of hepatitis B virus replication involves disruption of capsid Integrity through activation of NF-kappaB.* J Virol, 2003. **77**(7): p. 4033-42.
32. Ashkenazi, A., *Targeting death and decoy receptors of the tumour-necrosis factor superfamily.* Nat Rev Cancer, 2002. **2**(6): p. 420-30.
33. Cambiaggi, C., et al., *Constitutive expression of CD69 in interspecies T-cell hybrids and locus assignment to human chromosome 12.* Immunogenetics, 1992. **36**(2): p. 117-20.
34. Srour, E.F., et al., *Human CD34+ HLA-DR- bone marrow cells contain progenitor cells capable of self-renewal, multilineage differentiation, and long-term in vitro hematopoiesis.* Blood Cells, 1991. **17**(2): p. 287-95.
35. Freud, A.G. and M.A. Caligiuri, *Human natural killer cell development.* Immunol Rev, 2006. **214**: p. 56-72.

36. Freud, A.G., et al., *A human CD34(+) subset resides in lymph nodes and differentiates into CD56bright natural killer cells.* Immunity, 2005. **22**(3): p. 295-304.
37. Miller, J.S., C. Verfaillie, and P. McGlave, *The generation of human natural killer cells from CD34+/DR- primitive progenitors in long-term bone marrow culture.* Blood, 1992. **80**(9): p. 2182-7.
38. Waldmann, T., Y. Tagaya, and R. Bamford, *Interleukin-2, interleukin-15, and their receptors.* Int Rev Immunol, 1998. **16**(3-4): p. 205-26.
39. Freud, A.G., et al., *Evidence for discrete stages of human natural killer cell differentiation in vivo.* J Exp Med, 2006. **203**(4): p. 1033-43.
40. Tang, Q., et al., *Development of IL-22-producing NK lineage cells from umbilical cord blood hematopoietic stem cells in the absence of secondary lymphoid tissue.* Blood, 2011. **117**(15): p. 4052-5.
41. Borrego, F., et al., *Recognition of human histocompatibility leukocyte antigen (HLA)-E complexed with HLA class I signal sequence-derived peptides by CD94/NKG2 confers protection from natural killer cell-mediated lysis.* J Exp Med, 1998. **187**(5): p. 813-8.
42. Grzywacz, B., et al., *Natural killer-cell differentiation by myeloid progenitors.* Blood, 2011. **117**(13): p. 3548-58.
43. Lanier, L.L., et al., *The relationship of CD16 (Leu-11) and Leu-19 (NKH-1) antigen expression on human peripheral blood NK cells and cytotoxic T lymphocytes.* J Immunol, 1986. **136**(12): p. 4480-6.
44. Blom, B. and H. Spits, *Development of human lymphoid cells.* Annu Rev Immunol, 2006. **24**: p. 287-320.
45. Miller, J.S., K.A. Alley, and P. McGlave, *Differentiation of natural killer (NK) cells from human primitive marrow progenitors in a stroma-based long-term culture system: identification of a CD34+7+ NK progenitor.* Blood, 1994. **83**(9): p. 2594-601.
46. Mrozek, E., P. Anderson, and M.A. Caligiuri, *Role of interleukin-15 in the development of human CD56+ natural killer cells from CD34+ hematopoietic progenitor cells.* Blood, 1996. **87**(7): p. 2632-40.
47. Ma, A., R. Koka, and P. Burkett, *Diverse functions of IL-2, IL-15, and IL-7 in lymphoid homeostasis.* Annu Rev Immunol, 2006. **24**: p. 657-79.
48. Kennedy, M.K., et al., *Reversible defects in natural killer and memory CD8 T cell lineages in interleukin 15-deficient mice.* J Exp Med, 2000. **191**(5): p. 771-80.
49. Lodolce, J.P., et al., *IL-15 receptor maintains lymphoid homeostasis by supporting lymphocyte homing and proliferation.* Immunity, 1998. **9**(5): p. 669-76.
50. Koka, R., et al., *Interleukin (IL)-15R[alpha]-deficient natural killer cells survive in normal but not IL-15R[alpha]-deficient mice.* J Exp Med, 2003. **197**(8): p. 977-84.
51. Burkett, P.R., et al., *Coordinate expression and trans presentation of interleukin (IL)-15Ralpha and IL-15 supports natural killer cell and memory CD8+ T cell homeostasis.* J Exp Med, 2004. **200**(7): p. 825-34.

52. Dubois, S., et al., *IL-15Ralpha recycles and presents IL-15 In trans to neighboring cells.* Immunity, 2002. **17**(5): p. 537-47.
53. Dormady, S.P., X.M. Zhang, and R.S. Basch, *Hematopoietic progenitor cells grow on 3T3 fibroblast monolayers that overexpress growth arrest-specific gene-6 (GAS6).* Proc Natl Acad Sci U S A, 2000. **97**(22): p. 12260-5.
54. Roth, C., et al., *Stromal-cell regulation of natural killer cell differentiation.* J Mol Med, 2007. **85**(10): p. 1047-56.
55. Hoyne, G.F., *Notch signaling in the immune system.* J Leukoc Biol, 2003. **74**(6): p. 971-81.
56. Haraguchi, K., et al., *Notch activation induces the generation of functional NK cells from human cord blood CD34-positive cells devoid of IL-15.* J Immunol, 2009. **182**(10): p. 6168-78.
57. Yamane, T., et al., *Wnt signaling regulates hemopoiesis through stromal cells.* J Immunol, 2001. **167**(2): p. 765-72.
58. Sanchez, M.J., et al., *Identification of a common T/natural killer cell progenitor in human fetal thymus.* J Exp Med, 1994. **180**(2): p. 569-76.
59. Ramos, S.B., et al., *Phenotypic and functional evaluation of natural killer cells in thymectomized children.* Clin Immunol Immunopathol, 1996. **81**(3): p. 277-81.
60. Sihvola, M. and M. Hurme, *The development of NK cell activity in thymectomized bone marrow chimaeras.* Immunology, 1984. **53**(1): p. 17-22.
61. Ferlazzo, G., et al., *Distinct roles of IL-12 and IL-15 in human natural killer cell activation by dendritic cells from secondary lymphoid organs.* Proc Natl Acad Sci U S A, 2004. **101**(47): p. 16606-11.
62. Sykes, M., et al., *Hematopoietic cells and radioresistant host elements influence natural killer cell differentiation.* J Exp Med, 1993. **178**(1): p. 223-9.
63. Spanholtz, J., et al., *High log-scale expansion of functional human natural killer cells from umbilical cord blood CD34-positive cells for adoptive cancer immunotherapy.* PLoS One, 2010. **5**(2): p. e9221.
64. Kishimoto, S., et al., *Human stem cell factor (SCF) is a heparin-binding cytokine.* J Biochem, 2009. **145**(3): p. 275-8.
65. Clarke, D., et al., *Interaction of interleukin 7 (IL-7) with glycosaminoglycans and its biological relevance.* Cytokine, 1995. **7**(4): p. 325-30.
66. Najjam, S., et al., *Further characterization of the binding of human recombinant interleukin 2 to heparin and identification of putative binding sites.* Glycobiology, 1998. **8**(5): p. 509-16.
67. Hasan, M., et al., *IL-12 is a heparin-binding cytokine.* J Immunol, 1999. **162**(2): p. 1064-70.
68. Ruggeri, L., et al., *Effectiveness of donor natural killer cell alloreactivity in mismatched hematopoietic transplants.* Science, 2002. **295**(5562): p. 2097-100.
69. Leung, W., et al., *Inhibitory KIR-HLA receptor-ligand mismatch in autologous haematopoietic stem cell transplantation for solid tumour and lymphoma.* Br J Cancer, 2007. **97**(4): p. 539-42.
70. Miller, J.S., et al., *Successful adoptive transfer and in vivo expansion of human haploidentical NK cells in patients with cancer.* Blood, 2005. **105**(8): p. 3051-7.

71. Spiessens, B., et al., *An overview of group sequential methods in longitudinal clinical trials.* Stat Methods Med Res, 2000. **9**(5): p. 497-515.
72. Grzywacz, B., et al., *Coordinated acquisition of inhibitory and activating receptors and functional properties by developing human natural killer cells.* Blood, 2006. **108**(12): p. 3824-33.
73. Vivier, E., H. Spits, and T. Cupedo, *Interleukin-22-producing innate immune cells: new players in mucosal immunity and tissue repair?* Nat Rev Immunol, 2009. **9**(4): p. 229-34.
74. Siewe, B.T., et al., *In vitro requirement for periostin in B lymphopoiesis.* Blood, 2011. **117**(14): p. 3770-9.
75. Reijmers, R.M., et al., *Disruption of heparan sulfate proteoglycan conformation perturbs B-cell maturation and APRIL-mediated plasma cell survival.* Blood, 2011. **117**(23): p. 6162-71.
76. Ninomiya, H., et al., *Induction of Fc gamma R-III (CD16) expression on neutrophils affected by paroxysmal nocturnal haemoglobinuria by administration of granulocyte colony-stimulating factor.* Br J Haematol, 1993. **84**(3): p. 497-503.
77. Grzywacz, B., N. Kataria, and M.R. Verneris, *CD56(dim)CD16(+) NK cells downregulate CD16 following target cell induced activation of matrix metalloproteinases.* Leukemia, 2007. **21**(2): p. 356-9; author reply 359.
78. Bachanova, V., et al., *Activated notch supports development of cytokine producing NK cells which are hyporesponsive and fail to acquire NK cell effector functions.* Biol Blood Marrow Transplant, 2009. **15**(2): p. 183-94.
79. Moretta, L., et al., *Killer Ig-like receptor-mediated control of natural killer cell alloreactivity in haploidentical hematopoietic stem cell transplantation.* Blood, 2011. **117**(3): p. 764-71.
80. Saunders, B.M., et al., *Interleukin-6 induces early gamma interferon production in the infected lung but is not required for generation of specific immunity to Mycobacterium tuberculosis infection.* Infect Immun, 2000. **68**(6): p. 3322-6.
81. Spanholtz, J., et al., *Clinical-grade generation of active NK cells from cord blood hematopoietic progenitor cells for immunotherapy using a closed-system culture process.* PLoS One, 2011. **6**(6): p. e20740.