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Multiple aspects of natural killer cell expansion in relevance to immunotherapy for hematologic malignancies

Dominic Colosimo
University of Central Florida

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MULTIPLE ASPECTS OF NATURAL KILLER CELL EXPANSION IN

RELEVANCE TO IMMUNOTHERAPY FOR HEMATOLOGIC

MALIGNANCIES

by

DOMINIC A. COLOSIMO

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ABSTRACT

Natural Killer (NK) cells are a subset of lymphocytes that regulate adaptive immune responses and utilize "missing self" recognition to activate anti-tumor and anti-viral cytotoxicity. Clinical research, as well as murine and *ex vivo* models, have shown that a variety of NK cell applications have proven useful as immunotherapeutic treatments for patients with hematologic malignancies. However, the selective expansion of NK cells to yield relevant amounts of these lymphocytes has been a major hurdle in the development of methods for clinical therapeutic use. Here, we demonstrate a novel *ex vivo* expansion method utilizing k562 leukemic cell lines and soluble cytokines as well as a novel method utilizing isolated plasma membranes of genetically engineered tumor cell lines that could be of relevance to *in vivo* NK cell expansion. Also, the ligand expression by canonical feeder cell lines used for NK cell expansion and our isolated plasma membranes were compared via ligand quantification by western blot quantification of 4-1BB ligand. In an adjunct study, we sought to better characterize these expansion environments by investigating the glucose metabolism of NK cells using fluorescent glucose analog 2-(*N*-(7-Nitrobenz-2-oxa-1, 3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG) and the glycolysis inhibitor 2-Deoxy-D-Glucose (2-DG).

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INTRODUCTION

Hematologic Malignancies and Stem Cell Transplantation

For many patients with hematologic malignancies the dysfunction of the bone marrow limits the curative options to hematopoietic stem cell transplantation (HSCT). While HSCT has the ability to mitigate or eliminate cancers of the blood, its limitations are clearly visible in clinical data statistics such as 21-26% relapse rates, approximately 16% transplant-related mortality, and event-free survival in the 40% range [1, 2]. Complications of transplants include graft-versus-host disease (GVHD), failure to engraft, susceptibility to infection, and relapse due to inefficient tumor clearance. In close relation to these clinical risks, there are practical issues such as the existence of a suitable donor. Matched sibling donors (MSD) or siblings who share major HLA antigens with the recipient, have generally been regarded as the most effective source of stem cells [3-5]. However such donors may not always be present or quickly available. In such a case physicians have several options to find the second best stem cell donor which include HLA-disparate family members, phenotype matched unrelated donors, mismatched unrelated donors, and umbilical cord blood. While matched unrelated donors have shown to be a viable option, there are drastic shortages for minorities (Ten percent of African American patients in need of transplantation were able to receive treatment compared to 50% of Caucasians in need) [5]. However, *in vitro* and *in vivo* techniques for improving HSCT could mitigate the lengthy search for a perfect donor and allow for prompt treatment, regardless of HLA phenotype.

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Role of Natural Killer Cells

Natural killer cells are hematopoietic cells that provide a bridge between the innate and adaptive immune system and represent 7-15% of the lymphocyte population of the blood [6]. They provide an early reaction to both non-self pathogens and cells within the body undergoing stress such as viral infection or oncogenic mutations [7]. NK cells use two main effector functions to carry out such tasks: cytokine secretion (particularly IFN-γ) and direct cytotoxic mechanisms. The production and subsequent secretion of IFN-γ is an augmenting factor of the adaptive immune response via upregulation of antigen presentation in antigen presenting cells such as dendritic cells (DCs) [8]. IFN-γ can also interact with non-APC cells to induce an adaptive-innate combinatorial immune response via antibody dependent cell cytotoxicity (ADCC) [9]. This mechanism involves coating of a target cell with antibodies, whose Fc regions are able to interact with Fcγ-receptors (CD16) on NK cells to stimulate directed cytotoxicity [9]. Direct cell killing can be done by exocytosis of vesicles containing the lytic granules perforin and granzyme or by extracellular binding of death ligands, such as Fas and TRAIL, to corresponding target cell receptor which induces apoptosis. [9-11].

NK cells induce these effector functions differently from their adaptive immune system counterparts, T cells and B cells. While the latter utilize genomic variations followed by triggered clonal expansion to induce their cytotoxic functions, NK cells utilize a balancing system of inhibitory and activating receptors that sense the origin of interacting cells via cell-to-cell interaction [12, 13]. The goal of engaging these receptors is to identify foreign cells and the mechanism is known as "missing self" recognition. When an NK cell encounters a possible

target cell the NK cell's inhibitory receptor searches and binds to the specific coordinating HLA markers on the adjacent cell. In the absence of these correct "self" HLA markers, the NK is activated to kill. In addition to the action of the inhibitory receptors, activating receptors can lead to increased cytotoxic mechanisms by being bound by foreign ligands. The combination of activation and inhibition allows for a broad gradient of responses and for immediate cytotoxic activation.

Such an ability has lead to the investigation of NK cell utilization in hematological malignancies, particularly in combination or in lieu of HSCT [13]. The former, in the form of NK cell enriched donor lymphocyte infusions (DLI), has been investigated to alleviate complications caused by mismatched donors and improve graft-versus-tumor (GVT) effect [14-16]. In this manner, HLA mismatched donors could provide quick stem cell access as well as superior anti-tumor activity. In a study of 92 high-risk acute myeloid leukemia (AML) patients who received NK enriched DLI prior to HSCT from HLA-disparate family members, Ruggeri *et al.* showed that patients who had an inhibitory ligand incompatibility in the graft-versus-host direction had better rates of survival (60% versus 5%) than those who did not have the incompatibility. In addition to effect of NKs in combination with HSCT, NKs have also been used as a standalone treatment for AML patients in remission after chemotherapy with promising results [1].

<u>Expansion of Natural Killer Cells</u>

One of the major hurdles in the use of NK cells in immunotherapy for patients with hematologic malignancies is expansion of the NK cells. NK cells normally exist as 5-10% of the

lymphocyte population and are outnumbered by their cytotoxic counterparts, T-cells, which

mediate GVHD. Thus, lymphocyte infusions have shown to be most effective when the amount

of T-cells are depleted and the amounts of NK cells are elevated [13]. However, expansion of

NK cells has proven to be more difficult than traditional methods for expanding lymphocytes

such as exposure to stimulatory cytokines [17]. Currently, one of the main *in vitro* expansion

methods for NK cells is incubation with the leukemia cell line k562 engineered to express

membrane bound ligands 4-1BB and IL-15 on their surface (mb15 feeder cells) [17, 18] or

engineered to express membrane bound ligands 4-1BB and IL-21 (mb21 feeder cells) [19] which

have been treated to be non-proliferative. Inclusion of these two types of feeder cells increased

expansion compared to media conditions with only membrane bound cytokines alone and

increased cytotoxicity towards several tumor cell lines compared to untreated NK cells [17-19].

In addition to these methods, our group has shown a novel use of the k562 cell line alongside

soluble cytokines IL-21 and 4-1BBL as a valuable expansion method of NK cells capable of

matching the afore mentioned effect of engineered feeder cells. While the feeder cell methods

may be efficient for *ex vivo* expansion of NK cells, the use of cell lines for clinical *in vivo* NK

expansion is inapplicable. Thus, our group sought to test the expansion of NK cells with

membranes isolated from lysed mb15 and mb21 feeder cells. These membranes have the

potential to be injected as is or attached to biologically safe nanoparticles to allow for NK cell

expansion in the peripheral blood after or in absence of HSCT. This method would eliminate the

inherent proliferative harm of the cell lines and diminish the time that NK cells spent in an *ex*

vivo setting, where they are subject to human error, non-optimal conditions, and infection.

PBMCs were cultured with each of the prospective proliferation methods to monitor NK cell expansion. In addition, 4-1BB ligand expression by the feeder cells and the isolated membranes was quantified by western blotting to determine if the amount of ligand was a determining factor in NK cell proliferation.

Glucose Metabolism in Natural Killer Cells

While several groups have demonstrated cytotoxicity, viability, and expansion of NK cells for immunotherapy, the metabolism of proliferating NK cells has not yet been examined fully. Thus, we sought to characterize the NK cell metabolism during expansion with various k562 feeder cells. Utilizing a fluorescent glucose analog, 2-(*N*-(7-Nitrobenz-2-oxa-1, 3-diazol-4-yl)Amino)-2-Deoxyglucose (2-NBDG), glucose uptake by NK cells was monitored in various stages of *ex vivo* expansion over several weeks. During expansion, proliferation patterns were altered via feeder cell starvation and re-supplementation to observe the immediate effects of NK-feeder cell interaction on glucose uptake. Proliferating T-cells have been shown to increase glucose uptake during proliferation relative to quiescent T-cells, even in the presence of abundant oxygen [20, 21]. Known as the Warburg effect, this preference for glycolysis rather than the more efficient oxidative phosphorylation is characteristic of T-cells and cancer cells. We hope to begin exploring the possibility that NK cells also exhibit this effect during periods of expansive growth. Pertaining to this goal, the effect of 2-Deoxy-D-Glucose (2-DG), a non-cleavable glucose analog that inhibits the action of hexokinase in the glycolysis pathway, on the proliferation of NK cells was observed in various types of expansion methods.

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In addition to the study of how NK cells fuel metabolic processes during extended proliferation, we aimed to gain insight into the short term glucose uptake these lymphocytes utilize during cell killing. Uptake of 2-NBDG was measured for NK cells exposed to tumor cell lines over different periods of time. The cytotoxic efficiency of NK cells towards various tumor cell lines during 3, 8, and 24 hour exposure regiments was tested alongside the 2-NBDG uptake, in hopes of finding a relationship between cell killing ability and glucose uptake. The target cells in these assays included an array of mostly leukemic cell lines with differing phenotypic characteristics and could help increase knowledge of tumor microenvironments.

While the role of NK cells in fighting hematologic malignancies has shown to be important, the study of NK cells and their proliferation needs to be better understood to utilize immunotherapy efficiently. The small proportion of NK cells in the peripheral blood requires substantial expansion in order for their presence to make a difference in the prevention of GVHD and the induction of potent GVT effect after HSCT. In this study we seek to gain knowledge on how to maximize expansion of natural killer cells *in vivo* and *in vitro* and the contrast between different expansion methods, including a novel technique. In addition we demonstrate unstudied metabolic characteristics of expanding NK cells in regards to their glucose uptake. Collecting this new information is helpful in shedding new light on how NK cells fuel during proliferation and how feeder cells shape this fueling process.

METHODS

Cell Culture

Lymphocytes were expanded from peripheral blood mononuclear cells (PBMCs), which were isolated as follows: unused leukocyte source from anonymous donor's blood was bought from Florida Blood Center. Blood was diluted 1:1 with DPBS and pipetted upon Ficoll-Paque (GE Healthcare) reagent. Mixture was then centrifuged for 30 minutes at 500 x g. PBMC layer was removed via transfer pipette and washed 2X with DPBS. Then PBMCs were used fresh or frozen in stem cell growth media (SCGM) (CellGenix), 10 % fetal bovine serum (FBS) (Gibco), 10% dimethylsulfoxide (DMSO) (Protide) and stored in a liquid nitrogen tank. When culturing, natural killer cells were seeded at $1X10^5$ cells/mL in either SCGM growth media with 10% FBS, 2 mM GlutaMAX (Gibco) or in RPMI media (Cellgro) with 10% FBS. Feeder cells were treated to be non-proliferative via incubation with mitomycin-c (Sigma-Aldrich) at a concentration of 50 ng/mL for 30 minutes followed by several washes. For mb15 feeder cells, a ratio of 1.5:1 PBMC:mb15 feeder cells was used. If mb21 feeder cells were used they were originally seeded at a 10:1 ratio to NK cells. Cell cultures were maintained in humidified atmosphere of 1atm. Air/5% CO_2 at 37°C. Upon clearance of feeder cells (normally every 5-8 days), the cultures were re-supplemented with fresh feeder cells at 1:1 for mb15 feeder cells or 1:10 for mb21 feeder cells. Media color/pH and volume were monitored throughout culturing and half-media was replaced every several days, while cell density was adjusted to $1-2X10^6$ cells/mL when necessary. Cells were counted with a hemacytometer (Reicher, Hausser Scientific, Incyto). All cultures received IL-2 (Peprotech) at either 10 U/mL or 50 U/mL. Cultures in the 2-DG

experiments received 10 mM 2-DG (Acros Organic) daily. Cultures receiving cytokines IL-21 (Peprotech) and 4-1BBL (Peprotech) included them at concentrations of 50 ng/mL.

Natural Killer Cell Isolation

EasySep Negative Selection Human NK Cell Enrichment Kit (StemCell) and EasySep Magnet (StemCell) were used to isolate NKs from PBMCs according to the StemCell protocol.

Glucose Uptake Assay

Glucose uptake was analyzed as follows: NK cells were removed from culture media and diluted with culture media to a concentration no higher than 0.5×10^6 cells/mL in 48-well plates at a volume of 0.25 mL. Glucose uptake media used was RPMI, 10% FBS with appropriate cytokines used in culture media. 2-NBDG (Invitrogen) was then added at equivalent concentration to glucose in the media and then incubated at 37° C with 5% CO_2 for 3 hours. The cells were pelleted by centrifuging at 250 x g at 4°C for 7 minutes, then resuspended in cold DPBS, 0.5% (w/v) BSA, 2mM EDTA, CD56-PE-Cy7 (BD), CD3-APC (BD) and incubated on ice for 30 minutes. 100 μL of DPBS was then added to each tube before being analyzed by flow cytometry on a BD FACSCanto II.

Cytotoxicity Assay

Cytotoxicity was measured as follows: Target cells were stained with TFL4 dye by setting target cells to cell density less than 1×10^7 in RPMI with 10% FBS then incubating the cells with 1 μL 1:10 dilution of TFL4 and DMSO for 15 minutes at room temperature. Resulting cells were washed 3X with DPBS and transferred to new conical tubes. Effector cells alone,

target cells alone, and effector and target cells together at indicated ratios were incubated for allotted time in identical incubation settings to culture conditions. Afterwards, Aposcreen™ Annexin V-FITC Apoptosis Kit (Beckman Coulter) was used to detect the amount of dead or dying cells in all cultures, while SPHERO™ Accucount Fluorescent Particles (Spherotech Inc.) were used to normalize volumes. Cytotoxicity percentages were calculated by the amount of cells dead, dying, or missing in presence of NK as a proportion of would be viable cells as evident by the target only condition. Formula used was as follows: (% of FITC+ cells in effector + target condition) – (% of FITC+ cells in target only condition) x (# of APC+ cells in effector + target condition) = (Amount of target cells killed). (# of APC+ cells in target only condition) – (# of APC+ cells in effector + target condition) = (Amount of cells missing). (Amount of cells missing) + (Amount of cells killed) / (# of APC+ cells in target only condition) = % Cytotoxicity.

Plasma Membrane Isolation

Cells were resuspended in protease inhibitor buffer (50 mM HEPES (J.T. Baker), 2 mM EDTA (Gibco), 10 μg/mL Leupeptin(Applichem), 10 ug/mL Aprotinin (Applichem), 10 μM Pepstatin A (Applichem), and 1 mM AEBSF) and disrupted using Dounce homogenizer type b (Wheaton). Cell lysates were centrifuged 4°C for 30 minutes at 30,000 RPM in an SW50.1 swinging bucket rotor (Beckman). The resulting pellet was re-suspended and labeled as crude membranes (CM). Plasma membranes were isolated by preparative sucrose gradient centrifugation by layering crude membranes on a sucrose gradient and centrifuging for 12 hrs at 30,000 RPM, at 4° C with SW41 Ti swinging bucket rotor (Beckman). Plasma membranes were

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isolated at juncture of 25% and 43% sucrose mixtures. These purified membranes were then further centrifuged at 100,000 x g for 1 hour and re-suspended in protease inhibitor buffer. Membranes were quantified by their protein amount, as described below.

Flow Cytometry and Analysis

BD FACSCanto II was used for flow cytometry collection. Flow cytometry analysis was performed using FlowJo Version 7.6.5. Gating strategies were as follows. First, cell population was displayed on FSC-A vs. FSC-H dot plot and a linear gate was set to remove duplicate cells. Then lymphocytes were gated on a FSC-A vs. SSC-A dot plot. Then these lymphocytes were displayed on a CD-56 vs. CD-3 dot plot and the CD56(+)CD3(-) populations were considered to be NK cells. From here phenotypes were characterized using various described antibodies and glucose uptake using 2-NBDG. For glucose uptake assays, additional CD56 vs. 2-NBDG dot plot was used and cells examined were gated on CD56+ population. The following antibodies were used: anti CD56-PE (Miltenyi), anti CD56-PC7 (Beckman), anti CD3-APC (Beckman), anti CD19-PC7 (BD Biosciences), anti CD16-FITC (Beckman). Cells were analyzed using BD FACSCanto II flow cytometer (BD).

Protein Quantification

Cellular membranes were solublized using RIPA buffer (0.05 M Tris-HCl pH 7.4, 0.15 M NaCl, 1% Triton X-100, 1% Sodium Deoxycholate (Sigma), 0.1% SDS (Sigma), and 0.001 M EDTA) incubation for 30 minutes on ice. Protein concentration was determined using reducing agent-compatible version of Pierce BCA Protein Assay (Thermo Scientific). Samples were read on a VersaMax microplate reader (Molecular Devices).

<u>Gel Electrophoresis and Quantitative Western Blot</u>

Samples for gel electrophoresis were prepared by diluting each sample in 5X sample loading buffer (0.1% β-mercaptoethanol (Gibco), 3% SDS (Sigma), 35% Glycerol (Sigma), 125 mM Tris-HCl pH 6.8, 0.01% w/v bromophenol blue (Sigma)). Samples were loaded onto 12% Bio-Rad Protean® TGX™ precast gels of 15-well or 10 well depending on sample amount. Electrophoresis was carried out in BD Mini-Protean® Tetra Cell (Bio-Rad) utilizing BD Running Buffer and FB300 power source (Fisher Scientific) set to100 Volts for 60-75 minute run time. Proteins were transferred from gel onto PVDF membranes (Bio-Rad) using wet transfer technique in Mini Trans-Blot® Cell (Bio-Rad) submerged in ice. Transfer buffer used was BD Running Buffer with 20% methanol. Proteins were transferred at 100 V for 45-50 minutes. Membranes were then stained with 0.5% Higgins® Black Magic Waterproof Ink in Tris-buffered saline with Tween 20 (TBS-T) to visualize protein bands. After destaining with TBS-T, membranes were blocked with 5% non-fat milk in TBS-T for 60 minutes. Blocking buffer was used to dilute primary antibody, mouse anti human 4-1BBL (R&D Systems) to 1:500 and membranes were incubated with this mixture over night at 4° C. The next morning, membranes were washed 5X with 5 mL TBS-T. Then secondary antibody, goat anti mouse Ig IRDye 800CW (LI-COR) was added at dilution of 1:20000 and membranes were incubated for 60 minutes while protected from light. Membranes were then washed 5 times with 5 mL TBS-T and visualized using Odyssey® Infrared Imaging System (LI-COR). Image analysis and quantification was done using ImageJ 1.45s software according to protocol supplied by NIH.

RESULTS

k562-mb15-4-1BBL Plasma Membranes Expansion

To examine the possibility of expanding natural killer cells using plasma membranes isolated from mb15 feeder cells PBMCs were cultured alone or in the presence of mb15 feeder cells, increasing concentrations of non-purified mb15 plasma membranes (known as crude membranes or CM), or purified mb15 plasma membranes (PM) for 14 days. After fourteen days of culture natural killer cell expansion was the greatest in the presence of the mb15 feeder cells with a fold expansion of 168.4 ± 19.6 (Figure 1), and there was a positive relation between the NK cell expansion and the concentration of CM used (Figure 2). In addition to the dose-dependent relationship of the CM concentrations and the NK fold expansion, the purified plasma membranes were able to stimulate more expansion at lower concentrations than their non-purified counterparts.
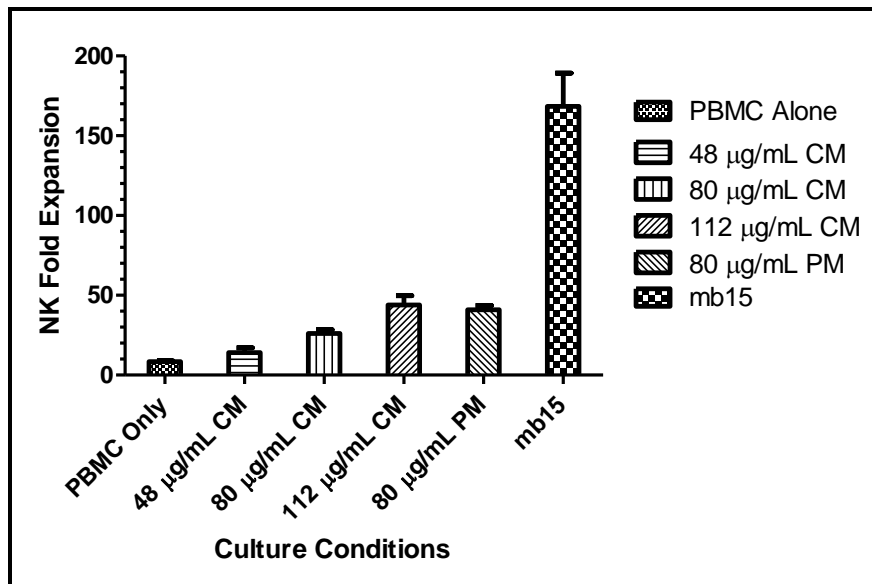
Figure 1 | mb15 14 Day Expansion Comparison. Culture conditions ran in duplicate.
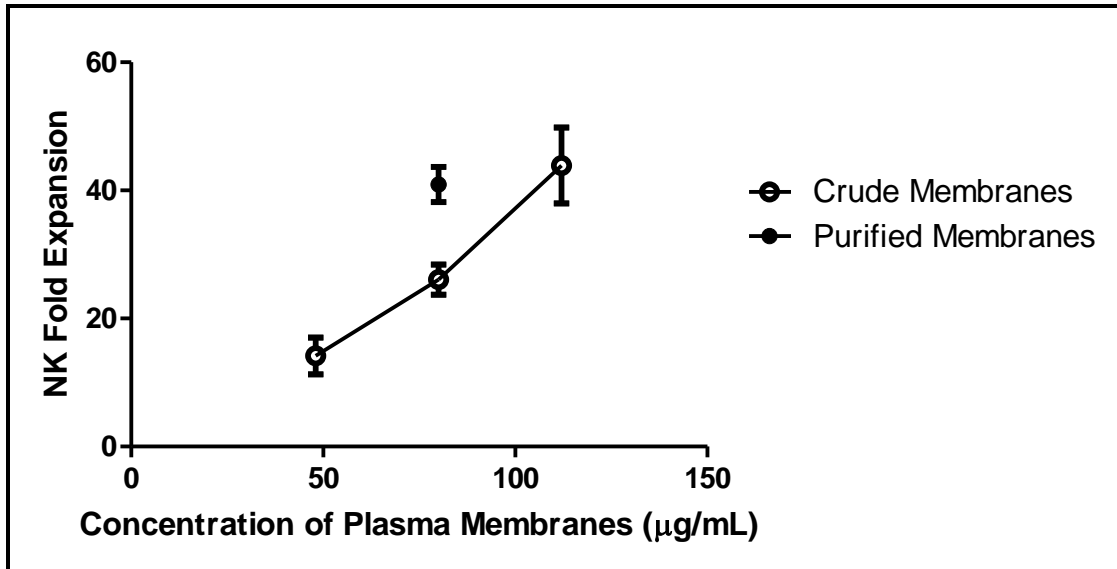
Figure 2 | mb15 Membrane Concentration vs. Expansion. Culture conditions ran in duplicate.

Over 21 days, however, the percentage of lymphocytes that were $CD56^+CD3^-$ continued to increase in the plasma membrane stimulated cultures to the extent of the mb15 feeder cell stimulated culture (Figure 3). The latter culture reached a NK proportion of 87.60% by Day 11, whereas 3 of the 4 membrane stimulated cultures did not obtain NK percentages above 80% until Day 21.
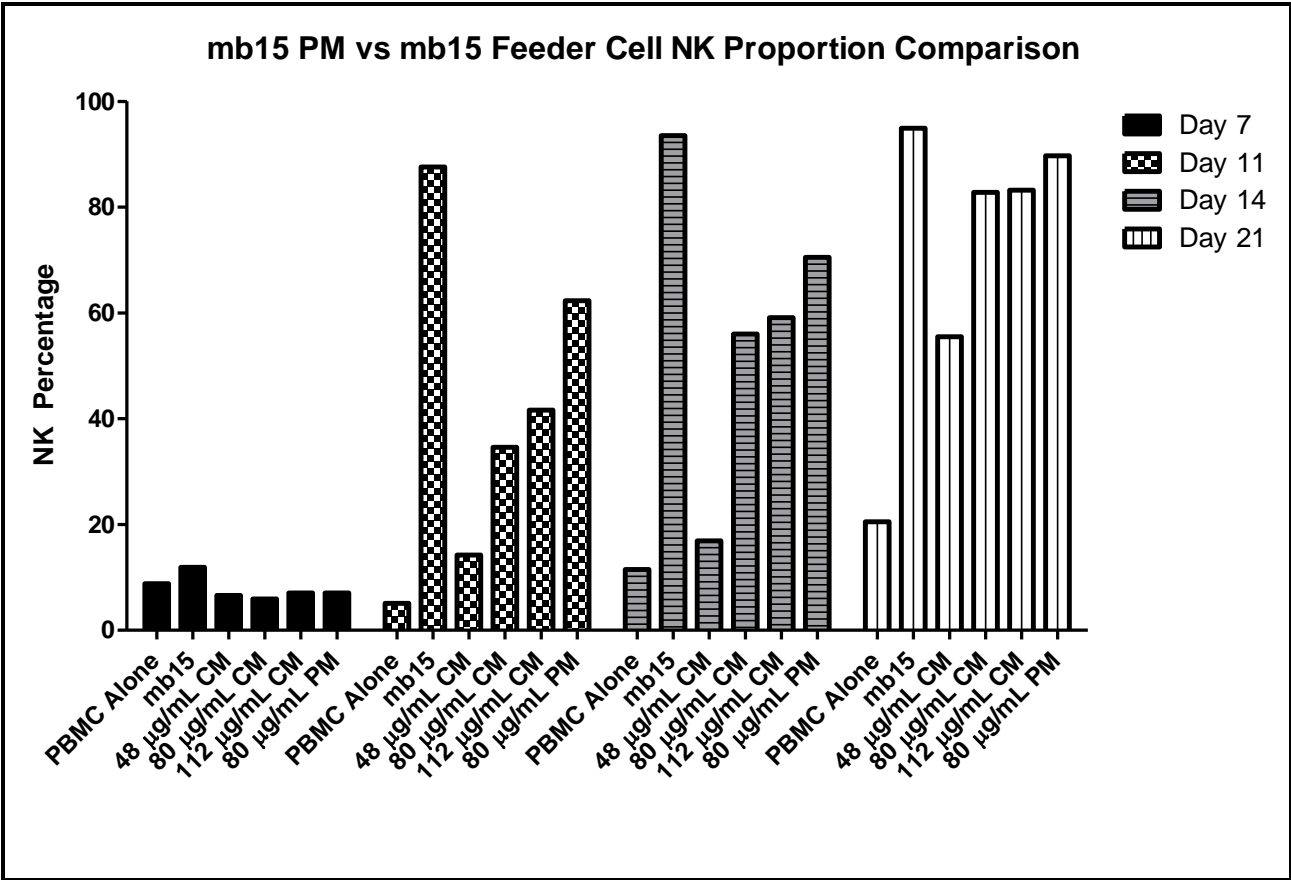
Figure 3 | mb15 PM vs. mb15 Feeder Cell NK Proportion Comparison. NK Percentage is indicative of the proportion of lymphocytes (gated on FSC vs. SSC) that were CD56$^+$CD3$^-$.

k562-mb21-4-1BBL Plasma Membranes Expansion

Alternatively, the second of the canonical feeder cells, mb21, was explored as a source of plasma membranes. PBMCs were cultured in various conditions including: without stimulation, with Mitomycin C treated mb21 feeder cells, or with various concentrations of purified plasma membranes (PM) from mb21 cells. After culturing for 14 days, the plasma membrane stimulated cultures were not able to reach the amount of NK expansion seen in the intact mb21 feeder cell

stimulated cultures (Figure 4). The 14 day NK fold expansion was 87.5 for the feeder cell culture, while the PM culture with the highest concentration only had an expansion of 24.5. The negative control of little to no growth, the PBMC only culture, was not able to sustain viable numbers after 14 days. As was seen in the mb15 membrane cultures the concentration of the plasma membranes had a dose-dependent relationship with the final fold expansion of natural killer cells (Figure 5). NK percentage of lymphocyte population was similar to that seen in the mb15 cultures (Data not shown).
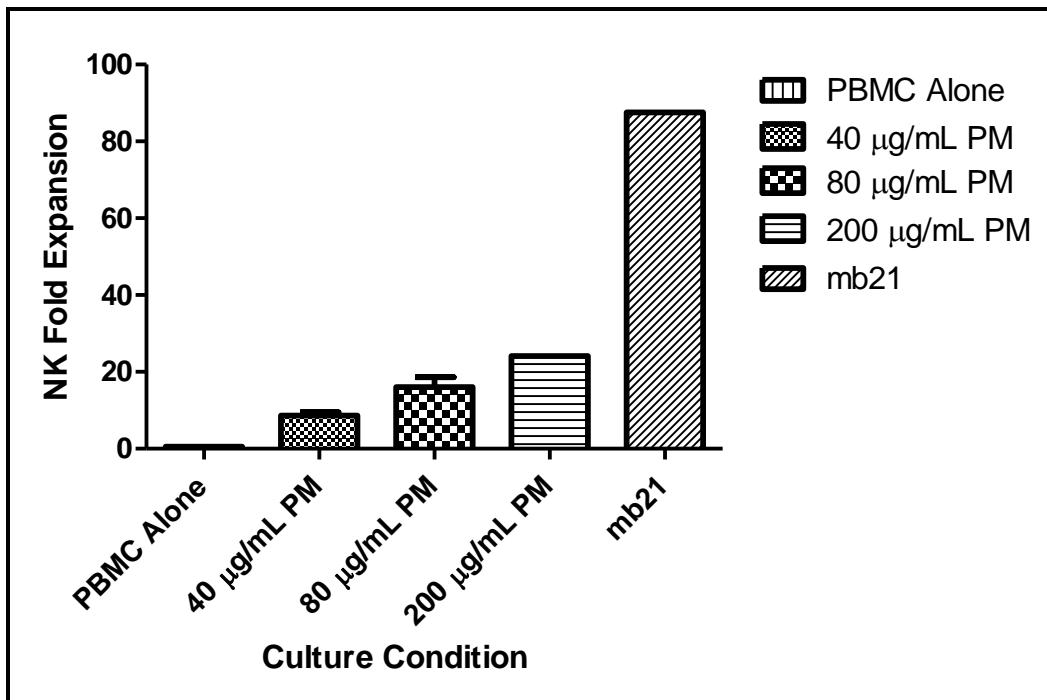
Figure 4 |mb21 14 Day Expansion Comparison.
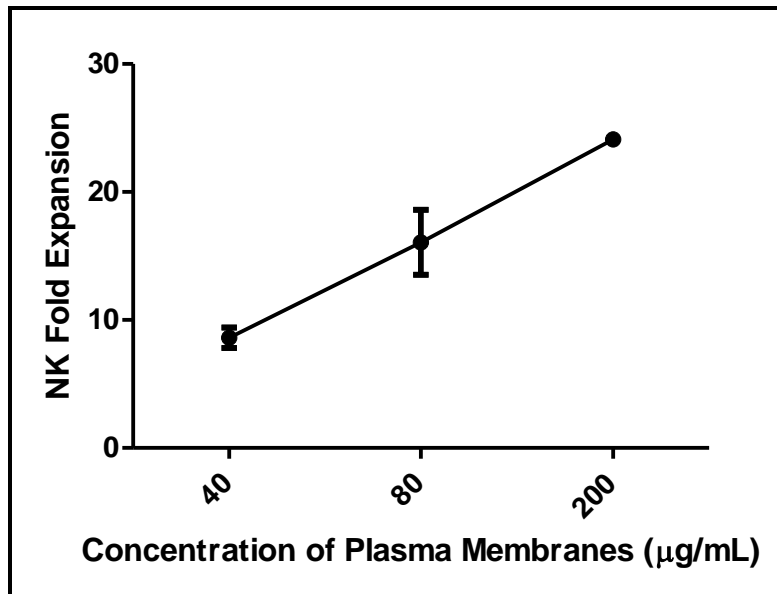
Figure 5 | mb21 Membrane Concentration vs. Expansion. Represented is the relationship between amount of mb21 PM and NK fold expansion.

4-1BBL Quantification Comparison

To compare the amount of 4-1BBL protein expressed by different stimulatory conditions, protein amounts proportionate to the conditions used previously in culture were quantified utilizing western blots (Figure 6). Purified plasma membranes expressed higher amounts of 4-1BBL than their whole cell counterparts (Figure 7).
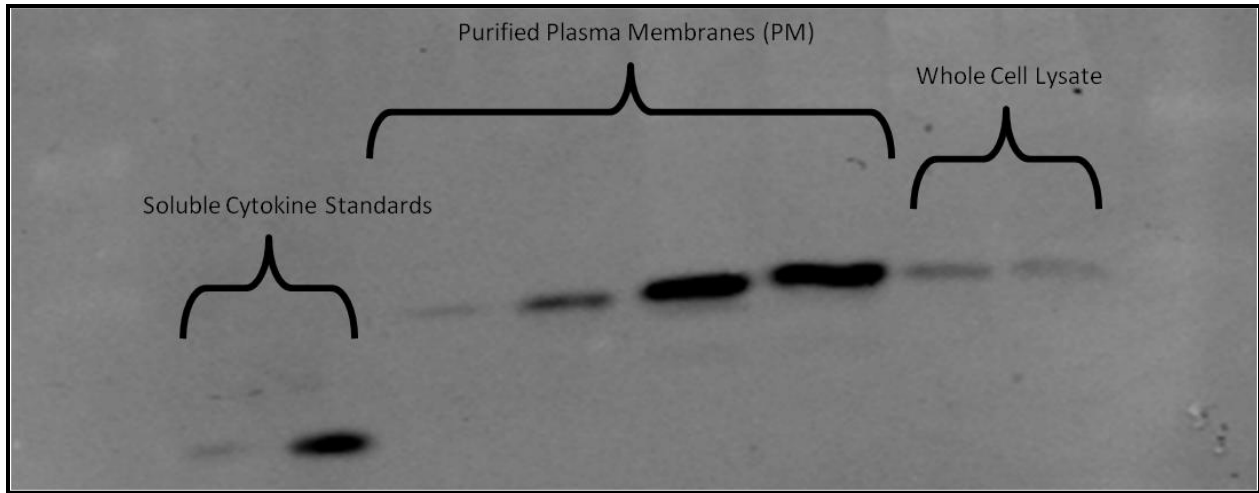
Figure 6 | Western Blot of 4-1BBL Protein Expression. Represented is the western blot image used to quantify 4-1BBL protein expression in various amounts of mb15 PM and whole mb15 cells. Cytokine standards represent 2.5 and 25 ng of IL-15, from left to right. Amounts of PM are indicative of four different culture condition concentrations; 50, 100, 200, and 400 ug/mL PM, from left to right. Whole cell lysate is representative of normal culture condition concentration of mb15 cells as well as a 1:1 dilution of this amount, from left to right.
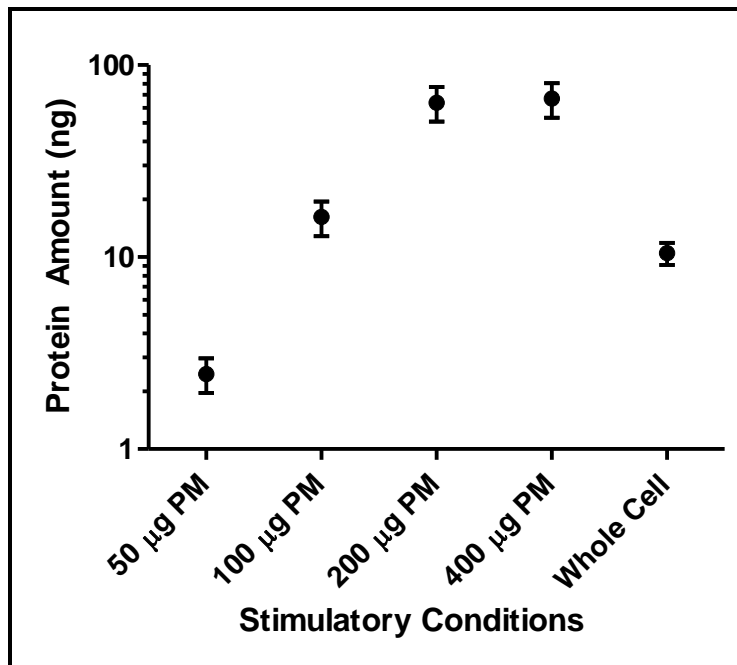
Figure 7 | Protein Quantification of 4-1BBL Protein in Various Stimulatory Conditions. Represented are the amounts of 4-1BBL protein, quantified via near-IR western blot visualization, contained within various amounts of mb15 PM and Whole mb15 cells. Y-axis is in log scale.

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Expansion of Natural Killer Cells with k562 and Soluble Cytokines

Utilizing k562 cell lines and soluble cytokines 4-1BBL and IL-21 (abbreviated S.C.), each at concentrations of 50 ng/mL, NK cells expanded more quickly than with canonical mb21 feeder cells (Figure 8). The number of NK cells in the presence of k562 and S.C. was at a maximum on Day 26 at 2,293, an approximately 47,767 fold expansion from Day 0. However, the mb21 feeder cells were able to maintain high periods of growth for longer periods of time and ultimately reached a maximum fold expansion of 282,385 at Day 35.
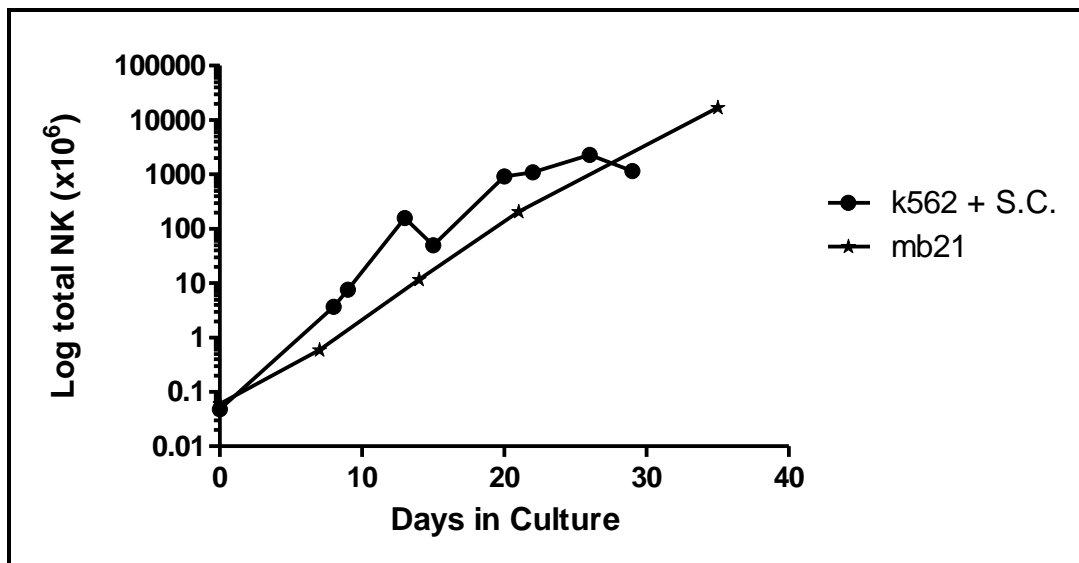
Figure 8 | NK Expansion Comparison. This graph represents the difference in NK expansion between cultures using k562 feeder cells and soluble cytokines 4-1BBL and IL-21 and mb21 feeder cells.

Figure 9 | Natural Killer Cytotoxicity at Effector: Target Ratio of 1:1. Cytotoxicity results are based on conditions ran in duplicate.
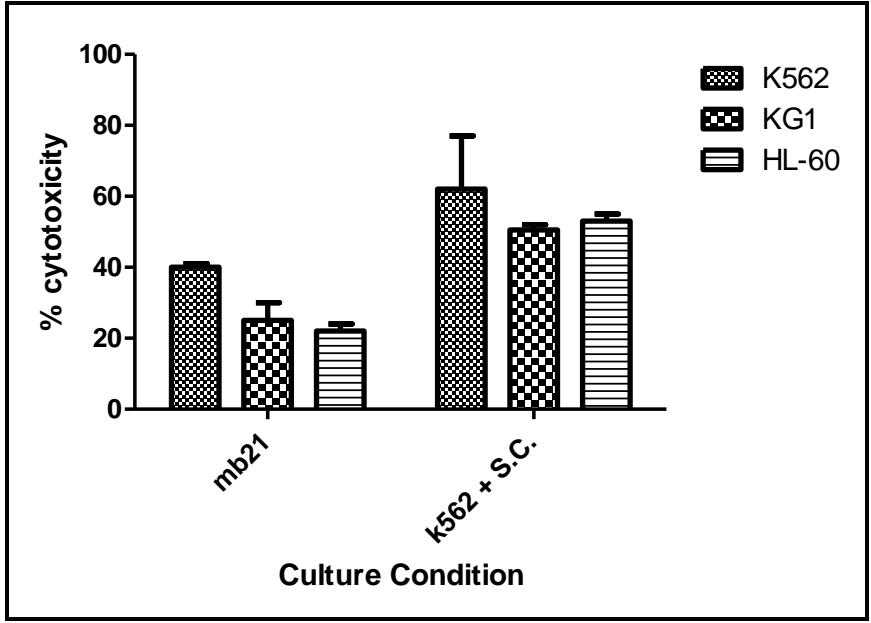
Figure 10 | Natural Killer Cytotoxicity at Effector: Target Ratio of 1:3. Cytotoxicity results are based on conditions ran in duplicate.

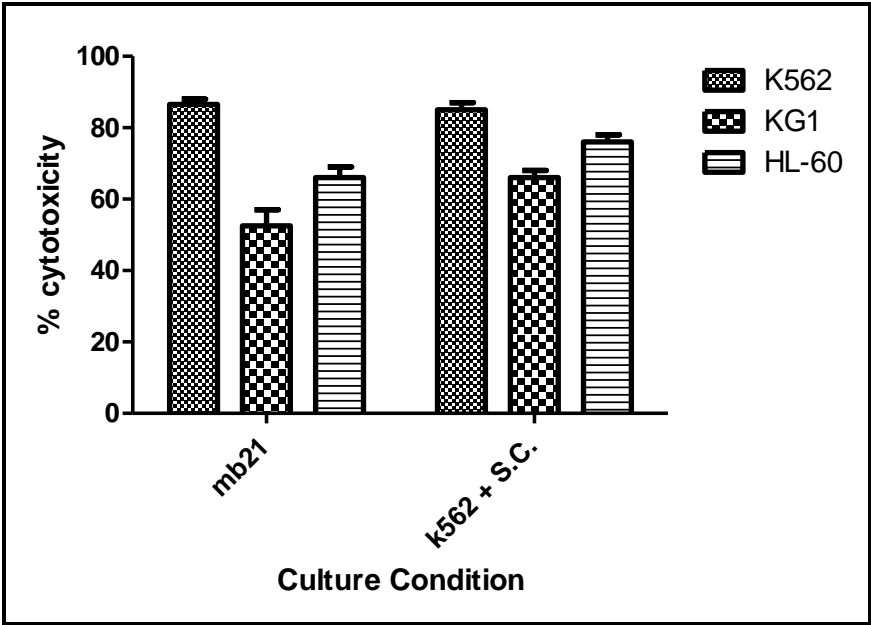In addition to the monitoring of their growth, the cytotoxicity of these NK cells against various tumor cell lines, including k562, KG1, and HL-60 was tested at two different effector-target ratios (E:T); 1:1 and 1:3 (Figure 9 and 10). At the former ratio, the NK cells expanded with either conditions showed relatively similar cytotoxicity towards k562 with percent cytotoxicity of 86.5 ± 1.5 and 85 ± 2 for mb21 and k562 + S.C. respectively. However, NK cells expanded with k562 + S.C. showed higher cytotoxicity against KG1 and HL-60. At a 1:3 ratio, k562 + S.C. expanded NK cells showed higher cytotoxicity across all tumor cell line targets.

Glucose Uptake of Expanding Natural Killer Cells

Using 2-NBDG, a fluorescent glucose analog, the glucose uptake of NK cells could be determined during their proliferation, or lack thereof, in the presence of different stimulatory conditions. Glucose uptake was monitored for both isolated NKs and NKs expanded from PBMCs as well as from frozen or fresh samples. Re-supplementation (Re-sup) refers to the addition of new feeder cells once previous feeders have been cleared, or are non-existent in culture. For the NKs expanded from frozen PBMCs in Figure 11, lymphocytes were in culture with k562 feeder cells and soluble cytokines IL-21 and 4-1BBL for 8 days, then once feeders were cleared, the culture was split into three conditions: not re-supplemented, re-supplemented with k562 feeder cells and soluble cytokines, and re-supplemented with mb21 feeder cells. Daily glucose uptake initially increased, then peaked on Day 4 and then dropped upon feeder cell clearance. After Day 8, glucose uptake increased in both re-supplemented cultures for several days, then dropped to the basal level after feeder clearance. In the not re-supplemented culture,

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glucose uptake did not increase but rather slowly declined over 6 days. NK fold expansion seen

in Figure 12 shows the disparity in proliferation between the re-supplemented and not re-

supplemented cultures.
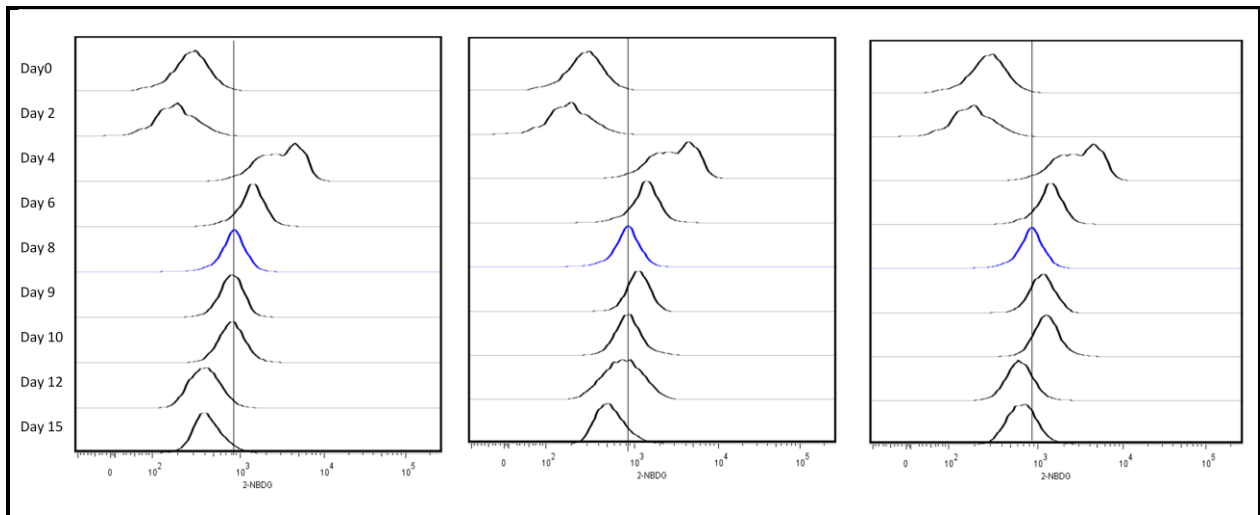
Figure 11| Glucose Uptake in Expanded NKs from Frozen PBMC Source. 2-NBDG uptake in first 8 days was indicative of a single parent culture. The blue peaks represent 2-NBDG reading immediately before splitting of cultures into three conditions: left – no re-sup, middle – re-sup with k562 + S.C., right – re-sup with mb21. Feeder cells cleared on Day 15 of both re-sup cultures.
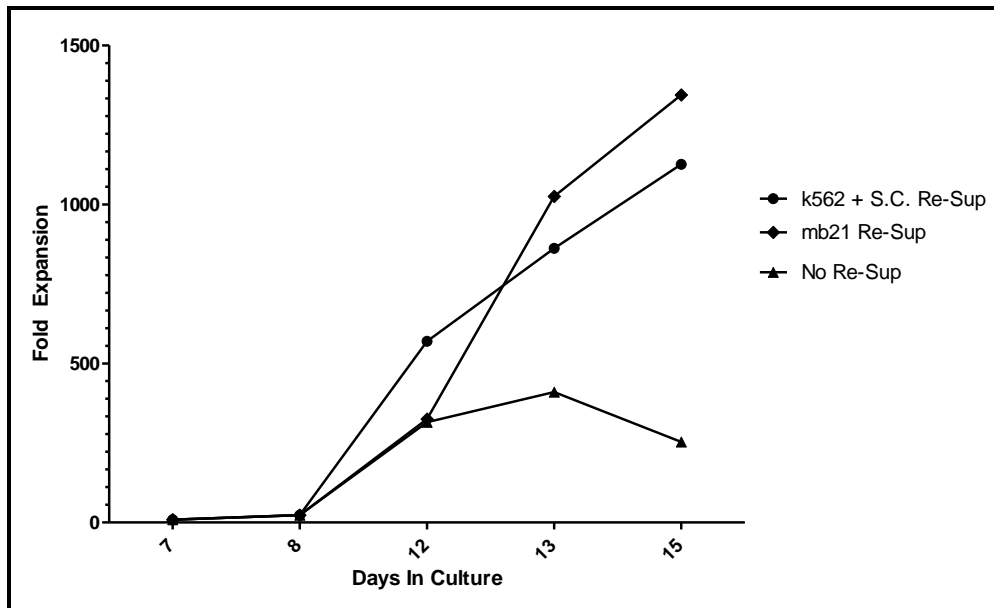
Figure 12 | NK Fold Expansion from Frozen Source. As in Figure 11, expansion numbers through Day 8 are indicative of a single parent culture.

Natural killer cells were isolated from fresh PBMCs via negative selection using complexes of dextran coated magnetic particles and antibodies against CD3, CD4, CD14, CD19, CD20, CD36, CD66b, CD123, HLA-DR, and glycophorin A. These complexes were then removed from NKs via magnetic binding. Isolated NK cells were cultured with mb21 or k562 feeder cells, then split into a re-supplementation or non re-supplementation condition after 10 days in culture. Their daily glucose uptake was measured as it was with the PBMC expanded NK cells. As can be seen in Figures 13 and 14, the NK cells increased glucose uptake immediately after re-supplementation for both feeder conditions, while glucose uptake decreased in the non-resupplemented cultures. Figure 15 demonstrates the difference in NK proliferation between the re-supplemented and non-resupplemented cultures.
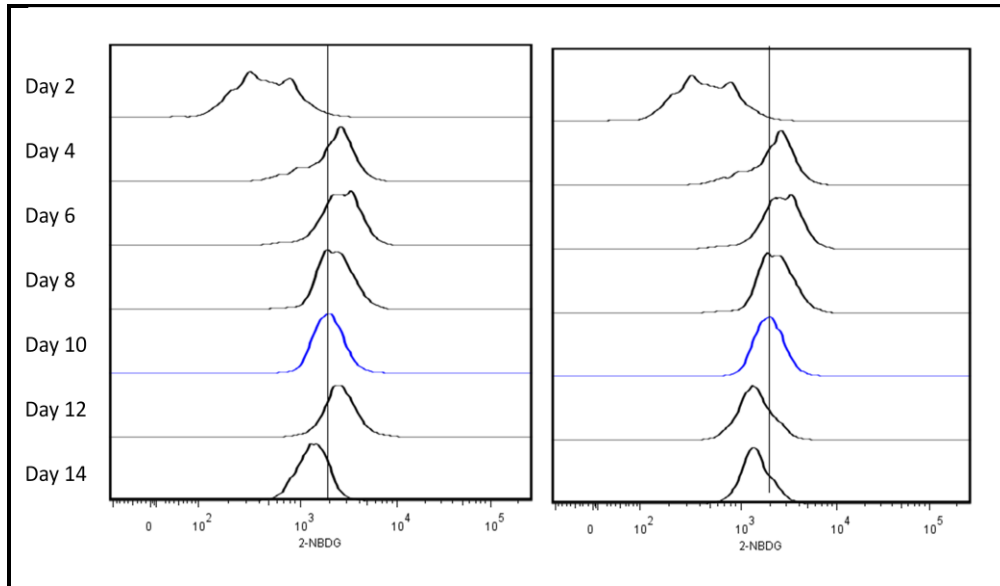
Figure 13 | Daily Glucose Uptake in Fresh Isolated NK Cells Expanded With k562 + Soluble Cytokines. The 2-NBDG readings from the first 10 days were indicative of a single parent culture. The blue peaks represent the 2-NBDG reading taken immediately before splitting of the cultures into re-supplemented condition (left) and non-resupplemented condition (right). Feeder cells of re-supplemented culture were cleared on Day 14.
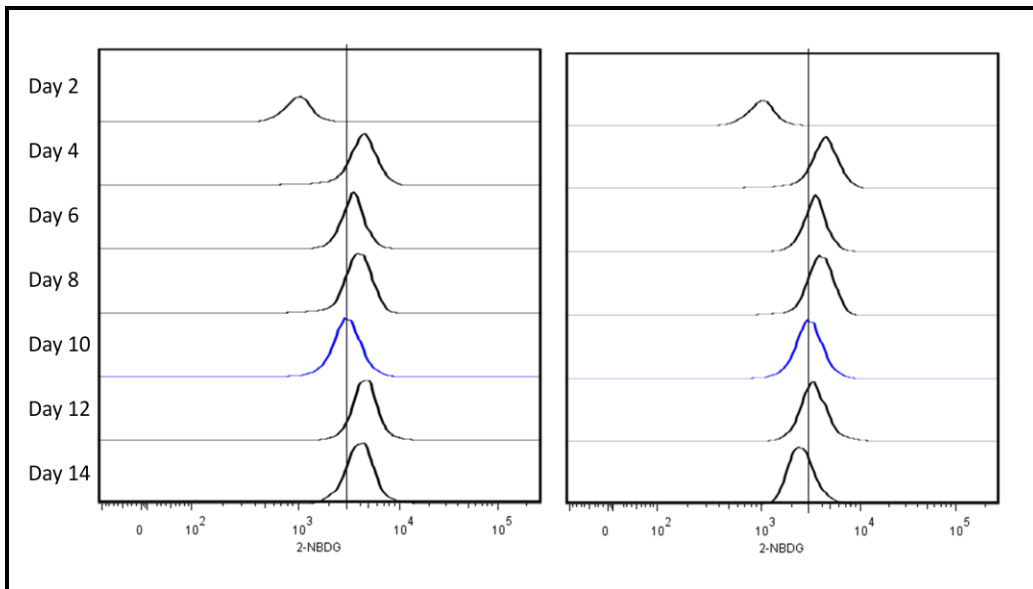
Figure 14 | Daily Glucose Uptake in Fresh Isolated NK Cells Expanded With mb21 Feeder Cells. The 2-NBDG readings from the first 10 days were indicative of a single parent culture. The blue peaks represent the 2-NBDG reading taken immediately before splitting of the cultures into re-supplemented condition (left) and non-resupplemented condition (right). Feeder cells of re-supplemented culture were cleared on Day 14.

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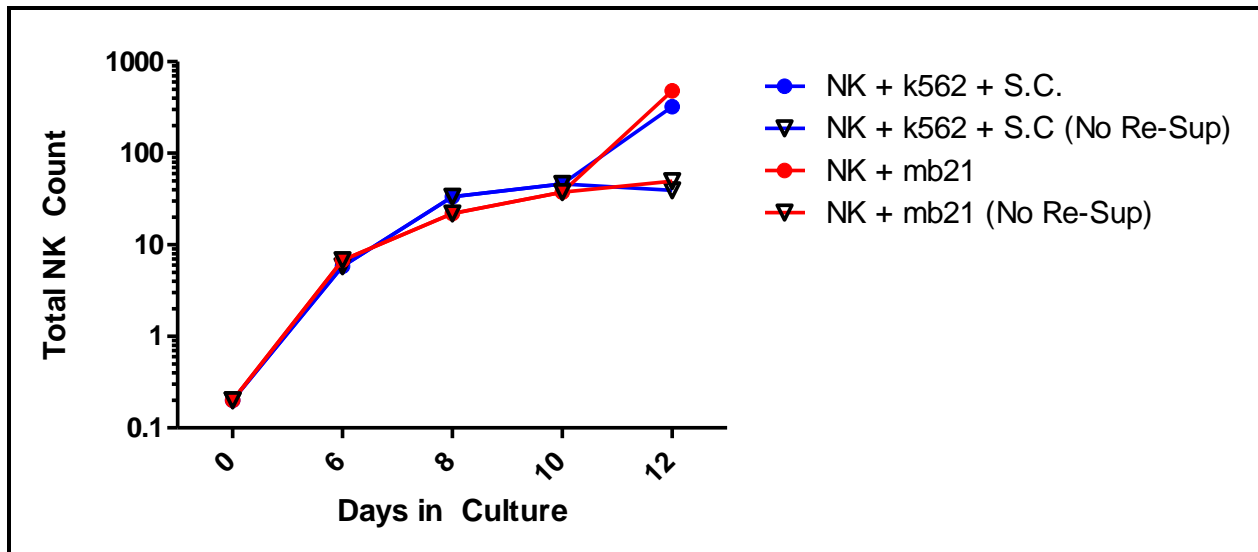
Figure 15 | NK Cell Proliferation Dependant on Re-Supplementation. This graph represents the alteration in the amount of total NK cells (shown in log scale on Y-axis) due to re-supplementation or lack thereof.

Glycolysis Inhibition of Expanding Natural Killer Cells

The effect of 2-DG on hexokinase, an integral enzyme in the beginning stages of glycolysis, on NK expansion was monitored in PBMCs exposed to several culture conditions: soluble cytokines 4-1BBL and IL-21 alone, mb21 feeder cells, and k562 feeder cells with soluble cytokines 4-1BBL and IL-21. Cultures were given daily doses of 2-DG for a week and their proliferation was measured (Figure 16 and 17). Natural Killer cell expansion was inhibited greatest for the highest proliferative condition, k562 and soluble cytokines 4-1BBL and IL-21. NK cells, in absence of 2-DG, were expanded less with the mb21 feeder cells; however these cultures proliferation was also inhibited less. The percent inhibition of maximum expansion for the mb21 feeder cell stimulated cultures was 76%±11, while the same statistic for k562 and soluble cytokine stimulated cultures was 95%±2 (Figure 18).

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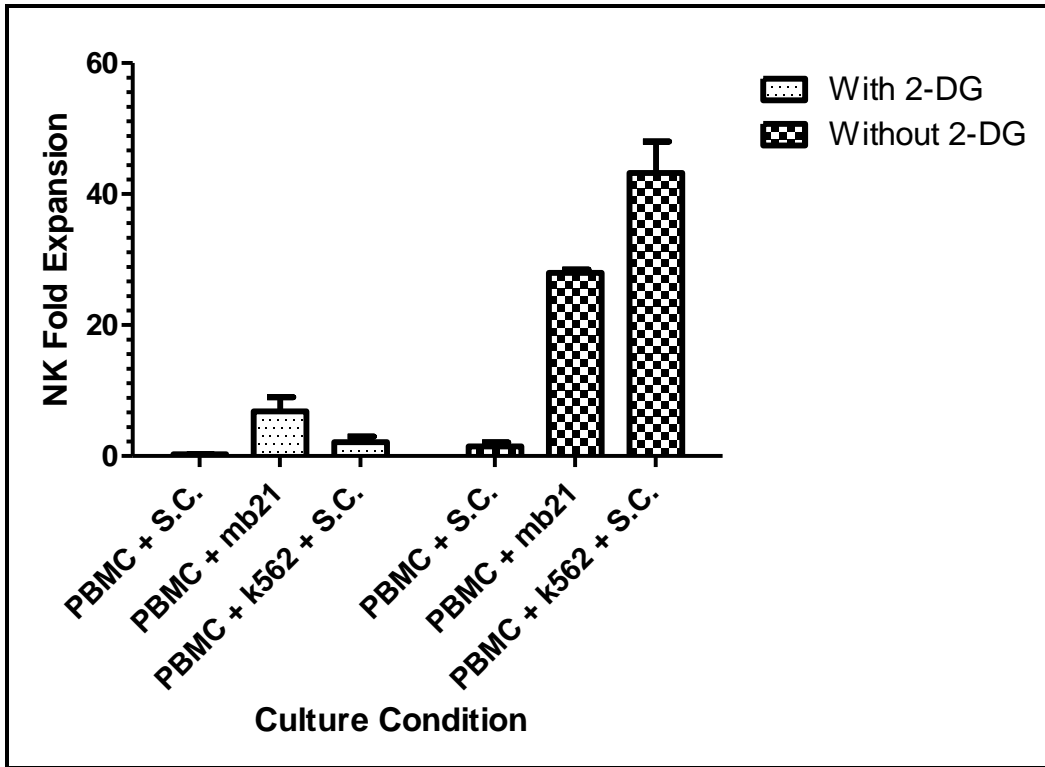
Figure 16 | Glycolysis Inhibition of NK Fold Expansion. This figure relates the difference in NK fold expansion between cultures in the presence (left group) or absence (right group) of glycolysis inhibitor, 2-DG. Results are indicative of cultures done in duplicate.
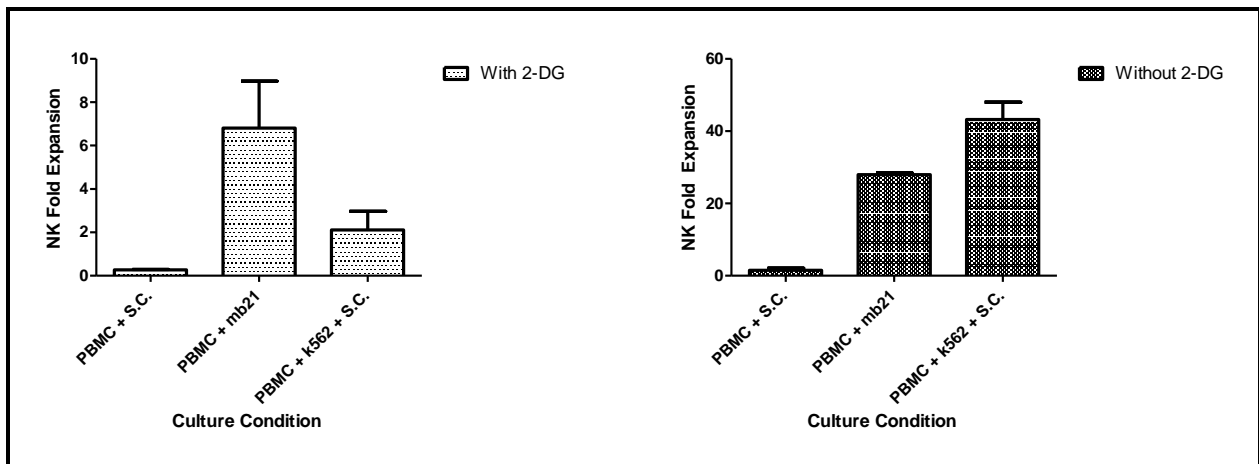
Figure 17 | NK Fold Expansion in Presence or Absence of 2-DG. This figure more accurately compares the different culture conditions and how the presence (left graph) or absence (right graph) of 2-DG affects the NK expansion of said conditions. Results are indicative of cultures done in duplicate.

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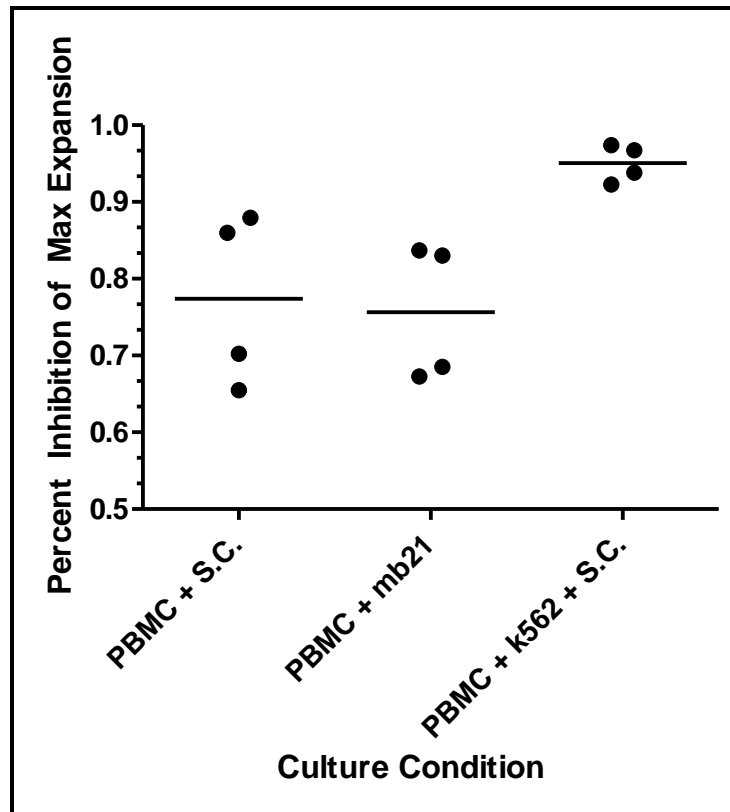
Figure 18 | Percent Inhibition of NK Expansion by 2-DG. This figure represents the proportion of maximum expansion inhibited in cultures in presence of 2-DG. Maximum expansion numbers were based on culture conditions in the absence of 2-DG.

Short Term Glucose Uptake and Associated Cytotoxicity

Expanded natural killer cells were incubated for 24 hours with several different tumor cell lines; the cytotoxicity patterns matched previous data in that k562 based cell lines were extremely susceptible to NK killing and showed high initial cytotoxicity that increased over time (Figure 19). The target cell lines HL-60 and KG1 showed a cytotoxicity plateau around 22.5% after 24 hours incubation with NK cells.
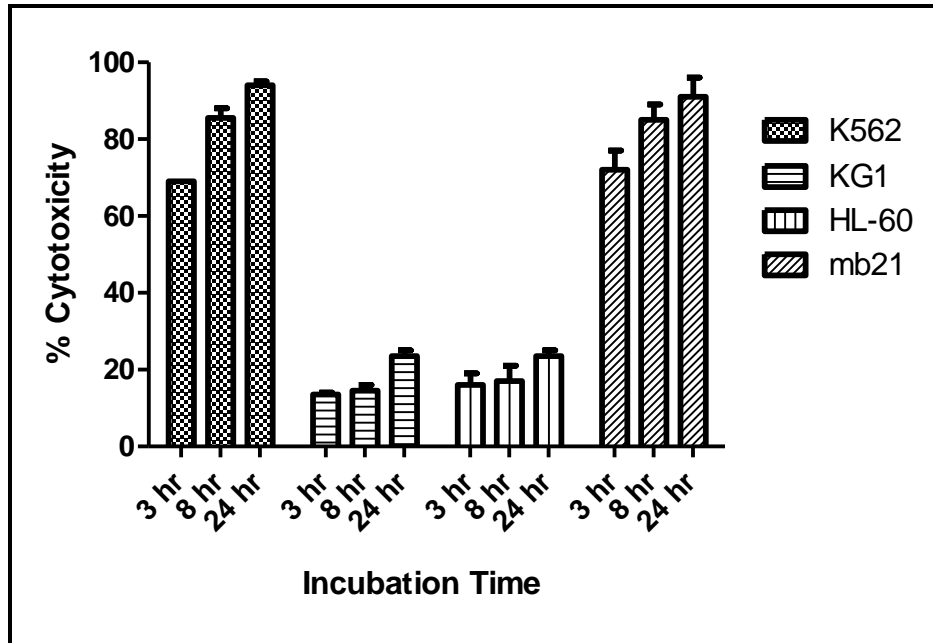
Figure 19 | Cytotoxicity of Natural Killer Cells Over 24 Hours. Cytotoxicity results are based on conditions ran in duplicate.

During this 24 hour time period the glucose uptake, based on 2-NBDG uptake assays, showed different trends for the different targets (Figure 20). For all target cells except KG1, the glucose uptake originally increased after three hours of exposure. The glucose uptake of NK cells exposed to k562, KG1, and HL-60 then seemed to diminish to a minimum at 8 hours, and then begin to show a rise at 24 hours. The natural killer cells exposed to mb21 cells however, diminished slowly over time to reach their minimum glucose uptake at 24 hours of incubation.
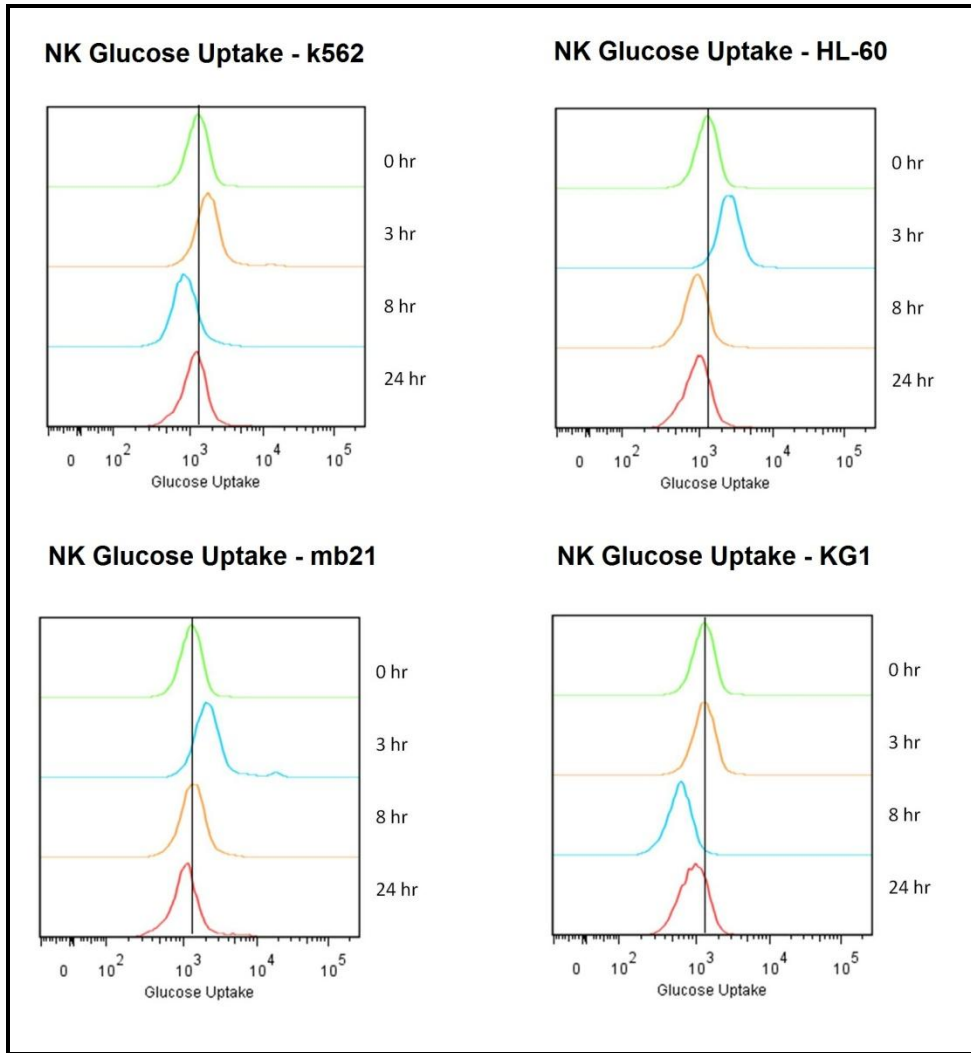
Figure 20 | Glucose Uptake of NK Cells Over 24 Hours of Exposure to Tumor Cell Lines. 0 hr 2-NBDG reading is indicative of single parent NK population.

DISCUSSION

Plasma Membrane Potentiality in Natural Killer Cell Expansion

The trends seen in the experiments with NK cell cultures stimulated with comparative plasma membrane and whole feeder cell additions seemed to suggest several important things. The NK cells in the presence of the CM or PM did not expand as rapidly or as greatly compared to the NK cells in the presence of intact feeder cells. However, the plasma membrane additions did expand NK cells to a considerable amount compared to stimulation with just IL-2 alone. Coinciding with the increase in NK cell amount, the percentage of NK cells in the lymphocyte population increased to the levels of the intact feeder cell cultures within 21 days. This ability to overcome T-cell dominance in the absence of direct anti-thymocyte agents illustrates the value of the plasma membranes in NK expansion. The concentration and purity of plasma membranes were determinate of the NK cell proliferation and their inducing ability can be improved by alteration of the product or increasing their concentration. In a direct test of PM and CM from the same source, the purified plasma membranes were able to expand NK cells to the level of crude membranes at double the concentration. The superior expansion inducing ability of PM was likely due to the fact that the concentrations of these membranes were judged using total protein quantification. Therefore PM and CM at the same concentration, have different proportions of plasma membrane proteins, which are the basis of the NK expansion. The CM contain impurities such as proteins found in the nuclear membrane that add to the total protein concentration, but do not contribute to NK cell expansion.

The inability of PM or CM to induce NK cell expansion to the level of the intact feeder

cells revealed a possible concern for the use of fragmented plasma membranes. Perhaps the

proteins used to stimulate NK cell growth diffused laterally out of the membranes or are being

degraded by the cell culture environment. In addition the relationship between membrane

concentration and expansion suggested the amount of protein on these membranes was an

expansion limiting factor. To judge if the proteins were still present in the plasma membranes

and to compare protein expression between the whole feeder cells and the plasma membranes we

utilized western blot analysis of the stimulatory protein 4-1BBL. The amount of PM or lysed

feeder cells on the blot were proportionate to amounts used in culture and were thus able to be

compared with each other. The stark contrast in 4-1BBL presence on the western blot showed

that although whole feeder cells were able to expand NK cells dramatically more than PM their

4-1BBL protein expression was limited. Assuming that the 4-1BBL expression was not an

anomaly, the presence of the protein proved the plasma membranes were not experiencing

dramatic protein loss. In regards to a comparison between feeder cells and plasma membranes, a

general conclusion could be reached that plasma membranes contain protein levels on par with

and exceeding that of the whole feeder cells.

While the presence of stimulatory proteins in the isolated plasma membranes is

indisputable, there is a glaring caveat to the application of the included western blot analysis.

Plasma membranes have the ability to fold in different orientations due to juxtaposed polar

environments. While the intent of isolating these plasma membranes is to allow for a reformation

of the cell exterior, it is possible that the plasma membranes could introvert creating an inside

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out orientation of the cell membrane. This could decrease the outward protein expression and limit the ability of stimulatory ligands to engage NK cells in receptor-ligand interaction. Therefore, while western blotting revealed higher protein levels in the isolated plasma membranes, it is possible these proteins are not externally accessible. To overcome this, it could be possible to use a solid support, such as nanoparticles, to fix these membranes with their stimulatory ligands facing outward. This could provide maximal stimulation while maintaining a cell line free method of expansion.

The protein ligands present on canonical feeder cells, referred throughout as mb15 and mb21, seem to make a large difference on the ability of NK cells to quickly and efficiently proliferate *ex vivo*, seeing as they provide a favorable receptor-ligand interaction. However, the fact that the plasma membranes with higher stimulatory ligand expression could not induce a similar amount of expansion leads to the notion that maximum stimulation goes beyond protein interaction. Multiple possibilities are apparent to explain such a missing link. One explanation is that the stimulation of NK proliferation requires a cytoskeletal reformation at the point of receptor-ligand binding to create an activation hub that works to positively regulate growth stimulation. While whole feeder cells have the ability to do so, the isolated plasma membranes would not have the intracellular components to cause such a reconfiguration. Another possible explanation is that there is some form of substrate transfer between the NK cell and the whole feeder cells and such a substrate could positively contribute to NK growth. Possible mechanisms could be engulfment-based or utilize passage through the lytic juncture. The substrate could be fundamental chemical building blocks or a specific non-essential stimulatory agent.

While some variables remain to be tested these results suggest that there may be an unknown exchange or interaction that is needed to achieve the highest level of NK cell expansion. Once these unknown variables become clear, not only can *ex vivo* expansion be optimized, but the use of plasma membranes *in vivo* could be a possibility for the expansion of highly cytotoxic NK cells capable of anti-leukemic effects.

Novel Natural Killer Expansion Method Has Potential for Rapid Growth

The acute myeloid leukemia cell line, k562, has been genetically altered to make potent stimulatory feeder cells. Most of the genetic changes are addition of soluble ligands in membrane bound form. The feeder cell line mb21 was engineered to express IL-21 and 4-1BB ligand as well as a variety of other receptors (CD64, truncated CD19, and CD86) on their membrane, to simulate NK stimulation by dendritic cells and other antigen presenting cells. While the membrane bound ligands have proven to be successful in NK expansion our group has evidence that soluble cytokines IL-21 and 4-1BBL alongside the unaltered k562 cell line in a non proliferative state, is also extremely effective. Natural killer cells expanded with our method tend to increase more rapidly in the first two weeks, however as described before in comparison to mb15 fed NKs [19], mb21 fed NK cells have the ability to maintain steady growth for longer periods of time. NK cells supplemented with only soluble cytokines IL-21 and 4-1BBL do not support extended growth (data not shown), leading to the belief that there is a significant interaction between "prey" feeder cells and the expanding NK cells, that goes beyond simple receptor-ligand binding. Such an interaction could involve transfer of necessary metabolites or unknown external stimuli, but remains unclear.

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What can be said is how different stimuli garner different phenotypes of NK cells, as measured by cytotoxicity and expansion patterns. Natural killer cells expanded with our novel method grew rapidly and were highly cytotoxic two weeks after culture, but reached a plateau in growth after three weeks. NK cells stimulated with mb21 feeder cells tend to expand at a comparable rate for longer periods of time, but do not seem to be as cytotoxic. In addition, the early growth of NKs with this method is less extensive. These gradual growth and effector function patterns could reflect specific signaling causing differentiation to a proliferative state rather than an effector state. Determining what causes the different trends seen in these two expansion methods could lead to the ability of selecting particular subsets of NK cells for exact functions in patients with hematologic malignancies or other diseases.

Our novel method of using soluble cytokines and canonical AML tumor cell lines to stimulate NK expansion is significant in several aspects. This method has shown to be more immediately effective than utilizing mb21 feeder cells and could be used to expand NK cells for lymphocyte infusions needed in a short period of time. In addition, the fact that soluble cytokines can be used with non-genetically engineered k562 cells reveals a method for rapid assessment of growth inducing ability of cytokine combinations. Instead of contributing large amounts of time to genetically altering tumor cell lines and testing their NK cell expansion efficiency, it is possible to test cytokines in a soluble form. Combinations of marked expansion inducing ability can then be pursued in the membrane bound or soluble form. Regardless of the application, the results from this study can make an impact in how NK cells are expanded and made available to patients receiving mismatched HSCT.

<u>Role of Glucose in Feeder Cell Mediated Natural Killer Cell Expansion and Cytotoxicity</u>

An important aspect of cell proliferation is metabolism and how cells obtain the energy they need to carry out the biochemical processes required for growth. By investigating the metabolism of NK cells *ex vivo*, the characteristics of NK cell energy production can be brought into the discussion of improving NK cell expansion. In this study, NK cells were expanded with our novel method or mb21 feeder cells until the feeder cells were not present in the culture. This parent culture was then either replenished with feeder cells or starved of feeder cells. By monitoring 2-NBDG uptake every several days, we were able to compare how the NK cells were uptaking glucose. NK cells increased their glucose uptake immediately after re-supplementation with feeder cells during periods of expansive growth. In contrast NK cells starved of feeder cells showed decreased glucose uptake over several days, while their growth also diminished. This was seen in both fresh and frozen natural killer cells, and from both isolated NKs and NKs expanded from PBMCs. These results suggested that glucose uptake, presence of feeders, and NK growth all were positively related. In the absence of feeder cells and growth, glucose uptake decreases. It is yet to be elucidated whether the change in glucose uptake is induced by the presence of the feeder cells, proliferation, or a combination of the two. One insight that would support the third scenario is the possibility that proteins are being transferred to the NK cells resulting in an increase in cellular nitrogen. If this is the case, the NK cells would increase carbon uptake to keep pace with the increased demand for fundamental chemical elements. In addition, the increased glucose uptake would provide energy needed for anabolic pathways.

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Therefore the feeder cells would induce glucose uptake in addition to the increased proliferation inducing glucose uptake.

One of the most important questions about this glucose uptake fluctuation is the end result of the glucose. To elucidate whether proliferating NK cells are primarily using oxidative phosphorylation in the mitochondria or fermenting glucose aerobically could help explain the nascent field of NK cell metabolism. The alternate cytotoxic lymphocytes, T cells, primarily ferment glucose in the presence of oxygen during growth, which is known as the Warburg effect. Due to the lack of NK metabolism knowledge, most of what is speculated about the field is assumed by observing cytotoxic T cells. Therefore, it would be of significance to demonstrate whether or not NK cells exhibit the Warburg effect. From the 2-NBDG uptake experiments, there remain several relevant questions, primarily whether the glucose uptake is a result of increased glycolysis, possibly due to the Warburg effect.

The first step in determining the extent of glycolysis during NK cell expansion was inhibiting glycolysis via 2-DG and monitoring NK cell growth. Expansion methods used were soluble cytokines IL-21 and 4-1BBL, mb21 feeder cells, and k562 feeder cells with IL-21 and 4-1BBL. The soluble cytokines alone were used to demonstrate low expansion of NK cells. NK fold expansion was limited most in the k562 and soluble cytokines stimulated culture, while the uninhibited version of this culture was the most proliferative. In comparison, the uninhibited mb21 fed NK cells expanded to a lesser extent, but their 2-DG inhibited culture was less inhibited by the presence of 2-DG. This could allude to the earlier notion that NK cells utilize different interactions from various stimulatory conditions to proliferate. One glaring fact is that

mb21 feeder cells have all excitatory ligands on their surface which could act as a hot spot for transfer of metabolites through a juncture. Perhaps, in the alternative expansion method NK cells are stimulated separately by soluble cytokines and k562 feeder cells, thus they do not have the opportunity to uptake metabolic substrates from the feeder cells as often. If mb21 fed NK cells are being "fed" an alternative source of metabolic power, their glucose uptake would not have to be upregulated for expansion. However, this conflicts with data in which 2-NBDG uptake increased during expansion. Another explanation for why the proliferation of the NK cells stimulated with k562 cells and soluble cytokines was inhibited to a greater extent than the mb21 feeder induced expansion could be the intensity of the initial proliferation. As evident in multiple experiments, the NK cells expanded with our novel method expand more rapidly and intensely than their mb21 fed counterparts. If glycolysis is indeed upregulated in expanding NKs, the inhibition of glycolysis could affect the greater expanding NKs more prominently. This notion conflicts with the fact that the soluble cytokine stimulated NK cells, whose expansion was minimal showed no difference in growth inhibition compared to the mb21 fed NK cells. The complexity of metabolism requires more studies to be done to determine why NK cells expanded using various methods react in different ways in relation to cytotoxicity, expansion, and ability to cope with glycolysis inhibitors.

During short term exposure to tumor cell lines, NK cells showed a variety of responses in terms of their uptake of 2-NBDG. All targets, except for KG1, seemed to elicit an increase in NK cell glucose uptake initially. All NK cells then decreased their 2-NBDG uptake over time. For all cell lines, cytotoxicity was maximal from 0-3 hours, during which time the NK cells experienced

an increase or stabilization of 2-NBDG uptake. Glucose uptake and cytotoxicity measurements after this time period seemed to suggest that most of the killing was done in the first several hours. Thus there seems to be a glucose uptake increase involved in cell killing, however more specific time points would be useful in more accurately describing the trends. As stated previously, more exact assessments of how the cell is generating ATP is needed.

Overall, metabolism of glucose and how cells provide themselves with energy is an important step in defining the expansion of NK cells for immunotherapy. In order to better understand how quantity and effector function of NK cells can be maximized, metabolism must be investigated. If the use of metabolic substrates could expand NK cells alongside cytokines, the presence of the feeder cell would not be necessary. This could provide robust *in vivo* expansion of natural killer cells. In juxtaposition, if feeder cells do provide a necessary and unmatched advantage to expansion, understanding how they influence the energy production of lymphocytes is crucial to maximizing potential.

Natural killer cells have the ability to transform dangerous mismatched stem cell transplantation into a successful treatment for patients with hematologic malignancies. For those without HLA matched siblings to provide the optimal donor source, NK cells have the ability to ensure a quick and efficient transplant. Perfecting the art of expanding NK cells both *in vivo* and *ex vivo* is an essential step in the utilization of NK cells in immunotherapy. In this study we have introduced a novel method for expanding NK cells *ex vivo* which out performs the most widely used method. In addition to its immediate clinical significance, the fundamental concept behind the methodology introduces a relationship between NK cell and feeder cell that is important to

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investigate. We have also shown a conceptual introduction to a novel *in vivo* method of expanding NK cells with the isolated plasma membranes of feeder cells. While the expansion *in vitro* was not as robust as feeder cell methods, the application of plasma membranes *in vivo* is a valuable clinical tool. With improvement it might be possible to increase these membranes effectiveness while maintaining their safety in patients. Beyond the methodologies developed novel NK cell characteristics, particularly their glucose uptake during proliferation, were elucidated. Due to the nascence of the NK field, any information about metabolic processes holds a certain value. Beyond that our findings show that metabolism needs to be taken into account when evaluating NK cells proliferation. Overall, if NK expansion can be improved patients with low quality transplants can reduce their susceptibility to GVHD, increase a GVT effect, and have a successful engraftment. All of these factors could lead to better survival and quality of life for patients suffering from hematologic malignancies.

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