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Expansion of NK cells for the treatment of multiple myeloma

Final Master's Thesis

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Abbreviations

ADCC – Antibody-dependent cell cytotoxicity

aNK – activated Natural Killer Cells

BM – Bone Marrow

DAMPs – Damage Associated Molecular Patterns

DCs – Dendritic Cells

eNK – Expanded NK Cells

FBS – Fetal Bovine Serum

IL – Interleukins

IS – Immunological Synapse

MM – Multiple myeloma

NK – Natural Killer

PAMPs – Pathogen Associated Molecular Patterns

PBMCs – Peripheral Blood Mononuclear Cells

PBS – Phosphate Buffered Saline

PRR – Pattern Recognition Receptors

RT – Room Temperature

Abstract

Chemotherapy and radiation therapy approaches to cancer treatment have a limited ability to discriminate between healthy and malignant cells. To increase specificity and decrease damage to healthy tissue, cancer immunotherapy is leading a paradigm shift in research and treatment development. Natural killer cells are part of our immune defense against transformed cells and are a promising option for use against cancer cells. For a viable treatment, human allogenic NK cells are expanded and activated before use on target cells. Previously developed protocols have shown the cytotoxic effect of these NK cells against a variety of hematological cancers.

The purpose of this study was to test the efficacy of expanded natural killer cells (eNKs) as a treatment against multiple myeloma (MM) cell lines and MM patient bone marrow aspirates. The cytotoxic effect of eNKs was studied alone and in combination with daratumumab, anti-CD38 monoclonal antibody approved for use in multiple myeloma patients. In multiple myeloma cell lines, eNKs were found to have a dramatic cytotoxic effect against the cells. For the patient samples, when eNKs were harvested during the optimum expansion period, they produced a cytotoxic effect against the MM cells. Daratumumab was also effective against some of the patient samples. However, when combined, eNKs and daratumumab produced a greater percent of specific cell death than both separately, on all cells tested. Combination therapy maximizes the effect of each individual treatment. This important finding supports the possible use of combination eNKs with daratumumab therapy against multiple myeloma in further trials.

1   Introduction

1.1   The Immune System

The immune system consists of a wide range of tissues, cells, and molecules designed to protect the body from harm, both of foreign and domestic origin. Composed of two branches, the innate and the adaptive, they produce an initially fast, generic response that is then attenuated and tailored to the specific pathogen. Together they work cooperatively in eradicating viruses, bacteria, and other errant cells.

1.1.1   The Innate Immune System

As the first line of defense, the innate immune system begins by using physical barriers, the epidermis being the largest, to keep out dangerous bacteria, viruses, and caustic molecules. Once breached, it mounts a rapid, generic response using cytokines, antigen presenting dendritic cells (DCs), phagocytes, complement, and natural killer (NK) cells. They are able to discriminate self and non-self, using pattern recognition receptors (PRR) such as Toll-like receptors (TLRs). The patterns recognized, pathogen associated molecular patterns (PAMPs) and damage associated molecular patters (DAMPs), are shared by specific group of microbes not found on host cells [1]. PRRs mediate proinflammatory signal transduction pathways that ultimately lead to the activation of transcription factors regulating genes involved in host defense and inflammation [2]. Cells of the innate immune system release inflammatory cytokines that provide activation signals for cells in both systems. Of the innate immune cells, this work focuses on human NK cells.

1.1.2   The Adaptive Immune System

Commencing in the later stages of infection, if the innate immune response has been insufficient, the adaptive immune response eliminates pathogens and generates cells with immunological memory. Characterized by a highly tailored response, it is initiated when an antigen is presented within a lymphoid organ. The system can be divided into a humoral response and a cell-mediated immune response. B cells are responsible for the humoral response as, once activated, they secrete antibodies, also known as immunoglobulins. The antibodies, each with a specialized function, can block and neutralize extracellular toxins and microbes. The cell-mediated response is primarily mediated by T cells further subdivided into T helper cells (CD4) and cytotoxic T cells (CD8). $CD4^+$ T cells and $CD8^+$ T cells are activated, in part, when their T cell receptors (TCRs) recognize and bind specific peptides

displayed by major histocompatibility complex (MHC) class II and MHC class I molecules, respectively. Once activated, CD4$^+$ T cells differentiate and are involved in cytokine production, homeostasis and tolerance maintenance, and control of intracellular pathogens. Cytotoxic T cells focus on elimination of infected cells. [1]

1.2　Natural Killer Cells

First described in 1975, Kiessling *et* al. characterized NK cells as small granular lymphoid cells that exhibited a cytotoxic function against Moloney leukemia cells. [3] They are able to induce cytotoxicity naturally without prior sensitization. [4] As they do not undergo germ-line Variable Diverse Joining receptor rearrangement as T and B cells, they are made part of the innate immune system. NK cells are large granular lymphocytes known for their ability to eliminate infected cells and comprise 5-10% of total lymphocytes found in blood.

1.2.1　CD56^{bright} and CD56^{dim} NK Cells

NK cells originate from hematopoietic stem cells and mature in the bone marrow [5]. Although, other differentiations sites have been reported [6] [7]. They express the NCAM-1 molecule, which clusters as CD56. Expression of CD56 and CD16 are how NK cells are regularly identified. Difference in CD56 expression intensity divides NK cells into two major subsets: CD56^{bright} and CD56^{dim} NK cells, both with unique functions and capabilities. CD56^{bright} produce proinflammatory cytokines and have low expression of killer immunoglobulin like receptors (KIR) [8]. These are reported to proliferate at a higher rate in vitro due to the presence of IL-2R ($\alpha\beta\gamma$), an IL-2 receptor complex, as IL-2 is used in NK cell cultures [9].

CD56^{dim} NK cells constitute the majority of NK cells in peripheral blood. As opposed to their counterparts, they express CD16 (FcγIII) in abundance thus mediate antibody-dependent cell-mediated cytotoxicity (ADCC). Additionally, CD56^{dim} NK cells kill target cells by either releasing perforin and granzyme by granule exocytosis or by inducing apoptosis following activation of the TNF-related apoptosis induced ligand (TRAIL) or Fas/FasL cell death pathways. Phenotypically, CD56^{dim} NK cells are also characterized by the high expression of KIR [10] [11].

1.2.2   Inactivating and Activating NK cell receptors.

KIRs and NKG2A are two major families of inhibitory NK cell receptors. KIRs bind to specific allelic variants of HLA -A, -B and -C encoding human major histocompatibility (MHC) class I molecules [12] [13]. When KIRs bind to their HLA ligand, an inhibitory signal is transmitted via immunoreceptor tyrosine-based inhibition motifs (ITIMs). This leads to decreased tyrosine phosphorylation of effectors in the activation pathways. This inhibits the release of lytic vesicles. The KIR-MHC class I interaction mediates NK cell tolerance to healthy autologous cells that express MHC class I, whereas cells that have downregulated MHC class I become vulnerable to NK cell lysis [14].

For a cytotoxic response to occur, NK cells need to be activated through the engagement of activating receptors they acquire during the early stages of maturation. NKG2D, DNAM-1 and NKp80 are examples of activating receptors that can directly mediate killing or act as co-receptors. NKG2D is a transmembrane receptor that is expressed as a homodimer and to several ligands including MHC class I related chain molecules A and B (MICA, MICB) [15]. These ligands are up-regulated on virus-infected cells, DNA damaged or transformed cells. Increase in tumor growth is seen in NKG2D deficient mice [16]. The points to the key role of activating receptors in in tumor surveillance and elimination [17].

Cytotoxic function of NK cells is carried out when activating receptors engage their ligands on target cells in the absence of inhibitory receptor/ligand interaction, the needed net activation signal is generated, thus lysing the target cell. Conversely when inhibitory receptors engage HLA in the absence of an activating receptor/ligand interaction, a net negative signal is generated, resulting in no target cell lysis (Figure 1). Activating and inactivating signals are also based on the net results of all activating ligand/receptor interaction versus all inhibiting ligand/receptor interactions of target cell. The net result is used when, for example, inactivating receptor/ligands are upregulated, amplifying the net inactivation signal to exceed the activating signal. In the absence of both inhibitory and activating signals, no NK cell activation occurs [18].
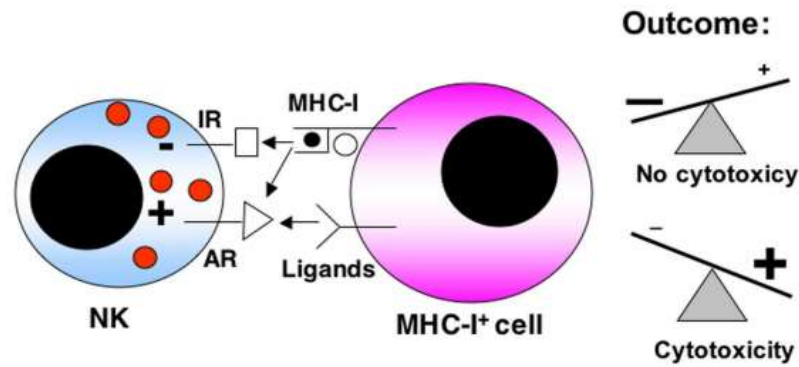
Figure 1. Balance between inhibitory and activating signals mediates NK cell cytotoxicity response. Inhibitory receptors (IR) on NK cells interact with MHC class I molecules expressed by healthy self-cells mediating an inhibitory signal leading to no cytotoxicity. NK cell mediated cytotoxicity is triggered when target cells (pink cells) that have downregulated expression of MHC class I and upregulated expression of activating ligands. Target cells that continue to express MHC class I molecules and also express activating ligands do trigger NK cell cytotoxicity because activating signal overrides the inhibitory signal. [18]

1.2.3 NK cell cytotoxicity function

NK cell cytotoxicity relies on the ability of transporting lytic granules to the immunological synapse (IS). During degranulation, the lysosome associated membrane protein (CD107a) is transported to the surface of NK cells facilitating the mobility of the lytic granules [19]. The Fc receptors expressed on the cell surface of NK cells bind to the Fc portion of immunoglobulins, transmitting activating signal within NK cells [20]. This is of what antibody dependent cell-mediated cytotoxicity (ADCC) consists and is used in in the treatment of cancer such as B-cell lymphoma and breast cancer as they overexpress unique antigens [21]. ADCC occurs through multiple pathways: proinflammatory cytokine release, tumor necrosis factor (TNF) family death receptor signaling, and as stated above, exocytosis of cytotoxic granules. Direct target cell apoptosis occurs only with cytotoxic granules through release perforin and granzyme and TNF signaling. CD56^{dim} NK cell subsets are responsible for ADCC but also produce IFN-ɣ after activation [22]. IFN-ɣ can also upregulate TRAIL expression on NK cells leading to another pathway of cytotoxicity [23].

1.2.4 Cytokine regulation of NK cell functions

Cytokines are soluble mediators released by innate or adaptive immune cells that play a key role in NK cell development, activation, expansion, trafficking and survival. NK cells express receptors for several cytokines that bestow different binding affinities and responsiveness.

IL-2, IL-15, and IL-21 are implicated in NK cell function. In addition, NK cells effectively respond to cytokines released by activated innate immune cells including IL-12 and IL-18. This serves to connect the innate immune response with the adaptive immune response. IL-2 was the first interleukin described in terms of T cell growth. Indeed, it is primarily released by activated T cells and supports T cell differentiation, growth and proliferation. In NK cells, IL-2 helps increase cytotoxic functions and in proliferation [24] [25]. IL-2 receptor (IL-2R) is constitutively expressed by all NK cells in varying states, either a dimeric receptor complex, or the trimeric receptor complex as in CD56^{bright} NK cells, described above. Low levels of IL-2 are adequate to deliver high affinity binding to CD56^{bright} NK cells and activate inflammatory cascades. CD56^{dim} NK cells have a dimeric receptor complex with lower binding affinity with IL-2. Thus, higher concentrations of IL-2 are needed for NK cells to respond [26]. Correct levels of IL-2 lead to proliferation, upregulation of activation receptors and enhanced target cell killing. Due to their potent effect, IL-2 was the first cytokine administered to enhance NK cell cytotoxicity against tumor cells in a clinical trial and continues to be used [27] [28].

Another interleukin that is essential for the function of NK cells is IL-15. It binds to IL-2Rβ and IL-2Rɣ subunits of the IL-2 receptor and thus shares many physiological properties with IL-2 [29] [30]. This is a lower affinity binding unlike its own receptor IL-15Rα [31]. Produced by many cells, such as DCs, macrophages and BM stromal cells, when bound to NK cells, it induces cell survival and proliferation [32]. The anti-apoptotic gene Bcl-2 is also upregulated [33]. Both interleukins can be used to maintain and proliferate NK cells.

1.3   Cancer Immunotherapy

Cancerous cells have evolved mechanisms to evade immune cells. They establish an immunoprivileged environment where immunosuppressive cytokines are produced. In order to combat this, new therapies have been developed to overcome an inadequate anti-tumor immune response. Tumor-specific monoclonal antibodies (mAbs) recognizing tumor-selective antigens on the surface of tumor cells are being used as cancer therapy. Some mAbs act by directing NK cells to kill target cells through ADCC. Tumor antigen targeting mAbs include rituximab (CD20), approved for use in non-Hodgkin's lymphoma, and cetuximab (EGFR), approved for metastatic colorectal cancer and head and neck cancer. Of interest in this project is daratumumab. It is a human IgG1kappa monoclonal antibody targeted against CD38. [34]. It exerts is antimyeloma mechanism through ADCC, complement

dependent cytotoxicity (CDC), antibody dependent phagocytosis (ADCP), upon secondary crosslinking direct induction of apoptosis, and enzymatic inhibition of CD38. CD38 is a 45-kD type II transmembrane glycoprotein that regulates cytoplasmic Ca^{2+} flux and mediates signal transduction in lymphoid and myeloid cells [35]. It is highly expressed on myeloma cells while having low expression on normal myeloid and lymphoid cells. CD38 was thus chosen as a target for multiply myeloma therapy as it can contribute to cell survival via adenosine production and calcium mobilization [36]. Daratumumab also has an immunomodulatory effect whereby CD38 expressing immunosuppressive cells (Tregs) are eliminated. However, NK cells also express CD38 in varying levels and exposure to daratumumab has been shown to cause NK cell death. Although effecting the NK cells by which it carries out ADCC, daratumumab is the first mAb approved for use in multiple myeloma and has had great clinical success. In the approval-granting phase II clinical trial SIRIUS, patients enrolled were heavily pretreated with relapsed or refractory myeloma. Overall response rate was 29.2% and mediation duration of response was 7.4 months. Of those who responded to the daratumumab, 25.8% had deepening response over time. Further trials have tested daratumumabs as a primary or secondary line of therapy with promising results. Daratumumab therapy is also being combines with proteasome inhibitors, bortezomib and carlfizomib, and immunomodulatory drugs such as lenalidomide and pomalidomide [37].

1.4   Multiple Myeloma

Multiple myeloma is a B-cell malignancy localized to the bone marrow characterized by the uncontrolled growth of plasma cells. The second most common hematologic malignancy in the United States, multiple myeloma accounts for approximately 18% of all hematologic malignancies and 2% of all newly diagnosed cancers. As MM is a highly heterogenous disease, pathogenic processes differ wildly among newly diagnosed patients and relapsed or refractory patients. An example of this is the deletion of 17p13 which is very common chromosomal abnormality [38]. While present in a small percentage of new cases, it is more frequently observed in refractory-relapsed patients.

MM exhibits many mechanisms of drug resistance such as the above genetic abnormality, deregulated signaling and drug transport pathways, and anti-apoptosis mechanisms that no singular drug has been able to overcome. Apart from drug resistance, MM cells also

increasingly escape immune surveillance as disease progresses. The progressive immune dysregulation impairs Band T cell functions, antigen presenting cells and NK cells. As stated previously, immunotherapeutic approaches to MM have been proposed included mAbs, chimeric antigen receptor T cell therapy (CAR-T cells), tumor vaccines, and immune checkpoint inhibitors [39].

1.4.1 NK Cell function in MM

NK cells not only play an important role in innate immunity, they are key in cancer surveillance. In multiple myeloma patients, the activation and cytotoxicity of NK cells is impaired. This immune escape is made possible by expression of activating and inhibiting NK cell ligands and immune checkpoint inhibitors. High levels of TGF-β is secreted by Tregs and plasma cells. This immunosuppressive cytokine has been shown to downregulating several NK-activating receptors and impair NK cell cytotoxicity [40]. NK cell affecting IL-6 is also produced. Other soluble factors inhibit activating signals transduces by NCR and NKG2D. These are only a few among several tumor microenvironmental factors that contribute to NK cell stagnation. Use of expanded allogenic NKs is a viable way to reboot NK cell mediated cytotoxicity in MM patients. [41]

2 Previous Work and Current Objectives

Julian Pardo's group optimized a protocol to activate human NK cells from healthy donors, employing a 5-day protocol using lymphoblastoid cells transformed by the Epstein Bar Virus (EBV+). In these studies, it was demonstrated that NK cells were able to eliminate mutant hematological and apoptotic-resistant cancer cell lines [42]. They continued on to use activated NK cells on patient-derived B-cell chronic lymphocytic leukemia cells demonstrating that eNK action was higher in patients with a worse prognosis [43]. Subsequently, Martin Villalba's group of the INSERM in Montepellier, France, proposed an expansion protocol of natural killer cells using umbilical cord cells instead of the previous peripheral blood mononuclear cells. Our protocol, below, was used in preparing NK cells for cytotoxicity experiments against B-cell chronic lymphocytic leukemia (B-CLL) patient samples. eNKs cells effectively eliminated patient sample cells. (Cavo, T. and Anel, A., personal communications)

Our group developed a natural killer cell expansion method using healthy donor PBMCs. NK cells were cultured with inactivated 721.211 feeder cells and the interleukins IL-2 and IL-15. Trials with and without IFN-α were conducted to ascertain role in the proliferation or activation of NK cells. As no significant benefit was found, IFN-α was eliminated from the protocol. PBMCs are made up of several types of lymphocytes and monocytes, T cells being the majority. In order to cut down on unneeded cell populations, a previous T cell depletion was performed at day 0. Traditionally, NK cells were isolated during these experiments employing immunomagnetic separation techniques from Miltenyi Biotech. Our lab tested both the elimination of T lymphocyte populations and the isolating of NK cells using products from Miltenyi and StemCell. The most successful NK cell expansions occurred with CD3+ cell depletion using StemCell products during the first day of expansion. On day 20, eNKs were best isolated through Miltenyi's CD56 MicroBeads [44].

Based on these studies, we sought to widen the usage of eNK cells and understand the molecular changes our expansion protocol wrought. The objectives consisted of the following:

- Successfully complete natural killer cell expansions based on the previous protocol and ascertain their cytotoxicity.

- Examine to what extent the anti-CD38 mAb, daratumumab, inhibits and is toxic to NK cells.
- Treat MM cell lines and fresh MM bone marrow samples with eNK, Daratumumab, and in combination to measure their cytotoxicity.
- Isolate mRNA from eNK cells at day 0 and day 20 for sequencing to find gene expression changes during the expansion protocol.

3 Material and Methods

3.1 Material

3.1.1 Reagents

Phosphate buffered saline, distilled water, MACS Buffer, StemCell Buffer, and ABB are all made in-house and autoclaved for sterility. RPMI-1640 is acquired from Gibco with 10% fetal bovine serum and 1% antibody (Pen-Strep) added. All flow cytometry antibodies (anti CD38 FITC and APC, anti CD138 PE and FITC, anti CD3 FITC, anti CD56 APC) were acquired from Miltenyi Biotech. Istoype controls for CD138 FITC was FITC Mouse IgG$_1$ (BD Bioscience) and for CD38 APC was APC labeled mouse IgG$_1$ (BD Bioscience). Daratumumab (Darzalex) was kindly provided by Dr. Luis Palomera, Hospital Clinico Universitario Lozanio Blesa, Zaragoza, Spain.

3.1.2 Blood & Bone Marrow Samples

Leukopaks were acquired from the Blood and Tissue Bank of Aragon (Banco de Sangre y Tejido de Aragon) and processed within 24 hours of extraction from donor. MM patient bone marrow aspirates were collected by the hematology departments of the Hospital Universitario Miguel Servet, Zaragoza, Spain and Hospital Clinico Universitario Lozano Blesa, Zaragoza, Spain, after gaining informed consent from each patient. Samples were picked up within 5 hours of extraction and processed on the same day.

3.1.3 Instrumentation and Software

All cellular experiments were carried out in a vertical, laminar flow hood (company) to maintain sterility. Cells were cultivated in a xx Incubator maintained at 37°C with 98% humidity, and 5% CO_2. Flow cytometry cell analysis was carried out via a BD FACSCalibur™ (BD Biosciences) and the CellQuest Pro software. Data was analyzed with FlowJO version 7 software. A NanoVue Plus™ Spectrophotometer (GE Healthcare) was used to quantify mRNA concentration and its purity.

3.2 Cell Cultivation

3.2.1 Cell Lines & Culture

All cell lines (MM1S, H929, U266, and 721.221) were cultured in the RPMI-1640 media and kept at 37°C as previously described (Section 3.1.1 & 3.1.3). Subcultures were realized every

3-5 days, as needed, to maintain adequate cell concentrations. 25 or 75 cm^2 flasks with $0.2\mu m$ filter were used to keep cells from contamination while allowing for gas flow.

3.2.2   Cell Viability and Quantification

Cell viability and quantification was carried out through a dye exclusion test using Trypan blue dye. This is based on the concept that dead cells are permeable thus able to absorb the dye, while healthy cells are unable because impermeability is maintained. A sample of the cell culture is mixed with Trypan blue at a 1:1 or 1:10 concentration in a 1mL Eppendorf tube. This mixture is applied to Neubauer slide and observed under a light microscope at 10x magnification whereby cells are visualized and counted accordingly.

3.2.3   721.221 Cell Inactivation

When needed for NK cell expansion, cultured 721.221 cells were treated with 1mL of Mitomycin C (0.5mg/mL) (SigmaAldrich) per $1x10^6$ cells. To prevent toxicity, cells were diluted - 2mL incomplete RPMI-1640 was added to every $1x10^6$ cells. They were incubated at 37°C for 2 hours and subsequently washed with fresh incomplete media for a further 30 minutes in the incubator, to release any residual mitomycin C.

3.2.4   PBMC Isolation

Leukopaks were decanted into 50mL conical centrifuge tubes and diluted with an equal volume of complete RPMI-1640. The diluted leukopak was gently layered above 15mL of Histopaque-1077 with a density of 1.077g/mL (Sigma Aldrich). Tube was centrifuged at 1,200xg for 20 minutes. PBMC layer was isolated using a 1000uL micropipette, transferred to a clean 50mL conical tube and washed twice with complete RPMI-1640 media at 1,200rpm for 5 minutes. PBMCs were re-suspended in complete RPMI-1640 media and counted.

3.3   NK Cell Expansion and Activation

3.3.1   CD3+ Cell Depletion

Using the EasySep™ Human CD3 Positive Isolation kit (StemCell Technologies), the CD3+ cell fraction is depleted in each PBMC samples in order to better culture NK cells. The method has been shown to be superior than direct NK cell isolation [44]. Using a 15mL conical centrifuge tube, cells are suspended in PBS (containing 2% FBS and 1 mM EDTA) at

a final volume of 1×10^8 cells/mL. The selection cocktail is added at 100μL Selection Cocktail per 1mL of sample. After a RT incubation of 3 minutes, 60 μL/mL of RapidSpheres™ is added and sample is again incubated at RT for 3 minutes. The volume of the sample is then increased to 2.5mL and conical tube is introduced into the EasySep™ Magnet for 3 minutes. In a swift continuous motion, tube and magnet together are inverted over a fresh tube decanting the supernatant and not collect residual drops. Remove tube from magnate, resuspend content to 2.5mL and insert into magnet, repeating previous steps twice. Supernatant washed in complete RPMI-1640 and ready to culture.

The supernatant contains the fraction of PBMCs without CD3$^+$ expression on their cell surface. Most of the CD3$^+$ cells, T lymphocytes and NKT cells, remain in the magnet and can be discarded.

3.3.2   Cell Proliferation Quantification

In order to calculate the quantity of specific cell subsets present in PBMC samples and subsequent cell cultures, flow cytometry was utilized. Cells were marked with the antibodies anti-CD3 conjugated with FITC and anti-CD56 conjugated with APC. Cells were plated at a density of 1×10^5 cells per well in a 96-well plate. The plate was then centrifuged at 3,500 RPM for 2 minutes. Once decanted, cells were re-suspended in 100μL of PBS with 5% FBS and 2μL each of the antibodies described above. Each cell sample had a duplicate well, left unmarked by the antibodies to act as the control. After the incubation period, the plate was centrifuged, decanted, each well re-suspended in 200μL and transferred to cytometry tubes. Each sample was processed through the flow cytometer and data analyzed.

3.3.3   NK Cell Expansion

After inactivation of 721.221 cells and PBMCs depletion described in previous sections, NK cells cultures were ready to be established. With the final NK cell percentage of each depleted PBMC donor being calculated, 1×10^6 NK cells were cultures in 25 cm^2 flask. Based off of the total final cell count, mitomycin c inactivated 721.221 cells were added at 1:5 concentration (721.221 cells: PBMCs). The flasks had a density of 2×10^6 cells/mL of RPMI-1640 with 10%FBS. For maintenance and expansion, IL-2 (100UI/mL) and IL-15(25IU/mL) based on the final culture volume.

Cultures were maintained for 20 days. Every 5 days, cultures were examined for cellular viability via Trypan blue (section 3.2.2) and population evolution was studied through flow cytometry. At these times, new inactivated 721.221 cells would be added at the above concentrations along with IL-2, IL-15 and fresh media, maintaining $2x10^6$ cells/ mL. From day 18-23, NK cells are viable for cytotoxicity experiments.

3.3.4   NK Cell Isolation

For use in cytotoxicity experiments and mRNA isolation, NK cells must be isolated from other lymphocytes within the cell culture. CD56 MicroBeads (Miltenyi Biotec) were used along with MACS Columns. An appropriate number of cells were centrifuged at 300xg for 10 minutes and decanted. The cell pellet was resuspended in 80μL MACS buffer per $1x10^7$ cells and 20μL of CD56 MicroBeads were added. After a 15-minute incubation at 4°C, cells would be washed by adding 2mL MACS buffer and centrifuging for 10 minutes at 300xg. Cells were decanted and re-suspended in 500μL MACS buffer. To prepare LS MACS Columns, column was placed in the magnetic field of the MACS Separator and rinsed with 3mL buffer. The cell suspension was applied to the column and washed 3 times with 3mL buffer. The flow-through was discarded and the column was removed from the magnetic field, 5mL of buffer were applied and a plunger was used to flush out the magnetically labeled cells. Isolated NK cells were then counted, washed, and ready for further experiments.

3.4   Cytotoxicity Experiments

For every cytotoxicity experiment, cells were plated at a 5:1 ratio (eNK cells:MM cells). For cell lines, 500,000:100,000 cells. For MM patient samples, the final number of cells was based on available cell number, always maintaining the 5:1 ratio.

3.4.1   eNK + Daratumumab

Once the eNK cells were isolated using the previously described technique, they were plated at a concentration of 500,000 cells/well in a 96-well plate. One well was used as control while the other was treated with 5μg/mL of daratumumab. Cells were suspended in 100uL of complete RPMI-1640 with 10% FBS and incubated at 37°C for 3 hours. After incubation, cells were centrifuged at 3,500RPM for 2 minutes, decanted, resuspended in 100μL of ABB and 4μL Annexin-V APC. After 20 minutes of incubation at RT, the plate was again

centrifuged, decanted, resuspended in 200μL of ABB, and transferred to flow cytometry tubes for analysis.

3.4.2   MM Cell Lines

After isolation, eNK cells were resuspended in 1mL PBS and 1μL of Cell Tracker Green followed by an incubation of 20 minutes at 37°C. Immediately after, cells were washed with RPMI-1640 at 10%FBS and incubated for a further 30 minutes at 37°C for the cells to expulse excess florescent marker from the cells. MM cell lines were harvested and seeded into 4 well in a 96-well plate in a final volume of 100μL RPMI-1640 with 10% FBS. Each well had the following conditions: control, marked eNK cells, daratumumab (5μg/mL), and marked eNK cells with daratumumab. Plate was incubated for 3 hours at 37°C. Once centrifuged and decanted, each well was re-suspended in 100μL of PBS with 5% FBS and 4μL of 7-AAD. Wells containing daratumumab were marked with 10μL of anti CD138. All other wells were marked with 10μL of anti CD38 and incubated for 20 minutes at RT. The cells were then prepared for flow cytometry by centrifuging the plate and resuspending in 200μL in ABB and transferring to cytometry tubes. This process was repeated for each of the three MM cell lines used: MM1S, U266, and H929.

3.4.3   MM Patient Bone Marrow Samples

Bone marrow samples were collected and consisted of 1-2mL bone marrow aspirate in a heparinized/EDTA tube. To isolate the bone marrow mononuclear cells (BMNCs), the same procedure as PBMC isolation was followed except at a volume of 15mL (section 3.2.4). Samples were also diluted and washed in RPMI-1640 10% FBS in place of PBS to maintain cell health. Once isolated, cells were counted and 100,000 cells were marked with antiCD38 FITC and processed through the flow cytometer to get the MM cell percentage within the sample. In a 5:1 ratio of isolated eNK to MM cells, although this ratio is skewed based on the percentage of other cells within the bone marrow sample. Cell were seeded into 4 wells in a 96-well plate in a final volume of 100μL RPMI-1640 with 10% FBS. Each well had the following conditions: mm cells alone, mm cells with eNK cells, mm cells with daratumumab (5μg/mL), and mm cells with eNK cells plus daratumumab. To each well, 1μL of IL-6 was added per 100,000 total cells to maintain the viability of MM cells. The plate was incubated for 3 hours at 37°C. Once centrifuged and decanted, each well was re-suspended in 100μL of ABB and 4μL of Annexin-V APC. Wells containing daratumumab were marked with 10μL

of anti CD138. All other wells were marked with 10µL of anti CD38 and incubated for 20 minutes at RT. The cells were then prepared for flow cytometry by centrifuging the plate and resuspending in 200µL in ABB and transferring to cytometry tubes.

3.5   mRNA isolation

At Day 0 and Day 20, $1x10^7$ cells NK cells were isolated (section 3.3.5) from 4 donors. Using the µMACS™ mRNA Isolation Kit (Miltenyi Biotec), the mRNA of these cells was isolated. Following kit indications, cells were washed in cold PBS, centrifuged at low speed and supernatant removed. Cells were lysed completely by resuspending in 1mL of Lysis/Binding Buffer and vortexing for 5 minutes. Mechanical shearing was performed by forcing lysate at high pressure 5 times through a 20G needle attached to a 2mL syringe. Lysate is then applied to the LysateClear Column within the centrifugation tube. The column is sealed and centrifuged at 13,000xg for 3 minutes to remove debris.

Meanwhile, the µMACS Column was placed inside the magnetic field of the thermos MACS Separator and rinsed with 100µL of Lysis/Binding Buffer. To the cleared lysate, Oligo(dT) MicroBeads were added at 50µL per 1mL of liquid. Mixed lysate was applied to top of the column matrix, flowing through while mRNA remained in the column. 200µL of Lysis/Binding Buffer was applied twice to column to remove DNA and proteins. To remove rRNA and remaining DNA, column was rinsed with 100µL of Wash Buffer 4 times. Elution Buffer, previously heated to 70°C in a XX, was passed through column and discarded. To fully elute the mRNA, 50µL Elution Buffer was placed on the column and flow-through collected in a RNase-free tube.

3.5.1   mRNA Quantification and Purity

To quantify and calculate the isolated mRNA, a NanoVue Plus™ Spectrophotometer (section XX) was used. 2µL of each sample was tested and the UV absorbance was measured at 260nm. The purity was obtained calculating the A260/280 ratio. The NanoVue Plus™ was calibrated using the Elution Buffer (nuclease-free H_2O). Samples were then stored at -80°C for further processing.

3.6   Statistical Analysis

Statistical data was obtained using GraphPad Prism (5.0). The statistical relevance of each study was first evaluated via ANOVA, analysis variance. For statistical significance ($p < 0.05$), Post-hoc Fisher's PLSD test was employed.

4 Results

4.1 Natural Killer Cell Expansions

In accordance with the protocol previously established in our lab [44], five expansions of natural killer cells were successfully completed. Although each leukopak donor exhibited a different initial CD56+CD3- cell % in the isolated PBMCs, all cultures began with $1x10^6$ NK cells. Quantity of interleukins and 721.221 feeder cells used at each time was tailored to the particular sample, while always maintaining the proper concentrations of IL-2 and IL-15 and the ratio of feeder cells indicated above.

4.1.1 NK Cell Expansion Results

To analyze the progression of expansion, the total number of NK cells was calculated every five days. Through flow cytometry analysis, the percent of marked CD3-CD56+ cells was determined and total number of cells in the culture was extrapolated. As shown in Figure 1, all donor NK cells proliferated after Day 0. The first five days were a period of acclimation to the new environment and little growth was recorded. By day 10, most donors NK cells had exponential growth ranging from 25 to 60-fold increase. In the case of donors 1 and 3, the exponential growth did not occur till day 15. Donor 10, while exhibiting a 2,500% increase on day 10, declined steadily and never recovered.
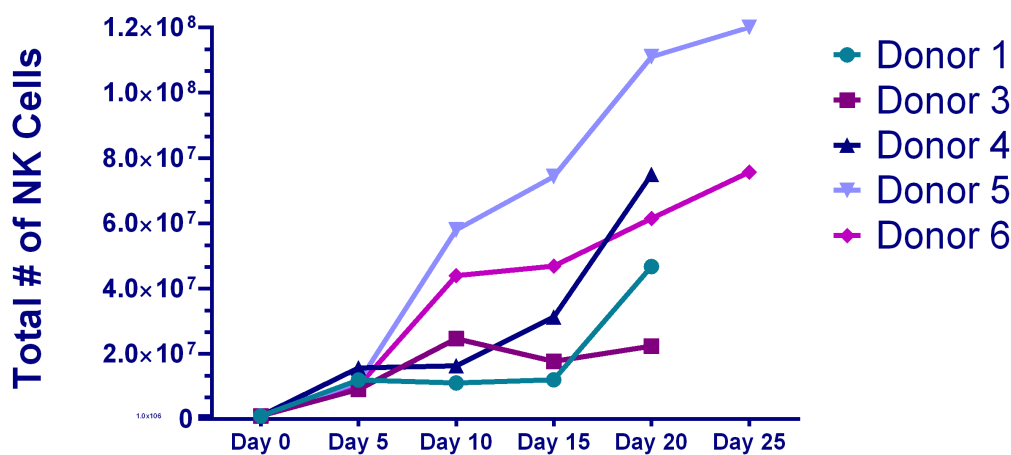

Figure 2. Expansion of eNKs based off of total number of NK cells in culture. For each expansion, data was collected every five days. Plotted is the total number of NK cells found in the flask, based off of the total number of cells present and the percent CD3-CD56+ cells analyzed by the flow cytometer. All expansions started with $1x10^6$ NK cells. Day 25 was only reached by donors 5 and 6.

The final expansion fold change is tabulated in Table 1. For donors 5 and 6, cell cultures were maintained past the standard 20 days, continuing to refresh the cells with interleukins and feeder cells for a further five-day period. As the cells continued to expand and remained viable -verified through flow cytometry analysis- cultures were conserved.

Donor	Final Fold Expansion
Donor 1	46.8
Donor 3	8.76
Donor 4	75
Donor 5	120
Donor 6	75.7
	65.3 Average

Table 1. Final fold expansion of each donor NK cells. Each donor began with the same total number of NK cells. After 20 days of treatment with 721.221 cells and interleukins, the final fold expansion was calculated. The growth from $1x10^6$ cells to final cell count constitutes the final fold expansion. The average growth of each expansion was 65.3 times the initial number of NK cells.

4.2 Expression of CD38 and CD138 on MM Cells.

4.2.1 CD38 signal loss caused by daratumumab

The most reliable marker for multiple myeloma cells is CD38. NK cells also have a minimal expression of CD38. During the cytotoxicity experiments, MM and NK cells were incubated with daratumumab. When marking with anti-human CD38 antibody for flow cytometry analysis, no signal was detected (Figure 2) Below, the boxes indicate the location of CD38+ multiple myeloma cell population in a patient sample. The first 2 panes present MM cells alone as control and MM cells together with eNK cells. The cluster of MM cells is clear. There are also subsets of other cells that express a lower level of CD38. Upon treatment with daratumumab, the signal is clearly lost as seen by the empty squares. The signal from the other CD38 expressing cells is also lessened. As the cytotoxicity of each parameter could not be measured when daratumumab was used, another marker had to be chosen. CD138 is also expressed on plasma cells including MM cells, although with decreased expression in

patients with relapsed/progressive disease. Daratumumab does not affect CD138 and blocked no signal.
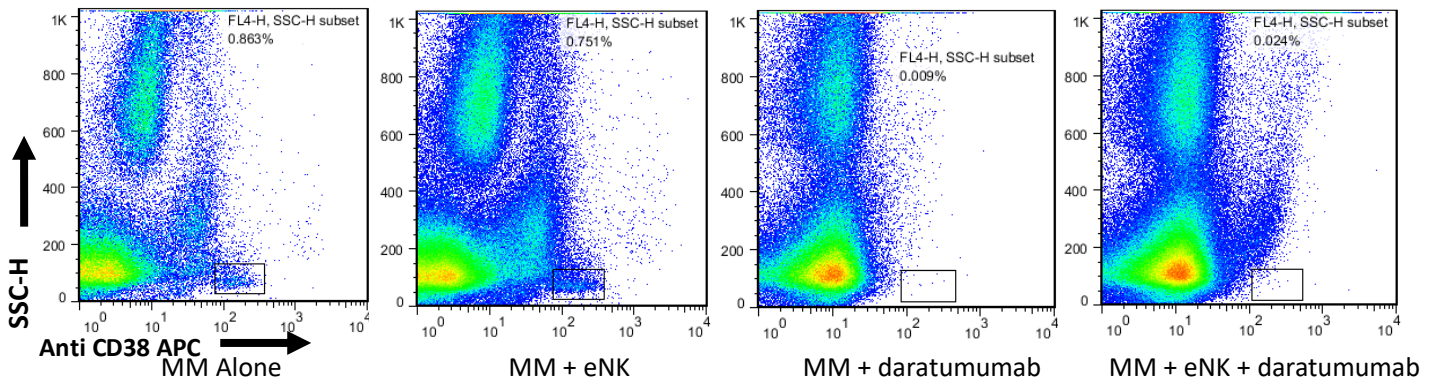

Figure 3 Flow cytometry results using anti-CD38 on multiple myeloma patient sample. Black square indicates location of CD38+ multiple myeloma cells. CD38 is expressed on the cell surface of multiple myeloma cells. When anti-CD38 APC is used to mark the cells, the signal is blocked by daratumumab, a CD38 mAb. The first two black squares show the MM population clearly marked with anti CD38 APC. In the last two squares where the samples are treated with daratumumab, the boxed area is empty. No MM appear marked by anti CD38 APC.

4.2.2 CD38 and CD138 expression in MM cell lines

We verified the expression of CD38 and CD138 on multiple myeloma cell lines to adjust flow cytometry markers. The cell lines employed were MM1S, H929 and U266. As seen in Figure 4, H929 and MM1S both express CD38 and CD138. U266 however only expresses CD138. This indicates that this cell line cannot be used with anti CD38. So as to maintain the same parameters for all cell line experiments, the eNK cells were marked with cell tracker green instead of usage of any anti-CD38 or anti-CD138 antibodies.
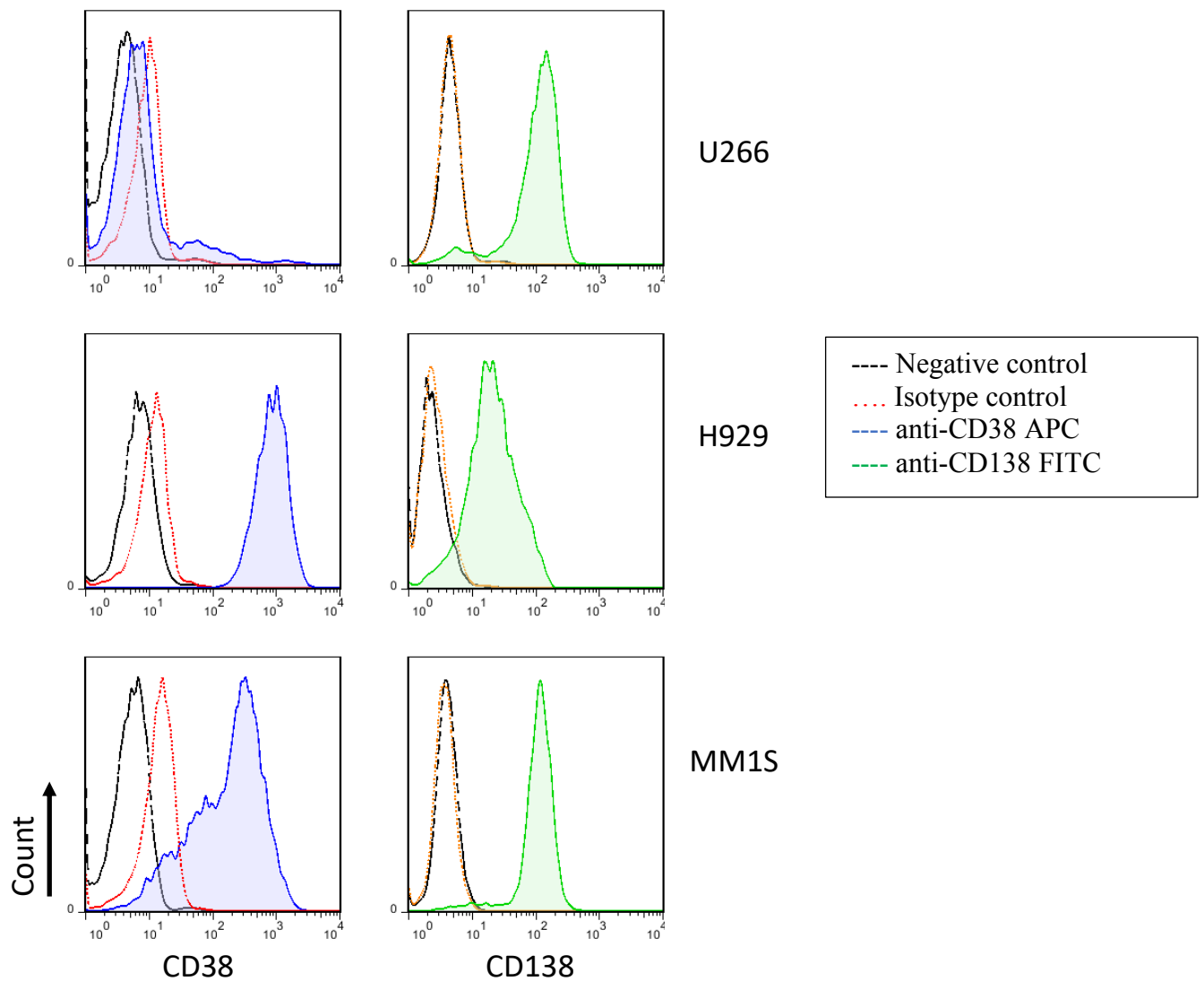
Figure 4. Flow cytometry results of CD38 and CD138 expression testing on multiple myeloma cell lines. Histograms show the cell surface expression of CD38 (blue tinted area) and CD138(green tinted area) on H929, U266, and MM1S using both negative (dashed black line) and isotype controls (dotted red lines, each antibody had its own isotype control.). U266 does not express CD38 but does express CD138. While both H929 and MM1S express CD38, MM1S has a higher number of receptors than H929. All histograms based on count of cells.

4.3   Effect of daratumumab on eNK cells.

As stated in the introduction, there are several published reports indicating daratumumab's cytotoxic effect on NK cells. To examine this possibility, we plated wells of isolated expanded NK cells alongside each multiple myeloma cytotoxicity experiment. Percent of specific cell death of eNKs was ascertained through Annexin-V staining. (Figure 5). There was no statistically relevant difference between the daratumumab treated eNK cells and their corresponding control well (p=0.67). eNKs tested originated from multiple expansions, although the majority came from the donor 5 expansion. Even with cells from a single expansion, isolated at different time points, the effect of daratumumab varied. When examined against the results of the mm cell cytotoxic experiments, there was no correlation between daratumumab mediated eNK cytotoxicity and decreased efficacy in eNK killing of MM cells. While daratumumab did bind and have a cytotoxic effect against the eNK cells, it did not significantly influence the efficacy of eNKs against MM cells.
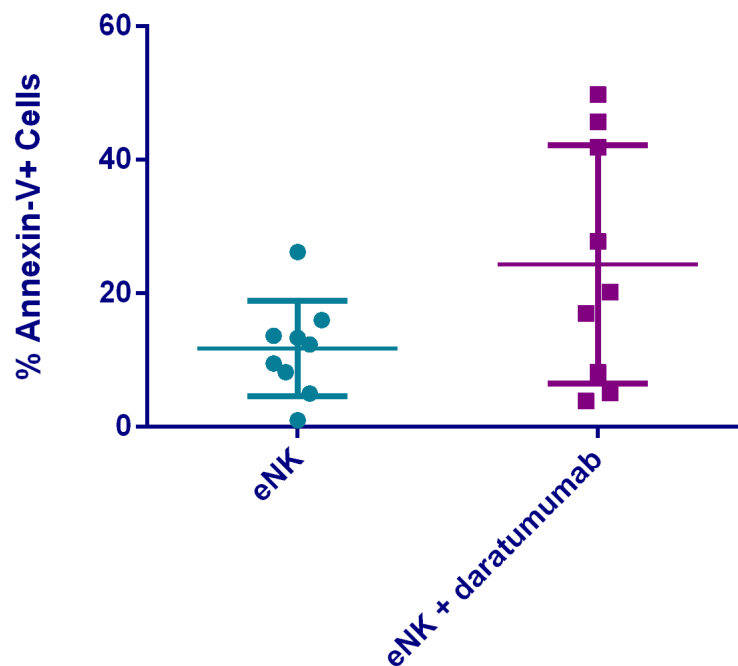

Figure 5. Percent of Annexin-V positive cells in eNK cells treated with daratumumab. Using the percent of Annexin-V positive cells in each sample, we plotted the cytotoxic effect daratumumab had on the eNK cells. For each use of eNKs against multiple myeloma cell lines or patient samples, eNKs were plated alongside and treated with or without daratumumab. Although daratumumab does cause cell death in NK cells, the percentage is not statistically significant and did not effect results of the MM cytotoxicity experiments.

4.4   Multiple Myeloma cell line cytotoxicity

To first examine the effects of eNK cells and daratumumab on multiple myeloma cells, we employed three traditional MM cell lines: MM1S, H929, U266. As described in section 2.4.2, cell lines were cultured with eNK and daratumumab alone and in different combinations. Figure 5 shows the flow cytometry results indicating the percent of 7-AAD positive cells meaning cell death in the population tested. In MM1S, eNK cells caused a 18% increase in specific cell death when compared to un-treated cells. This pattern was followed in H929 and U266 cell lines with a 60 and 64% specific cell death increase, respectively. When daratumumab was added to the eNK cells, no increase in specific cell death was noted for the MM1S cell line, attributable to their lack of CD38 expression. In the H929 and U266 cell lines, there was a minute increase in cell death (1% in both). However, daratumumab had no effect when used singularly against MM cell lines (data not shown). This may be due to decreasing viability of the daratumumab sample. Experiment will be repeated to verify results.
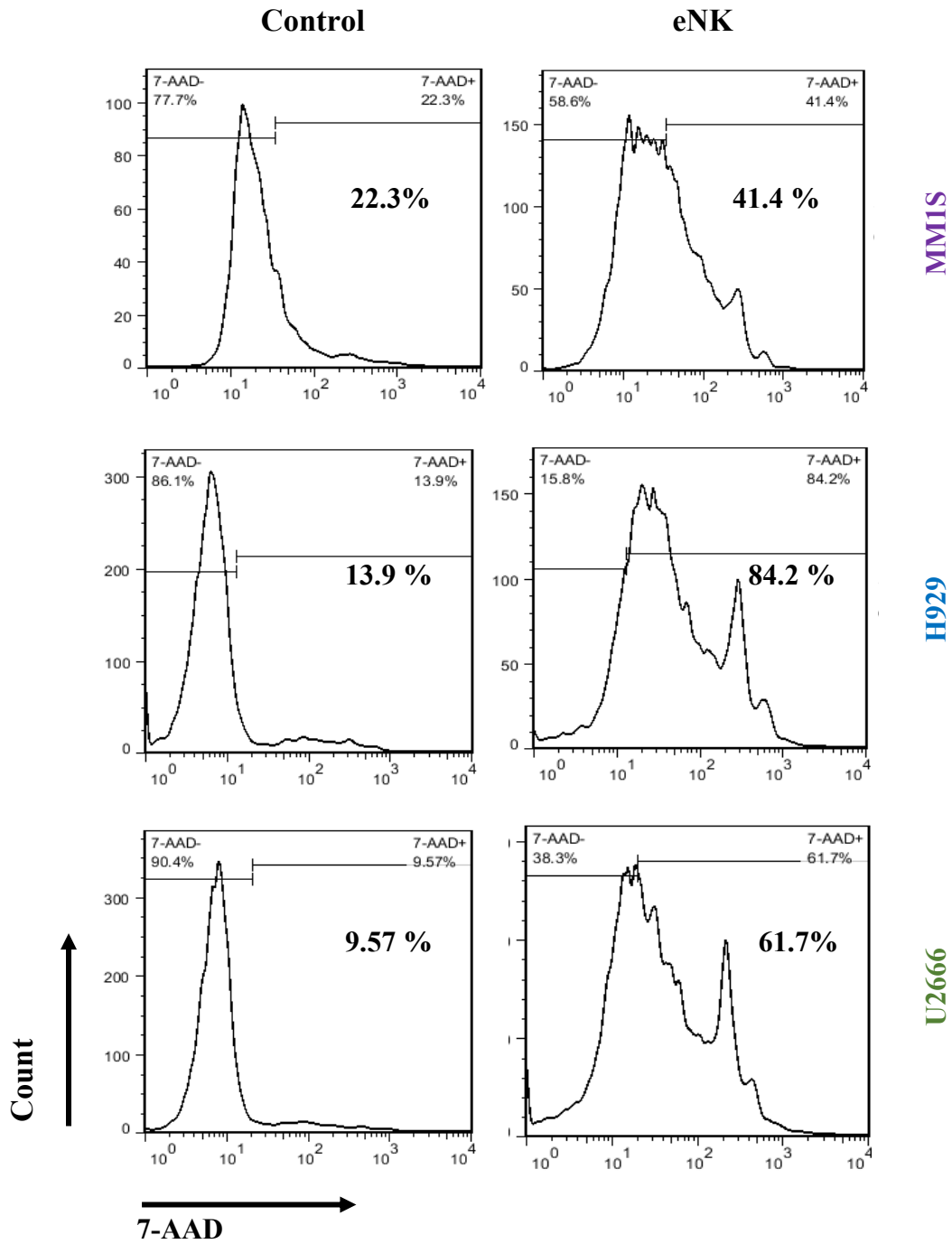
Figure 6. Specific cell death of MM cell lines as measured by 7-AAD expression. Histograms show the count of 7-AAD positive cells. All gates based on control. When the three MM cell lines were treated with eNKs, the percent of 7-AAD positive cells increased dramatically, indicating high percent of specific cell death. The most susceptible cell line was H929 with a 70.3% increase in specific cell death.

Experiments on these and other cell lines need to be repeated to statistically show that eNK cells have a cytotoxic effect against MM cells and are a promising avenue of therapy. The figure below summarized the quantified results of the cell line experiments. The dramatic effect of eNK cells on multiple myeloma cell lines.
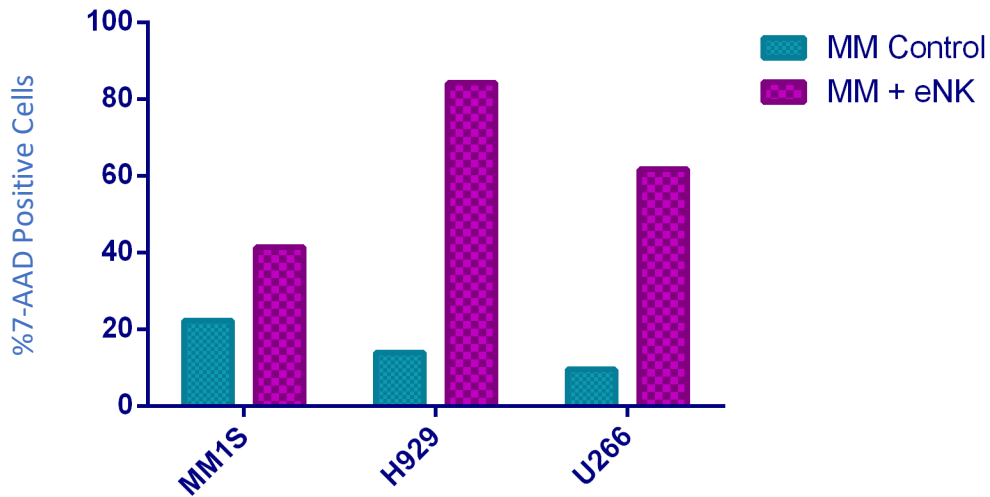

Figure 7. Percent of 7-AAD positive cells in multiple myeloma cell lines. When treated with eNKs, the percent specific cell death, as indicated by 7-AAD positive cells, increases dramatically. In all multiple myeloma cell lines, eNKs had a cytotoxic effect and were able to kill the cells effectively. Study will need to be repeated to ascertain statistical significance

4.5   Multiple Myeloma patient cytotoxicity.

4.5.1   Patient Clinical Data

After the experiments were carried out, the patient clinical data was received, in order to maintain a single-blind study. Due to difference in sample location and processing, not all data is available for all samples. In the chart is a summary of all data obtained. There was no statistically relevant data that correlated to cytotoxicity assay outcomes. A greater number of samples is needed in order to find a trend or correlative cause that influences our data. Currently, no clear relationship between the patient clinical data and the experimental treatment outcomes.

Patient Clinical Data

Table 2. Clinical data of multiple myeloma patients. Data was collected by the Hematology department at each patient's

Column1	HC30	HMS1	HMS8	HMS10	HMS11	HMS12	HMS13	HMS15
Age	75	73	66	73	64	63	65	51
Diagnosis	MM BJ	MM	MM BJ	MM smoldering	MM	MM	MGUS	MM
Previous Treatments		No	Bortezomib, dexamethasone, auto BM transplant (twice)	No	No	Bortezomib, cyclophosphamide, dexamethasone. Auto BM transplant. Bortezomib, lenalidomide, dexamethasone	No	No
Current Treatments		Bortezomib, Melfalan	Daratumumab, dexamethasone	Lenalidomide, dexamethasone	Bortezomib, thalidomide, dexamethasone	Autologous bone marrow transplant	No	No
Cytogenicity	17p (TP53)	normal	14q32IGH, +1q, del13q14	No	p53(17p13)	normal	normal	+1q
% plasma cells	57	0	3.5	7.99	12.88	9.14	2.92	25.39
Myeloid Progenitors					0.68		1.4	0.58
Mononuclear Phagocytic Fragment		6.04		9.56	4.11		4.76	4.66
Progression Free Survival		7.26		33.98	28.01	49.89	14.29	47.14
T Lymphocytes		4.18		19.99	10.82	18.68	7.83	8.08
B-cell progenitors		1.51		0.71	1.3	13.74	1.26	1.37
B-lymphocytes		0.06	1.3	1.09	0.97	4.99	1.77	2.32
NK lymphocytes		1.27		4.2	2.04	3.34	0.51	9.98
Plasma Cells	57	0.24	3.49	7.99	12.88	9.14	2.92	25.39
Immunophenotype of Plasma Cells							CLONE 1/CLONE 2	
CD45		-/+	-	-	-/+ (80%)	+	+	+ weak
CD38		+ weak	+	++	++	+ weak	+ weak	+ weak
CD138		+ overexpression	+	+ strong	-/++ (37%)		++	
CD56		++	++	+	-	++	++ (1) - (2)	+
CD19		-	-	-	-	-	-	-
CD10		-		-		-	-	-
CD117		-	+ weak	-	-/+ 29%	-	++	+
CD33		++	-	-	-	-	-	-/+ 4%
CD27		+ strong	-	-	-/+ 61 %	-	-	-/+ 31 %
CD28		-	-	-	+ weak	+ weak	-/+	-
CD20				-/+				-
CD200				++	++	++	+	++
CD81		-	-	-	+ weak	-	-/+	-
Intracytoplasmic light chains	kappa +		kappa +				lambda ++ (1) kappa ++ (2)	

4.5.2   Multiple Myeloma Patient Cytotoxicity Results

Following the positive results from the cell line experiments, we obtained bone marrow aspirates from multiple myeloma patients. Once isolated, we treated the BMNCs with previously described parameters to test the cytotoxic effect of the eNK cells along with daratumumab. The eNK cells used were from different expansions and harvested on the same day as sample extraction. Cells varied from day 20 harvesting up to day 34. For the samples that were treated with daratumumab, anti-CD138 antibody was used, while the control and eNK treated samples remained with anti-CD38 marking antibodies to identify the MM cell population.

In Figure 8, we see numerated the percent of annexin-V positive cells, interpreted as percent specific cell death. While not statistically significant, eNK and daratumumab singularly had a limited cytotoxic effect on MM cells. However, in combination, they had an exponential increase in percentage of specifc cell death ($p < 0.05$).
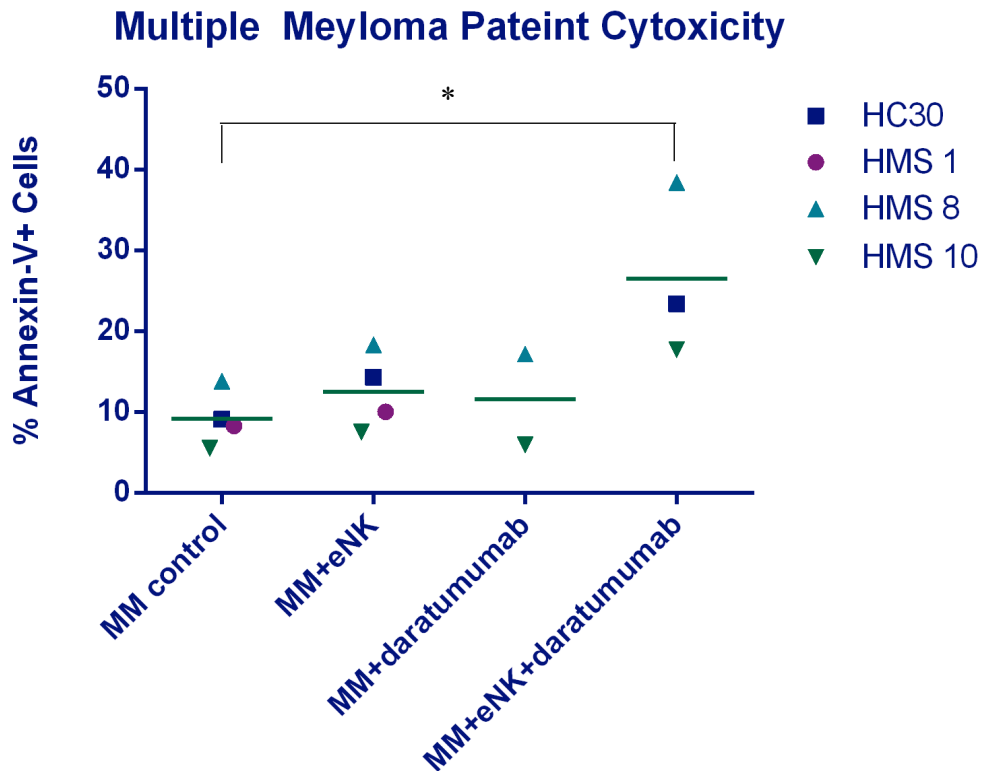
Figure 8. Percent specific cell death in multiple myeloma patient cytotoxicity experiments. Each shape indicates a patient sample. Patient samples had four treatments and the resulting cell death was analyzed using annexin-v staining. While treatment with eNK and daratumumab alone resulted in increased percent of specific cell death from the untreated group, the results were not statistically significant. However, when eNK was combined with daratumumab against patient samples, there was a statistically significant increase in percent of specific cell death ($p<0.05$). Only patient samples that were treated with viable eNKs were used.

The 2-to-3-fold increase in cytotoxicity caused by combination treatment can be seen in patients HC30, HMS 8, HMS10, and to a far lesser extent, HMS11. eNK cells alone also caused specific cell death in these patients, slightly more than daratumumab alone (Figure 8). They maintained efficacy against the myeloma cells when used alone and in combination.
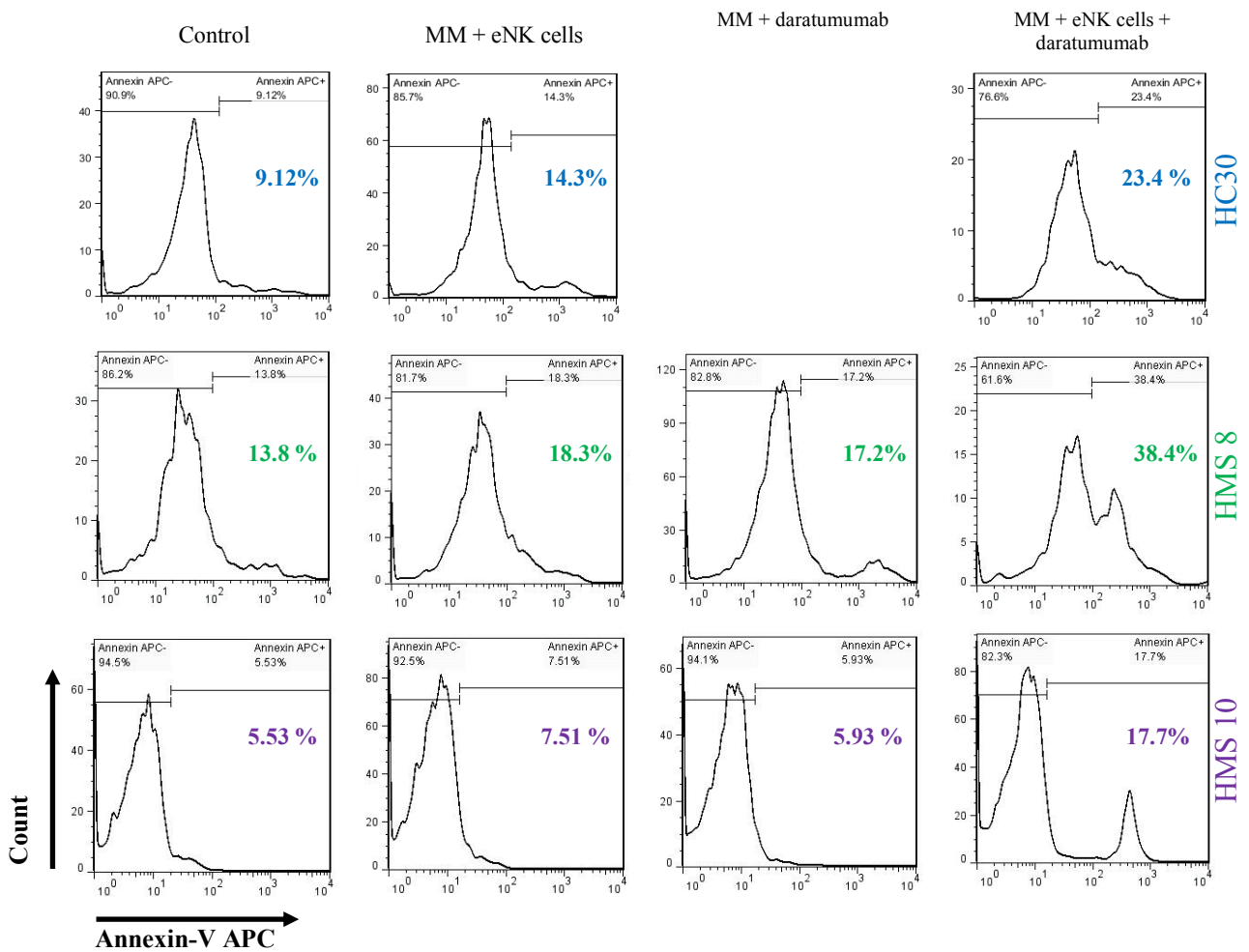
Figure 9. Flow cytometry data analysis of percent specific cell death in MM patient samples HC30, HMS 8 and HMS10. Histograms show the count for the cell death produced in each treatment for each patient. Cell death is measured by percent of annexin positive cells. Gates are taken from the control of each patient sample. Patient cells were treated with three conditions – eNK, Daratumumab, and combination of eNK and daratumumab. Due to low cell count, there is no data on the treatment of daratumumab alone for HC30. While eNK and daratumumab alone caused an increase in cell death from baseline, the combination therapy caused a 2-3-fold increase in cell death. For these samples, the eNKs were isolated during the optimal period before day 28.

The increase in cytotoxicity was dependent upon day of eNK cell isolation. We found that at day 28, NK cells began to lose their cytotoxic effect against MM cells, both in singular treatment and in combination with daratumumab. The results can then properly be separated into pre and post-day 28 eNK cell expansion. The 10-day period between day 18-28 can be considered optimal time for eNK cell cytotoxic effect (Figure 9). Either before or after, eNKs exhibit little to no killing ability.
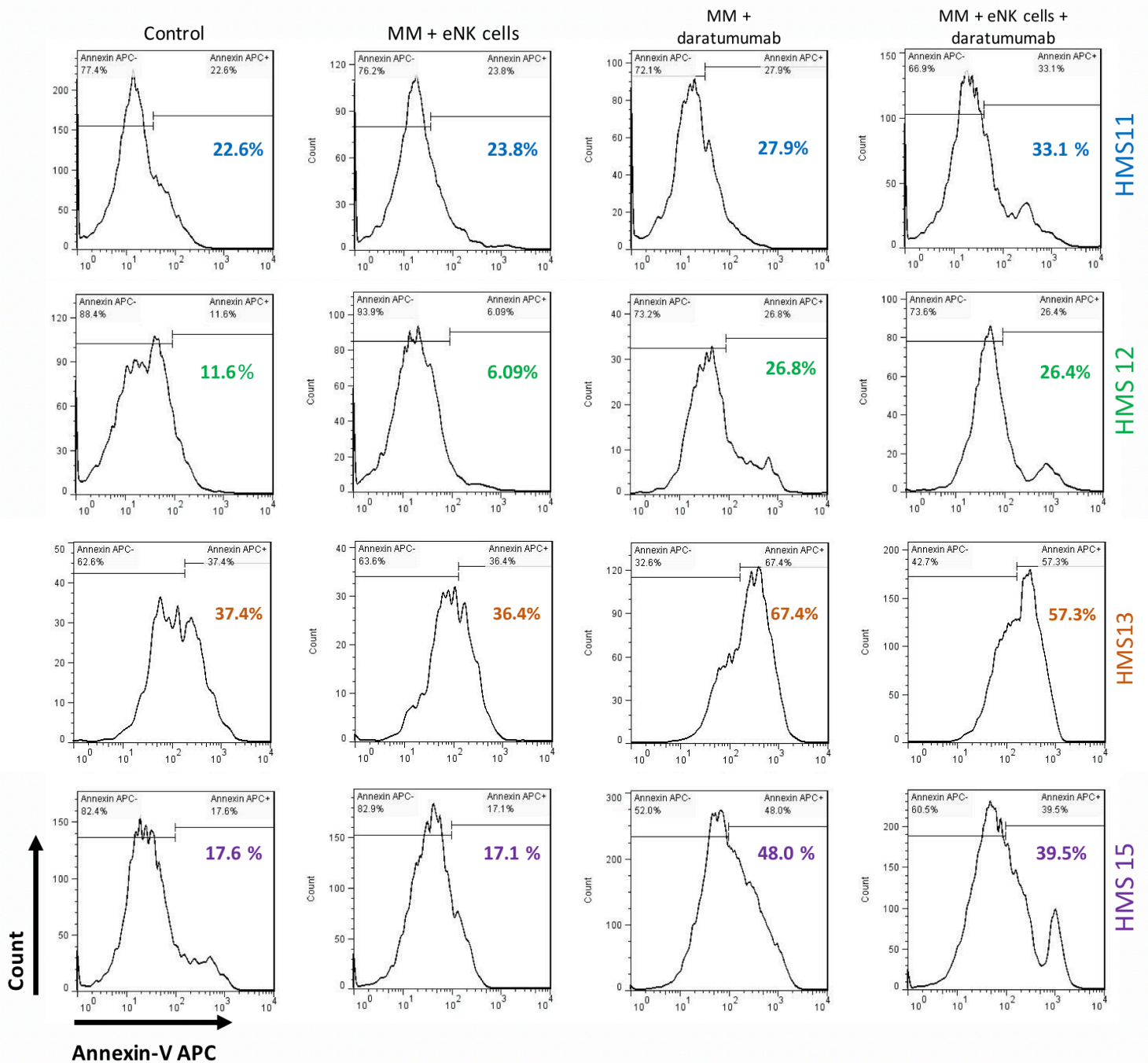
Figure 10. Flow cytometry data analysis of percent specific cell death in MM patient samples HC30, HMS 8 and HMS10. These are three examples of the results with eNKs isolated passed day 28. As the histograms show, the previously seen increase in percent specific cell death with use of only eNKs is no longer present, nor is the exponential effect of eNK and daratumumab joint treatment. In HMS11, only daratumumab caused a noted increase in cell death from baseline. In HMS 12, the eNK treated cells showed a decrease in cell death from baseline and cell death due to combination treatment was equal to specific cell death due to daratumumab alone. Interestingly, daratumumab alone caused a greater cell death than any other treatment in samples HMS 13 and HMS15 while daratumumab treatment presented no change from baseline MM cell death levels.

eNK cells isolated on day 28 show a dramatic decrease in their ability to act against the transformed patient plasma cells (Figure 10). Patient samples HMS 11, 12, 13 and 15 used eNKs isolated on day 28, 32, 33, and 34. In the case of HMS 11, specific cell death caused by eNK cells was less then 1%. Daratumumab continued to work against the MM cells although there was only a 14% increase in specific cell death when combined with eNKs in cells from this patient. In previous samples, the lowest increase was over 20%. However, for samples HMS12-15, there was no increase in cell death when combination treatment was used.

Sample HMS12, when treated with eNKs, on day 32 of expansion, showed a marked reduction (52.5%) in annexin-v positive cells as compared to control. The annexin-v percent in both the daratumumab and combination treatment wells were equal, 26.8 and 26.4% respectively. In samples HMS 13 and HMS 15, cell death percentages from the eNK treated sample always equaled that of control. In samples 12 and 13, combination cytotoxicity equaled that of daratumumab alone, indicating eNKs had no effect. Only in HMS13 and HMS15 did we see a decrease in cell death when daratumumab and eNKs combined. For sample HMS 15, daratumumab alone had 48% specific cell death. The combination treatment caused only an increase of 39.5% from baseline. The presence of these eNKs alone caused an unexpected decrease in the cytotoxic effect of daratumumab. Perhaps their expression of CD38 was elevated and bound more daratumumab, leaving less available to carry out cytotoxicity against MM cells. Further investigation is needed into the possible deleterious effects of sub-optimal eNKs.
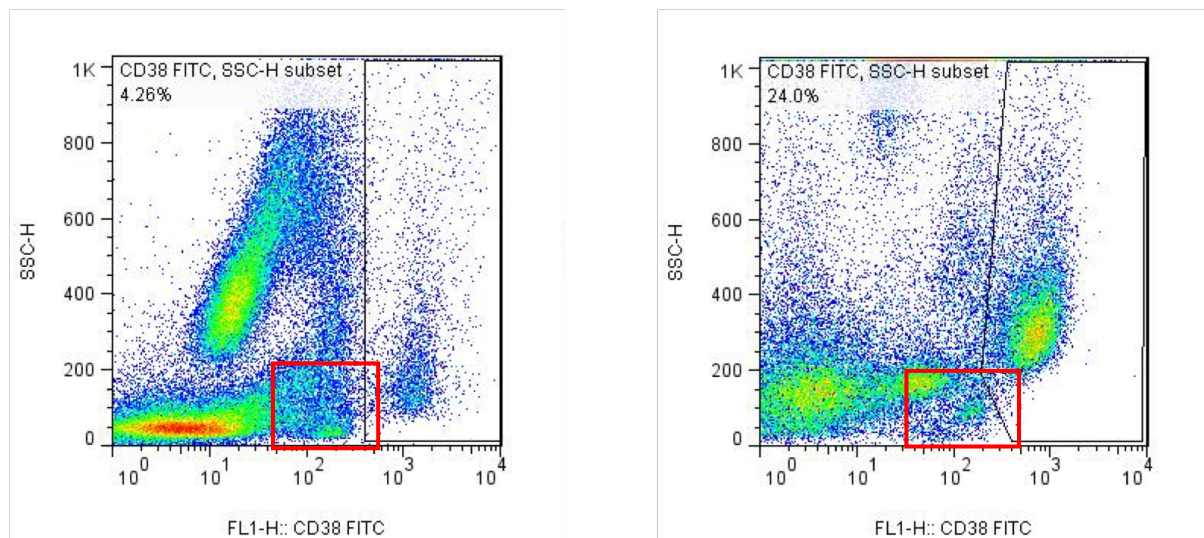
Figure 11. Flow cytometry data from HMS13 and HMS15. On the left is the control for HMS13 and the right is the control for HMS15. Both patient samples have a visible nucleus of autologous NK cells outlined by the red box. These samples were the only two that showed a marked increase in specific cell death when samples were treated with only daratumumab. Presence of an active tumor microenvironment in patients may augment the effect of daratumumab.

It is interesting to remark that in patients HMS13 and HMS15, the effect of daratumumab alone was very substantial, associated with a clearly detectable NK cell population (CD38^{dim}) in the patient sample (Figure 11). This demonstrates that daratumumab's action would be more effective in MM patients with a more immunologically active tumor microenvironment.

4.6   mRNA isolation results.

To further study the effects the 20-day expansion protocol on donor natural killer cells, their mRNA was isolated at day 0 and day 20. The mRNA isolation was successful and final concentrations are stated in the Table 3. Sequencing data is pending and can perhaps elucidate functional changes in these cells and what leads to their cytotoxic effect on days 18-28. After the results from the patient samples, the mRNA isolation can be extended to natural killer cells past day 20.

	Donor 1		Donor 3		Donor 5		Donor 6	
	Day 0	Day 20	Day 0	Day 20	Day 0	Day 20	Day 0	Day 20
mRNA concentration (μg)	0.3	8	1.1	6	3.5	3.6	1	2.4
A260/280 ratio (purity)	1.7	2	1.9	2.1	2.3	2	1.7	1.6

Table 3. mRNA concentration and purity. Table indicates the concentrations calculated by the Nanovue at absorbance 260 for mRNA concentration and the ratio of A260 to A280 for purity of mRNA sample.

5 Discussion

Based on the results obtained, it would be fair to conclude that donor NK cells, expanded according to our protocol, serve as effective treatments against multiple myeloma cells. As seen in section 4.1.1, using mitomycin inactivated 721.221 feeder cells, IL-2, and IL-15 over a 25-day period, properly expand NK cells, increasing their cytotoxic effect. This cytotoxicity can only be utilized during an optimal period of days 20 to 27. The decrease efficacy of eNKs older than 28 days was clearly shown. Once expanded, a 65-fold increase should be the mean expected. With each expansion, there are enough eNKs to treat either cell lines or multiple myeloma patient samples.

As effective as eNKs are alone when used on MM cell lines, when combined with daratumumab, the percent of specific cell death produced against MM patient samples is significant. The risk of using daratumumab lies in its cytotoxic effect against NK cells. Although a small percent cell death was produced in NK treated with daratumumab, there existed no statistically significant relationship between the daratumumab susceptible eNKs and their ability to act upon the MM patient samples.

Interestingly, in the two patients with clearly detectable NK cell populations, treatment with daratumumab alone had a substantial percent of specific cell death. The presence of an active tumor microenvironment is perhaps an indicator of the positive potential efficacy of daratumumab.

Ultimately, eNKs, when used during their optimal period, exhibit a cytotoxic effect against patient multiple myeloma cells. Daratumumab, as an approved MM treatment, also kills the patient cells. However, eNKs in combination with daratumumab proved to have an exponentially greater cytotoxic effect on the same cells. This opens an exciting new treatment approach for multiple myeloma patients.

6 Conclusions

- The NK cell expansion protocol used in the present work, using IL-2, IL-15 and 721.221 feeder cells reaches mean expansions of 65-fold, being this expansion enough to treat one patient with cells from one allogenic donor.

- Multiple myeloma cell lines H929 and U266 express both CD38 and CD138. MMIS only express CD138.

- Daratumumab does not have a significant cytotoxic effect on eNK cells.

- When used during the optimal expansion period, eNK cells are highly cytotoxic against multiple myeloma cell lines, causing 20% of specific cell death on MM1s cells, 50% on U266 cell and 70% on H929 cells.

- In bone marrow samples from multiple myeloma patients:
  - eNKs isolated from day 20-27 caused moderate cell death when used as only treatment.
  - eNKs isolated after day 27 have little to no cytotoxic ability against MM cells.

- The combination of eNKs obtained in the optimal expansion period and daratumumab caused a substantial and significant increase in cytotoxicity (27% of mean specific cell death).
- The present data, although obtained in a reduced number of patients, suggests that the combination of eNK cells with daratumumab could be a promising approach to the treatment of multiple myeloma.

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